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FACTORS RELEASED FROM ENDOCARDIUM OF THE FERRET AND PIG MODULATE MYOCARDIAL CONTRACTION

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SUMMARY

1. In isolated heart muscle preparations, selective removal of the endocardium results in a characteristic and unusual negative inotropic effect. Possible mechanisms for this effect were investigated in this study.

2. In endocardium-intact preparations of ferret papillary muscle, 8-bromo-cyclic GMP, sodium nitroprusside, atrial natriuretic peptide (ANP) and substance P each induced changes in contractile behaviour similar to selective endocardial removal, and each significantly elevated myocardial cyclic GMP levels. Substance P failed to elevate myocardial cyclic GMP levels following removal of endocardium or in the presence of haemoglobin, suggesting that it may act by releasing endothelium-derived relaxing factor (EDRF) from endocardium. However, there was no change in myocardial cyclic GMP levels following endocardium removal alone.

3. In cascade bioassay experiments, it was confirmed that porcine cultured endocardial cells released an unstable humoral agent whose effects on an endothelium-denuded pig coronary artery were indistinguishable from EDRF.

4. The negative inotropic effects of endocardium removal were reversed in bioassay experiments where an endocardium-denuded papillary muscle was exposed to the effluent from a column of porcine cultured endocardial cells on microcarrier beads. This demonstrates for the first time the release of a 'contraction prolonging factor' from endocardium, the tonic release of which would explain the negative inotropic effect of endocardium removal.

5. It is concluded that elevation of ferret papillary muscle cyclic GMP (as for example with EDRF) produces changes in contractile performance similar to those induced by endocardium removal. We also demonstrate that superfused porcine cultured endocardial cells release a humoral agent (provisionally named 'endocardin') which causes reversal of the changes in mechanical properties seen after endocardial removal.

INTRODUCTION

In isolated heart muscle preparations, selective damage to the endocardium results in an unusual type of negative inotropic effect characterized by reduction of contractile twitch duration and peak isometric tension without any change in maximum unloaded shortening velocity (V_{max}) (Brutsaert, Meulemans, Sipido & Sys,

1988). This differs from other inotropic interventions such as reduction or increase in extracellular calcium (Chappell, Henderson & Lewis, 1986; Lee & Allen, 1989), changed frequency of stimulation (Chappell *et al.* 1986; Henderson, Brutsaert, Parmley & Sonnenblick, 1969; Lee & Allen, 1989), hypoxia (Tyberg, Yeatman, Parmley, Urschal & Sonnenblick, 1970; Henderson & Brutsaert, 1973; Allen, Lee & Smith, 1989), acidosis (Fry & Poole-Wilson, 1981; Allen & Orchard, 1983; Lee & Allen, 1989) or reduction of cyclic AMP-mediated effects (Tada & Katz, 1982; Winegrad, 1984), all of which are associated with reduction in V_{max}. The mechanism(s) underlying these effects of endocardium removal are unknown.

In the present study, we have investigated the possibility that endocardium might influence contraction of subjacent myocardium by release of chemical messengers in a manner analogous to endothelial regulation of vascular smooth muscle tone (Griffith, Lewis, Newby & Henderson, 1988). We show that elevation of myocardial cyclic GMP levels is associated with changes in contractile performance of isolated ferret papillary muscle preparations, similar to those induced by endocardium removal. We also show that superfused porcine cultured endocardial cells release both a humoral agent with properties identical to those of endothelium-derived relaxing factor (EDRF), and an unidentified substance(s) provisionally named 'endocardin' which in endocardium-denuded papillary muscles causes reversal of the changes in mechanical properties seen after endocardial removal.

METHODS

Papillary muscle preparations

Right ventricular papillary muscles ($n = 58$) from pentobarbitone-anaesthetized ferrets were carefully isolated and mounted vertically in tissue baths containing oxygenated (95% O_2, 5% CO_2) Krebs–Ringer solution (composition in mM: NaCl, 118; KCl, 4·7; $MgSO_4.7H_2O$, 1·2; $NaHCO_3$, 24; KH_2PO_4, 1·1; glucose, 10; $CaCl_2.2H_2O$, 1·25; acebutolol, 0·004) at 29 °C as previously described (Chappell *et al.* 1986; Brutsaert *et al.* 1988). The baseline characteristics of the muscles used in this study are shown in Table 1. Muscles were stimulated homogeneously with 5 ms rectangular pulses at 0·2 Hz, 10% above threshold voltage, via a pair of longitudinal platinum electrodes. The force–length transducer enabled imposition of variable preloads and afterloads, and measurement of displacement was by an optico-electronic system and of force by a unidirectional force-sensing feedback circuit. After mounting the muscles they were allowed to equilibrate for 2–3 h before experiments were begun, which were all performed at l_{max}, the muscle length at which active force development was maximal. The following contractile properties were measured from an isotonic zero-loaded twitch and an isometric twitch: resting tension (R tension required to stretch muscle to l_{max}), maximum unloaded shortening velocity (V_{max}), peak isometric twitch tension (TT), peak rate of tension development ($+dT/dt$), time-to-peak isometric twitch tension (t_{TT}) and time to half-isometric twitch tension decline ($RT_{0·5}$). For V_{max} measurements, the loading on the stimulated muscle preparation was suddenly changed from the preload to zero load immediately after the latency period (Brutsaert & Claes, 1974). All test contractions under steady-state conditions were separated by at least eight isotonic preloaded twitches to eliminate the residual effects of length and load during preceding contractions (Brutsaert & Claes, 1974).

Values for V_{max} were normalized for length at l_{max}, and isometric parameters for muscle cross-sectional areas (CSA). The latter was calculated at the end of experiments by dividing the lightly blotted wet weight by length at l_{max} and the specific gravity (1·05). Muscles with resting tension greater than 20% of total isometric tension at l_{max} were excluded from the study.

Selective removal of endocardium

Endocardium was selectively removed as previously described (Brutsaert *et al.* 1988). Briefly, a muscle was immersed for 2 s in 0·5% Triton X-100 dissolved in Krebs–Ringer solution while in its mounted working position. Immediately after this procedure, all the Triton X-100 was rapidly and

TABLE 1. Baseline characteristics of ferret papillary muscles (29 °C, 1·25 mM-calcium)

Group	n	l_{max} (mm)	CSA (mm^2)	R/TT (%)	TT ($mN\ mm^{-2}$)	$+dT/dt$ ($mN\ mm^{-2}\ s^{-1}$)	t_{TT} (ms)	$RT_{0.5}$ (ms)	V_{max} ($ML\ s^{-1}$)
Endocardial removal	15	5·0 ± 0·2	0·79 ± 0·08	15·1 ± 1·0	46·3 ± 5·0	336·6 ± 35·5	200·1 ± 15·5	329·3 ± 7·4	1·53 ± 0·08
8-Bromo-cyclic GMP	5	5·0 ± 0·4	0·67 ± 0·07	14·0 ± 1·5	52·2 ± 8·8	341·4 ± 52·4	222·8 ± 7·2	375·2 ± 19·1	1·27 ± 0·11
SNP	6	5·2 ± 0·5	0·75 ± 0·12	15·3 ± 1·7	56·0 ± 12·9	366·4 ± 71·6	221·7 ± 16·9	385·7 ± 35·0	1·36 ± 0·10
SNP − E	4	5·5 ± 0·5	0·63 ± 0·12	13·2 ± 2·5	52·7 ± 8·4	390·7 ± 64·9	215·5 ± 8·4	390·5 ± 33·1	1·33 ± 0·16
ANP	6	4·3 ± 0·4	0·93 ± 0·12	13·7 ± 0·5	40·4 ± 3·9	302·0 ± 29·5	199·7 ± 3·2	326·3 ± 12·3	1·58 ± 0·05
SP	5	4·6 ± 0·2	0·57 ± 0·10	10·2 ± 1·0	66·2 ± 11·1	440·6 ± 75·6	236·4 ± 11·8	435·6 ± 27·9	1·60 ± 0·11
SP + Hb	6	4·6 ± 0·2	0·78 ± 0·12	18·3 ± 1·1	42·0 ± 6·4	308·0 ± 29·9	209·7 ± 5·9	335·0 ± 9·6	1·32 ± 0·10
SP − E	5	5·6 ± 0·9	0·72 ± 0·11	13·4 ± 1·7	49·6 ± 3·9	352·4 ± 18·9	216·8 ± 11·0	359·2 ± 16·8	1·40 ± 0·09
Endocardial cell bioassay	6	5·3 ± 0·2	0·62 ± 0·08	16·2 ± 1·5	48·7 ± 8·4	372·3 ± 64·9	190·0 ± 5·8	321·3 ± 13·9	1·74 ± 0·10

Values given as means ± S.E.M.

thoroughly rinsed off using at least 500 ml of freshly warmed Krebs–Ringer solution, and the muscle was then allowed to stabilize for at least 20 min.

After equilibration, some muscles not exposed to Triton X-100 ($n = 3$) and some following exposure to Triton X-100 ($n = 3$) were removed from the tissue bath and prepared for examination by transmission electron microscopy. Each muscle was placed in 2·5% glutaraldehyde in 0·15 M-cacodylate buffer (pH 7·2) at room temperature and cut into 1 mm thick slices. After 2 h, the specimens were rinsed with 0·1 M-cacodylate buffer (pH 7·2) and stored overnight at 4 °C in this solution. After further rinsing, they were post-fixed in cold 0·5% osmium tetroxide in 0·1 M-cacodylate buffer (pH 7·2) for 1 h at room temperature. The excess osmium tetroxide was rinsed off using cacodylate buffer followed by distilled water. A standard dehydration schedule was used using increasing strengths of ethanol followed by propylene oxide. The specimens were embedded using propylene oxide epoxy resin LX112 followed by polymerization at increasing temperatures.

After sectioning, specimens were examined using a Leitz AMR-1200-B electron microscope at 15 kV.

Endothelial and endocardial cell culture

Endothelial cells from pig aortae were isolated as described by Smith & Lang (1990) and cultured in six-well plates (three per aorta) as described below for endocardium.

Intact pig hearts were obtained from the local abattoir and, keeping conditions as sterile as possible, transported back to the laboratory in sterile isotonic saline (0·9% NaCl) containing 0·1% bovine serum albumin, benzylpenicillin (200 U ml^{-1}) and streptomycin (200 $\mu g\ ml^{-1}$). In the laboratory, the hearts were transferred to a laminar flow hood, where both atria and the left ventricle were cut away to leave the right ventricle in the form of a pouch attached to the ventricular septum. The right ventricle was flushed with sterile isotonic saline and wherever possible all blood clots, valve tissue and chordae tendineae removed. The ventricle was then mounted upright in an expanded polystyrene container, filled with 0·2% collagenase (type II) in medium 199 with Earle's salts and incubated at 37 °C for 45 min. The inside of the ventricle was then gently rubbed with a glass rod to remove any loosely attached cells, and the collagenase and suspended cells aspirated with a syringe. The ventricle was filled with sterile isotonic saline, rubbed again, and the saline aspirated off. The combined collagenase and saline wash were centrifuged (200 *g*, 4 min) to sediment the cells, and the cell pellet resuspended in medium 199 supplemented with 10% fetal bovine serum, 10% newborn bovine serum, glutamine (6 mM), benzylpenicillin (200 U ml^{-1}), streptomycin (200 $\mu g\ ml^{-1}$) and kanamycin (100 $\mu g\ ml^{-1}$) (known hereafter as complete medium).

The suspensions from three or four hearts were pooled, placed in culture flasks of area 75 cm^2 or seeded into six-well culture plates of area 9·6 cm^2, and incubated in a 5% CO_2, 20% O_2 atmosphere at 37 °C. The culture medium was renewed every 2 days until cell confluence was achieved. The cells from three of the flasks were harvested using 0·05% trypsin in 0·9% saline containing 0·02% sodium EDTA, centrifuged and the cells resuspended in complete medium. The cells were then placed in a stirring cell culture vessel with 3 ml of microcarrier beads and 125 ml of complete medium, and gassed with 20% O_2, 5% CO_2, 75% N_2. The flask was gassed for 2–3 min daily with this mixture, and 50–80% of the medium replaced every other day for up to 3 weeks. The microcarrier bead suspension was stirred continuously in an incubator at 37 °C. For bioassay experiments, 0·7–0·8 ml of uncovered (naked) or cell-covered beads were washed in isotonic saline at room temperature and placed in 1 ml polypropylene syringes and superfused with oxygenated Krebs–Ringer solution (containing indomethacin, 1 μM) at 35 °C at a flow rate of 3–4 ml min^{-1}.

Myocardial, endothelial and endocardial cyclic GMP radioimmunoassay

For measurement of cyclic GMP content, papillary muscles were rapidly removed from tissue baths 10 min after any intervention, blotted dry and snap-frozen in liquid nitrogen. They were subsequently weighed to the nearest hundreth of a milligram, taking care that they did not thaw out, and then stored for up to 1 month at −70 °C. Each muscle was homogenized in 1 ml ice cold 5% perchloric acid. The homogenate was neutralized by vortex mixing for 2 min with 2 ml of 0·5 M-tri-*n*-octylamine in 1,1,2-trichloro-trifluoroethane (Freon). The mixture was centrifuged at 700 *g* for 5 min at 4 °C, and a sample of the upper aqueous layer assayed for cyclic GMP content by radioimmunoassay using a commercially available kit (Amersham International, Cardiff). Cyclic GMP content is expressed as pmol cyclic GMP (mg muscle wet weight)$^{-1}$.

For measurement of cyclic GMP content in cultured endothelial and endocardial cells (in six-well plates), the tissue culture medium was aspirated and the cells washed twice, and 2 ml Krebs solution of the following composition (mM): NaCl, 94·8; KCl, 4·7; $MgSO_4$, 1·2; $CaCl_2$, 2·5; KH_2PO_4, 1·2; $NaHCO_3$, 25; glucose, 11 added. The mixture was incubated at 37 °C in an atmosphere of 5% CO_2 in air for at least 90 min. After the appropriate time following the addition of drugs (see experimental protocol), the Krebs solution was rapidly removed and frozen for subsequent assay of cyclic GMP content. The cells were immediately extracted with 0·5 ml of ice cold 6% trichloroacetic acid (TCA), scraped from the well and, together with the TCA, aspirated into plastic tubes. Each well was extracted with a second 0·5 ml ice-cold 6% TCA. The combined extracts were centrifuged at 10000 *g* for 2 min and pellet and supernatant separated. The DNA content of the cell pellet was measured by the fluorimetric method of Kissane & Robins (1958). The supernatant was neutralized as described for the papillary muscles. The cyclic GMP content of the aqueous upper layer of the cell homogenate and of the Krebs solution aspirated off the cells was measured by radioimmunoassay and expressed as fmol(μg DNA)$^{-1}$.

Experimental protocol

Effects of cyclic GMP-elevating agents on papillary muscles. The effects of single concentrations of sodium nitroprusside (1 μM) or human atrial natriuretic peptide (ANP; 0·4 μM), stimulants of soluble and particulate guanylate cyclase respectively (Katsuki, Arnold, Mittal & Murad, 1977; Waldman, Rapoport & Murad, 1984) or substance P (1 μM), which releases endothelium-derived relaxing factor (EDRF) from vascular endothelium (Zawadzki, Furchgott & Cherry, 1981), on contraction of ferret papillary muscles were measured 10 min after addition, for comparison with the effects of 8-bromo-cyclic GMP (0·1 mM). Each muscle specimen was then prepared for cyclic GMP assay. These drug concentrations were chosen on the basis of pilot experiments. Only one intervention was studied per muscle preparation. The effects of sodium nitroprusside and substance P on contraction and myocardial cyclic GMP content of endocardium-damaged preparations were also studied, and of substance P additionally in the presence of haemoglobin (10 μM), a selective inhibitor of EDRF activity and scavenger of nitric oxide (Martin, Villani, Jothianandan & Furchgott, 1985).

Effects of cyclic GMP-elevating agents on cultured cells. Following incubation of the cultured endothelium and endocardium for 90 min with 2 ml Krebs solution, sodium nitroprusside (10 μM) or ANP (0·1 μM) were each added to the culture wells for 3 min. In some experiments haemoglobin (10 μM) was added to the wells 20 min before the nitroprusside or ANP.

Acetylated low-density lipoprotein uptake by cultured cells. Acetylated low-density lipoprotein labelled with 1,1-dioctadecyl-1-3,3,3′3′-tetramethylindocarbocyanine perchlorate (Di I-Ac-LDL; 10 μg ml^{-1}), was dissolved in complete medium, 2 ml added to each well containing confluent endothelium or endocardium, and incubated with the cells for 4 h at 37 °C as described previously (Voyta, Via, Butterfield & Zetter, 1984). Cells were washed with isotonic saline, fixed for 20 min in phosphate-buffered formalin, washed again with isotonic saline, and the fluorescence examined with a Nikon Diaphot-TMD inverted microscope using an excitation wavelength of 535 nm.

Bioassay studies. Papillary muscle bioassay studies were performed in one of two ways. Effluent from naked or cell-covered beads was either collected and transferred as a single volume (following temperature, pH and P_{O_2} equilibration) to a 10 ml tissue bath containing an endocardium-denuded papillary muscle, i.e. complete bath transfer, or was continuously infused directly into the bottom of the tissue bath at 3 ml min^{-1}. Using this second method the delay between the column of cells (or naked beads) and the bath was 11 s; the bath contents overflowed slowly and this resulted in a complete bath exchange over approximately 3·5 min. No changes in temperature (29 °C) of the tissue bath occurred during infusion at this relatively slow rate. For these direct infusion experiments, freshly warmed and oxygenated Krebs–Ringer solution perfused over naked microcarrier beads was continuously infused into the bath before switching over to the effluent from the cell-covered microcarrier beads.

For the vascular bioassay experiments, effluent from the cells (or naked beads) was allowed to superfuse an endothelium-denuded pig coronary artery ring preparation mounted as previously described (Christie & Lewis, 1988). This was partially pre-constricted with prostaglandin $F_{2\alpha}$ (1 μM) and 5-hydroxytryptamine (1 μM) infused distal to the cells. Changes in isometric tension of the coronary ring were recorded on a chart recorder.

Materials

All the chemical agents used in this study were purchased from Sigma Chemical Co. Ltd, Poole except where otherwise stated. Tissue culture reagents were obtained from Flow Laboratories, Rickmansworth. Acebutolol hydrochloride was obtained from May & Baker Ltd, Dagenham, L-*N*-monomethyl arginine from Ultrafine Chemicals, Manchester and Di I-Ac-LDL from Biogenesis Ltd, Bournemouth. Haemoglobin was reduced to the ferrous form as previously described (Smith & Lang, 1990).

RESULTS

Effects of interventions on papillary muscles

Electron microscopic examination of all preparations treated with 0·5% Triton X-100 showed destruction of the endocardial monolayer with characteristic multiple perforations in the endocardial cells. Subjacent myocardial cells were, however, undamaged by this treatment. Only when higher concentrations of Triton X-100 were used (e.g. 2–5%) were signs of damage to the subendocardial myocytes observed.

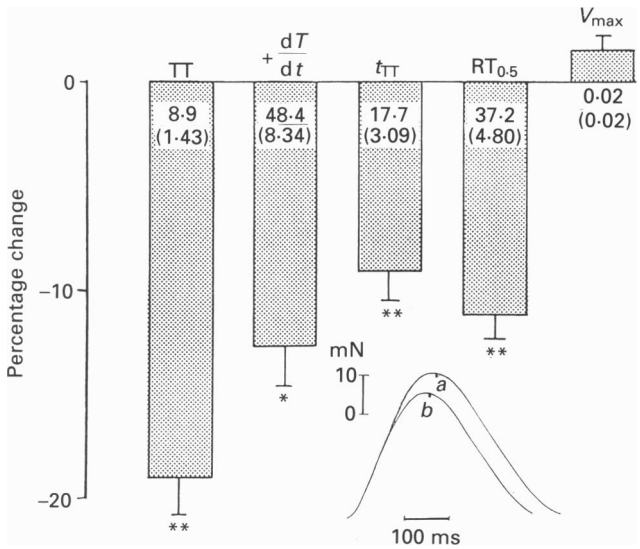

Fig. 1. Mean percentage (±S.E.M.; columns) and absolute (±S.E.M.; figures inside columns) changes in peak isometric twitch tension (TT; mN mm^{-2}); maximum rate of isometric tension development ($+dT/dt$; mN mm^{-2} s^{-1}); time to peak isometric twitch tension (t_{TT}; ms); time to half-isometric twitch tension decline ($RT_{0·5}$; ms); and maximum velocity of unloaded shortening (V_{max}; muscle lengths (ML) s^{-1}) of isolated ferret papillary muscle preparations following selective endocardial removal (*$P < 0·05$; **$P < 0·01$; comparisons on absolute values by Student's t test; $n = 15$). The inset shows representative isometric twitches of a ferret papillary muscle before (*a*) and after (*b*) selective endocardial removal with 0·5% Triton X-100.

Selective endocardial damage to endocardium of the papillary muscle preparations resulted in a characteristic alteration of contractile twitch (Fig. 1) similar to that previously described in cat papillary muscles (Brutsaert *et al.* 1988) with reduction in twitch duration and peak isometric tension but no change in V_{max}. The threshold voltage for stimulation of the muscle did not alter after this intervention.

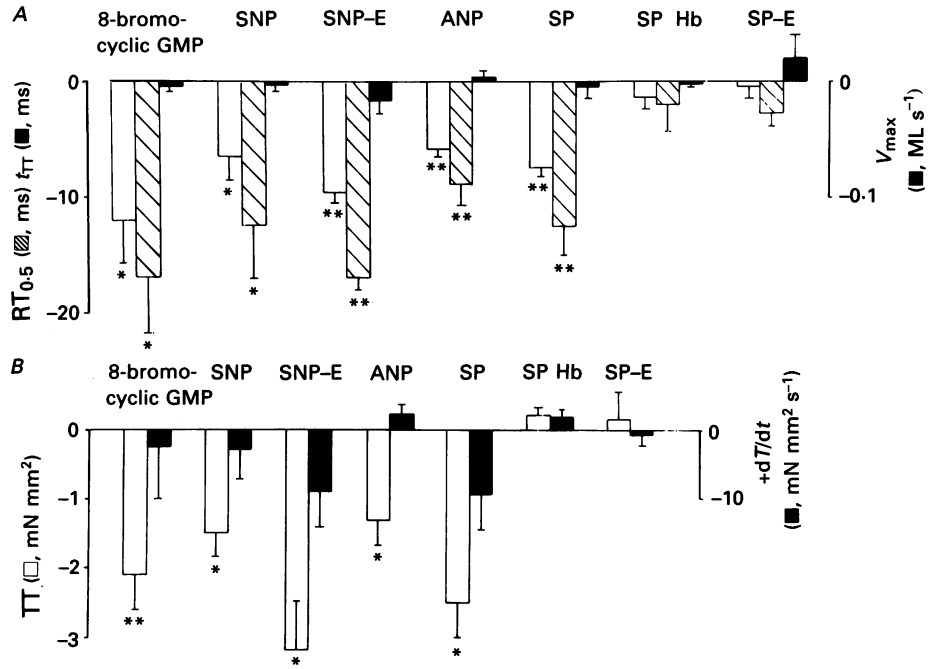

Fig. 2. Mean (±S.E.M.) change in t_{TT}, $RT_{0\cdot5}$, V_{max} (*A*), TT, $+dT/dt$ (*B*) of ferret papillary muscle preparations following incubation with 8-bromo-cyclic GMP (0·1 mM); sodium nitroprusside (SNP; 1 μM); atrial natriuretic peptide (ANP; 0·4 μM); and substance P (SP; 1 μM). Substance P had no effect on contraction of endocardium-denuded preparations (−E), or in the presence of haemoglobin (Hb; 10 μM) (*$P < 0\cdot05$; **$P < 0\cdot01$, cf. baseline values; Student's t test; $n > 5$). Abbreviations and conventions as for Fig. 1.

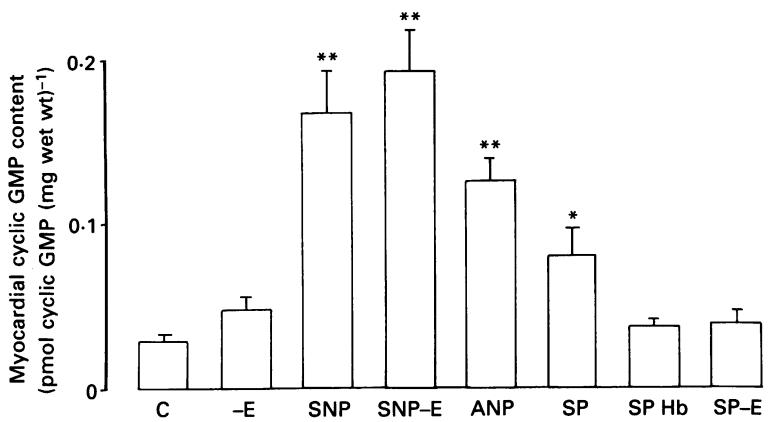

Fig. 3. Mean (±S.E.M.) myocardial cyclic GMP content of ferret papillary muscles. Cyclic GMP levels were similar with (C) or without (−E) endocardium but were significantly increased in endocardium-intact preparations 10 min after addition of sodium nitroprusside, ANP or substance P, and in endocardium-denuded muscles after SNP. In the presence of haemoglobin or in endocardium-denuded muscles, cyclic GMP levels after addition of substance P were similar to baseline values. Concentrations of drugs and conventions as in Fig. 2 (*$P < 0\cdot05$, cf. baseline values; ANOVA; $n > 5$).

In endocardium-intact preparations, sodium nitroprusside, ANP and substance P each induced rapid changes in contractile behaviour similar to 8-bromo-cyclic GMP and to selective endocardium removal (Fig. 2). The effects of substance P on contraction were blocked by the EDRF inhibitor haemoglobin or by previous removal of endocardium. By contrast, the 'contraction-abbreviating' effect of sodium nitroprusside was still observed in endocardium-damaged muscles (Fig. 2).

Myocardial cyclic GMP levels in endocardium-intact preparations were significantly increased by sodium nitroprusside, ANP or substance P (Fig. 3). In the presence of haemoglobin or following endocardium removal, substance P failed to increase cyclic GMP levels above baseline values, but sodium nitroprusside still induced a significant increase in endocardium-damaged muscles. Myocardial cyclic GMP levels did not alter following endocardium removal alone (Fig. 3).

Morphological and biochemical characterization of cultured endocardium

When viewed by phase-contrast microscopy, endocardial cells appeared slightly larger and more granular than endothelial cells. In both cell types, more than 98% of the cells were observed to fluoresce following incubation with the fluorescently labelled acetylated low-density lipoprotein Di I-Ac-LDL. Endothelium and endocardium showed similar rates of growth in culture, but endocardium had a greater tendency to transform to fibroblast-like cells. No myocytes were observed in the endocardial cultures.

Figure 4*A* and *B* shows the effects of stimulants of soluble and particulate guanylate cyclase on the cyclic GMP content of endothelial and endocardial cells. Endothelial cells responded to both sodium nitroprusside and ANP with an increase in cell cyclic GMP content (Fig. 4*A*). Haemoglobin pre-treatment inhibited the sodium nitroprusside-induced increase, but did not affect the ANP-induced increase. The cyclic GMP content of the medium bathing the cells did not change with any of the drug treatments.

Endocardial cells did not respond to nitroprusside, but did respond to ANP with an increase in cell cyclic GMP content (Fig. 4*B*). The ANP-induced increase was not inhibited by haemoglobin pre-treatment, and there was no change in the cyclic GMP content of the medium with any of the drug treatments.

Cultured endocardial cell bioassay

In bioassay experiments (Fig. 5) the cultured endocardial cells released a vasodilator substance under basal conditions, the action of which was increased when superoxide dismutase (30 U ml^{-1}) and catalase (100 U ml^{-1}) (which increase the half-life of EDRF (Gryglewski, Palmer & Moncada, 1986) and prevent effects from hydrogen peroxide generated by superoxide dismutase respectively) were added to the cell superfusate. Addition of bradykinin (0·1 μM) which releases EDRF from vascular endothelium (Furchgott, Zawadzki, Jothianandan & Cherry, 1982) also enhanced the vasodilator effects of the cell effluent. The vasorelaxant effect of the cell effluent was inhibited, however, by perfusion of the cells with either L-*N*-monomethyl arginine (*L*-NMMA) or N^{ω}-nitro-L-arginine, inhibitors of EDRF production (Palmer, Rees, Ashton & Moncada, 1988; Moore, al-Swayeh, Chong, Evans & Gibson, 1990) but was restored by perfusing the cells with the amino acid

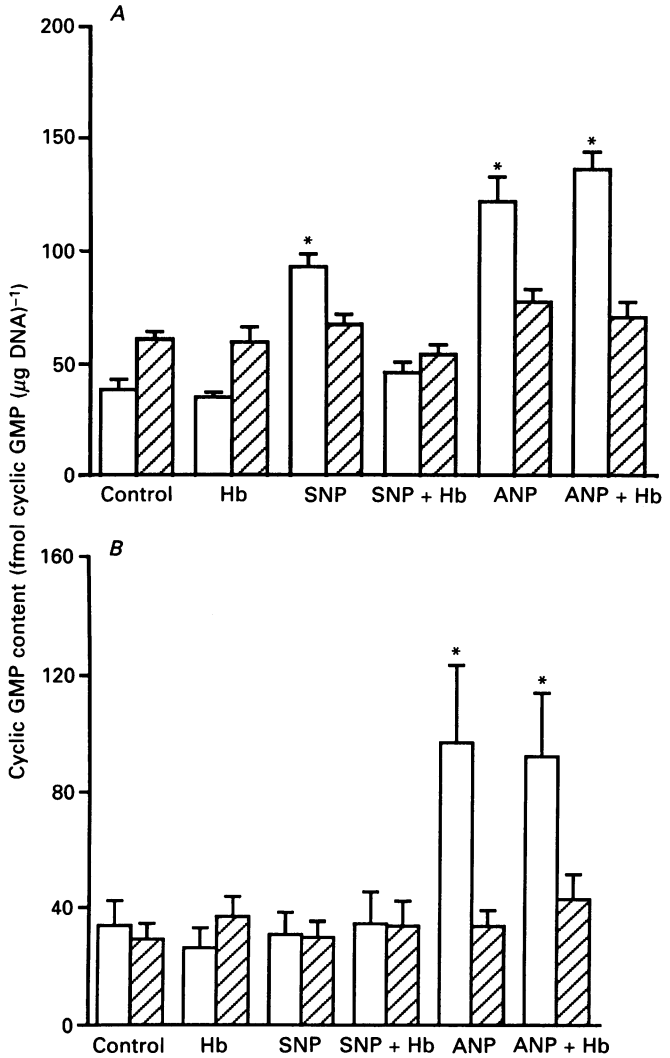

Fig. 4. *A*, mean (±S.E.M.) cyclic GMP content (fmol $(\mu g\ DNA)^{-1}$) of porcine endothelial cells (open columns) and their bathing medium (hatched columns). The pairs of columns show basal cyclic GMP content (control) and following pre-treatment with haemoglobin (Hb), sodium nitroprusside (SNP), sodium nitroprusside and haemoglobin (SNP + Hb), atrial natriuretic peptide (ANP), or atrial natriuretic peptide + haemoglobin (ANP + Hb) (*$P < 0.001$, cf. control; $n > 8$). *B*, mean (±S.E.M.) cyclic GMP content (fmol $(\mu g\ DNA)^{-1}$) of porcine endocardial cells (open columns) and their bathing medium (hatched columns). The pairs of columns show basal cyclic GMP content (control) and following pre-treatment with Hb, SNP, SNP + Hb, ANP or ANP + Hb. Conventions as in *A*. (*$P < 0.05$, cf. control; $n > 8$).

L-arginine, the precursor of EDRF (Palmer, Ashton & Moncada, 1988). Vasodilatation was also abolished by including haemoglobin (0·5 μM) in the cell superfusate (data not shown). The extent of relaxation observed varied with different

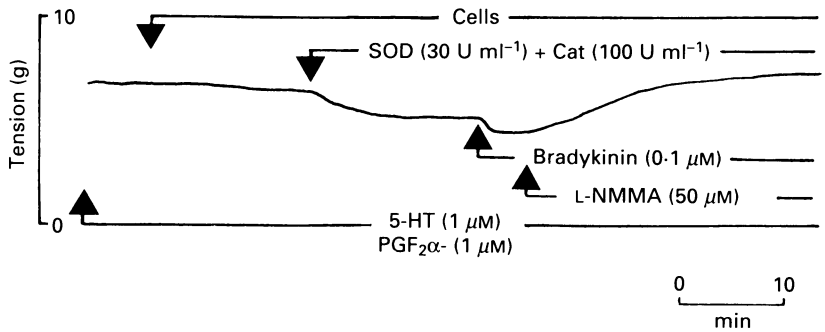

Fig. 5. Representative recording of changes in tension of a partially pre-constricted, endothelium-denuded pig coronary artery ring superfused with effluent from a column containing cultured porcine right ventricular endocardial cells. The small decline in tension on exposure to cell effluent was increased on addition of superoxide dismutase (SOD; 30 U ml^{-1}) and catalase (Cat; 100 U ml^{-1}) to the superfusing fluid. Relaxation was further enhanced when the cells were superfused with bradykinin (0·1 μM), and was reversed following superfusion of the cells with L-*N*-monomethyl arginine (L-NMMA; 50 μM).

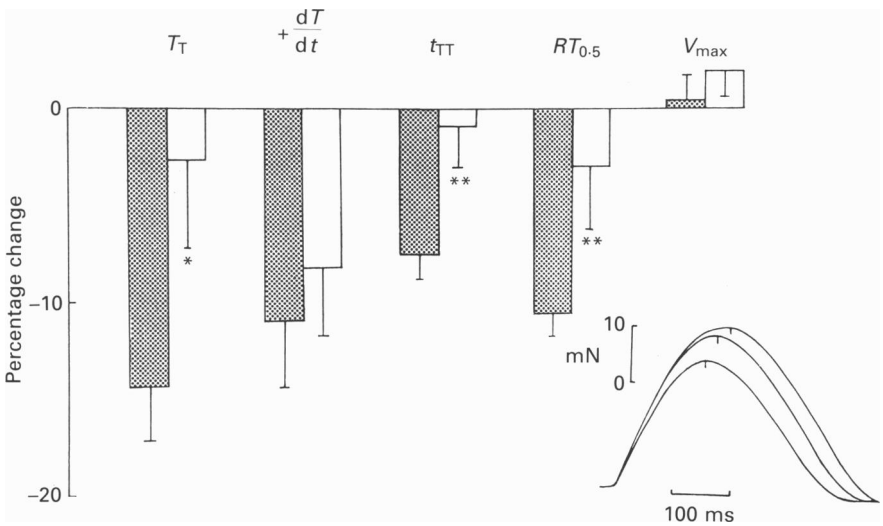

Fig. 6. Mean (±S.E.M.) percentage change compared with baseline values in TT, $+dT/dt$, t_{TT}, $RT_{0.5}$ and V_{max} of ferret papillary muscles following endocardial removal (stippled columns) and following addition of effluent from cultured porcine endocardial cells ($n = 6$; open columns) using the technique of single bath-volume replacement ($n = 4$) or direct infusion from the cell column ($n = 2$). Note the recovery in TT, t_{TT} and $RT_{0.5}$. Abbreviations as for Fig. 1 (*$P < 0.05$; **$P < 0.01$, cf. values after endocardial removal; comparisons on absolute values by Student's t test). The inset shows representative isometric twitches of a ferret papillary muscle before endocardial removal (upper trace); after selective endocardial removal (lowest trace); and following replacement (by infusion) of tissue bath fluid with effluent from column containing porcine cultured endocardial cells (middle trace). Vertical lines indicate time of peak isometric tension.

batches of endocardial cells but was seen every time during twenty experiments, always being reversed with L-NMMA ($n = 18$) and in two experiments with N^{ω}-nitro-L-arginine (20 μM).

In bioassay experiments using endocardium-denuded ferret papillary muscles as recipients, transfer of endocardial cell effluent to the papillary muscle bath, either by a single bath-volume transfer or by direct infusion, resulted in a similar effect on contraction in every experiment (Fig. 6). There was a reversal, either complete or partial, of the effects of selective endocardial removal. No such effect was observed with effluent from naked beads. Note that there was no change in V_{max} after addition of endocardial cell effluent, but significant recovery of TT, t_{TT} and $RT_{0.5}$. Peak rate of tension development did not recover to the same extent as the other indices.

With single bath-volume replacement, the effect of endocardial cell effluent was apparent within 30 s and peaked at approximately 2 min, thereafter declining gradually and usually disappearing by 7–10 min. In direct infusion experiments, changes in contraction were apparent about 3 min after commencement of infusion and peaked at about 6–8 min, thereafter remaining unchanged for up to 30 min. Exposure of the endocardial cells for 30 min to N^{ω}-nitro-L-arginine did not alter the subsequent response of denuded papillary muscles to the cell effluent ($n = 3$).

DISCUSSION

This study shows that the characteristic 'contraction-abbreviating' effect of selective endocardium removal is mimicked by, but not itself associated with, elevation of myocardial cyclic GMP. It also shows that an EDRF-like agent can be released from endocardium of ferret papillary muscles (by substance P) and from cultured endocardial cells of the pig (by bradykinin); in the papillary muscles EDRF results in elevation of myocardial cyclic GMP. Furthermore, the results demonstrate for the first time that cultured porcine endocardial cells also release an as yet unidentified factor(s) which can reverse most of the effects of selective endocardium removal on myocardial contractile performance. Because 'endocardium-derived contraction prolonging factor' is rather cumbersome, we propose that the substance be named 'endocardin' until it is identified.

The effect of endocardium removal on myocardial contraction was similar to that previously described by Brutsaert and colleagues in cat papillary muscles (Brutsaert *et al.* 1988). The changes resulting from endocardial removal are unlikely to be due to inadvertent myocardial damage for a number of reasons. Firstly, the myocardium appeared morphologically normal in transmission electron microscopy. Secondly, there was no significant reduction in V_{max}. Thirdly, the pattern of change in contraction was 'physiological' in that its magnitude was related to underlying conditions. We have previously reported that endocardium removal caused a greater reduction in TT and $+dT/dt$ at low concentrations of extracellular calcium, than at high concentrations of extracellular calcium when contraction was augmented and relatively slower (Shah, Smith & Lewis, 1990). Duration of contraction was however reduced to a similar extent at all calcium concentrations studied. Fourthly, no change in resting tension nor in stimulation threshold was observed in any muscle preparation following endocardial removal. Fifthly, the magnitude of the observed change was independent of muscle cross-sectional area. Sixthly, once established the phenomenon was completely irreversible and was not followed by further deterioration in t_{TT} or TT. Seventhly, a second exposure to 0·5% Triton X-100 resulted in quite different contractile effects (a large decrease in V_{max} and peak

performance without change in twitch duration) due to damage of subendocardial myocytes. Each of these features argues against true myocardial depression.

The phenomenon is also unlikely to be a cyclic GMP-mediated effect since the myocardial levels of cyclic GMP did not change following endocardium removal. It is likely however that the contractile changes are the result of loss of tonic release of a contraction-prolonging factor following endocardial damage. This was confirmed in the bioassay experiments when reversal of the effect of endocardium removal was seen.

Since in the bioassay experiments porcine cultured endocardial cells were used as the 'donor' tissue, it was important to establish firstly the purity of the cell cultures used and secondly whether these cells differed in any way from cultured endothelium from the same species. Evidence that the cultures were not contaminated by myocytes is provided by the observation that none were detected on light microscopic examination of the cultures. Furthermore fluorescently acetylated LDL compound was incorporated into 98% of the cells, a feature not seen with myocytes, but characteristic of endothelial cells and macrophages (Voyta *et al.* 1984). Endothelial cells and endocardial cells were similar in their uptake of fluorescently acetylated LDL and in forming a 'cobblestone-like' monolayer in culture. However, microscopic and biochemical differences were observed between cultured endothelial and endocardial cells. The latter were slightly larger and more granular and unable to respond, in terms of cyclic GMP elevation, to a stimulant of soluble guanylate cyclase, i.e. sodium nitroprusside. These features therefore provide strong evidence that the endocardial cells used in the bioassay studies were free of contaminating myocytes and were both morphologically and biochemically distinct from cultured porcine aortic endothelial cells.

Neither the nature nor the mechanisms of action of the contraction-prolonging factor 'endocardin' is known at present. The substance is unlikely to be endothelin since this peptide has quite different effects on ferret papillary muscles, causing a symmetrical increase in all contractile parameters including V_{max} but with no change in t_{TT} or $RT_{0.5}$ (Shah, Lewis & Henderson, 1989). The factor is relatively stable and appears to be acting in an opposite manner to the effects of cyclic GMP elevation. The factor released from the porcine cultured endocardial cells successfully reversed the effects of endocardium removal in the ferret muscles indicating that it is unlikely to be species selective. Interestingly, reversal of the changes in dT/dt was not as great as the reversal seen with the other indices of contraction. The reason for this is not known but could be a concentration-related phenomenon, or may represent species differences.

Although endocardium of both ferret and pig could be stimulated to release an EDRF-like agent, basal release could be demonstrated only from the cultured cells. The reason for this difference is unknown but may be related to the presence of flow in the cell column, which is a stimulus for EDRF release from endothelium (Rubanyi, Romero & Vanhoutte, 1986). It is possible in the intact heart that the endocardium might release EDRF in response to the high blood flow it is subjected to during each cardiac cycle.

In view of the relatively small numbers in each experimental group, no attempt was made to correlate changes in myocardial cyclic GMP levels with changes in

contractile performance. Generally, however, each of the agents which increased cyclic GMP caused similar changes in contractile performance. The mechanism of the cyclic GMP effects is unknown at present. The mechanical changes resemble those which occur immediately following a reduction in muscle length (Hibberd & Jewell, 1982; Allen & Kentish, 1985) and in an analogous way may result from a reduced affinity of the contractile proteins for calcium.

The physiological roles of these two endocardial factors in the intact heart are unknown at present. The biological half-life of EDRF is so short that it is unlikely to penetrate the myocardium well enough to significantly influence myocardial contraction, except perhaps where the myocardium is thin, e.g. the atria, parts of the right ventricle and in the fetal heart. EDRF has both platelet anti-aggregatory and anti-adhesive properties (Furlong, Henderson, Lewis & Smith, 1987; Radomski, Palmer & Moncada, 1987) and its main role *in vivo* therefore may be a local one of inhibition of platelet adhesion to the endocardial surface and prevention of thrombus formation in the cardiac chambers, rather than an effect on myocardial contraction. In view of the greater stability of the contraction-prolonging factor, as evidenced by the cell bioassay experiments, it is possible that it could influence myocardial contraction both by direct diffusion and also by transport from the cardiac chambers into the coronary circulation thus reaching the myocardium via this route.

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