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Activation of calcineurin in human failing heart ventricle by endothelin-1, angiotensin II and urotensin II

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1 The calcineurin (CaN) enzyme–transcriptional pathway is critically involved in hypertrophy of heart muscle in some animal models. Currently there is no information concerning the regulation of CaN activation by endogenous agonists in human heart.

2 Human right ventricular trabeculae from explanted human (14 male/2 female) failing hearts were set up in a tissue bath and electrically paced at 1 Hz and incubated with or without 100 nM endothelin-1 (ET-1), 10 μM, angiotensin-II (Ang II) or 20 nM human urotensin-II (hUII) for 30 min. Tissues from four patients were incubated with 200 nM tacrolimus (FK506) for 30 min and then incubated in the presence or absence of ET-1 for a further 30 min.

3 ET-1 increased contractile force in all 13 patients ($P<0.001$). Ang II and hUII increased contractile force in three out of eight and four out of 10 patients but overall nonsignificantly ($P>0.1$). FK506 had no effect on contractile force ($P=0.12$).

4 ET-1, Ang II and hUII increased calcineurin activity by 32, 71 and 15%, respectively, while FK506 reduced activity by 34%. ET-1 in the presence of FK506 did not restore calcineurin activity ($P=0.1$).

5 There was no relationship between basal CaN activity and expression levels in the right ventricle. Increased levels of free phosphate were detected in ventricular homogenates that were incubated with PKCε compared to samples incubated without PKCε.

6 Endogenous cardiostimulants which activate Gαq-coupled receptors increase the activity of calcineurin in human heart following acute (30 min) exposure. PKC may contribute to this effect by increasing levels of phosphorylated calcineurin substrate.
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Abbreviations: Ang II, angiotensin-II; BCA, bicinchoninic acid; CaMK, Ca^{2+}/calmodulin-dependent kinase; CaN, calcineurin; CGP 20712A, 2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl) 1H-imidazole-2-yl) -phenoxy)propyl) amino)ethoxy)-benzamide monomethane sulfonate; DTT, DL-dithiothreitol; ET-1, endothelin-1; hUII, human urotensin-II; ICI 118, 551 erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylamino-butano-2-ol; L-158809, 5,7-dimethyl-2-ethyl-3-[[2′-(1H-tetrazol-5-yl)-[1,1′]-biphenyl-4-yl]methyl]-3H-imidazo[4,5-b]pyridine; NF-ATs, nuclear factors of activated T-cells; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; RII substrate, Asp–Leu–Asp–Val–Pro–Ile–Pro–Gly–Arg–Phe–Asp–Arg–Arg–Val–pSer–Val–Ala–Ala–Glu; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; tacrolimus, FK506

Introduction

The failing heart is the scene of complex and extensive biochemical, molecular and structural changes, which collectively pursue a nonsustainable course (Braunwald & Bristow, 2000). Despite progress, such as, for example, the use of angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin II type I (AT_1)-receptor blockers together with the widespread implementation of β-adrenergic receptor antagonist (β-blocker) therapy with improved clinical outcomes, the prognosis of heart failure is still poor, probably reflecting an incomplete understanding of factors contributing to heart failure. Thus, further advances in the treatment of heart failure are likely to come from efforts to understand and inhibit activated deleterious neurohormonal systems and consequent signaling pathways. More recently, a critical link between calcium-sensitive type IIB phosphatase, calcineurin (CaN) and the development of hypertrophy was described (Molkentin *et al*., 1998). Its specific targets, cytoplasmic phosphorylated nuclear factors of activated T-cells (NF-ATs), were shown to localize to the nucleus following de-phosphorylation, and to combine with the zinc-finger nuclear transcription factor GATA4 to initiate fetal cardiac gene programs (Molkentin *et al*., 1998). A role for CaN in human heart hypertrophy was proposed following the observation of increased calmodulin-immunoprecipitated CaN in left ventricular extracts of patients with heart failure compared to those without heart failure (Lim & Molkentin, 1999). Furthermore, in left

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ventricular outflow tract samples obtained from patients undergoing aortic valve replacement for aortic stenosis and other patients undergoing transaortic subvalvular myotomy–myectomy, CaN phosphatase activity was increased (Ritter *et al*., 2002). Expression of a CaN fragment containing the catalytic domain was increased while expression of a fragment containing the autoinhibitory domain was reduced, and NF-AT was relatively de-phosphorylated in comparison to ventricle from healthy donor hearts (Ritter *et al*., 2002). Collectively, these data were interpreted as indicating a role for CaN in human heart hypertrophy and confirmed earlier data showing increased expression of CaN catalytic subunit and reduced expression of the autoinhibitory subunit (Tsao *et al*., 2000). In patients with terminal dilated cardiomyopathy compared to donors, the left ventricle showed increased CaN activity, translocation of NFAT-3 from the cytosol to the nucleus and increased nuclear expression of the nuclear transcription factor GATA-4 (Diedrichs *et al*., 2004). Thus, CaN and its associated biochemical partners are implicated in various stages of heart failure including terminal heart failure.

Both endothelin-1 (ET-1) and angiotensin-II (Ang II) receptor systems are coupled to phospholipase C -Gαq protein signaling pathways, resulting in activation of protein kinase C isoforms and inositol phosphates, and both systems induce pathological hypertrophy accompanied by contractile dysfunction and poor clinical outcomes (Braunwald & Bristow, 2000). In a rat model of heart failure induced by left coronary artery ligation, blockade of ET_A receptors prevented hypertrophy, reduced contractile dysfunction and prolonged survival (Sakai *et al*., 1996). Blockade of the effects of Ang II by AT_1-receptor blockade with candesartan prolongs survival in patients with heart failure (Pfeffer *et al*., 2003), with additional benefit obtained in patients already taking ACE inhibitors (McMurray *et al*., 2003). AT_1-receptor blockade with 5,7-dimethyl-2-ethyl-3-[[2′-(1H-tetrazol-5-yl)-[1,1′]-biphenyl-4-yl]methyl]-3H-imidazo[4,5-b]pyridine (L-158809) in rats with left coronary artery ligation attenuated increases in cardiac hypertrophy and left ventricular end diastolic pressure (Yoshida *et al*., 2001). We investigated multiple $G\alpha_q/G\alpha_{11}$-receptor systems because these are likely to work in parallel with complete cardiomyocyte-specific abrogation of hypertrophic responses obtained in $G\alpha_q/G\alpha_{11}$-protein-deficient mice (Wettschureck *et al*., 2001). Although increased CaN activity and expression has been observed in human hypertrophy and heart failure patients, the mechanism is not clear. In this study, we investigated whether ET-1 and Ang II receptor systems evoke activation of CaN in the human heart using ventricular trabeculae muscle obtained from explanted hearts. Moreover, elevation of human urotensin-II (hUII) in plasma and the hearts of patients with congestive heart failure has been observed (Douglas *et al*., 2002; Russell *et al*., 2003; Russell, 2004) together with coupling of its receptor to activated protein kinase C-dependent pathways in human heart (Russell *et al*., 2004). hUII causes a hypertrophic response in rat neonatal cardiomyocytes and cultured H9c2 cardiomyoctyes transfected with urotensin-II receptors (Tzanidis *et al*., 2003; Johns *et al*., 2004; Russell, 2004). Therefore, in addition to ET-1 and Ang II, we also investigated whether hUII and its receptor system also affect CaN activity in human heart.

In this study, we showed that acute (30 min) exposure of human heart ventricular muscle to ET-1, Ang II or hUII was sufficient to cause an increase in the activity of CaN. CaN activity was increased in tissue homogenate supernatants that were incubated with PKCε compared to samples incubated without PKCε, possibly indicating phosphorylation of CaN substrate proteins(s).

Methods

Patients

Right ventricular trabeculae were obtained from explanted hearts from 16 consecutive patients undergoing heart transplantation at The Prince Charles Hospital (Table 1, The Prince Charles Hospital human ethics committee approval no. EC9876).

Preparation of ventricular trabeculae

Upon surgical removal of the failing heart, the heart was immediately placed in ice-cold pre-oxygenated (95% O_2/5% CO_2) incubation medium (mM:Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 32, HPO_4^{2-} 1, EDTA 0.04). The right ventricular free wall was rapidly separated from the remaining heart. A small segment of right ventricle was immediately frozen with liquid nitrogen pre-cooled clamps, transported to the laboratory in liquid nitrogen and stored at −80°C and later used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and PKC experiments (below). The remaining right ventricle was placed in a sealed container with incubation medium and transferred to the laboratory.

Right ventricular trabeculae were dissected in incubation medium under continuous oxygenation, set up on tissue-electrode blocks, attached to strain-gauge transducers and driven with square wave pulses (1 Hz, 5 ms duration, just over threshold voltage) (Kaumann *et al*., 1999). Tissues were incubated with incubation medium that was supplemented with (mM:Na^+ 15, fumarate 5, pyruvate 5, L-glutamate 5 and glucose 10). Tissues were incubated for 70 min with prazosin (1 μM) and either 200 nM (−)-propranolol (Sarsero *et al*., 2003, patients 1, 2, 4–6, 12–14, 16, Table 1) or the combination of 300 nM 2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl) 1H-imidazole-2-yl) -phenoxy) propyl) amino) ethoxy)-benzamide monomethane sulfonate (CGP 20712A) + 50 nM erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylamino-butano-2-ol (ICI 118,551) (Kaumann *et al*., 1999; Molenaar *et al*., 2000, patients 3, 7–11, 15, Table 1) to block α-adrenoceptors and β_1- and β_2-adrenoceptors (n.b. Earlier (chronological) experiments used (−)-propranolol and later experiments used the combination of CGP 20712A and ICI 118,551. Both conditions were used to prevent endogenous catecholamine occupation of β_1- and β_2-adrenoceptors as previously used in our laboratory.) ET-1 (100 nM), Ang II (10 μM) or hUII (20 nM) were then added to some tissues and incubated for 30 min, while others were used as time-dependent 'controls'. Each experimental condition was carried out in quadruplicate for each patient.

Concentrations of peptides were designed to produce maximal effects based on previous contractility experiments carried out in human right ventricular trabeculae (ET-1, Burrell *et al*., 2000; hUII, Russell *et al*., 2001) or right or left ventricular trabeculae (Ang II, Moravec *et al*., 1990). Maximal

Table 1 Patient details and corresponding basal calcineurin activity

Patient	*Disease*	*Age*	*Sex*	*EF*	*CI*	*LVEDV*	*RVSP*	*Drug treatment*	*CaN activity*
1	IHD	54	F	52	2.6	—	—	A,Bc,C,D,E,F,G	0.006
2	Aortic Valve Disease	43	M	32	—	195	64	A,Bm,D,E,H,Ia	0.006
3	IHD	32	M	13	1.9	190	50	A,Bc,C,D,E,Idb,Idp,K,L,M,N,O	0.007
CI measured while on dobutamine. Carvedilol ceased 6 weeks prior to transplant.									
4	IDC	48	M	40	—	446	43	A,C,D,K,L,M,O,P,Q	0.008
5	IDC	63	M	9	1.3	429	61	A,Bc,D,K,L,P,S,T	0.008
6	IHD[a]	62	M	43	2.3	174	50	A,Ba,C,E,G,Ia,Ic,U,V,W	0.008
7	IHD	34	M	37	2.1	—	—	A,Bm,C,D,E,Ia,M,O,P,	0.009
8	DC	23	M	23	2.2	208	—	A,D,H,Jdb,K,L,T,U,Y	0.016
Dilated cardiomyopathy secondary to anthracycline. Previous history of ASD repair 15 years prior to transplantation. CI measured while on dobutamine infusion.									
9	Idiopathic CCF	27	M	11	1.6	—	—	A,Jdb,K,L,P,S,Z	0.017
Dobutamine infusion ceased 1 day prior to transplant, milrinone commenced.									
10	IHD	52	M	28	—	—	—	Bc,I,Jdp,L,O,X,	0.025
Carvedilol discontinued 27 days prior to transplant, dopamine commenced 27 days prior to and continued to transplant.									
11	DC	57	F	12	2.8	—	53	Bc,D,K,L,N,Q,R,X,T,AAg,AAi,AB	0.025
Dilated cardiomyopathy secondary to end-stage rheumatic disease.									
12	Becker's muscular dystrophy	21	M	15	2.2	223	74	A,Bc,C,D,Ia,M,O,Q,	0.029
13	DC	42	M	20	1.4	169	35	A,Bc,D,K,O	0.03125
LVAD present at time of transplant.									
14	IHD	59	M	36	—	150	—	A,C,D,G,H,K,L,P,Q,V,	0.0377
15	Single ventricle	26	M	32	—	—	—	A,Bc,C,Ia,L,G,V,O,P,R,Y	0.0403
Previous Fontan procedure.									
16	Familial DC	28	M	11	1.6	493	51	A,Bc,D,K,L,O,P,T,Y,	0.0525

EF, left ventricular ejection fraction; CI, cardiac index ($ml\,min^{-1}\,m^{-2}$); LVEDV, left ventricular end diastolic volume (ml); RVSP, right ventricular systolic pressure (mmHg); calcineurin activity (free phosphate $nmol\,\mu g\,protein^{-1}\,30\,min^{-1}$); IHD, ischemic heart disease; IDC, idiopathic dilated cardiomyopathy; DC, dilated cardiomyopathy; CCF, congestive cardiac failure; LVAD, left ventricular assist device. A, angiotensin-converting enzyme inhibitor; B, β-blocker, a = atenolol, c = carvedilol, m = metoprolol; C, nitrate; D, diuretic; E, hypolipidemic; F, inhalational asthma medication (β_2-adrenoceptor agonist with or without muscarinic receptor antagonist); G, combined L-type calcium/K^+ channel antagonist (perhexiline); H, proton pump inhibitor; I, platelet aggregation inhibitiors (a Aspirin, c clopidogrel); J, β-adrenoceptor agonist infusion (db dobutamine, dp dopamine); K, spironolactone; L, warfarin; M, hydralazine; N, darbepoietin; O, digoxin; P, amiodarone; Q, allopurinol; R, H_2-receptor antagonist (ranitidine); S, 5-HT_3 receptor antagonist (ondansetron); T, thyroxine; U, 5-HT uptake inhibitor (citalopram); V, ATP-sensitive K^+ channel opener (nicorandil); W, cyclooxygenase 2 antagonist (celecoxib); X, AT_1 receptor antagonist (irbesartan); Y, enoxaparin; Z, milrinone; AA, hypoglycemic (AAi insulin, AAg glipizide); AB, quinine.
[a]Syndrome X.

concentrations were used initially to increase the probability of observing an effect on CaN and were continued throughout the study. Other trabeculae were incubated with 200 nM tacrolimus (FK506) for 30 min and then incubated with or without 100 nM ET-1 for another 30 min. Recordings of contractility were made on eight- or 12-channel Graphtec linearcorders. At the completion of incubation periods, tissues were snap frozen with liquid nitrogen and stored at $-80^\circ C$ until required for determination of CaN activity.

CaN activity assay

Protein extraction The right ventricle segment was homogenized in 400 μl lysis buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.2% Nonidet P-40 (NP-40), 1 mM DL-Dithiothreitol (DTT), 5 mM ascorbic acid, protease inhibitor cocktail) using a Polytron (Polytron PT 2100, 7 mm aggregate, setting 26, 3×2 s bursts, Kinematica, Littau-Lucerne, Switzerland). DTT, ascorbic acid and protease inhibitor cocktail (Boehringer, Castle Hill, Australia) were freshly added before use to the above final concentration. NP-40 (0.2%) was added after two homogenization cycles. The samples were centrifuged at $4^\circ C$, $10{,}000 \times g$ for 10 min. The supernatant was collected and 350 μl supernatant was added to a chromatography column (BioMol, PA, U.S.A.) to separate the free phosphate from the extract. The eluent from the column was collected and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, IL, U.S.A.).

CaN activity assay CaN activity was determined using BioMol CaN assay kit (BioMol). A portion of eluent (equivalent of 20 μg protein) was used for the enzymatic activity assay. Samples (20 μg protein in each sample) were incubated with 0.15 mM Asp–Leu–Asp–Val–Pro–Ile–Pro–Gly–Arg–Phe–Asp–Arg–Arg–Val–pSer–Val–Ala–Ala–Glu (RII substrate) for 30 min, at 30°C in 2× assay buffer (100 mM Tris, pH 7.5, 200 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40, 1 mM $CaCl_2$, 250 nM calmodulin, 500 nM okadaic acid), or in 2× EGTA buffer (100 mM Tris, pH 7.5, 20 mM EGTA, 200 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40, 500 nM okadaic acid). Released free phosphate was detected on a micro-plate reader at OD 620 nm based on the classic malachite green assay (Martin *et al*., 1985; Harder *et al*., 1994). The difference in free phosphate released from reactions in assay buffer or in EGTA buffer indicates specific CaN activity. All samples were run in quadruplicate and a mean value was calculated.

SDS–PAGE

Epicardial connective tissue was removed from human right ventricular myocardium. Cardiac tissues were matched for mass, minced using a scalpel blade and homogenized in 0.3 ml lysis buffer (Tris-HCl, 20 mM; NaCl, 150 mM; deoxycholate, 1%; SDS, 0.1%; NP-40, 1%; activated orthovanadate, 1.0 mM; sodium fluoride 1.0 mM and one tablet of Complete Mini protease inhibitor per 7 ml, pH 7.4) using a Polytron PT2100 with 5 mm aggregate (5 s, setting 30, Kinematica). Lysates were spun (10,000 × *g*, 5 min, 4°C) and supernatants were collected and matched for protein levels. Samples were combined with 2× loading buffer, heated (95°C, 5 min), loaded onto 10% polyacrylamide gels (150 V for 70 min) and transferred to Immobilon-P membranes (100 V, 70 min). Membranes were incubated with rabbit polyclonal antibodies raised against peptides with amino-acid residues 237–257 (Stressgen Biotechnologies, Canada), residues 247–449 (BD Transduction Laboratories, U.S.A.), and residues 312–521 (Santa Cruz Biotechnology, U.S.A.) corresponding to human CaN Aα. Proteins were detected using Phototope-HRP Western Blot Detection System (Cell Signaling Technology). Semiquantitative determination of protein levels was performed using a Bio-Rad Molecular Imager, FX.

PKC contribution to CaN activation

Tissue preparation Samples from heart numbers 1, 3–7 (Table 1, PKCε experiments), 1–7 (Table 1, PKC$\alpha\beta\gamma$ experiments), were weighed and minced using a scalpel blade and homogenized in MOPS buffer (1 g ml^{-1}; 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, one tablet of Complete Mini protease inhibitor). Homogenates were spun (10,000 × *g*, 5 min at 4°C), supernatants were collected and frozen in liquid nitrogen or used immediately for incubation with recombinant PKC.

Contribution of PKC to CaN-dependent free phosphate formation Supernatants were incubated with 1.14 μg ml^{-1} recombinant PKCε, 0.9 μg ml^{-1} recombinant PKC$\alpha\beta\gamma$ (Upstate Biotechnology, NY, U.S.A.) or the PKC diluent for control, for 30 min at 30°C with gentle shaking. Reaction mixtures included PKC lipid activator (containing 110 μg ml^{-1} phosphatidylserine, 11 μg ml^{-1} diacylglycerol, 0.23 mM $CaCl_2$; Upstate Biotechnology, NY, U.S.A., sonicated for 1 min on ice prior to use), PKA/Ca^{2+}/calmodulin-dependent kinase (CaMK) inhibitor cocktail (containing 0.45 μM PKA inhibitor peptide, 4.5 μM compound R24571; Upstate Biotechnology, NY, U.S.A.), 0.11 mM ATP and assay dilution buffer according to the manufacturer's recommendation. Samples were loaded onto the chromatography columns, and eluents were collected after centrifugation (3000 × *g*, 10 min, 4°C).

CaN activity assays CaN activity was determined as described above, with minor modification. Samples were incubated without the RII phosphopeptide, to investigate possible PKC-dependent phosphorylation of endogenous substrate proteins. Free phosphate formation was determined using the BioMol Green reagent.

Statistics

Data are presented as individual results for each patient or means ± s.e.m. Student's paired *t*-test was used to determine the significance of differences between basal values and effects. Spearman rank correlation was used to determine the relationship between CaN activity values, *in vivo* human heart functional data and protein expression. *P*-values of <0.05 were considered significant.

Results

Effects of ET-1, Ang II and hUII on contractility in human right ventricle

ET-1 (100 nM), Ang II (10 μM) and hUII (20 nM) increased contractile force of right ventricular trabeculae from 13/13, 3/8 and 4/10 patients, with an overall significant increase by ET-1 (basal 8.8 ± 1.0 mN; ET-1 12.0 ± 1.4 mN, $n=13$ patients, $P<0.001$) but not Ang II (basal 4.9 ± 1.1 mN; Ang II 5.2 ± 1.1 mN, $n=8$ patients, $P=0.3$) or hUII (basal 11.2 ± 2.7 mN; hUII 11.4 ± 2.6 mN, $n=10$ patients, $P=0.8$) (Figure 1). There was no change in the time to reach peak force or time to reach 50% relaxation for any of the peptides.

The addition of 200 nM FK506 to ventricular trabeculae did not alter contractile force (basal 4.9 ± 0.8 mN; FK506 5.14 ± 0.7 mN, $n=4$ patients, $P=0.1$). In the presence of FK506, ET-1 100 nM increased contractile force in 3/4 patients, but overall this was not quite significant (FK506 (basal) 5.9 ± 1.0 mN; + ET-1 6.7 ± 1.3 mN, $n=4$ patients, $P=0.05$).

CaN activity in human right ventricle

Basal CaN activity ranged from 0.006 to 0.031 nmol μg protein^{-1} 30 min^{-1} free phosphate (mean 0.020 ± 0.004, $n=16$ patients) in right ventricular trabeculae, set up in the tissue bath and stimulated to contract and relax, but not exposed to the cardiostimulants (ET-1, Ang II or hUII) or FK506 (Table 1). CaN activity was not correlated with right ventricular systolic pressure ($P=0.8$, $n=8$ values, Table 1) or left ventricular end diastolic volume ($P=0.09$, $n=10$ values, Table 1).

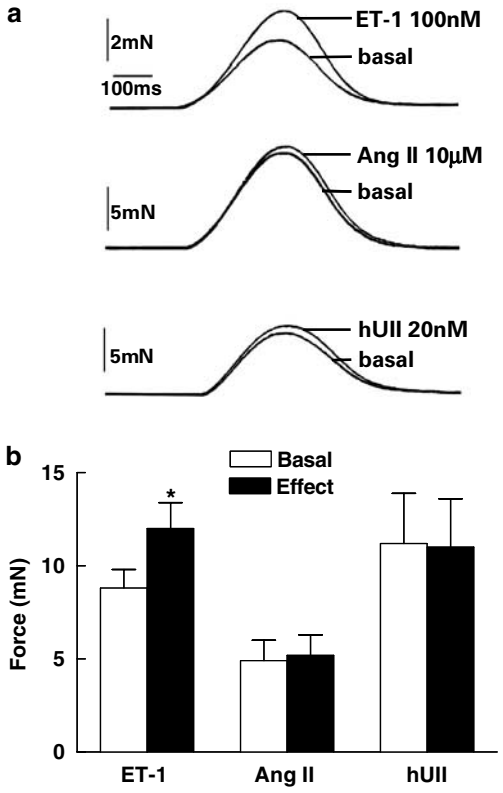

Figure 1 Effects of ET-1, Ang II and hUII on contractility of human right ventricular trabeculae from failing hearts. (a) Superimposed representative fast-speed recordings of individual contractions before (basal) and after the addition of peptides. Mean data are shown in (b). Values given in (b) refer to trabeculae/patient numbers. Bar graphs show mean ± s.e.m. Values from 13 (ET-1), eight (Ang II) and 10 (hUII) patients. $^*P<0.001$.

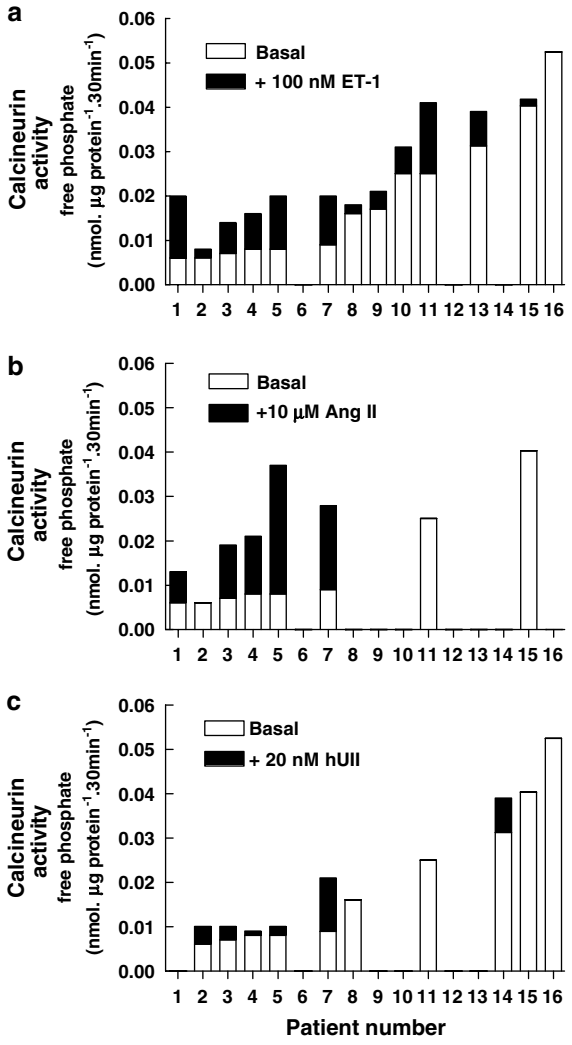

Figure 2 CaN activity in the right ventricular trabeculae from failing hearts in the absence and presence of ET-1 (a), Ang II (b) and hUII (c). Basal CaN activities are shown by the open columns and are arranged from lowest to highest. Increased calcineurin (CaN) activity levels in the presence of ET-1, Ang II and hUII are indicated by the filled boxes. CaN enzyme activity is expressed in terms of free phosphate liberated over a period of 30 min, nmol μg protein^{-1} 30 min^{-1}. Data for individual patients are given. Patients 6 and 12 were only used to record basal CaN activity (Table 1).

CaN activity was increased in ventricular trabeculae incubated with ET-1 (100 nM), Ang II (10 μM) or hUII (20 nM) for 30 min (Figure 2, Table 2). Incubation of tissues for 30 min with the immunosuppressant drug FK506 (200 nM) caused a reduction in CaN activity compared to trabeculae not exposed to FK506 (Figure 3, Table 2, $P<0.0001$, $n=4$ patients). The subsequent addition of 100 nM ET-1 for 30 min (FK506 (total time 60 min) + ET-1 (total time 30 min)) did not alter CaN activity compared to tissues incubated with FK506 only ($P=0.1$, $n=4$ patients) and the activation remained low compared to basal CaN activity ($P=0.005$, $n=4$ patients).

Expression of CaN in human right ventricle

To determine whether basal CaN activity values (Table 1, Figure 2) reflected different expression levels of CaN protein, protein levels were determined. There was no difference in the level of the 58 kDa protein corresponding to full-length CaN A protein determined with an antibody directed against residues 247–449 (Figure 4) and two other antibodies raised against different CaN A residues (not shown). There was no relationship between CaN activity and expression levels ($P=0.3$, not shown) and no difference in CaN expression in tissues incubated with or without ET-1 ($P=0.34$, $n=3$, not shown).

PKC contributes to CaN-dependent free phosphate formation

A putative mechanism for the increase in 'CaN activity' by ET-1, hUII and Ang II was investigated by examining the effect of PKC on free phosphate formation in the absence of exogenously added RII peptide. Okadaic acid was used to inhibit protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A) activity, and the combination of okadaic acid and EGTA to inhibit PP1, PP2A and CaN. In the presence of okadaic acid, free phosphate formation was greater when the ventricular preparation was pre-incubated with PKCε compared to samples that were not pre-incubated with PKC (Figure 5a). CaN-dependent formation of free phosphate [OA−(OA + EGTA)] was detected in samples that

Table 2 Effect of ET-1, Ang II, hUII and FK506 on calcineurin activity in human right ventricular trabeculae

Experimental condition	*CaN activity*	n *(patients)*	*Statistical comparison*	P-*value*
Basal	0.019±0.004	13	Basal vs ET-1	0.001
ET-1	0.025±0.004			
Basal	0.014±0.004	8	Basal vs Ang II	0.03
Ang II	0.024±0.004			
Basal	0.020±0.005	10	Basal vs hUII	0.04
hUII	0.023±0.005			
Basal	0.021±0.002	4	Basal vs FK506	0.001
FK506	0.014±0.003		FK506 vs FK506+ET-1	0.1
FK506+ET-1	0.015±0.003		Basal vs FK506+ET-1	0.02

Calcineurin activity (free phosphate, nmol μg^{-1} 30 min^{-1}). ET-1 100 nM, Ang II 10 μM, hUII 20 nM, FK506 200 nM.

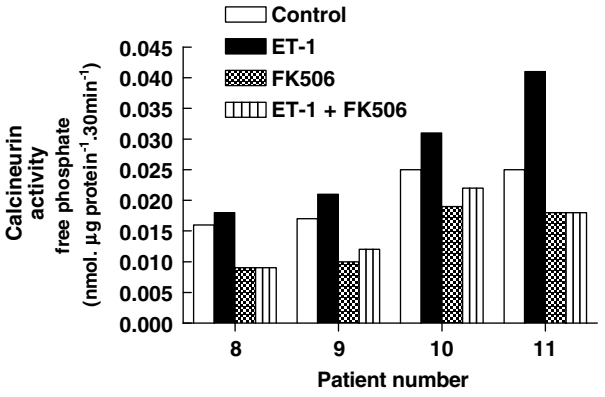

Figure 3 Effect of FK506 on CaN activity levels in the right ventricular trabeculae from failing hearts. Pre-incubation of trabeculae with FK506 for 30 min prevented ET-1 from increasing CaN activity values. Data obtained from four patients, numbered 8–11.

were pre-incubated with PKCε, but not in control samples (Figure 5b). A nonsignificant trend for increased PKCα,β,γ-stimulated CaN-dependent free phosphate compared to control was observed (data not shown).

Discussion

In this study, our primary interest was in determining whether a link could be demonstrated between endogenous peptides, ET-1 and Ang II, which are implicated in the development of human heart hypertrophy and progression to heart failure, and the activation of CaN, an enzyme also implicated in human heart hypertrophy and failure. We were also interested in whether CaN could be activated by hUII, another peptide for which immunoreactivity is increased in heart failure patients (Russell *et al*., 2003). We showed that acute exposure of contracting human right ventricular heart muscle to ET-1, Ang II and hUII *in vitro* is sufficient to increase the ability of CaN to liberate free phosphate.

ET-1, Ang II and hUII cause activation of specific $G\alpha_q$/$G\alpha_{11}$-protein-coupled receptors with subsequent activation of phospholipase Cβ. The signaling cascade diverges with the generation of inositol phosphates and diacyglyerol, with the latter resulting in the activation of protein kinase C isoforms. The complete abrogation of a hypertrophic response in $G\alpha_q$/$G\alpha_{11}$-protein-deficient hearts in mice with aortic banding (pressure overload) indicates critical hypertrophic signaling pathways through these G-proteins. It has been suggested that hypertrophic signaling through $G\alpha_q$/$G\alpha_{11}$ proteins may in part involve CaN as indicated by an incomplete inhibition of hypertrophy by the chronic administration of the CaN inhibitor cyclosporine A (CsA) in mice with constitutively active $G\alpha_q$ protein (Mende *et al*., 1998). Activation of CaN by agonists such as Ang II and ET-1 has been observed in Wistar rats treated with Ang II (Goldspink *et al*., 2001) and in cultured neonatal cardiomyocytes (Taigen *et al*., 2000). This study has taken a novel approach in introducing agonists directly to human ventricular muscles to investigate their effect on CaN.

The concentrations of ET-1, Ang II and hUII used in this study were maximal for their ability to induce positive inotropic effects in human ventricular muscle preparations *in vitro*. The relationship between concentration, time and CaN activity has not been determined at this stage. Circulating levels of endogenous peptides in patients without heart failure are ~6 pM ET-1 (healthy volunteers, antecubital vein; McMurray *et al*., 1992), ~6 pM Ang II (healthy volunteers, blood sample vessel not specified; Ménard *et al*., 1995) and hUII ~16 pM (patients investigated for coronary artery disease, aortic root; Russell *et al*., 2003). Thus, based on concentrations required to cause positive inotropic effects, the plasma concentrations of ET-1 and Ang II would not be expected to be effective. However, plasma levels of the peptides may not be indicative of peptide concentrations in equilibrium with their respective receptors. It was argued previously (Burrell *et al*., 2000) that although plasma levels of ET-1 are insufficient to stimulate human heart muscle for positive inotropic effects, it is possible that locally synthesized and abluminally released ET-1 (Wagner *et al*., 1992) could directly stimulate heart muscle (Burrell *et al*., 2000). The same may be true for activation of CaN. Plasma concentrations of hUII could, however, be expected to be active. The same considerations concerning plasma and effective concentrations of peptides may be valid in human heart failure despite increases for ET-1 (~12 pM ET-1; McMurray *et al*., 1992) and Ang II (~7.5–10 pM, venous blood sample, patients administered angiotensin-converting enzyme inhibitors and diuretics; Rousseau *et al*., 2002).

The current clinical management of heart failure as yet does not incorporate the concept that multiple $G\alpha_q$/$G\alpha_{11}$-protein-

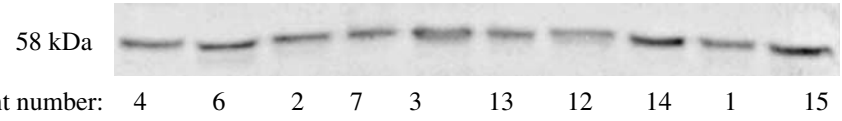

Figure 4 Expression levels of CaN A protein determined with an antibody raised against residues 247–449 in 10. There was no relationship between expression levels (optical density values) and basal CaN activity values (Table 1, $P=0.3$).

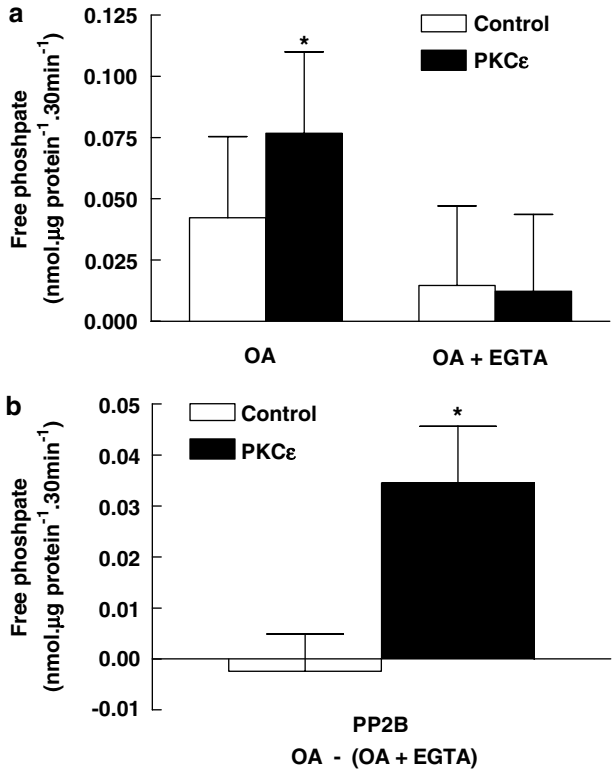

Figure 5 Effect of PKC on CaN-dependent free phosphate formation in human right ventricular homogenates. Okadaic acid (OA)-insensitive free phosphate formation was greater in homogenates that were pre-incubated with PKCε (a). Pretreatment of homogenates with PKCε caused an increase in CaN-dependent free phosphate formation [OA−(OA + EGTA), calculated from individual experiments] (b). Values are mean ± s.e.m. Statistical comparisons used the Student's *t*-test. * $P<0.05$; $n=7$. OA, okadaic acid.

coupled receptor systems may contribute to hypertrophy. In our patient cohort of successive patients presenting for heart transplantation, all were managed with either an ACE inhibitor or AT_1 receptor antagonist, irrespective of etiology. Additional therapeutic options to block $G\alpha_q/G\alpha_{11}$-protein-coupled receptors or downstream signaling effectors are currently not available for clinical practice. Thus, it is possible that these patients were exposed to an incomplete blockade of $G\alpha_q/G\alpha_{11}$-protein signaling possibly involving CaN during the development of hypertrophy and progression of heart failure.

Basal CaN levels ranged nearly 10-fold, from 0.006 to 0.031 nmol μg protein^{-1} 30 min^{-1} in our cohort of patients. This may reflect the heterogeneity of our patient population which represented consecutive patients presenting for heart transplantation with differing etiologies. Importantly, it is possible it may also reflect differing contributions of CaN to ongoing hypertrophy in these patients. This study showed that pre-incubation of human right ventricular heart muscle with the CaN enzyme inhibitor, FK506 (tacrolimus) reduced CaN basal activity. FK506 must first form a complex with the immunophilin protein FKBP12, which then binds to and inhibits CaN. The complex is most likely retained throughout processing of ventricular tissues for the *in vitro* CaN assay. Pre-incubation with FK506 also abolished the ability of ET-1 to activate CaN. Thus, inhibition of CaN may ultimately provide an additional therapeutic option to reduce multiple receptor-signaling pathways involved in the development of hypertrophy and heart failure. FK506 itself is not suitable for this purpose because of toxicity, but development of novel drugs which directly bind to CaN in the same manner as the immunophilin protein – FK506 complex may be beneficial.

Earlier work in B lymphocytes indicated that NFAT was activated by prolonged low-amplitude calcium plateaux, presumably through CaN activation (Dolmetsch *et al*., 1997). It has been accepted that CaN is activated by the increase in intracellular calcium caused by most, if not all, cardiac stimuli. However, the importance of differing calcium sources for activation of CaN in human heart has not been investigated yet. In this study, we utilized electrically stimulated ventricular trabeculae in order to simulate *in vivo* conditions as closely as possible. This includes maintenance of fluctuating calcium fluxes associated with oscillating systolic and diastolic states of the heart. Upon extrapolation to human heart muscle, CaN may preferentially detect elevated levels of diastolic calcium associated with diastolic disorders in heart failure in preference to more 'transient' elevated levels of calcium associated with systole. We were not able to fully test this hypothesis in the present study because we used heart muscle from the right ventricle where direct measures of diastolic function were not available.

ET-1 consistently caused increases in contractile force in the right ventricular trabeculae from patients with heart failure, but the effects of both Ang II and hUII were more variable. This is consistent with previous reports for hUII (Russell *et al*., 2001) and Ang II in ventricle (right and left ventricular trabeculae; Moravec *et al*., 1990). Changes in contractile force for these peptides were not associated with changes in the duration of contraction as previously observed (Moravec *et al*., 1990; Burrell *et al*., 2000; Russell *et al*., 2001). ET-1 caused increases in contractile force in the presence of 200 nM FK506 in 11 out of 16 trabeculae from three out of four patients, suggesting that the ability of ET-1 to alter the contractile state and to activate CaN in human heart muscle probably represents a divergence in signaling, possibly in part at the level of protein kinase C isoforms and their target proteins. Activated protein kinase C may stimulate the Na^+/H^+ exchanger, cause intracellular alkalinization and sensitization of sarcomeric proteins to Ca^{2+} (Russell & Molenaar, 2000), and phosphorylate myosin light chain (hUII; Russell & Molenaar, 2004).

The mechanism through which ET-1, Ang II and hUII increases the activity of CaN in the absence of changes in

expression levels is unclear. We have attempted to determine a possible mechanism by which CaN activity was increased involving PKC. The *in vitro* tissue bath experiments examined the effects of ET-1, hUII and Ang II on endogenous CaN activity using conditions (addition of Ca^{2+}/calmodulin) that facilitated stimulation of the phosphatase. The exposure of tissues to the agonists was acute (30 min), thus presumably precluding substantive effects on transcriptional (not tested) or translational regulation. The mechanism for increased agonist-stimulated CaN activity, in an environment in which CaN was maximally activated, was therefore investigated. Activation of ET-1, hUII and Ang II receptors causes the production of diacylglycerol and inositol 1,4,5 trisphosphate (IP_3). Although IP_3 can increase intracellular Ca^{2+} levels, it cannot explain the increased CaN activity that was observed because high concentrations of exogenous Ca^{2+} (and calmodulin) were added to the assay buffer. Conventional and novel isoforms of PKC are activated by diacylglycerol, stimulating phosphorylation of substrate proteins that are expressed in the heart (Nishikawa *et al*., 1997). PKC isoforms (α, βI, βII, δ, ε and η) have been detected in particulate fractions of right ventricle obtained from patients with nonfailing and failing hearts (Shin *et al*., 2000). The assembly of PKC and CaN with G-protein-coupled receptors may be facilitated by scaffold molecules such as A-kinase anchoring proteins (Malbon *et al*., 2004). We therefore investigated whether agonist-activated receptors could lead to PKC-dependent phosphorylation of proteins that were also a substrate for CaN activity by examining the effect of co-incubating human heart homogenates with recombinant PKC.

The investigation of the effect of PKC on free phosphate formation was carried out in the absence of the synthetic CaN substrate RII phosphopeptide, and in the presence of okadaic acid to inhibit PP1 and PP2A. Under these conditions, free phosphate concentration was higher in samples that were pre-incubated with PKCε, compared to samples that were incubated without PKC, indicating that PKC could plausibly phosphorylate endogenous proteins that are substrates for a non-PP1, non-PP2A phosphatase. Samples were co-incubated with okadaic acid and EGTA for additional inhibition of CaN activity. In samples which were pre-incubated with PKCε, free phosphate formation was significantly higher in the presence of okadaic acid than the combination of okadaic acid and EGTA, indicating CaN-dependent activity. Together, these findings suggest a mechanism by which ET-1, hUII and Ang II could conceivably increase free phosphate formation through activation of PKC. Activation of PKC by diacylglycerol results in phosphorylation of endogenous proteins which, together with exogenously added RII phosphopeptide, serve as substrates for CaN.

Interestingly, CaN is also a substrate protein for PKC-dependent phosphorylation (Tung, 1986; Hashimoto & Soderling, 1989). Therefore, an alternate hypothesis may be proposed whereby PKC modulates CaN activity *via* direct phosphorylation of the PKC consensus sequence on CaN, independent of the phosphorylation of other substrate proteins within the right ventricular homogenate. However, the CaN assay measures maximal CaN activity with the addition of high Ca^{2+}/calmodulin concentrations, and it is therefore unlikely that PKC could further increase CaN activity directly. In addition, phosphorylation of CaN by PKC caused either no change in CaN activity (Tung, 1986), or an $\sim$2-fold increase in K_m and no change in V_{max}, reflecting inhibition of CaN activity by PKC (Hashimoto & Soderling, 1989). Together, the findings indicate that PKC-induced increased free phosphate formation was attributed to an increase in phosphorylation of endogenous proteins which served as additional substrate(s) for CaN. The identity of the endogenous substrate proteins that might be phosphorylated by PKC in this study remains to be determined. Interestingly, proteins such as MARCKS and neuromodulin that are expressed in heart or cardiac nerves have been identified as targets for PKC-dependent phosphorylation (Herget *et al*., 1995; Seki *et al*., 1995), and substrates for CaN-dependent dephosphorylation (Seki *et al*., 1995).

This study has provided a direct association between endogenous peptides which activate $G\alpha_q/G\alpha_{11}$-protein-coupled receptors and CaN activity in human heart. *In vitro* experiments using a homogenate preparation showed that activation of PKC can phosphorylate endogenous substrates which could contribute to the 'activity' of CaN.

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