Structural characterization and effects on corticosteroid secretion of endothelin-1 and endothelin-3 from the frog *Rana ridibunda*

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ABSTRACT

Despite the intensive study of endothelin (ET) in mammals, the primary structure and biological activity of the peptide is not known for any species of non-mammalian tetrapod. Extracts of the stomach and the liver of the European green frog *Rana ridibunda* contained ET-like immunoreactivity measured by RIA using an antiserum raised against human ET-1. The amino acid sequence of the peptide that was isolated in pure form from the stomach extract was identical to that of human ET-1 and the peptide purified from the liver extract was identical to human ET-3 except for a single amino acid substitution (Phe⁴→Tyr). These observations demonstrate that the amino acid sequences of ET family peptides have been very strongly conserved during evolution of tetrapods and suggest that the pathway of post-translational processing of preproendothelin in the frog is similar to that in

mammals. Both frog/human ET-1, frog ET-3 and human ET-3 produced a concentration-dependent increase in the production of corticosteroids from perfused slices of the frog interrenal gland. The maximum responses produced by the peptides (approximately 2-fold increase over basal levels for both corticosterone and aldosterone production) were not significantly different. The potency of ET-1 ($-\log EC_{50}=9.81 \pm 0.01$ (S.E.M.) for corticosterone and 9.52 ± 0.29 for aldosterone production) was significantly ($P<0.01$) greater than that of frog ET-3 ($-\log EC_{50}=8.13 \pm 1.6$ for corticosterone and 8.15 ± 0.33 for aldosterone production) but the potencies of frog ET-3 and human ET-3 ($-\log EC_{50}=8.29 \pm 0.34$ and 7.87 ± 0.18) were not significantly different.

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INTRODUCTION

The endothelin (ET) family of peptides (ET-1, ET-2 and ET-3 (Inoue *et al.* 1989)) play important and diverse roles in the regulation of mammalian physiological processes and in the pathophysiology of human diseases (Rubanyi & Polokoff 1994). However, our understanding of the structural and biological properties of ETs from non-mammalian species is very limited. There is strong evidence that the tissues of Anura (frogs and toads) synthesize ET-related peptides and express ET receptors and that ET displays similar biological actions in

amphibians as in mammals. Using an ET-3 specific antiserum in immunohistochemistry, ET-3-like immunoreactivity was detected in gonadotrophs distributed throughout the pars distalis of the pituitary of the female bullfrog, *Rana catesbeiana* (Suzuki *et al.* 1997). High affinity ET-1 binding sites have been identified in liver (Nambi *et al.* 1994), heart (Kumar *et al.* 1994) and oocytes (Kumar *et al.* 1993) of the toad *Xenopus laevis* and recently two *Xenopus* ET receptor subtypes have been cloned and characterized: ET_{AX} from heart (Kumar *et al.* 1994) and ET_C from dermal melanophores (Karne *et al.* 1993).

Functional studies have shown that the vascular tissues of amphibia are extremely sensitive to ET, suggesting that the cardiovascular role of this peptide has been highly conserved during the course of vertebrate evolution. For example, human ET-1 potently ($EC_{50} < 10$ nM) constricts isolated vascular rings prepared from arteries and veins of the frog *R. pipiens* (Poder *et al.* 1991), and the toad *Bufo viridis* (Doi & Fujimoto 1993). In several species of mammal (Cozza *et al.* 1989, Morishita *et al.* 1989, Belloni *et al.* 1996, Rossi *et al.* 1997), ET-1 stimulates steroidogenesis in dispersed cells from the glomerulosa zone of the adrenal cortex and it has been shown that synthetic human ET-1 stimulates both corticosterone and aldosterone production by perfused frog interrenal slices from the frog *R. ridibunda* (Delarue *et al.* 1990).

Prior to the onset of our program of study, the only non-mammalian ET-like peptides to have been characterized structurally were the sarafotoxins, a family of five isoforms isolated from the venom of the snake, *Atractaspis engaddensis* (Takasaki *et al.* 1988). The sarafotoxins, like the ETs, comprise 21 amino acid residues, possess the same pattern of disulfide linkages and exhibit strong vasoconstrictor activity, but the evolutionary relationships between the two families is unclear (Takasaki *et al.* 1992). In most mammalian tissues, ET is secreted by the constitutive pathway (Nakamura *et al.* 1990) with the result that steady-state concentrations of the peptide are very low. This poses a challenge to the peptide chemist to obtain sufficient pure material to permit structural characterization, but recent advances in the instrumentation of microsequence analysis allow amino acid sequence determination of very low picomole amounts of peptide. The present study extends our understanding of the evolution of the ET family of peptides by describing the purification, structural characterization and steroidogenic activity in the species of origin of ET-1 and ET-3 from the European green frog *R. ridibunda*.

MATERIALS AND METHODS

Materials

Human ET-1 was synthesized as previously described (Forget *et al.* 1996). (3-[125 I]Iodotyrosyl) ET-1 (specific activity 74 TBq/mmol) was supplied by Amersham Life Science, Arlington Heights, IL, USA).

Tissue extraction

Adult specimens of the European frog (*R. ridibunda*) were obtained from a commercial source

(Couetard, St-Hilaire de Riez, France). Whole stomachs and livers were collected from 2000 animals and immediately frozen on dry ice. The frozen tissues (stomach 1.1 kg wet weight; liver 1.5 kg wet weight) were separately homogenized in ethanol/0.7 M HCl (3:1 v/v; 8 ml/g tissue) at 0 °C using a Waring blender. After centrifugation (1600 g, 30 min, 4 °C), ethanol was removed from each supernatant under reduced pressure. After a further centrifugation (1600 g, 30 min, 4 °C), peptide material was isolated from the supernatants by passage through fifteen Sep-Pak C18 cartridges (Waters Associates, Milford, MA, USA) connected in series as previously described (Chartrel *et al.* 1995). Bound material was eluted from the cartridges with 70% (v/v) acetonitrile/water and lyophilized.

RIAs

ET-like immunoreactivity (ET-LI) was measured using an antiserum raised against human ET-1 that shows 60% cross-reactivity with human ET-2, 70% cross-reactivity with human ET-3, but only 0.1% reactivity with 'big' human ET-1 (1–38) (Takahashi *et al.* 1991). 125 I-Labeled human ET-1 was used as tracer. The minimum detectable concentration using human ET-1 as standard was approximately 2 fmol/tube. Corticosterone and aldosterone concentrations were determined by RIA without prior extraction, as previously described (Delarue *et al.* 1990). The intra- and interassay coefficients of variation were lower than 4 and 10% respectively.

Purification of ET-1 from frog stomach

The stomach extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid/water (10 ml) and chromatographed on a 2.5×100 cm column of Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 48 ml/h and fractions (8 ml) were collected. Absorbance was measured at 280 nm. The concentration of ET-LI in the fractions was determined by RIA at a dilution of 1:30. Fractions containing ET-LI were pooled and pumped onto a 1×25 cm Vydac 218TP510 (C-18) reverse-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% over 10 min, held at this concentration for 30 min and raised to 49% over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min)

were collected. The fraction containing ET-LI was successively chromatographed on a 0.46×25 cm Vydac 214TP54 (C-4) column, a 0.46×25 cm Vydac 219TP54 (phenyl) column and a 0.46×25 cm Vydac 218TP54 (C-18) column at a flow rate of 1.5 ml/min using the elution conditions indicated in Fig. 1.

Purification of ET-3 from frog liver

The liver extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid/water (10 ml) and subjected to gel permeation chromatography on Sephadex G-25 under the same conditions used for the stomach extract. Frog ET-3 was purified to near homogeneity by successive chromatographies on a 1×25 cm Vydac 218TP510 (C-18) reverse-phase HPLC column and on 0.46×25 cm Vydac 214TP54 (C-4), Vydac 219TP54 (phenyl) and Vydac 218TP54 (C-18) columns under the same conditions used for purification of ET-1 from the stomach extract.

Structural analysis

The primary structures of the peptides were determined by automated Edman degradation using a Procise 491A sequenator (Perkin-Elmer, Foster City, CA, USA). Mass spectrometry of the peptide was performed on a Voyager RP MALDI-TOF instrument (Perspective Biosystems Inc., Framingham, MA, USA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction and the accelerating voltage in the ion source was 25 kV. Approximately 10 pmol of sample was used and the accuracy of the mass determinations was at least 0.05%.

Peptide synthesis

Frog ET-3 was synthesized using a manual solid-phase synthesizer according to Fmoc chemistry protocols. A hydroxymethylated resin (Wang resin) with a substitution of 1.12 mmol/g, was used as a solid support. Every coupling was monitored with the use of the ninhydrin test. The peptide was cleaved from the solid support with liquid hydrofluoric acid (10 ml/g) in the presence of *m*-cresol (1 ml/g) and dimethylsulfide (1 ml/g) as scavengers. The crude material was purified by preparative reverse-phase HPLC using a linear gradient of trifluoroacetic acid/water (0.06%) and acetonitrile for elution. The fractions corresponding to the purified linear ET were pooled and lyophilized. To cyclize the peptide, the lyophilized

material was dissolved in degassed aqueous acetic acid (80%), and a solution of iodine (50 equivalents) in methanol was added dropwise. The reaction was monitored by analytical HPLC and it was stopped by the addition of ascorbic acid. The peptide was purified by preparative HPLC and its identity confirmed by electrospray mass spectrometry.

Biological activity

The effects of synthetic frog ET-1 and ET-3 on the secretion of corticosterone and aldosterone by perfused adrenal glands of the frog *R. ridibunda* were carried out as previously described (Delarue *et al.* 1990). In brief, the adrenal (interrenal) glands were carefully dissected, freed of kidney tissue, sliced with scissors and preincubated in 5 ml Ringer's solution (15 mM Hepes buffer, 112 mM NaCl, 2 mM CaCl_2 , 2 mM KCl, and 15 mM NaHCO_3) supplemented with 2 mg/ml glucose and 0.3 mg/ml BSA. The Ringer's solution was gassed with O_2/CO_2 (95%/5%), and the pH was adjusted to 7.4. The tissue slices were rinsed three times with fresh medium and layered between several beds of Bio-Gel P2 into perfusion chambers (equivalent of 12 glands per chamber). The adrenal tissue was continuously perfused with gassed Ringer's solution at a constant flow rate (200 $\mu\text{l}/\text{min}$) and temperature (24 °C). The experimental procedure commenced after a stabilization period of 2 h. ET-1 or ET-3 was dissolved in gassed Ringer's solution and infused into the columns at the same flow rate as Ringer's solution alone. Fractions of perfusate effluent were collected every 5 min and immediately frozen until assay. Concentration–response curves were constructed using a SigmaPlot program (Jeandel Scientific, San Rafael, CA, USA) and potencies ($-\log \text{EC}_{50}$ values) and efficacies (percent increase over mean basal rates of secretion) were calculated using the program. Basal rates were calculated as the mean of eight samples (40 min) collected at the beginning of the perfusion experiment. Data points show means ($\pm$ S.E.M.) for between three and eight independent experiments. Data were compared using ANOVA and the Tukey–Kramer multiple comparisons test. A *P* value of <0.05 was considered to be significant.

RESULTS

Purification of the peptides

The extract of frog stomach, after partial purification on Sep-Pak cartridges, was subjected to gel permeation chromatography on a Sephadex G-25 column. ET-LI was eluted as a broad peak with

FIGURE 1. Purification of ET-1 from an extract of frog stomach by reverse-phase HPLC on (A) semi-preparative Vydac C-18, (B) analytical Vydac C-4, (C) analytical Vydac phenyl and (D) analytical Vydac C-18 columns. The columns were eluted at a flow rate of 1.5 ml/min (2 ml/min in (A)) with linear gradients of increasing acetonitrile concentrations, denoted by the dashed lines. The fractions denoted by the bars contained ET-LI and the arrows show where peak collection began and ended.

K_{AV} between 0.55 and 0.75. These fractions were pooled and chromatographed on a semi-preparative Vydac C-18 column and the elution profile is shown in Fig. 1A. ET-LI was associated with the fraction denoted by the bar. Frog ET-1 was purified to near homogeneity, as assessed by peak symmetry, by successive chromatographies on an analytical Vydac C-4 column (Fig. 1B), an analytical Vydac phenyl column (Fig. 1C) and an analytical Vydac C-18 column (Fig. 1D). The final yield of pure peptide was approximately 30 pmol.

The ET-LI in the extract of frog liver, after partial purification on Sep-Pak cartridges, was also eluted from a Sephadex G-25 gel permeation column as a broad peak with approximately the same elution volume as the gastric ET. Frog ET-3

was purified to near homogeneity by reverse-phase HPLC under the same conditions of chromatography used to purify frog ET-1. The elution profiles are shown in Fig. 2A–D. The final yield of pure peptide was approximately 40 pmol.

Structural characterization

The primary structures of frog ET-1 and ET-3 were determined by Edman degradation using an automated microsequence analyzer. The amino acid sequence of frog ET-1 was established as: Xaa-Ser-Xaa-Ser-Ser-Leu-Met-Asp-Lys-Glu-Xaa-Val-Tyr-Phe-Xaa-His-Leu-Asp-Ile-Ile-Trp. No phenylthiohydantoin-coupled amino acid derivatives were detected during cycles 1, 3, 11 and 15. This is

FIGURE 2. Purification of ET-3 from an extract of frog liver by reverse-phase HPLC on (A) semi-preparative Vydac C-18, (B) analytical Vydac C-4, (C) analytical Vydac phenyl and (D) analytical Vydac C-18 columns. The conditions of chromatography were the same as those shown in Fig. 1. The fractions denoted by the bars contained ET-LI and the arrows show where peak collection began and ended.

consistent with the presence of cystine residues at these positions and so Xaa is assumed to be cysteine. The structure of frog ET-1, including the presence of two disulfide bridges, was confirmed by mass spectrometry using internal calibrants. The observed molecular mass of the peptide was 2492.0 ± 0.2 compared with a calculated average molecular mass of 2492.0 for the proposed structure.

The primary structure of frog ET-3 was established as: Xaa-Thr-Xaa-Tyr-Thr-Tyr-Lys-Asp-Lys-Glu-Xaa-Val-Tyr-Tyr-Xaa-His-Leu-Asp-Ile-Ile-Trp. Again, no phenylthiohydantoin-coupled amino acid derivative was detected during cycles 1, 3, 11 and 15. The proposed amino acid sequence, including the presence of two cystine bridges was confirmed by mass spectrometry without internal calibration (observed

average molecular mass 2658 ± 1 ; calculated average molecular mass of 2659).

Effect of ET-1 and ET-3 on corticosteroid secretion

It has been previously shown that repeated pulses of ET-1 cause attenuation of the response of frog adrenal tissue to the peptide (Delarue *et al.* 1990). To avoid tachyphylaxis, a single pulse of ET-1 or ET-3 was administered to perfused adrenal slices. Administration of graded concentrations of ET-1 (10^{-11} to 10^{-8} M), human ET-3 (10^{-10} to 5×10^{-7} M) and frog ET-3 (10^{-10} to 5×10^{-7} M) to perfused adrenal slices stimulated corticosterone and aldosterone secretion in a dose-dependent manner (Fig. 3). The potencies and efficacies (percent maximum response over basal levels) of the

FIGURE 3. Semi-logarithmic plots comparing the effects of increasing concentrations of frog/human ET-1 (10^{-11} to 10^{-8} M), frog ET-3 (10^{-10} to 5×10^{-7} M), human ET-3 (10^{-10} to 5×10^{-7} M) on corticosterone (A) and aldosterone secretion (B) by perfused frog adrenal explants. Results are expressed as a percentage of the basal secretory rate. Mean basal levels of corticosterone and aldosterone secretion in these experiments were 12.1 ± 1.0 and 5.5 ± 0.4 pg/min per adrenal gland (frog/human ET-1), 9.0 ± 0.5 and 3.7 ± 0.3 pg/min per adrenal gland (frog ET-3), and 11.3 ± 0.5 and 3.5 ± 0.4 pg/min per adrenal gland (human ET-3) respectively.

three peptides are compared in Table 1. The data show that the potency of frog ET-3 in stimulating corticosterone and aldosterone production was not

significantly different from human ET-3. Frog/human ET-1 was significantly more potent than frog ET-3 and human ET-3 in stimulating corticosterone ($P < 0.01$) and aldosterone ($P < 0.05$). The efficacies of the three peptides on corticosteroid output (approximately a 2-fold increase over basal rates of secretion) were not significantly different.

DISCUSSION

In a preliminary experiment using an antiserum that reacted strongly with the three isoforms of human ET in RIA, it was shown that ET-LI was present in all frog tissues examined (stomach, liver, kidney, interrenal gland, heart, brain and pituitary) with the exception of the skin. In view of the greater mass of the liver and stomach, and therefore greater amounts of ET in terms of pmol/organ, it was decided to utilize these two tissues as a source of ET for purification and characterization. Although it was possible to isolate sufficient material to permit structural characterization, the origin of the ET isolated from the frog stomach and liver is uncertain. While the purified material probably represents, at least in part, peptides that are synthesized in these tissues, it may also represent circulating ET that has been internalized by the organs through receptor-mediated endocytosis. Receptor-mediated uptake of circulating ET-3 by the rat liver has been described (Anggard *et al.* 1989) and the lysosomal compartment of bovine pulmonary endothelial cells has been shown to contain high concentrations of ET (Nakamura *et al.* 1990).

The amino acid sequence of each ET isoform has been strongly conserved among those mammalian species yet studied (human, pig, dog, rabbit, ox and rat, reviewed in Rubanyi & Polokoff (1994)) with the only species-related sequence difference being the substitution Ser⁴→Asn in mouse ET-2 (Saida *et al.* 1989). Our study has demonstrated that the primary structures of the peptides have been very strongly conserved throughout the evolution of tetrapods. As shown in Fig. 4, frog ET-1 is identical in structure to human ET-1, and frog ET-3 is the same as human ET-3 except for a single amino acid substitution (Phe⁴→Tyr). A plausible hypothesis explaining the evolution of the ET family of peptides is that duplication of the gene encoding an ancestral ET gave rise to a gene encoding ET-3 and a second gene encoding the ancestor of ET-1 and ET-2. A subsequent duplication of this latter gene gave rise to separate genes encoding ET-1 and ET-2 (Landan *et al.* 1991). As all mammalian species yet studied synthesize the three ET isoforms, both gene

TABLE 1. Effect of increasing concentrations of frog/human ET-1, frog ET-3 and human ET-3 on the rates of secretion of corticosterone and aldosterone by perfused frog adrenal glands. Potency refers to the $-\log EC_{50}$ values and efficacy is expressed as the percent increase over mean levels of spontaneous steroid output (100%). Values are given as mean $\pm$ S.E.M. for between three and eight independent experiments

	Corticosterone		Aldosterone	
	Potency	Efficacy	Potency	Efficacy
Peptide				
Frog/human ET-1	9.81 $\pm$ 0.01	198 $\pm$ 1	9.52 $\pm$ 0.29	222 $\pm$ 30
Frog ET-3	8.13 $\pm$ 0.16*	190 $\pm$ 9	8.15 $\pm$ 0.33 ⁺	203 $\pm$ 19
Human ET-3	8.29 $\pm$ 0.34*	173 $\pm$ 25	7.87 $\pm$ 0.18 ⁺	221 $\pm$ 17

* $P < 0.01$; ⁺ $P < 0.05$ compared with the effects of frog/human ET-1.

duplications are presumed to have occurred before the appearance of mammals. Our failure to isolate ET-2 from the frog tissues examined is consistent with, but of course does not prove, the view that the second gene duplication took place after the appearance of the Amphibia. Further work is clearly required, using specific probes for the frog ET isoforms in immunohistochemistry and/or *in situ* hybridization, to study the expression of ET genes in frog tissues.

The biosynthesis of the ET isoforms in mammals is unusual in that post-translational processing of preproendothelin at the site of dibasic amino acid residues by the well-characterized prohormone convertases produces a 'big' ET, of between 38 and

41 amino acids depending on the species, that has low biological potency (Kido *et al.* 1998). Big ET is further processed by a highly selective enzyme, ET-converting enzyme, that exists in several isoforms and cleaves at the Trp²¹-Val²² bond in big ET-1 and ET-2 and at the Trp²¹-Ile²² bond in big ET-3 (Shimada *et al.* 1995). The isolation of frog ET-1 and ET-3 in 21 amino acid residue forms suggests that preproendothelins are processed in amphibia by a pathway similar to that in mammals. Our data do not exclude the possibility that big ET isoforms are present in frog tissues, as the antiserum used in this study for detection does not recognize big ET.

The effects and mechanism of action of ET on steroidogenesis in the perfused interrenal gland of *R. ridibunda* have been studied in detail using synthetic human ET-1 and ET-3. The data show ET-1 produces a concentration-dependent increase in the production of corticosterone and aldosterone (Delarue *et al.* 1990) that involves the activation of the cyclooxygenase, the phospholipase C and the adenylate cyclase transduction pathways (Delarue *et al.* 1990, Cartier *et al.* 1999). It has also been shown that the steroidogenic action of human ET-1 requires an intact microfilament network in the interrenal gland, whereas microtubules and intermediate filaments are not involved (Remy-Jouet *et al.* 1994). In mammals, the biological actions of ET are mediated through interaction with two well-characterized receptors. The ET_A receptor is selective for ET-1 and ET-2 and binds ET-3 with approximately 100-fold less affinity, whereas the ET_B receptor exhibits similar affinities for all three isopeptides (Sokolovsky 1995). Previous studies have indicated that the receptor mediating the effects of ET on the frog interrenal gland resembles the mammalian ET_A subtype more closely than the

Frog ET-1:	CSCSS	LMDKE	CVYFC	HLDI	W
Frog ET-3	-T-YT	YK---	---Y-	-----	-
Human ET-1	-----	-----	-----	-----	-
Human ET-2:	-----	WL---	-----	-----	-
Mouse ET-2	---N-	WL---	-----	-----	-
Human ET-3:	-T-FT	YK---	---Y-	-----	-
Sarafotoxin-a:	---KD	MT---	-LN--	-N-V-	-
Sarafotoxin-b:	---KD	MT---	-LY--	-N-V-	-
Sarafotoxin-c:	-T-ND	MT-E-	-LN--	-N-V-	-
Sarafotoxin-d:	-T-KD	MT---	-LY--	-N---	-
Sarafotoxin-e:	-T-KD	MT---	-LY--	-NG--	-

FIGURE 4. A comparison of the amino acid sequences of the naturally occurring ET-related peptides. The five isoforms of sarafotoxin were isolated from the venom of a snake. - - denotes that the amino acid residue has been conserved.

ET_B subtype. In this tissue, the ET_A receptor antagonist, BQ-485, inhibited the steroidogenic effect of ET-1, whereas the selective ET_B receptor agonist, IRL 1620, was without effect (Cartier *et al.* 1997).

The present study has compared the effects of frog/human ET-1, frog ET-3 and human ET-3 on corticosterone and aldosterone production using perfused interrenal glands of frogs that were bred and housed under identical conditions. The observation that frog ET-1 is between 33 times (aldosterone) and 49 times (corticosterone) more potent than frog ET-3 supports the earlier conclusion that the steroidogenic action of ET is mediated through a receptor subtype that resembles the mammalian ET_A receptor more closely than the mammalian ET_B receptor. A third receptor subtype (ET_C) that binds ET-3 with higher affinity than either ET-1 or ET-2 has been identified in *X. laevis* dermal melanophores (Karne *et al.* 1993), but its physiological significance is unclear. The lower potency of frog ET-3 compared with ET-1 indicates that the ET_C receptor does not appear to be important in mediating steroidogenesis in *R. ridibunda*. The observation that frog ET-3 is equipotent with human ET-3 demonstrates that the substitution (Phe⁴→Tyr) is not important in determining binding of ligand to the amphibian ET_A receptor. This result was expected in the light of the observation that replacement of Ser⁴ by Ala in human ET-1 had only minimal effect on the ET_A-receptor mediated constriction of vascular rings from the rat pulmonary artery (Nakajima *et al.* 1989).

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