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Activation of a thioesterase specific for very-long-chain fatty acids by adrenergic agonists in perfused hearts

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Abstract

We have recently described an acyl-CoA thioesterase specific for very-long-chain fatty acids, named ARTISt, that regulates steroidogenesis through the release of arachidonic acid in adrenal zona fasciculata cells. In this paper we demonstrate the presence of the protein as a 43 kDa band and its mRNA in cardiac tissue. The activity of the protein was measured using an heterologous cell-free assay in which it is recombined with adrenal microsomes and mitochondria to activate mitochondrial steroidogenesis. Isoproterenol and phenylephrine activate the enzyme in a dose-dependent manner (10^{-10}–10^{-6} M). Both propranolol (10^{-5} M) and prazosin (10^{-5} M) block the action of isoproterenol and phenylephrine respectively. Antipeptide antibodies against the serine lipase motif of the protein and the Cys residue present in the catalytic domain also block the activity of the protein. Taken together, our results confirm the presence of ARTISt in heart and provide evidence for a catecholamine-activated regulatory pathway of the enzyme in that tissue.

Keywords: Thioesterase; Arachidonic acid; Heart; Isoproterenol; Phenylephrine; Steroidogenesis

1. Introduction

Arachidonic acid (AA) and its eicosanoic metabolites (e.g. prostaglandins, leukotrienes and epoxides) play critical roles in the initiation or modulation of a broad spectrum of physiological responses and certain abnormal processes in mammalian cells [1–3]. AA is not freely stored in the cells, instead, it is esterified to cellular phospholipids mainly at the *sn*-2 position [4]. A number of different pathways for the mobilization of AA have been proposed, including phospholipase C in concert with glycerol lipases, lysophospholipase and phospholipase A_2.

Recently, a novel mechanism for the release of AA has been proposed. This mechanism involves the release of AA by hydrolysis of the thioester linkage of the fatty acid to CoA [5] by a thioesterase with substrate specificity for very-long-chain fatty acids [6]. The first demonstration of such mechanism was obtained by studying peptide hormone regulation of steroid synthesis. We have isolated [7], cloned and sequenced [5] a protein that plays an obligatory role in the activation of steroidogenesis, through

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the release of AA, from adrenal zona fasciculata cells. The protein has been characterized as a thioesterase using arachidonoyl-CoA as substrate and named arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt) [5]. The activity of the protein is controlled by a hormone-regulated protein phosphorylation mechanism, which may then constitute an alternative regulatory mechanism for the release of AA.

A similar very-long-chain acyl-CoA thioesterase has been isolated and sequenced from liver [8]. This enzyme is located in liver mitochondria and can be induced by peroxisome proliferators.

ARTISt messenger is expressed in steroidogenic as well as in non-steroidogenic tissues such as kidney, liver and heart [8,9], indicating that the protein could actually consist of a family of proteins related to the regulation of fatty acid release acting in different tissues.

The effects of AA and its metabolites in cardiac tissue are various and profound. Recent studies indicate that the release of AA in response to physiological agonists may be an important paracrine signaling event in heart, leading to alterations in cardiac contractility [10]. AA also causes the uncoupling of cells by closing gap junction channels [11] and it was demonstrated to induce changes in the inotropic state of isolated perfused heart [12]. In its turn, stimulation of AA release by adrenoceptors has been demonstrated in a variety of cells [13] and it has been suggested that release of AA in response to receptor activation by endogenous mediators or pathological stimuli may be involved in mediating inotropic response in cardiac muscle [14,15]. In addition, heart muscle is one of the tissues in which lipoxygenase products have been shown to exert a pathophysiological prominence with the onset of ischemia [16,17].

Given the presence of ARTISt in heart and the involvement of AA in the regulation of cardiac function, we investigate whether ARTISt can be regulated in isolated perfused hearts by catecholamines. The model used for this study includes a heterologous recombination assay, where adrenal mitochondria are challenged by ARTISt isolated from catecholamine-stimulated hearts.

2. Materials and methods

2.1. Materials

9-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (dexamethasone) and the inhibitors 2-methyl-1,2-di-3-pyridyl-1-propanone (Metopyrone, 21-hydroxylase inhibitor) and 1-(β-guanidinoethyl)-3-(4-pyridyl)indol sulfate (Ba 40.028, 11β-, 18- and 19-hydroxylase inhibitor) were a kind gift from Ciba Geigy (Basel, Switzerland). Isoproterenol (ISOP), phenylephrine (PHE), propranolol (PROP), prazosin (PRAZ), nordihydroguayaretic acid (NDGA), protein kinase A inhibitor (PKI), alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G, 5-bromochloro-3-indolylphosphate, nitroblue tetrazolium and Ponceau S were purchased from Sigma (St. Louis, MO, USA). All other reagents were commercial products of the highest grade available.

2.2. Animals

Adult Wistar rats (200–300 g) were used throughout. For heart perfusion experiments, rats were fasted overnight but given ad libitum access to water. For adrenal preparations, rats had free access to both rat chow and water, and were supplied with dexamethasone (10 μg/ml, ad libitum) in the drinking water 16 h before sacrifice.

2.3. Heart perfusion

Animals were anesthetized with ether and received heparin. The thorax was opened, and the heart removed and placed in Krebs-Henseleit buffer (KHB) containing the following components: 114 mM NaCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.7 mM KCl, 25 mM $NaHCO_3$ and 5.5 mM glucose, equilibrated with 95% oxygen-5% CO_2.

Hearts were perfused via the aorta according to the method of Langendorff, using a universal organ perfusion system (Harvard Apparatus, Millis, MA, USA). The hearts were perfused at a constant rate of 8 ml/min with KHB using a peristaltic pump (Harvard Apparatus). The water jacket surrounding

the organ was warmed to 37°C by a heater pump (Techne, Cambridge, UK). After equilibration the hearts were perfused during 15 min with 10^{-6}–10^{-10} M of either ISOP or PHE, and with 10^{-5} M PROP or PRAZ prior to 10^{-7} M ISOP or PHE perfusion respectively. Control hearts were perfused only with KHB.

2.4. Preparation of cardiac cytosol

After perfusion, the hearts were chopped with scissors and washed with ice cold buffer A (270 mM mannitol, 10 mM Tris-HCl buffer, pH 7.4) to remove all of the blood. Next, the hearts were transferred to a Teflon, motorized Ultraturrax homogenizer and homogenized in 5 ml of buffer A per gram of chopped heart. The homogenate was centrifuged at $800\times g$ for 10 min. The supernatant was centrifuged at $9000\times g$ for 20 min. The supernatant was centrifuged at $105\,000\times g$ for 60 min to obtain the cytosol and the microsomal fraction.

2.5. Preparation of adrenal mitochondrial and microsomal fractions

Animals were sacrificed by decapitation and adrenal glands were excised and kept on ice. Adrenal mitochondrial and microsomal fractions were obtained as described elsewhere [18]. Briefly, adrenal zona fasciculata tissue from dexamethasone-treated rats was homogenized in 0.2 ml of buffer A per adrenal gland. The homogenate was centrifuged at $800\times g$ for 10 min. The mitochondrial fraction was obtained by centrifugation of the $800\times g$ supernatant at $9000\times g$ for 20 min. The obtained pellet was rinsed with buffer A, then resuspended in 0.2 ml of fresh buffer A/adrenal gland. The $9000\times g$ supernatant was centrifuged at $105\,000\times g$ for 60 min to obtain the $105\,000\times g$ pellet (microsomal fraction) and the cytosol. The pellet was rinsed with buffer A and resuspended in 0.1 ml/adrenal gland of buffer A.

2.6. In vitro heterologous recombination assay

An in vitro modified recombination assay was performed as described elsewhere [18]. Briefly, cardiac cytosol (0.10 ml) and adrenal microsomal fraction (0.05 ml) were recombined with 0.10 ml of the adrenal mitochondrial fraction in a final volume of 0.7 ml in the presence of 0.27 mM Metopyrone and 1.6 mM Ba 40.028 as inhibitors of progesterone and pregnenolone metabolism. The mixture was incubated for 10 min at 37°C and stopped by cooling the tubes on ice/water and by addition of 2 ml cold methanol/2.5 mM HCl (1:1, v/v)/tube. Steroids were extracted and progesterone production determined by radioimmunoassay (RIA). The RIA for pregnenolone was considered less specific than that for progesterone since a cross-reaction with lipophilic pregnenolone esters present in adrenal tissue could not be excluded. Therefore, the determination of progesterone was chosen as the main measurement of side chain cleavage [18] and expressed as pg of progesterone per incubation.

2.7. Incubation with ARTISt antibodies

Cytosol of ISOP 10^{-7} M perfused heart was incubated overnight with ARTISt antipeptide antibodies [7] or rabbit normal serum (NS) at 4°C. The ability of the antibody incubated cytosols to activate progesterone synthesis in the presence of unstimulated adrenal mitochondria and microsomes was measured.

2.8. Western blot analysis

For Western blot analysis [19], cardiac cytosol (10 μg protein) was loaded and resolved on a 5–17% gradient SDS-polyacrylamide gel under non-reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes using 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol. The transfer was performed at a constant voltage of 6 V for 150 min. Protein transfer was monitored by staining of the nitrocellulose membrane with Ponceau S. Membranes were then incubated with 5% fat-free powdered milk in NaCl/Pi (2.7 mM KCl, 140 mM NaCl, 10 mM Na_2HPO_4, pH 7.4), 0.5% Tween 20 for 30 min at room temperature with gentle shaking, rinsed twice in NaCl/Pi, Tween 20 and incubated overnight with appropriate dilutions of antibody at 4°C. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G with 5-bromochloro-

3-indolylphosphate and nitroblue tetrazolium as substrate.

2.9. Northern blot analysis

Total RNA from cardiac tissue was extracted by the method of Chomczynski [20] and quantified spectrophotometrically at 260 nm. RNA samples (24 μg) were denatured at 65°C for 15 min in 45% formamide/5.4% formaldehyde and electrophoresed at room temperature on a 1.2% agarose/2.2 M formaldehyde gel. RNA was transferred by capillarity to nylon membranes in 20×SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate) and fixed by baking of the membranes for 2 h at 80°C. Blots were prehybridized for 4–5 h at 42°C in 10 ml of 50% formamide, 1% SDS, 1×Denhardt's reagent, 5×SSC and 100 μg/ml of denatured salmon sperm DNA. Hybridization was performed overnight at 42°C as previously described [5]. They were then subjected to autoradiography using intensifying screens for 18–72 h at −80°C.

2.10. Protein determination

Protein concentration was determined by the method described by Lowry [21] using bovine serum albumin as standard.

2.11. Data analysis

Results are shown as the mean ± S.E.M. Statistical significance was evaluated using ANOVA followed by Tukey's test; $P < 0.05$ was considered significant.

3. Results

3.1. Detection of ARTISt and its mRNA in cardiac tissue

The presence of ARTISt mRNA was detected in heart, as two transcripts, by Northern blotting using a 795 bp PCR amplification product as a probe (Fig. 1A).

ARTISt was found in cardiac cytosol, using different antibodies against several peptides of the protein. All tested antibodies specifically detected the protein as a 43 kDa band as shown for adrenal ARTISt [7]. Shown in Fig. 1B is the band obtained using an antipeptide antibody raised against the lipase serine motif.

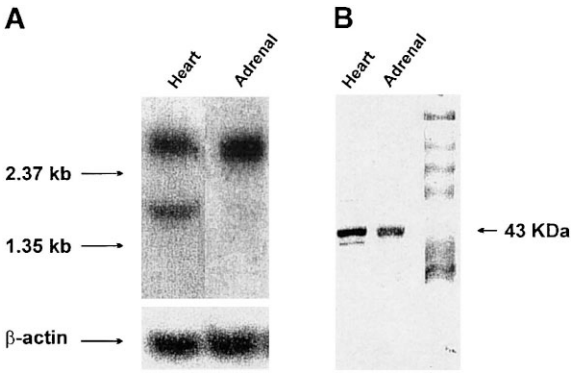

Fig. 1. Northern and Western blots of ARTISt in cardiac tissue. (A) RNA (24 μg) from cardiac and adrenal tissues was electrophoresed and transferred to nylon membranes. A 795 bp PCR product from a 1.4 kb fragment was used as a probe as described elsewhere [5]. They were then subjected to autoradiography using intensifying screens for 18–72 h at −80°C. (B) Myocardial and adrenal cytosol (10 μg) were electrophoresed, electrotransferred to nitrocellulose membranes and probed using an antipeptide antibody (G11K) directed against the lipase serine motif of ARTISt.

3.2. Effect of ISOP on the stimulation of acyl-CoA thioesterase activity (ARTISt)

The activity of cardiac ARTISt was analyzed using a heterologous recombination assay as described in Section 2.

In this assay, the protein from adrenal gland was demonstrated to induce AA release that after further metabolism to leukotrienes will stimulate steroid synthesis in unstimulated adrenal mitochondria. In addition the effect of the protein is blocked by the addition of inhibitors of AA such as NDGA (a lipoxygenase pathway inhibitor) [7,22,23].

ISOP (10^{-10}–10^{-6} M) produced a dose-dependent increase of cardiac ARTISt activity measured as the capacity of the protein to stimulate steroid synthesis (Fig. 2). The effect became significant above 10^{-8} M. ISOP effect was blocked by addition of PROP. These results suggest that ISOP-stimulated heart could activate ARTISt activity via a β-adrenoceptor-associated mechanism.

3.3. Effect of NDGA

In order to study whether the activity of ARTISt from cardiac tissue is also affected by inhibition of AA metabolism, the adrenal microsomal fraction was preincubated with 5 μM NDGA, during 30 min at 37°C prior to the heterologous recombination. As expected, preincubation of the microsomal fraction with NDGA resulted in a significant inhibition of progesterone synthesis (Fig. 3). Moreover, the increase in progesterone synthesis is observed only in the presence of adrenal microsomes, the source of the lipoxygenase enzyme.

3.4. Effect of ARTISt antipeptide antibodies

To confirm that the activity of cardiac cytosol was due to the presence of activated ARTISt, we studied the effect of specific antipeptide antibodies on enzyme activity. The activity of cardiac cytosol was blocked by addition of antibodies against the N-terminal sequence of ARTISt (FG7), and against internal peptides of the protein (FR7, G11K and A10A).

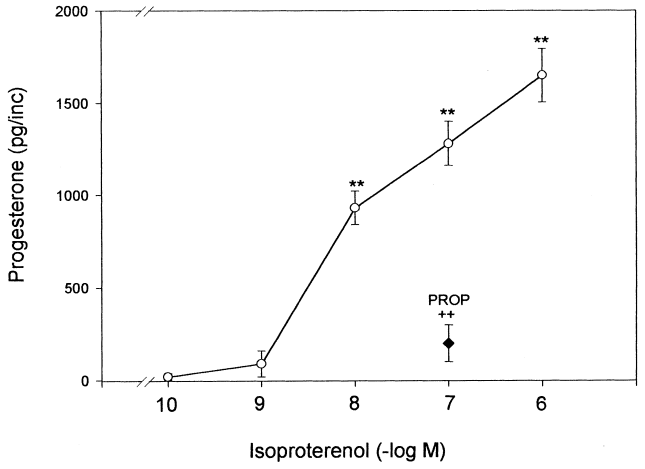

Fig. 2. Effect of ISOP and PROP. Hearts were perfused for 15 min with different concentrations of ISOP (○) or with the β-adrenoceptor antagonist PROP (10^{-5} M) and then for another 15 min with 10^{-7} M ISOP (◆). Cytosols from perfused hearts were recombined with adrenal mitochondria and microsomes in an in vitro recombination assay. Progesterone was extracted from the assay medium with 1.5 ml of cyclohexane and its content was determined by RIA. Data are shown as the mean ± S.E.M., $n=4$. **Significantly different ($P<0.01$) from basal values; $^{++}$significantly different ($P<0.01$) from 10^{-7} M ISOP. Results shown are representative of five independent experiments.

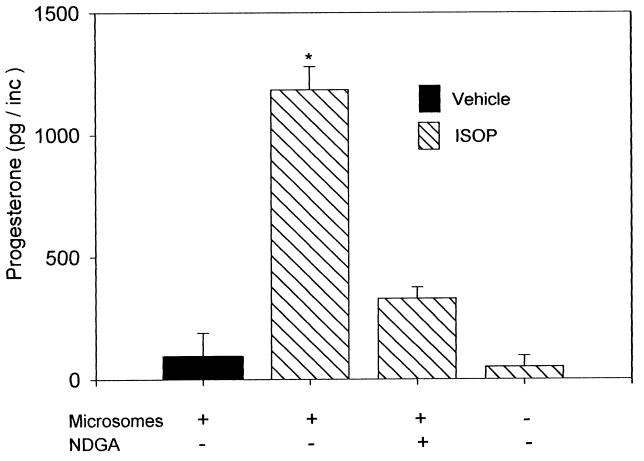

Fig. 3. Effect of NDGA. Adrenal microsomes were preincubated for 30 min at 37°C with NDGA 5 μM or buffer only. Cytosol from ISOP and KHB perfused heart was recombined with or without the preincubated microsomes and adrenal mitochondria in a heterologous recombination assay and progesterone production was measured. Data are shown as the mean ± S.E.M., $n=4$. *Significantly different from control values ($P<0.05$). Results shown are representative of three independent experiments.

The activity was unaffected by addition of normal rabbit serum and of the antipeptide antibody V15Q (Fig. 4).

3.5. Effect of protein kinase A inhibitor

In order to exclude that the effect of ISOP is due to the addition of activated cAMP-dependent protein kinase (PKA) present in the cytosol, the heterologous in vitro recombination assay was performed in the presence of a PKA inhibitor (PKI). As shown in Table 1, addition of the inhibitor had no effect on the activity of ARTISt measured as the capacity of the protein to stimulate progesterone synthesis.

3.6. Effect of PHE on the stimulation of acyl-CoA thioesterase activity (ARTISt)

As for ISOP, PHE (10^{-10}–10^{-6} M) produced a dose-dependent increase of ARTISt activity measured as the capacity of the protein to stimulate steroid synthesis (Fig. 5). Again, the effect became significant at concentrations above 10^{-8} M.

PHE effect was blocked by addition of 10^{-5} M PRAZ suggesting the involvement of an α-adreno-

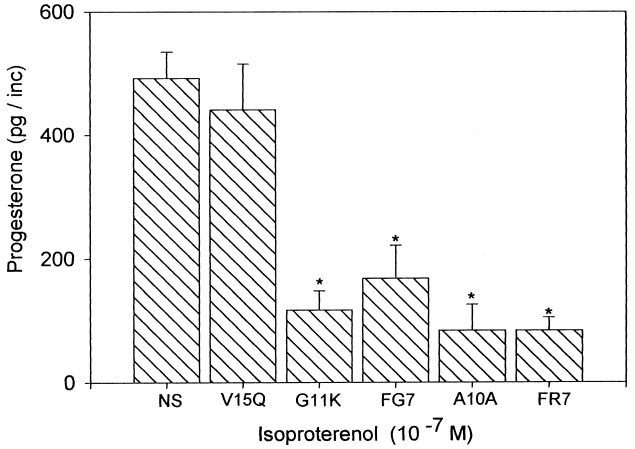

Fig. 4. Incubation of the ISOP-stimulated cytosol with ARTISt antipeptide antibodies. Rat hearts were perfused with 10^{-7} M ISOP for 15 min. Cytosols from perfused hearts were obtained. Aliquots of 100 μl (400 μg of protein) of cytosol were incubated overnight with different ARTISt antipeptide antibodies (1:100) and with rabbit normal serum (NS) (1:100) at 4°C. After incubation cytosols were recombined with adrenal mitochondria and microsomes and net progesterone synthesis was measured by RIA. Data are shown as the mean ± S.E.M., $n=4$. *Significantly different from NS values ($P<0.05$). Results shown are representative of three independent experiments.

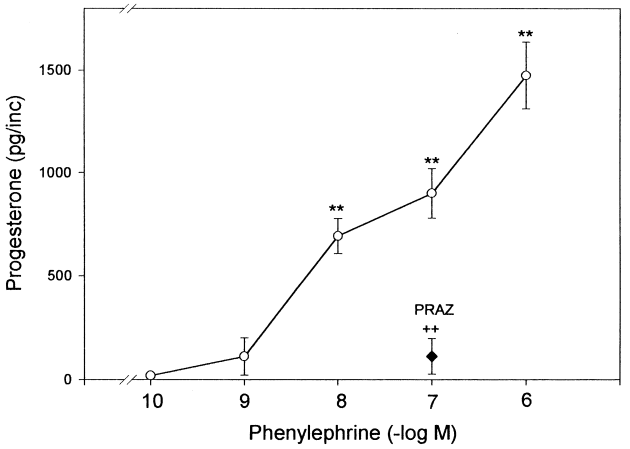

Fig. 5. Effect of PHE and PRAZ. Hearts were perfused with different concentrations of PHE (○) or with the α-adrenoceptor antagonist PRAZ (10^{-5} M) and then for another 15 min with 10^{-7} M PHE (♦). Cytosols from perfused hearts were recombined with adrenal mitochondria and microsomes in an in vitro recombination assay. Net progesterone production in adrenal mitochondria was determined by RIA. Data are shown as the mean ± S.E.M., $n=4$. **Significantly different from controls ($P<0.01$); $^{++}$significantly different from 10^{-7} M PHE ($P<0.01$). Results shown are representative of three independent experiments.

ceptor-associated mechanism in the activation of ARTISt.

4. Discussion

Our results show that an acyl-CoA thioesterase specific for very-long-chain fatty acids and its mRNA are expressed in cardiac tissue. We also demonstrate that the activity of this protein, named ARTISt, can be regulated by ISOP and PHE in a mechanism that involves both β- and α-adrenoceptors.

We have previously shown that the function of ARTISt in steroidogenic tissues was determined using an in vitro recombination cell-free assay [18]. Using that assay, the protein was demonstrated to induce AA release that after further metabolism to leukotrienes will stimulate steroid synthesis in unstimulated adrenal mitochondria. The activity of ARTISt from cardiac tissue was studied here by means of a similar recombination cell-free assay using the cytosol of cardiac tissue as a source of protein. Using this assay we demonstrate that ISOP could regulate ARTISt activity. Our results show that the activity of cardiac cytosol was due to the presence of active ARTISt since specific antipeptide antibodies against the protein completely abolished the activity present in cytosols isolated from ISOP-perfused hearts.

We are confident of the specificity of the effect of ISOP described in this study since the effect was dose-dependent, in a concentration range known to exert physiological responses and blocked by specific β-adrenoceptor antagonists. ARTISt from hearts perfused in the absence of ISOP produced no effect.

Table 1
Effect of protein kinase A inhibitor

Cytosol	PKI	Progesterone (pg/inc.)
Control	−	ND[a]
	+	ND
ISOP	−	810 ± 60
	+	780 ± 30

Cytosols obtained from control and ISOP (10^{-7} M)-perfused hearts were preincubated either with PKI (100 μg/ml) or buffer only, and recombined with adrenal microsomal and mitochondrial fractions

Progesterone production was determined as previously described.

[a]ND, not detectable.

It is known that ARTISt is regulated by PKA in LH-stimulated Leydig cells and in ACTH-stimulated adrenal zona fasciculata cells [22,24]. Since ISOP increases PKA activity [25] a possible effect of activated PKA generated after β-adrenergic agonism was ruled out on the basis of the results obtained with PKI.

We cannot conclude from our results that ARTISt in cardiac tissue will follow the same mechanism of action as in steroidogenic tissue, e.g. AA release. However, we conclude that ISOP is able to stimulate the acyl-CoA thioesterase activity. This is supported by: (a) antibodies against the lipase serine motif of ARTISt (G11K) and against the Cys residue present in the catalytic domain block the activity of the cardiac protein as has been reported for LH and ACTH [5,9]; (b) the effect of ARTISt from cardiac tissue is blocked by NDGA as was demonstrated in adrenal tissue. Further support comes from the observation that ARTISt from cardiac tissue releases AA from arachidonoyl-CoA in vitro in an ISOP-regulated manner (manuscript in preparation).

Taken together, our results will suggest that the acyl-CoA thioesterase will release AA or another fatty acid that will act as a signal transducer in response to adrenergic agonism in cardiac tissue. The question as to how fatty acids esterified to CoA are produced and stored in cardiac tissue to serve as substrate for this enzyme is a topic that still remains to be elucidated.

There are several types of acyl-CoA synthetase (ACS) that are expressed to various degrees in different tissues. ACS1 is the well characterized ACS abundant in liver, adipose tissue and heart and exhibits a broad fatty acid specificity [26–28]. A novel arachidonate-preferring synthetase (ACS4) is present in steroidogenic cells of rat adrenal, ovary and testis [29]. This protein, although at low amounts, is also expressed in heart [29]. Interestingly, in tissues where ARTISt is expressed, such as steroidogenic tissues, liver, kidney, heart and brain, an acyl-CoA synthetase is found that is able to catalyze the activation of very-long-chain fatty acids to produce acyl-CoA. It may then be possible for ARTISt to release AA or another fatty acid in cardiac tissue to serve as a signal transduction mechanism as it does in steroidogenic cells.

The results presented in this paper constitute the first evidence indicating that catecholamines could regulate the activity of a thioesterase specific for very-long-chain acyl-CoA.

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