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Species-dependent differences in inotropic effects and phosphoinositide hydrolysis induced by endothelin-3 in mammalian ventricular myocardium

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1 Species-dependent variations in the positive inotropic effect (PIE) of endothelin-3 (ET-3), and the relationships between the PIE and specific binding sites for [^{125}I]-ET-3 and the PIE and the acceleration of phosphoinositide hydrolysis by ET-3, were studied in ventricular muscles from the rat, guinea-pig, rabbit, ferret and dog.

2 ET-3 in the presence of (±)-bupranolol (0.3 μM) and prazosin (0.3 μM) elicited a concentration-dependent PIE in the ventricular muscle from the rat, guinea-pig, rabbit and ferret. The potency of ET-3 and its efficacy in inducing a PIE were highest in the rabbit, intermediate in the rat and guinea-pig and lowest in the ferret. ET-3 did not have any inotropic effect on ventricular muscle from the dog.

3 Specific high-affinity binding of [^{125}I]-ET-3 was observed with membrane fractions derived from the ventricular muscle of the five species. The maximal specific binding (B_{max}) of ET-3 was highest in the rat and guinea-pig, intermediate in the rabbit and ferret and lowest in the dog. The values of K_D in the rabbit and dog (33 and 52 pM) were lower than those in the rat, guinea-pig and ferret (141–221 pM).

4 In slices of ventricular muscle from all five species, ET-3 increased the accumulation of [^{3}H]-inositol monophosphate (IP_1) in a concentration-dependent manner. The extent of accumulation of IP_1 was highest in the rat, intermediate in the guinea-pig and rabbit and lowest in the ferret and dog.

5 The results demonstrate the wide range of variations in the PIE of ET-3 on mammalian ventricular muscles. The variations in the coupling processes subsequent to the acceleration of the hydrolysis of PI, triggered by the binding of ET-3 to its receptor, might be important in these species-dependent differences in the PIE of ET-3.

Keywords: Endothelin-3; positive inotropic effect; species difference; binding of [^{125}I]-endothelin-3; hydrolysis of phosphoinositide; ventricular muscle

Introduction

Endothelin (ET), a polypeptide of 21 amino acids, was originally described as a potent endogenous vasoconstrictor (Yanagisawa *et al*., 1988b). Recently, genes for three distinct isoforms of ET, designated ET-1, ET-2 and ET-3, have been cloned (Inoue *et al*., 1989). ET-2 is very similar to ET-1, while ET-3 differs from ET-1 at 6 out of 21 positions (Yanagisawa *et al*., 1988a; Inoue *et al*., 1989). ET-1 has a pronounced positive inotropic effect (PIE) in the hearts of several mammalian species, such as the rat (Baydoun *et al*., 1989; Moravec *et al*., 1989), guinea-pig (Ishikawa *et al*., 1988; Takanashi & Endoh, 1991), ferret (Shah *et al*., 1989), rabbit (Endoh & Takanashi, 1991; Takanashi & Endoh, 1991) and man (Moravec *et al*., 1989). The PIE of endothelin is mediated by specific receptors for endothelin that are densely distributed on the membranes of myocardial cells. However, the PIE of ET-1 varies widely among mammalian species, a phenomenon that cannot be ascribed soley to differences in the distribution of endothelin receptors between species (Takanashi & Endoh, 1991). Compared to the PIE of ET-1, the action of ET-3 on myocardial contractility has been studied in less detail. The limited data published to date indicate that ET-3 elicits a PIE with an efficacy similar to that of ET-1 in rabbit ventricular muscle (Takanashi & Endoh, 1992). While ET-3 has no cardiotonic effect on the ventricular wall of the adult rat, the density of specific binding sites for [^{125}I]-ET-3 in the rat ventricle (Ishikawa *et al*., 1991) is much higher than that in the rabbit ventricle (Kasai *et al*., 1994). These data suggest that diverse coupling processes, subsequent to the activation of the receptor for endothelin, might be responsible for the wide range of interspecies variation.

Activation of endothelin receptors in cardiac muscle is coupled to several signal-transduction pathways, such as stimulation of phospholipase C with resultant acceleration of the hydrolysis of phosphoinositide (Galron *et al*., 1990), an increase in the sensitivity of the myofilaments to Ca^{2+} ions (Kelly *et al*., 1990), mobilization of intracellular Ca^{2+} ions and stimulation of transmembrane Ca^{2+} currents (Kelly *et al*., 1990), and alkalinization of the intracellular milieu (Krämer *et al*., 1991). In the rabbit ventricular muscle, ET-3 elicits a PIE with an efficacy similar to that of ET-1 and a close relationship exists between the hydrolysis of phosphoinositide and the PIE induced by ET-1 (Takanashi & Endoh, 1992) and by ET-3 (Endoh *et al*., 1996). However, the ET-1-induced and ET-3-induced PIE and the hydrolysis of phosphoinositide might be mediated by different subtypes of endothelin receptor in the rabbit (Kasai *et al*., 1994; Endoh *et al*., 1996). The relationship between the ET-3-induced hydrolysis of phosphoinositide and contractility in other mammalian species remains unclear. In the present study, species-dependent differences in the ET-3-induced PIE, in relation to the specific binding of [^{125}I]-ET-3 and the acceleration of the hydrolysis of phosphoinositide induced by ET-3, were examined in the ventricular muscle of the rat, guinea-pig, rabbit, ferret and dog. A part of this work has recently been presented as an abstract (Yang *et al*., 1996).

Methods

Measurement of inotropic effects

Male Wistar rats (300–360 g), Hartley guinea-pigs (380–450 g), albino rabbits (1.8–2.2 kg), ferrets (0.8–1.2 kg) and mongrel dogs (7–9 kg) of either sex were used for the experiments. The animals were anaesthetized with pentobarbi-

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tone sodium (60 mg kg^{-1}, i.p., for rats and guinea-pigs; 50 mg kg^{-1}, i.v., for rabbits) or with ether (ferrets), and then the hearts were removed and the papillary muscles were excised immediately from the right ventricle in oxygenated Krebs-Henseleit solution. The dogs were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.) and right ventricular trabeculae were excised. Muscles were mounted immediately in 20 ml organ baths that contained Krebs-Henseleit solution (with 0.057 mM ascorbic acid and 0.027 mM disodium EDTA to prevent autoxidation of the compounds examined). The solution was bubbled with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). The concentrations (mM) of the various components of the solution were as follows: Na^+ 142.9, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, SO_4^{2-} 1.2, Cl^- 127.8 and glucose 11.1. For rat papillary muscles, the concentration of Ca^{2+} ions was reduced to 1.25 mM. The muscles were stimulated electrically by square-wave pulses of 5 ms duration at a voltage that was about 20% above the threshold and at a frequency of 1 Hz in rats and guinea-pigs and at 0.5 Hz in rabbits, ferrets and dogs. The force of isometric contraction was measured by force-displacement transducers (Shinkoh UL 10 GR; Minebea, Tokyo, Japan). During the 60 min equilibration, the muscles were initially stretched by a tension of 5 mN and the length was then adjusted to give 90% of the maximal contractile force. β- and α-adrenoceptor blockade was achieved by the addition of 0.3 μM ($\pm$)-bupranolol (Kaumann *et al*., 1980) and 0.3 μM prazosin (Hiramoto *et al*., 1988), respectively. ($\pm$)-Bupranolol and prazosin were allowed to act for 30 min before the administration of ET-3 and both were present in the solution in the organ bath throughout the experiments. At the end of each experiment, the maximum contractile force was determined in each muscle by the cumulative administration of isoprenaline (Iso) after washing out of other drugs for at least 2 h and until the basal force of contraction had returned to the control level. The inotropic responses to the agonists were expressed as percentages of the maximal response to Iso.

Radioligand binding assay

The binding assay with [^{125}I]-ET-3 was carried out as described in detail elsewhere (Takanashi & Endoh, 1991). In brief, pieces of right and left ventricular muscle, including free walls and septum, were excised from the hearts of the rats, guinea-pigs, rabbits, ferrets and dogs and homogenized in ten volumes of ice-cold buffer (0.25 M sucrose containing 5 mM Tris-HCl and 1 mM $MgCl_2$, pH 7.4) in a Polytron (PT-10; Kinematica, Luzern, Switzerland) three times for 15 s each at setting 7. Each homogenate was then centrifuged at $500 \times g$ for 15 min at 4°C. The supernatant was filtered through a single layer of cheesecloth and centrifuged at $50{,}000 \times g$ for 20 min at 4°C. The resulting pellet was washed twice with ice-cold incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) by repeated resuspension and recentrifugation. The final pellet was resuspended in ice-cold incubation buffer that contained 1 mg ml^{-1} bovine serum albumin.

The assay of specific binding was performed in an incubation mixture that contained 150 μl of a suspension of membranes (approximately 50–100 μg of protein), 50 μl of a solution of [^{125}I]-ET-3 at various concentrations (specific activity, 2,000 Ci $mmol^{-1}$) and 50 μl of incubation buffer that contained 1 mg ml^{-1} bovine serum albumin without unlabelled ET-3 (for assays of total binding) or with unlabelled ET-3 (for assays of nonspecific binding). The reaction was started by the addition of the membrane fraction, and the reaction mixture was incubated for 90 min at 25°C. The reaction was terminated by addition of 2 ml of ice-cold incubation buffer. Then the mixture was rapidly filtered through a GF/C glass filter (Whatman International Ltd., Maidstone, U.K.) in a cell harvester (M-24R; Brandel, Gaithersburg, MD, U.S.A.). Each filter was washed rapidly with 12 ml (3×4 ml) of ice-cold incubation buffer. After the filter had been dried for 1 h at 90°C, radioactivity bound to the filter was quantitated. Nonspecific binding of [^{125}I]-ET-3 was defined as the binding detected in the presence of 1 μM unlabelled ET-3. Specific binding of [^{125}I]-ET-3 was defined as the total radioactivity minus the radioactivity due to nonspecific binding. Each binding assay was carried out in duplicate. Protein was quantitated by the method of Lowry *et al*. (1951) with bovine serum albumin.

Quantitation of [^{3}H]-inositol phosphates

Hearts were quickly removed from rats, guinea-pigs, rabbits, ferrets and dogs under anaesthesia, as described above, and placed in Krebs-Henseleit solution, bubbled with 95% O_2 and 5% CO_2, at 37°C to wash out the blood. The experimental procedure was the same as that described previously (Yang & Endoh, 1994). Slices of ventricular muscle (0.5 mm thick) were prepared with a tissue slicer (Arthur H. Thomas Company, Philadelphia, PA, U.S.A) in cold (4°C) Krebs-Henseleit solution. After being weighed, the slices were equilibrated in Krebs-Henseleit solution for 30 min at 37°C and then the slices were preincubated with 10 μCi ml^{-1} *myo*-[^{3}H]-inositol in Krebs-Henseleit solution for 120 min. After the preincubation, slices were washed with fresh Krebs-Henseleit solution that contained 5 mM *myo*-inositol and 10 mM LiCl. All succeeding procedures were performed in Li^+-containing solutions. The protocol employed for examining the effect of ET-3 on the accumulation of [^{3}H]-inositol monophosphate (IP_1) was similar to that described in the functional study. Thirty min after the administration of ET-3, slices were quickly blotted and put into 1 ml of a mixture of chloroform, methanol and 12 N HCl (100 : 200 : 1, v/v) to terminate the reaction. The tissue was homogenized and then the homogenate was centrifuged at $1{,}400 \times g$ for 20 min to separate the aqueous and organic phases. An aliquot of the aqueous layer was applied to a column that contained a 50% slurry of AG1-X8 (anion-exchange resin; 100–200 mesh; formate form; Bio-Rad, Richmond, CA, U.S.A.). The column was washed first with 20 ml of distilled water and then glycerophosphoryl esters were eluted with 8 ml of a solution contained 5 mM sodium tetraborate and 60 mM sodium formate (Berridge *et al*., 1983). [^{3}H]-IP_1 was collected and the radioactivity was quantitated in a scintillation mixture (ACS-II; Amersham, Arlington Heights, IL, U.S.A.) with a scintillation counter (TRI-CARB 1500; Packard, Downers Grove, IL, U.S.A.) at a counting efficiency of 66%. The accumulation of IP_1 was used as an indicator of the hydrolysis of phosphoinositide because IP_1 provides a good measure of the extent of stimulation of the hydrolysis of phosphoinositide that is induced by activation of receptor (Endoh *et al*., 1996).

Chemicals

The drugs and reagents used were (−)-isoprenaline hydrochloride, *myo*-inositol, lithium chloride and bovine serum albumin (Sigma, St. Louis, MO, U.S.A.); ET-3 (Peptide Institute, Osaka, Japan); prazosin hydrochloride (Pfizer Taito, Tokyo, Japan); ammonium formate (Wako Pure Chemicals Co., Osaka, Japan); ($\pm$)-bupranolol hydrochloride (Kaken Pharmaceutical Co., Tokyo, Japan); pentobarbitone sodium (Abbott Laboratories, North Chicago, IL, U.S.A.); [^{125}I]-ET-3 (specific activity, 2,000 Ci $mmol^{-1}$) and *myo*-[2-^{3}H]-inositol (specific activity, 86 Ci $mmol^{-1}$; Amersham, Buckinghamshire, U.K.). The stock solution of isoprenaline was prepared in a 0.1% (w/v) solution of ascorbic acid, kept ice-cold, and diluted with a 0.9% (w/v) solution of NaCl just before use.

Statistical analyses

Data are presented as means $\pm$ s.e.mean for the given number of experiments. Statistical analysis of data was performed by one-way or repeated analysis of variance with the analytical software STATVIEW J-4.5 (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). A P value smaller than 0.05 was considered to be

significant. In saturation binding assays, the dissociation constant (K_D) and the maximal binding capacity (B_{max}) for binding of [^{125}I]-ET-3 to endothelin receptors were determined from Scatchard plots.

Results

Inotropic effects of ET-3 on ventricular muscles from different mammalian species

The effects, in the ventricular muscles isolated from the rat, guinea-pig, rabbit, ferret and dog, of ET-3 on contractile force in the presence of 0.3 μM ($\pm$)-bupranolol and prazosin are shown in Figure 1. The pD_2 values (values of $-\log_{10}$ 50% effective concentration (EC_{50})) and the maximal responses to ET-3 for the various preparations are presented in Table 1. The action of ET-3 varied widely between the mammalian species. A concentration-dependent PIE of ET-3 was observed in right ventricular papillary muscles from the rat, guinea-pig, rabbit and ferret, with the potency being: rabbit >> guinea-pig > rat >> ferret. In fact, EC_{50} could not be determined in the ferret because of the low potency and limited availability of ET-3. The maximum response to ET-3 in rabbits was 72.9$\pm$7.3% of that to Iso, and it was more than 3 fold higher than the value in the rat and the guinea-pig. By contrast, ET-3 has no inotropic effect in the dog ventricular trabeculae at concentrations of up to 300 nM.

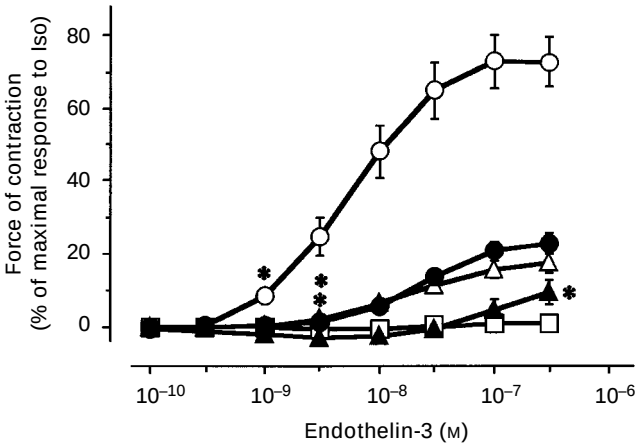

Figure 1 Concentration-response curves for the effects of endothelin-3 on the force of contraction in the presence of 0.3 μM ($\pm$)-bupranolol and 0.3 μM prazosin in rabbit (○, $n=7$), rat (●, $n=5$), guinea-pig (△, $n=6$) and ferret (▲, $n=6$) isolated papillary muscles and in dog ventricular trabeculae (□, $n=5$). Iso, isoprenaline. Asterisks indicate the threshold concentration for the positive inotropic effect ($P<0.05$). Vertical lines show s.e.mean.

Characteristics of the specific binding of [^{125}I]-ET-3 to membrane fractions derived from ventricular muscles of different mammalian species

Figure 2 shows the results of representative analyses of the saturation binding of [^{125}I]-ET-3 to ventricular membrane fractions from the rat (Figure 2a), guinea-pig (Figure 2b), rabbit (Figure 2c), ferret (Figure 2d) and dog (Figure 2e). In all species, Scatchard analysis indicated that the specific binding of [^{125}I]-ET-3 was saturable and there was apparently only a single class of binding sites. The values of B_{max} and K_D for the specific binding of [^{125}I]-ET-3 to the membrane preparations derived from these species are summarized in Table 2. The B_{max} values for [^{125}I]-ET-3 were the highest in the guinea-pig and rat, intermediate in the ferret and rabbit and lowest in the dog. By contrast, the affinity of [^{125}I]-ET-3 for specific binding sites was higher in the rabbit and the dog than in the rat, guinea-pig and ferret ($P<0.01$).

Effects of ET-3 on the accumulation of [^{3}H]-IP$_1$ in ventricular muscles from different mammalian species

Concentration-response curves for the accumulation of [^{3}H]-IP_1 in response to ET-3, determined 30 min after the addition of ET-3, in slices of ventricular muscles of the various species are shown in Figure 3. ET-3 at 1 nM and higher in the rat, guinea-pig and rabbit, and at 10 nM and higher in the ferret and dog, caused a concentration-dependent increase in the accumulation of [^{3}H]-IP_1. The extent of the ET-3-induced accumulation of [^{3}H]-IP_1 was the highest in the rat (299.1$\pm$21.4% of the basal level, $n=4$), intermediate in the guinea-pig and rabbit (227.6$\pm$21.8%, $n=4$, and 204.2$\pm$9.8% of the basal level, $n=6$, respectively) and lowest in the ferret and dog (148$\pm$5.1% and 160.8$\pm$10.6% of the basal level, respectively, $n=5$ each). The pD_2 value for ET-3 for stimulation of the accumulation of IP_1 was 8.34$\pm$0.09 in the rabbit, a value that is consistent with the value of pD_2 for induction of the PIE by ET-3 in the rabbit (Table 1). The pD_2 values could not be calculated in the other species because it appeared that the maximal responses were probably not achieved at the highest concentration of ET-3 tested (Figure 3).

Figure 4 shows the relationships between the PIE of ET-3 and the accumulation of [^{3}H]-IP_1 that was induced by ET-3 in the ventricular muscles of the rat, guinea-pig and rabbit. There was clearly a close correlation between the ET-3-induced PIE and the accumulation of [^{3}H]-IP_1 in these three species ($r=0.96$ in the rat and the rabbit and $r=0.95$ in the guinea-pig). However, the relationships obtained from experiments with the ventricular muscles from the ferret and the dog were quite different: in the ferret the PIE was very small as compared to the accumulation of [^{3}H]-IP_1 in response to various concentrations of ET-3; in the dog the accumulation of [^{3}H]-IP_1 was not associated with a PIE (Figure 5).

Discussion

The inotropic effect of ET-3 varied widely in ventricular myocardium isolated from various mammalian species: ET-3

Table 1 Effects of endothelin-3 on the contractile force in mammalian ventricular myocardium

			Endothelin-3		*Isoprenaline*
Species	n	*Basal force* (mN mm^{-2})	*Maximal response* (mN mm^{-2})	*pD_2*	*Maximal response* (mN mm^{-2})
Rat	5	3.10$\pm$0.68	9.46$\pm$1.89	7.64$\pm$0.06	25.8$\pm$4.3
Guinea-pig	6	2.56$\pm$0.65	5.92$\pm$1.20	7.86$\pm$0.06*	20.2$\pm$4.0
Rabbit	7	4.54$\pm$0.92	33.2$\pm$6.3	8.22$\pm$0.07***	37.5$\pm$4.6
Ferret	6	32.2$\pm$5.1	36.3$\pm$5.8	–	54.3$\pm$8.8
Dog	5	3.40$\pm$0.83	–	–	28.2$\pm$6.1

Values are means$\pm$s.e.mean; n, number of experiments. *$P<0.05$, ***$P<0.001$ vs the corresponding value for the rat.

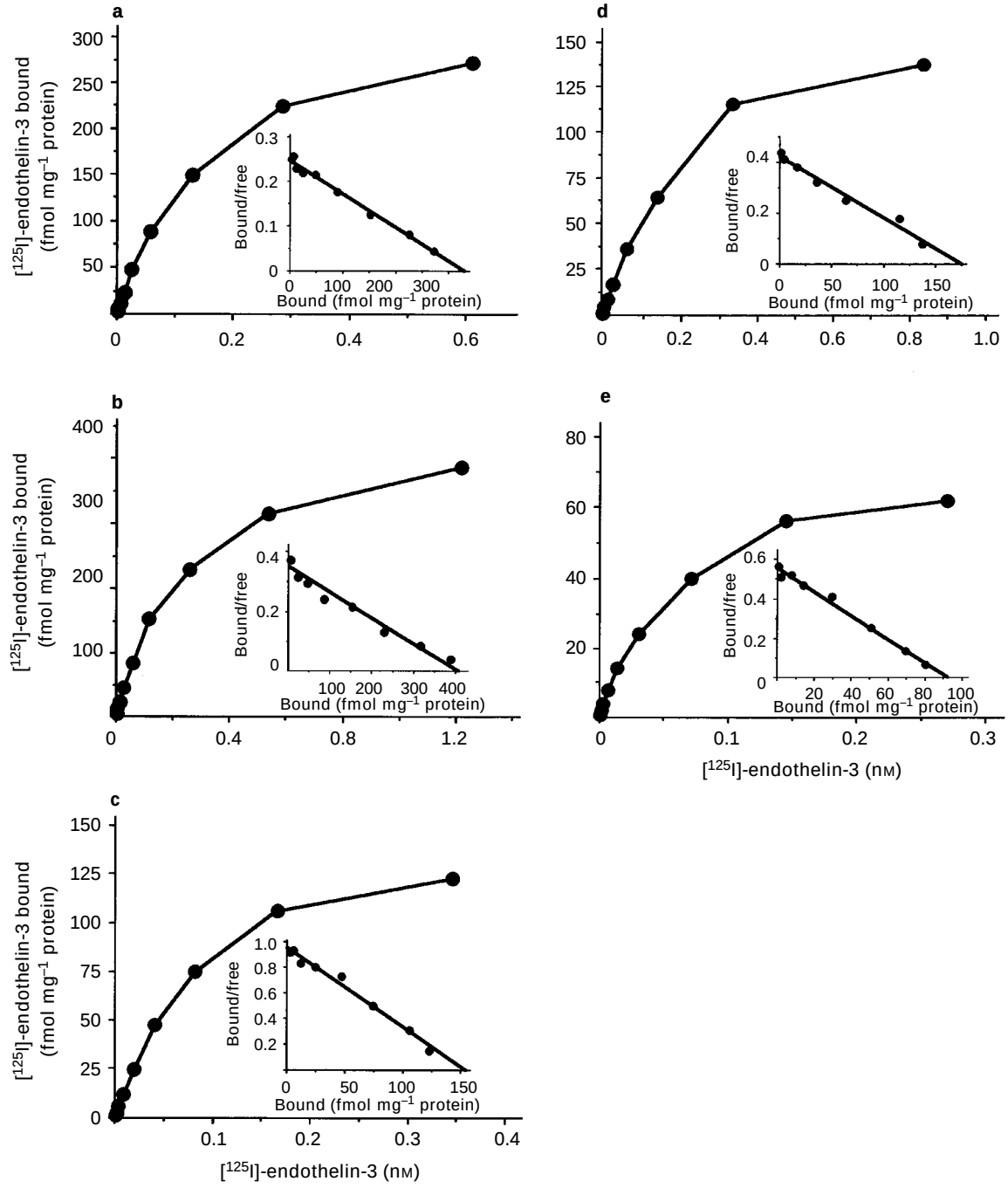

Figure 2 Saturation curves for binding of [^{125}I]-endothelin-3 to membrane fractions derived from the right ventricular myocardium of the rat (a), guinea-pig (b), rabbit (c), ferret (d) and dog (e). Inset: Scatchard analysis of the data. The slope of the regression line was determined by the least-squares methods. Values presented are means of duplicate determinations in representative experiments.

had a pronounced PIE in rabbit papillary muscle; it had a weak but significant effect on papillary muscles from the rat, guinea-pig and ferret; and it had no inotropic effect on ventricular trabecula from the dog. Numerous steps in signal transduction processes, triggered by the binding of ET-3 to its receptor and involving the density and affinity of the receptors, the subtypes of receptor and the coupling to effectors, which together lead to the ultimate inotropic response, could be responsible for these variations.

Characteristics of receptors and their subtypes

In the membrane fractions derived from the ventricular muscles of all species examined in the present study, endothelin receptors were found at high densities and with high affinity when they were analysed in terms of the specific binding of [^{125}I]-ET-3. The density of binding sites for ET-3 was even higher than that of adrenoceptors, determined by the same experimental procedure (Endoh *et al.*, 1991), indicating the potentially significant role of the receptors for endothelin in the regulation of physiological and/or pathophysiological events in the mammalian ventricular myocardium. The values of B_{max} were highest in the guinea-pig and rat, intermediate in the ferret and rabbit, and lowest in the dog (Table 2), while ET-3-induced PIEs in the rat and the guinea-pig were much weaker than that in the rabbit. Moreover, in the dog ventricular myocardium, ET-3 did not elicit a PIE even though there were abundant specific binding sites for [^{125}I]-ET-3. Therefore, as a whole, the density of receptors did not reflect the magnitude of the PIE induced by ET-3 in these species, an

Table 2 Values of B_{max} and K_D for the specific bindings of [^{125}I]-endothelin-3 to membrane fractions derived from the ventricular myocardium of the rat, guinea-pig, rabbit, ferret and dog

Species	n	B_{max} (fmol mg^{-1} protein)	K_D (nM)
Rat	5	307.4±29.1	0.141±0.017
Guinea-pig	4	359.2±21.1	0.221±0.047
Rabbit	5	171.5±22.9	0.033±0.006
Ferret	4	186.7±14.3	0.186±0.029
Dog	4	72.3±12.0	0.052±0.009

Values are means±s.e.mean; *n*, number of experiments; B_{max}, maximal binding capacity; K_D, equilibrium dissociation constant.

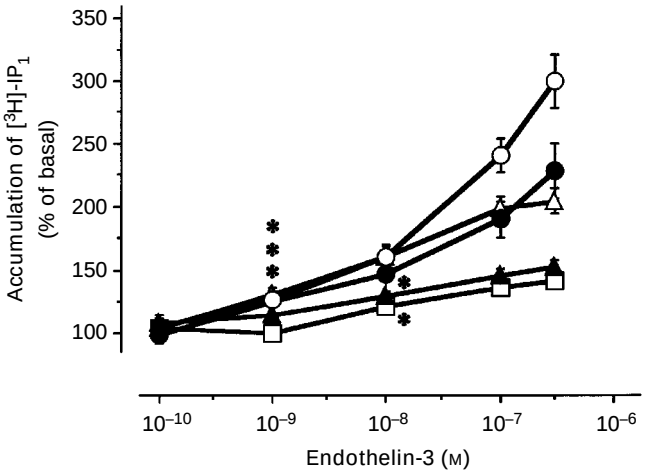

Figure 3 Endothelin-3-induced accumulation of [^{3}H]-inositol monophosphate ([^{3}H]-IP$_1$) in ventricular slices from the rat (○), guinea-pig (●), rabbit (△), dog (▲) and ferret (□). Data are given as percentages of the corresponding basal levels and were determined 30 min after the addition of endothelin-3 in the presence of 10 mM LiCl, 0.3 μM (±)-bupranolol and 0.3 μM prazosin. Each value indicates the mean of the results from four to six experiments: vertical lines indicate s.e.mean. Asterisks indicate the threshold concentrations for accumulation of [^{3}H]-IP$_1$ ($P<0.05$).

indication that differences in receptor density cannot account for the species-dependent variations in the ET-3-induced inotropic response.

Scatchard analysis of the results of saturation-binding studies indicates that [^{125}I]-ET-3 bound to an apparently single class of receptors in a monophasic manner, the values of K_D for the specific binding of [^{125}I]-ET-3 being relatively high in the rabbit and dog and low in the rat, ferret and guinea-pig (Table 2). These findings imply that at least two different subtypes of the receptor are involved in the specific binding of [^{125}I]-ET-3 in mammalian ventricular muscle.

Receptors for endothelins are classified into three subtypes in terms of the rank order of the efficacy and potency of endothelin isopeptides: the ET_A subtype is predominantly activated by ET-1 and the ET_B subtype is activated nonselectively by ET-1 and ET-3; the ET_C subtype responds preferentially to ET-3 (Masaki *et al*., 1994). The ET_A and ET_B subtypes coexist in cardiac tissue and the distribution of subtypes differs between species (Arai *et al*., 1990; Molenaar *et al*., 1993). The wide range of variations in the ET-3-induced PIE might provide a functional basis for the differences in the distribution of endothelin subtypes between species. However, the receptor subtype that mediates the PIE of ET-3 has not previously been compared under the same experimental conditions in different species. In the rabbit, ET-3 elicited a pronounced PIE that was similar to that of ET-1 (Takanashi & Endoh, 1991) and that

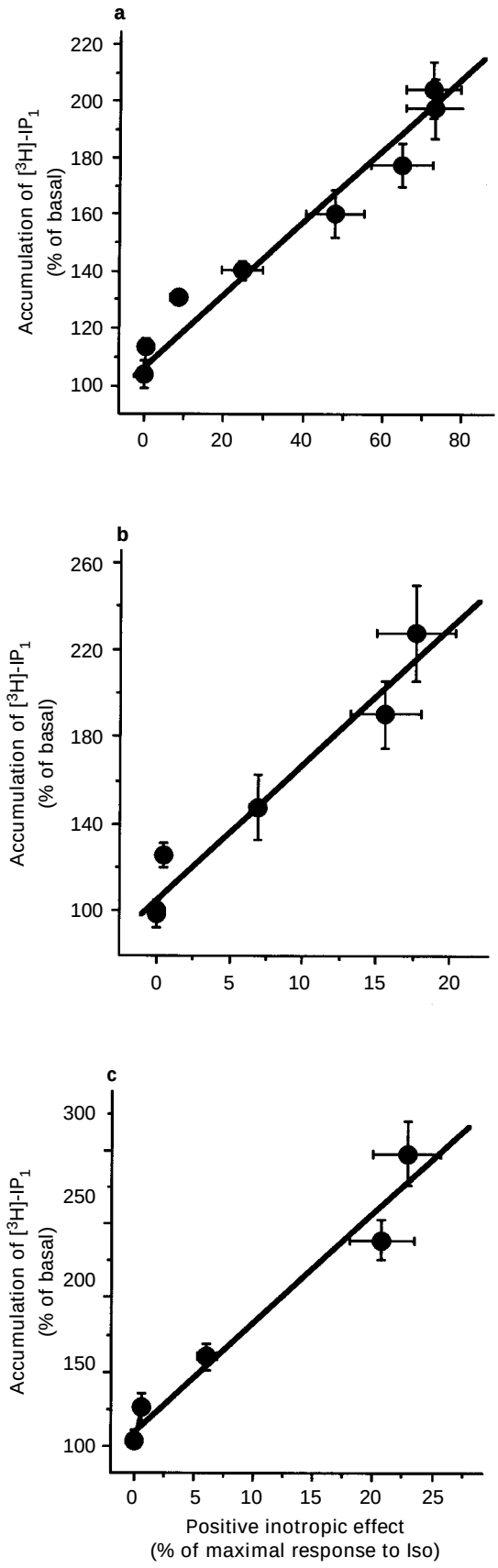

Figure 4 Correlation between the positive inotropic effect of endothelin-3 and the accumulation of [^{3}H]-IP$_1$ induced by endothelin-3 in the ventricular muscle of the rabbit (a), guinea-pig (b) and rat (c). Data obtained from Figure 1 and Figure 3 were used for analysis. Vertical and horizontal bars indicate s.e.mean of the results of four to seven experiments. Iso, isoprenaline.

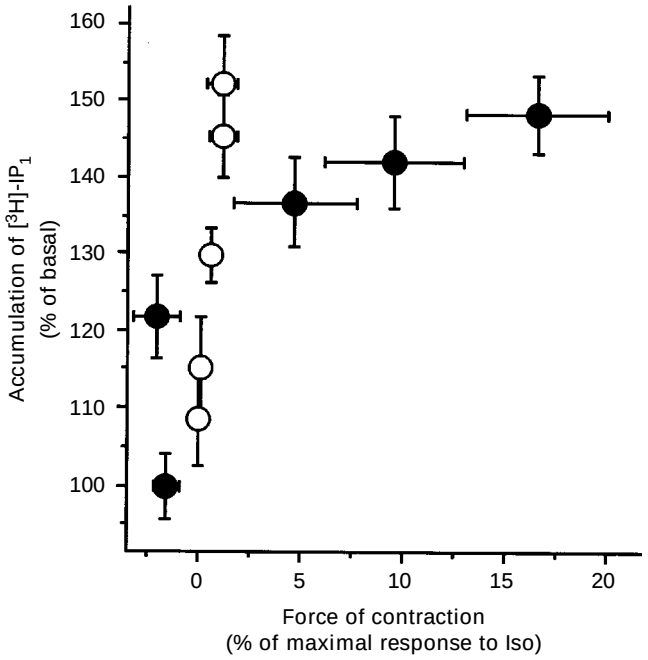

Figure 5 Relationships between the positive inotropic effect of endothelin-3 and the accumulation of [^{3}H]-IP$_1$ induced by endothelin-3 in ventricular muscle of the dog (○) and the ferret (●). Data obtained from Figure 1 and Figure 3 were used for the analysis. Vertical and horizontal bars indicate s.e.mean of the results from five to six experiments. Iso, isoprenaline.

was very different from the results with other mammalian species. Although it appears that, in the rabbit, the ET_B subtype plays a crucial role in the ET-3-mediated PIE, detailed pharmacological analysis, with selective antagonists of endothelin receptors, showed that the PIE of ET-3 in the rabbit ventricular myocardium is mediated by a receptor subtype that is sensitive to the selective antagonists of ET_A receptor, BQ 123 and FR 139317 (Kasai *et al*., 1994; Endoh *et al*., 1996). These findings indicate that the ET_{A1} receptor that is sensitive to BQ 123 (Sudjarwo *et al*., 1994) might mediate the PIE of ET-3 in the rabbit ventricle. It is noteworthy that, in the rabbit, the PIE of ET-1 from 1 nM to 100 nM was not inhibited by these selective antagonists of ET_A receptors, an indication that the ET_{A2} subtype of receptor that is resistant to BQ 123 (Sudjarwo *et al*., 1994) might predominantly mediate the PIE of ET-1.

By contrast to the findings in the rabbit ventricular myocardium, in other species our comparisons of the effectiveness of endothelin isopeptides and pharmacological analysis with selective antagonists of endothelin receptors indicate that the conventional ET_A subtype is responsible for the PIE of endothelin isopeptides. Therefore, the pronounced PIE of ET-3 in the rabbit could be due to the existence of an atypical subtype of ET_A receptor that responds to ET-3 in this species. It has been shown that the receptors for endothelin that are involved in facilitation of cardiac function are of the ET_A subtype in tissues of other species, including the ventricle of the adult rat (Hilal-Dandan *et al*., 1994) and the papillary muscle of the ferret (Shah *et al*., 1989; Wang *et al*., 1991; Evans *et al*., 1994). These findings imply that the difference in distribution of endothelin subtypes in these species might partially account for the species-dependent variations in the ET-3-induced PIE. However, the difference in receptor subtypes, which can be differentiated by radioligand binding assays, is unlikely to play a crucial role in the variations in the intropic response because ET-3 elicited the most pronounced PIE in the rabbit but no PIE in the dog, even though the affinity of binding sites for ET-3 was identical in these two species.

The hydrolysis of phosphoinositide and the PIE of ET-3

Isopeptides of endothelins stimulate the hydrolysis of phosphoinositide in cardiac tissues, such as neonatal ventricular myocytes (Galron *et al*., 1990) and cardiac muscles of the rat (Krämer *et al*., 1991), rabbit (Takanashi & Endoh, 1992; Endoh *et al*., 1996), ferret (Wang *et al*., 1993) and man (Zerkowski *et al*., 1993). It is currently unclear whether the products that result from the stimulation of the hydrolysis of phosphoinositide are responsible for the induction of the PIE of endothelin isopeptides. ET-3 accelerated the hydrolysis of phosphoinositide in all species examined. In the rabbit, we found a close relationship between the hydrolysis of phosphoinositide and the PIE induced by ET-3 (Figure 4), and the relationship was essentially similar to that observed with ET-1 (Takanashi & Endoh, 1992). In ventricular muscles of the rat and guinea-pig, a close relationship between these two parameters was also observed (Figure 4). These observations suggest that the stimulation of hydrolysis of phosphoinositide and the PIE might be mediated by identical receptors in each species.

In the dog, stimulation of the hydrolysis of phosphoinositide was not associated with the PIE. Furthermore, the extent of the accumulation of [^{3}H]-IP$_1$ was much higher in the rat than in the rabbit, but the maximal inotropic response to ET-3 in the rat was less than one-third of the response in the rabbit. In the ferret, ET-3 stimulated the hydrolysis of phosphoinositide and elicited the PIE but the PIE of ET-3 was less pronounced (Figure 5) than might be expected from the increase in accumulation of [^{3}H]-IP$_1$, when the increase is compared with that in other species (Figure 4). Therefore, even if the hydrolysis of phosphoinositide were to play a critical role in the PIE of ET-3, a difference in the signal transduction process subsequent to the acceleration of the hydrolysis of phosphoinositide in different species must be responsible, in part, for the species-dependent variations in the PIE of ET-3 in mammalian ventricular muscles. It is noteworthy that dissociation of the inotropic response from the density of receptors and/or the hydrolysis of phosphoinositide has also been observed with other isopeptides of endothelin (Takanashi & Endoh, 1991), with angiotensin II (Ishihata & Endoh, 1995) and with agonists of α-adrenoceptors (Endoh *et al*., 1991) in the dog ventricle, while stimulation of these receptors consistently induced a PIE, in association with acceleration of the hydrolysis of phosphoinositide, in the rabbit ventricular muscle (Takanashi & Endoh, 1992; Ishihata & Endoh, 1993; Yang & Endoh, 1994).

One potential candidate for an intracellular mediator of the PIE of ET-3 is diacylglycerol, a product of the hydrolysis of phosphoinositide and an activator of protein kinase C. Activated protein kinase C phosphorylates troponin I and troponin T, but the phosphorylation appears to be related to a decrease in Ca^{2+}-stimulated MgATPase activity and, thus, to the negative inotropic effect of tumour-promoting phorbol esters and/or to the inhibition of a receptor-mediated PIE (Noland & Kuo, 1993; Strang & Moss, 1995). There are multiple isoenzymes of protein kinase C, each with different catalytic properties, in cardiac muscle (Steinberg *et al*., 1995), and several types of receptor, including receptors for endothelins (Krämer *et al*., 1991) and angiotensin II (Dösemeci *et al*., 1988) as well as α_1-adrenoceptors (Brown *et al*., 1985), which are coupled to the activation of protein kinase C in cardiac muscle. Therefore, the coupling subsequent to the activation of protein kinase C might be more complex than we currently suspect. In other words, activation of different isoenzymes of protein kinase C might occur in different species, which might lead to a positive and/or negative inotropic response to endothelin isopeptides in the various species.

It has been found that, in guinea-pig ventricular myocytes, stimulation of ET_A receptors inhibits L-type Ca^{2+} currents that have previously been enhanced by isoprenaline through the action of a pertussis toxin-sensitive G-protein (Xie *et al*., 1996). In rat cardiac myocytes, activation of ET_A receptors suppresses the production of adenosine 3′:5′-cyclic monopho-

sphate (cyclic AMP) by inhibition of adenylate cyclase via the action of G_i protein (Hilal-Dandan *et al*., 1994). In the dog ventricular myocardium, ET_A receptors are also involved in the inhibition of the PIE induced by isoprenaline (Zhu *et al*., 1996). Coupling to such an inhibitory process might also contribute to the variations in the PIE of ET-3 and such a possibility awaits further study.

In summary, ET-3 had a pronounced PIE in the rabbit, a weak PIE in the rat and guinea-pig, a much weaker PIE in the ferret and no PIE in the dog. In all species, we found specific binding sites with high affinity for ET-3 and ET-3 stimulated the hydrolysis of phosphoinositide. The difference in the density and in the affinity of specific binding sites for ET-3 between the species cannot, therefore, account for the species-dependent differences in the inotropic effects of ET-3. The differences in the extent of stimulation of the hydrolysis of phosphoinositide might be responsible, in part, for the variations in the ET-3-induced PIE between species, while differences between coupling processes subsequent to stimulation of the hydrolysis of phosphoinositide might be critical to the species-dependent variations in the ET-3-induced PIE.

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