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The role of cAMP in the frequency-dependent changes in contraction of guinea-pig cardiomyocytes

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Abstract

Objectives: β-Receptor desensitisation, low basal cAMP, and a negative force–frequency relationship are characteristic changes in human heart failure. Isolated cardiomyocytes from noradrenaline-treated guinea pigs also show these features. We tested the hypothesis that low basal cAMP underlies the loss of contractile response to increasing stimulation frequency in this model. **Methods**: Isolated cardiomyocytes were obtained from noradrenaline-treated (NA) and sham-operated (SHAM) guinea pigs. They were stimulated from 0.1–2 Hz and contraction amplitude was monitored with a video edge-detection system. **Results**: NA cells had less positive amplitude–frequency responses (AFR) compared to SHAMs at 2 mM ($P = 0.002$, $n = 17$), or midrange Ca^{2+} concentrations (EC40-EC60) ($P < 0.001$, n = 13). When the cAMP agonist, 8-CPT-cAMP (CPT, 10 μM) or high Ca^{2+} (above EC75) was added to NA cells the AFR was normalised to that of SHAM myocytes (NA vs. SHAM P = ns). In control experiments the cAMP antagonists, Rp-cAMPS (Rpc) and Rp-8-CPT-cAMPS (Rp8, 100 μM), blocked the positive inotropic effects of CPT at 0.5 Hz (control $pD_2 = 4.36 \pm 0.06$, Rp8 $pD_2 = 3.68 \pm 0.08$, $P < 0.0001$, $n = 6$ paired). Rpc (100 μM) completely but reversibly blocked the effect of maximal isoprenaline in control experiments ($P < 0.0001$). Neither antagonist reduced the AFR compared to time-matched controls (P = ns, $n = 6$). Blockade of SERCA2a with thapsigargin resulted in a significant reduction in the AFR (ANOVA $P < 0.0001$). **Conclusions**: The results are consistent with sarcoplasmic reticulum (SR) function being a more important determinant of the amplitude–frequency relationship than tonic levels of cAMP under basal conditions. Reversal of AFR depression by CPT may result from stimulation of SR Ca^{2+} uptake. © 1998 Elsevier Science B.V.

Keywords: Heart failure; Myocyte; Basal cAMP; Contractile function; Guinea pig; Rp-cAMPS

1. Introduction

The loss of the positive force–frequency response or Bowditch Treppe in failing human myocardium has been well established in vivo [1,2], and in muscle strips [3–6]. We have shown the same phenomenon in isolated human myocytes [7], indicating that this is a cellular effect, independent of extracellular conditions such as fibrosis, loading conditions or other haemodynamic variables.

The mechanisms for impaired frequency-dependent responses in human heart failure are linked to abnormal calcium handling [8], are closely related to SR Ca^{2+}-ATPase (SERCA2a) activity [9] and may be mimicked by blockade of SERCA2a with thapsigargin [10]. However, while SERCA2a activity is reduced in heart failure, protein levels are found by some to be unchanged [11,12].

The positive force–frequency relationship can be restored in muscle strips from failing myocardium with isoprenaline, although only at low concentrations [13], or by forskolin [14]. In addition, normal β-adrenergic dependent increases in force–frequency response are impaired in heart failure models [15]. This has lead to the hypothesis

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that the force–frequency response in non-failing hearts is positive due to tonically high levels of cAMP under basal conditions. High cAMP leads to PKA dependent phosphorylation of phospholamban and L-type Ca^{2+} channels and this may be further increased during exercise or conditions of stress by β-agonist induced cAMP formation [16]. In heart failure there are reduced levels of basal cAMP [17–19] and this may lead to reduced phospholamban phosphorylation and low SERCA2a activity [11] and therefore a flattening of the force–frequency relationship.

Low levels of cAMP are likely to be due to β-adrenoceptor desensitisation with concomitant downregulation of β_1-receptor number, increases in Gi and reduced adenylate cyclase activity [20–24]. In heart failure, β-receptor desensitisation and reduced basal cAMP are thought to be a consequence of chronic sympathetic activation with resulting increases in noradrenaline levels — a hormone which has a strong inverse correlation with prognosis.

In normal guinea pig myocytes there is also evidence for a central role for cAMP in the regulation of the frequency-dependent changes in calcium current [25]. The noradrenaline-treated guinea pig is a model of β-receptor desensitisation in which we have previously demonstrated that myocytes have low basal cAMP and blunted amplitude–frequency responses [26,27]. This model has a reduced contractile response to isoprenaline which may be partially overcome with phosphodiesterase inhibition but retains the same response to increasing extracellular calcium as controls [28]. In noradrenaline-treated guinea pigs, basal cAMP levels in yield-matched preparations were 20.1 ± 3.6 pmol mg^{-1} protein in controls and 8.8 ± 0.9 pmol mg^{-1} protein in noradrenaline-treated myocytes ($P < 0.02$, $n = 7$) [26]. This reduction in basal cAMP is comparable to that seen in human heart failure [24].

We sought to test the hypothesis that low basal cAMP underlies the flat frequency response in this model by examining the negative effect of chronic in vivo noradrenaline treatment on contraction amplitude of isolated myocytes at various stimulation frequencies (amplitude–frequency response — AFR) and attempting to reverse the observed changes by stimulating protein kinase A with the cAMP analogue 8-CPT-cAMP (CPT). In addition we blocked protein kinase A with the membrane permeable cAMP analogues Rp-cAMPS (Rpc) and Rp-8-CPT-cAMPS (Rp8) to see if this reduced the AFR. We established in control experiments their effectiveness in blocking responses to isoprenaline and CPT but not calcium. Finally we determined the effects on the AFR of increasing extracellular calcium and of blocking sarcoplasmic reticulum (SR) calcium re-uptake with thapsigargin.

This study demonstrates that noradrenaline treatment is effective in reducing the amplitude–frequency response in guinea pig myocytes and this could be reversed by supplementing basal cAMP with CPT or by increasing extracellular calcium. Concentrations of cAMP antagonists capable of completely blocking the maximum contraction with isoprenaline had little effect on the AFR under basal conditions. This was in contrast to thapsigargin, which was effective in reducing the AFR.

2. Methods

2.1. Treatment of animals with noradrenaline

Osmotic mini-pumps (model 2001, Alzet, Alza Corp., CA, USA) were filled with (−)-noradrenaline bitartrate dissolved in sterile isotonic saline containing 1 mM ascorbate and preincubated in sterile isotonic saline at 37°C for 4 h before implantation. The pumps were implanted subcutaneously in the necks of male Dunkin–Hartley guinea pigs (weight 300–400 g) following sedation with 2% Hypnorm (0.5 ml kg^{-1}) and local anaesthesia with 2 ml kg^{-1} of lignocaine hydrochloride. A 2 cm incision was made in the skin between the scapulae and a small pocket was formed by spreading apart the subcutaneous tissues. The pump was inserted into the pocket with the flow moderator pointing away from the incision. The skin was then closed with sutures. The infusion rate from the mini-pump was 0.97 μl h^{-1}, delivering 700–900 μg kg^{-1} h^{-1} of noradrenaline for a period of up to seven days. Sham-operated guinea pigs underwent an identical operative procedure except for the implantation of a pump. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8523, revised 1985).

2.2. Isolation of animal ventricular myocytes

After 6–7 days of noradrenaline treatment the guinea pigs were sacrificed by cervical dislocation following intraperitoneal administration of heparin (1000 U kg^{-1}). The hearts were quickly removed and placed into ice-cold Krebs–Henseleit (KH) solution (119 mM NaCl, 1.0 mM $CaCl_2$, 4.7 mM KCl, 0.94 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.5 mM glucose, previously bubbled with 95% O_2, 5% CO_2 to maintain the pH at 7.4 and containing 1 mM Ca^{2+}) with 1000 U added heparin.

Single cardiac myocytes were prepared by enzymatic dissociation by retrograde perfusion of the heart via the aorta with a modified Langendorff technique. The heart was first perfused with a low Ca^{2+} solution (120 mM NaCl, 5 mM $MgSO_4$, 12–15 μM Ca^{2+}, 5 mM pyruvate, 20 mM taurine, 10 mM HEPES and 20 mM glucose in AnalaR water and bubbled with 100% O_2) for 5 min. This was followed by protease and collagenase plus hyaluronidase digestion according to our previously described protocol [29]. The cell suspension was filtered through a 300 μm gauze. Undigested tissue was given further collagenase digestion. The filtrate was then centrifuged gently and the pellet was washed and resuspended in modified low Ca^{2+} solution containing 200 μM Ca^{2+}.

2.3. Contraction experiments

Myocytes were placed in a cell bath over an inverted microscope as previously described [29]. The cells were perfused at 2 ml min^{-1} with KH and field stimulated to contract at 0.5 Hz. Experiments involving cAMP antagonists required a modification of the apparatus to allow recirculation of solutions. This was necessary due to the cost of the antagonists. Control experiments were performed to establish the stability of the cells and experimental drugs under these circumstances compared to the standard protocol (not shown). Unloaded shortening was measured using a video edge-detection method. Data on amplitude of contraction, maximal rate of contraction and relaxation, time-to-peak contraction (TTP), time-to-50% relaxation (R50) and time-to-90% (R90) relaxation were collected.

Concentration–response curves for isoprenaline and the cAMP agonist CPT were established in noradrenaline treated (NA) and sham operated (SHAM) guinea pigs and the maximum cell shortening was expressed as a fraction of the response to maximal calcium (isoprenaline:calcium and CPT:calcium ratios). This was used as a measure of β-receptor and post-receptor desensitisation respectively. Maximum isoprenaline and CPT were defined by the concentration at which there was a less than 10% increase in contraction for a half log increment in agonist concentration or the concentration at which an arrhythmia developed in which case peak contraction prior to the arrhythmia was taken as maximum shortening. Maximum calcium was defined in the same way except using 2 mM increments in concentration. The negative logarithms of the concentrations evoking a 50% of maximum response to isoprenaline (pD_2) or CPT were calculated for NA and SHAM concentration–response curves.

Control experiments were performed to establish that the two antagonists, Rpc (100 μM) and Rp8 (100 μM), blocked the positive inotropic effects of both the cAMP agonist, CPT, and isoprenaline. Amplitude–frequency response (AFR) curves were obtained in NA and SHAM cells by measuring contraction at increasing stimulation frequencies (0.1, 0.2, 0.33, 0.5, 0.66, 1, 1.42 and 2 Hz).

2.4. Statistics

Contraction data were normalised to the length of each cell measured (% shortening) and expressed as means and standard errors of mean. All *n* numbers refer to numbers of animals. Where experiments have been repeated in a single animal replicate data have been averaged before further analysis. Student's *t* test was used to compare paired and unpaired group means including pD_2 and isoprenaline:calcium and CPT:calcium ratios. Where multiple comparisons are made Bonferroni's correction to *P* values has been applied. Repeated measures analysis of variance (RM ANOVA) using STATVIEW software was used in comparisons between amplitude–frequency response curves.

3. Materials

(−)-Noradrenaline bitartrate (norepinephrine), Protease, type XXIV, Hyaluronidase, type I-S and 8-(4-chlorophenylthio) adenosine-3′,5′-monophosphate, were obtained from Sigma Chemicals, Poole, UK and Collagenase type II from Worthington, New Jersey, USA. Mini-osmotic pumps, model 2001 (1.0 μl h^{-1}, 7 days) were obtained from Alzet, Alza Corp., CA, USA. Adenosine-3′,5′-monophosphorothioate, Rp-isomer (Rp-cAMPS), 8-(4-chlorophenylthio) adenosine-3′,5′-monophosphorothioate, Rp-isomer (Rp-8-CPT-cAMPS), were obtained from Biolog, Bremen, FRG.

4. Results

4.1. Effect of noradrenaline treatment on isoprenaline:calcium and CPT:calcium ratios

As we have previously shown, exposure to noradrenaline in vivo for 7 days produced marked β-adrenoceptor desensitisation in myocytes isolated from hearts of NA-treated animals. In the present series, the ratio between maximum response to isoprenaline to maximum Ca^{2+} was reduced from 0.92 ± 0.05 in cells from sham-operated animals (SHAM, $n = 14$) to 0.63 ± 0.05 in those from noradrenaline-treated guinea pigs ($n = 21$, $P < 0.001$) (Fig. 1a). The isoprenaline concentration–response curve was also right-shifted with pD_2 values 6.89 ± 0.10 in NA ($n = 21$) and 8.74 ± 0.11 in SHAMs ($n = 8$, $P < 0.001$). Noradrenaline-treated cells required more isoprenaline to achieve a maximal response (SHAM, $-\log[\text{Iso}]_{max} = 8.45 \pm 0.27$, NA, $-\log[\text{Iso}]_{max} = 6.51 \pm 0.24$, $P < 0.01$). There was no significant reduction in the maximum response to maximum Ca^{2+} in NAs at the baseline stimulation frequency (0.5 Hz) and there was no difference in the concentration of calcium required to achieve a maximal response (SHAM, 6.84 ± 0.55 mmol/l; NA, 7.54 ± 0.55 mmol/l, P = ns). The NA animals used in the present study therefore show a β-adrenoceptor desensitisation quantitatively similar to those we have previously described [26,30].

The maximum response to the cAMP analogue CPT was also reduced in myocytes from NA guinea pigs (CPT:calcium ratios SHAMs 0.89 ± 0.10, NA 0.61 ± 0.05, $n = 6$, $P < 0.05$). Noradrenaline-treated myocytes required a higher concentration of CPT to achieve a maximal contractile response (SHAM, $-\log[\text{CPT}]_{max} = 4.20 \pm 0.12$; NA, 3.66 ± 0.14, $P = 0.01$). As shown in Fig. 1b, the quantitative reduction in the CPT:calcium ratio was not significantly different to that of the isoprenaline:calcium

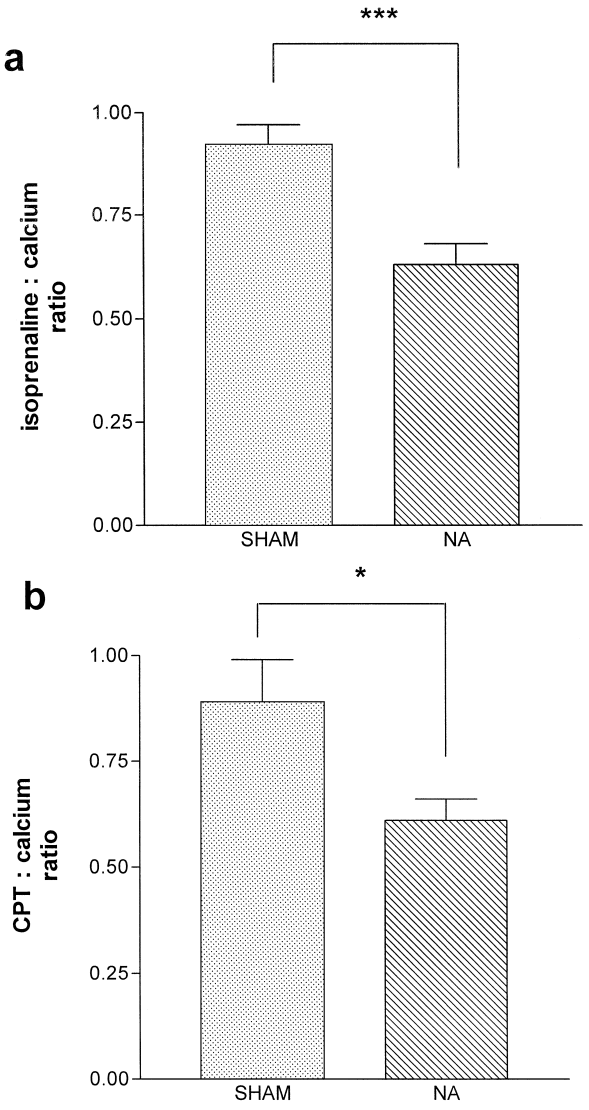

Fig. 1. (a) Maximum response to isoprenaline expressed as a fraction of maximum response to Ca^{2+} (isoprenaline:calcium ratio), (NA $n = 21$ vs. SHAM n = 14, $^{***}P < 0.001$). ($[Ca^{2+}]_{max}$ = SHAM, 6.84 ± 0.55 mmol/l; NA, 7.54 ± 0.55 mmol/l, P = ns. $-\log[Iso]_{max}$ = SHAM, 8.45 ± 0.27, NA, 6.51 ± 0.24, $P < 0.01$). (b) CPT:calcium ratio. Maximum response to CPT as a fraction of maximum response to Ca^{2+} (NA $n = 7$ vs. SHAM $n = 6$, $^{*}P < 0.05$). SHAM, $-\log[CPT]_{max} = 4.20 \pm 0.12$; NA, 3.66 ± 0.14, $P = 0.01$. Bars represent mean $\pm$ s.e. mean.

ratio. This suggests there is a post-receptor reduction in sensitivity in the NA guinea pig myocytes compared to SHAMs distal to cAMP generation.

4.2. Effect of noradrenaline treatment on amplitude–frequency response (AFR) and normalisation with CPT

Comparison of the AFRs of NA and SHAM animals showed a significant reduction in shortening at high frequencies in noradrenaline-treated animals (repeated measures ANOVA $P < 0.001$). The experiments shown in Fig. 2a. were at intermediate Ca^{2+} concentrations (defined as between the EC40-EC60 for Ca^{2+} response, mean Ca^{2+} 6.52 ± 2.02 in noradrenaline-treated animals and 6.35 ± 0.41 in controls, P = ns). A similar reduction in AFR was seen at 2 mM Ca^{2+} ($P < 0.01$, $n = 17$). Addition of 10 μM CPT (a threshold concentration for increases in contraction in normal myocytes at 0.5 Hz — see Fig. 3) increased the AFR in both SHAM and NA cells, abolishing the initial difference between these groups (Fig. 2a). On repeating the experiments at higher Ca^{2+} concentrations (> EC 75 for Ca^{2+} response) there was no difference between NA and SHAMs, showing that increasing extracellular Ca^{2+} also normalised frequency responses (Fig. 2b).

4.3. Effect of cAMP antagonists on concentration–response curves to CPT

Control experiments were designed to demonstrate the action of the cAMP antagonists in blocking responses to the agonist CPT and isoprenaline (but not maximum Ca^{2+}) to confirm the site of action as PKA. Each experiment consisted of an initial control CPT concentration–response curve (control 1) followed by either a second time-matched control curve (control 2) or one in the presence of Rp8 (100 μM) or Rpc (100 μM). There was no significant difference between the first and second control concentration–response curves to CPT either when comparisons were made for group data or when results were paired for

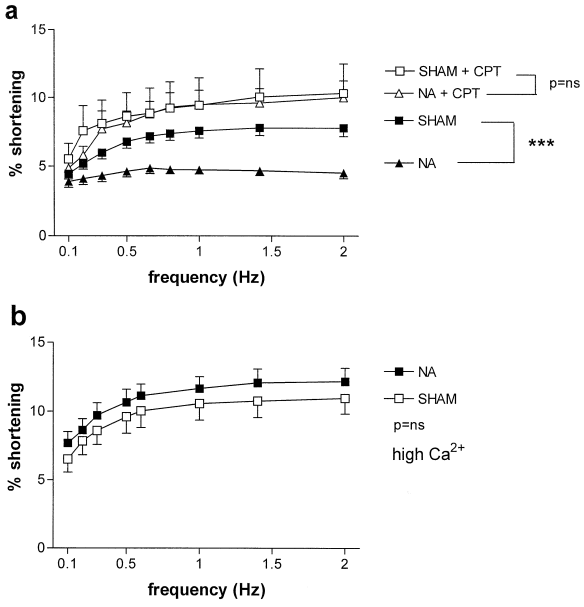

Fig. 2. (a) The amplitude–frequency response in myocytes from noradrenaline-treated guinea pigs and sham-operated controls (SHAM $n = 22$, NA $n = 13$, repeated measures (rRM) ANOVA $^{***}P < 0.001$) and in the presence of 10 μM CPT (a cAMP agonist). Stimulation frequency is plotted against contraction amplitude as a percentage of cell length (% shortening) which is expressed as mean $\pm$ s.e. mean. (b) AFR of NA and SHAM cells at high Ca^{2+} (SHAM, mean $Ca^{2+} = 6.52 \pm 2.02$ mmol/l in NAs, $n = 9$ and 6.35 ± 0.41 mmol/l in controls, $n = 7$, P = ns).

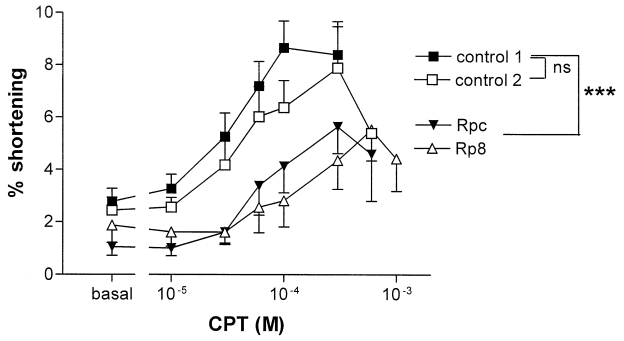

Fig. 3. CPT concentration–response curves. CPT (M, log scale) is plotted against % shortening expressed as means ± s.e. mean. Control 1: initial control curve. Control 2: second control curve time-matched to antagonist experiments (control 1 vs. control 2, P = ns). Rpc: CPT concentration–response curve in the presence of 100 μM Rp-cAMPS. Rp8: CPT concentration–response curve in the presence of 100 μM Rp-8-CPT-cAMPS (control1 vs. Rpc or Rp8, $^{***}P < 0.001$, $n = 6$ in each group). Bars represent mean ± s.e. mean.

individual cells. This indicates that there is little time-dependent decrease in response and that prior exposure to CPT does not produce desensitisation of subsequent effects. The presence of either Rpc or Rp8 significantly depressed the concentration–response curve for CPT ($n = 6$ time-matched controls (control 2), 8 Rp8 and 6 Rpc, $P < 0.001$ vs. control 2, RM ANOVA, Fig. 3). At 60 μM CPT (the highest concentration for which there was data for all cells) contraction amplitude (% shortening) was reduced from 6.84 ± 0.54% in the absence of antagonists to 2.56 ± 0.97% with Rp8 ($P < 0.01$) and 3.38 ± 1.12% with Rpc ($P < 0.05$). In addition Rp8 (but not Rpc) significantly shifted the CPT curve to the right (pD_2 control 1 4.50 ± 0.11, Rp8 3.85 ± 0.32, $P < 0.05$).

4.4. Effect of cAMP antagonists on isoprenaline responses

Myocytes stimulated at 0.5 Hz were exposed to increasing concentrations of isoprenaline until a maximal (sufficient to induce an arrhythmia in the cell) or close to maximal (less than 10% increase in contraction with a half log increment in isoprenaline concentration) response was reached. This was achieved with concentrations of isoprenaline ranging from 3 to 30 nM ($-\log[\text{Iso}]_{max} = 8.15 \pm 0.14$, $n = 7$). The basal contraction increased from 2.50 ± 0.44% to 11.5 ± 1.0% with maximal isoprenaline. The effect of adding Rpc (100 μM) was then measured. The maximum reduction in contraction was achieved between 14 and 40 min (mean = 30 ± 4). There was no significant difference between basal contraction and contraction in the presence of Rpc (3.90 ± 0.33%). Returning to the initial concentration of isoprenaline alone, the amplitude rose again to 10.1 ± 0.7% — this was not significantly different from the original maximum (Fig. 4). In two experiments an arrhythmia was induced during the initial isoprenaline response. The maximum contraction immediately prior to the development of the arrhythmia has been taken as the maximum isoprenaline response in the analysis. It is notable that in both these experiments the addition of Rpc abolished the arrhythmia after a few minutes and that arrhythmia returned after the antagonist was washed off. The antagonist was effective in reducing isoprenaline-induced contraction at both 0.5 and 1.42 Hz.

4.5. Effect of cAMP antagonists on response to maximal calcium

To investigate whether the two antagonists might be having direct inhibitory effects on excitation–contraction coupling, cellular responses in high calcium (Ca^{2+} SHAM, 5.82 ± 0.33 mmol/l; NA, 5.64 ± 0.36 mmol/l, P = ns) in the presence and absence of Rpc (100 μM) were compared. After 40 min of stimulation at 0.5 Hz there was no significant reduction in cell shortening (before addition of Rpc 7.07 ± 1.49%, after 40 min Rpc 6.83 ± 1.73%, $n = 5$, P = ns). We concluded that the significant reduction in isoprenaline responses with Rpc were unlikely to be due to changes in excitation–contraction coupling.

4.6. Effect of cAMP antagonists on amplitude–frequency response

SHAM and NA guinea pigs cells were stimulated at increasing frequencies to provide an initial control AFR. This frequency response was then repeated after 40 min of exposure to Rp8, Rpc (100 μM) or no antagonist (time-matched control). The exposure time was determined from the results of the isoprenaline experiments in which the Rpc had taken up to 40 min to produce a maximum

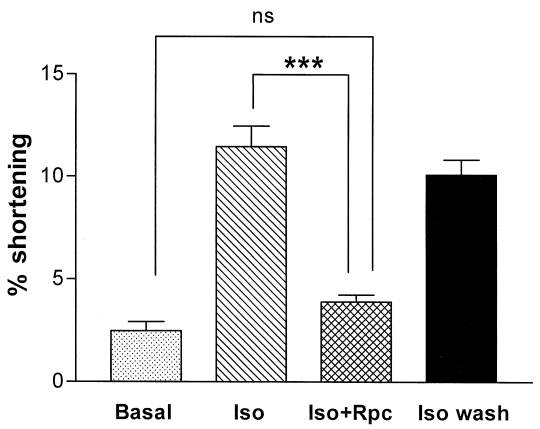

Fig. 4. Bar chart of the effect of Rp-cAMPS (100 μM) on maximal contractile response to isoprenaline. Basal: contraction amplitude (% shortening) in the absence of isoprenaline. Iso: % shortening in the presence of maximal isoprenaline. Iso + Rpc: % shortening after addition of Rp-cAMPS (100 μM) to maximal isoprenaline (Iso vs. Iso + Rpc, $^{***}P < 0.001$, P = ns compared to basal). Iso wash: cells perfused with initial maximum isoprenaline solution ($n = 7$). Bars represent mean ± s.e. mean.

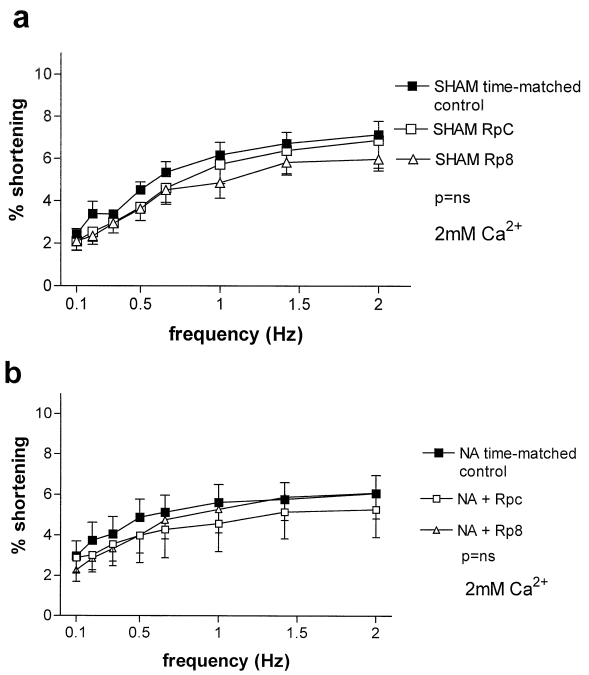

Fig. 5. (a) Effect of Rpc (100 μM) and Rp8 (100 μM) on the AFR in SHAM cells at 2 mmol/l Ca^{2+} compared to time-matched controls (rRM ANOVA, P = ns, $n = 6$). (b) The effect of Rpc and Rp8 on the AFR in NA myocytes at 2 mmol/l Ca^{2+} compared to time-matched controls (RM ANOVA P = ns, $n = 6$). % shortening is expressed as mean ± s.e. mean.

inhibitory effect. Paired tests of the initial control AFRs and the time-matched controls in SHAM and NA groups showed a small but significant reduction with time in contraction amplitude, but not in the slope of the AFR, over 40 min at 2 mM Ca^{2+} (not shown). Cells exposed to antagonists also demonstrated reduced AFRs after 40 min but this effect was not significant when compared to time-matched controls (Fig. 5a). Similarly NA treated animals showed no reduction in AFR compared to time-matched controls at 2 mM Ca^{2+} (Fig. 5b). There was no reduction in AFR with Rpc (100 μM) at high calcium in

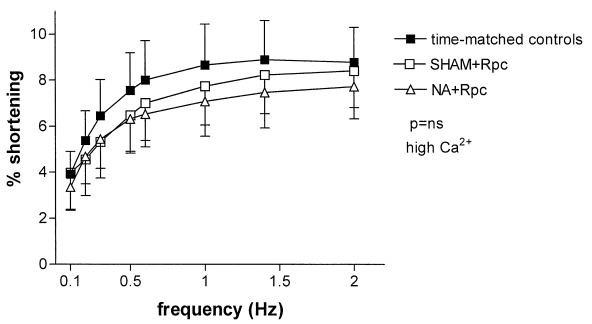

Fig. 6. Effect of Rpc (100 μM) on the AFR of SHAM and NA treated cells at high Ca^{2+} (Ca^{2+} SHAM, 5.82 ± 0.33 mmol/l; NA, 5.64 ± 0.36 mmol/l, $n = 6$, P = ns) compared to time-matched SHAM controls (RM ANOVA, P = ns). Bars represent mean ± s.e. mean.

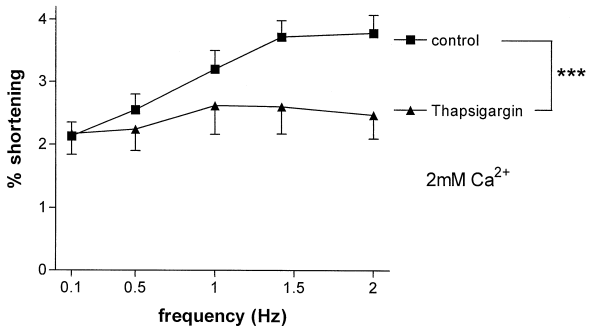

Fig. 7. Effect of thapsigargin on the AFR in normal cells. Control: amplitude–frequency response before thapsigargin. Thapsigargin: AFR in the presence of thapsigargin (3 μM) (RM ANOVA, $P < 0.001$, $n = 12$). % shortening is expressed as mean and s.e. mean.

either NA or SHAM groups (Ca^{2+} SHAM, 5.82 ± 0.33 mmol/l; NA, 5.64 ± 0.36 mmol/l, P = ns, Fig. 6). This suggests that the antagonists have no significant effect on amplitude–frequency response under basal conditions at concentrations capable of blocking completely the effect of maximal isoprenaline.

4.7. The effect of thapsigargin on frequency response

Thapsigargin blocks the SR Ca^{2+}-ATPase (SERCA2a) and therefore prevents re-uptake of cytosolic Ca^{2+} into the sarcoplasmic reticulum. The consequence of this is the removal of the SR as a functional organelle involved in intracellular Ca^{2+} cycling. In this series of experiments there was a marked and significant reduction in the AFR in both amplitude and slope on addition of 3 μM of thapsigargin for 10 min ($n = 12$, $P < 0.001$, Fig. 7). This is consistent with a central role for the SR and SERCA2a in the regulation of the amplitude–frequency response.

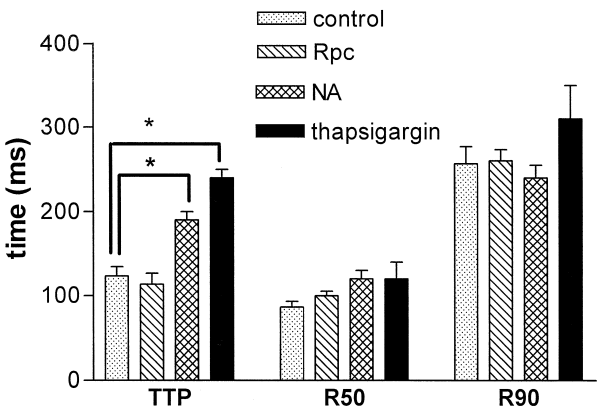

Fig. 8. Bar chart of the effects of Rp-cAMPS (100 μM) in SHAMs, NA treatment and thapsigargin on contraction and relaxation times compared to SHAM controls at 0.5 Hz at 2 mM Ca^{2+}. TTP: time-to-peak contraction in ms (ANOVA $^{*}P = < 0.01$). R50: time to 50% relaxation. R90: time-to-90% relaxation ($n = 6$, P = ns). Bars represent mean ± s.e. mean.

4.8. Time-to-peak contraction and relaxation

Rpc (100 μM) had no effect at any frequency on times-to-peak contraction (TTP), times-to-50% relaxation (R50) and times-to-90% relaxation (R90) in SHAM or NA cells (SHAMs at 0.5 Hz, control and Rpc respectively — TTP 123 ± 11 vs. 113 ± 13 ms, R50 86.7 ± 6.7 vs. 100 ± 5 ms, R90 257 ± 20 vs. 260 ± 13 ms, $n = 6$, P = ns, Fig. 8). This result was consistent at low and high calcium. Noradrenaline treatment and thapsigargin significantly increased the time-to-peak contraction but not the relaxation times compared to controls (TTP-control, 123 ± 11; NA, 190 ± 10, thapsigargin, 240 ± 10, ANOVA $P < 0.01$).

5. Discussion

This study does not support the hypothesis that the reduction in amplitude–frequency response following β-adrenoceptor desensitisation is secondary to low basal cAMP. Although the slope of the AFR was reduced in NA cells and this could be reversed by CPT, raising Ca^{2+} was equally effective in providing reversal. Further, lowering the tonic effect of basal cAMP with PKA antagonists did not mimic the depression in AFR.

5.1. Noradrenaline treatment desensitised responses to isoprenaline and CPT

In this series guinea pigs exposed to noradrenaline treatment had reduced pD_2 values for isoprenaline, required more isoprenaline to achieve a maximum response and had lower isoprenaline:Ca^{2+} ratios, indicating β-receptor desensitisation. Although cAMP was not measured in this study, this desensitisation was quantitatively similar to our previous NA guinea pig series in which a 56% reduction in basal cAMP was demonstrated [26]. This parallels other studies in failing human myocardium where reductions in unstimulated basal cAMP of 60–70% have been shown [19,24]. The contractile response of desensitised guinea pig cells to high Ca^{2+} was unaffected at all frequencies.

Whilst part of the reduction in sensitivity to isoprenaline might be ascribed to β-receptor loss or phosphorylation and increases in Giα, there was also a reduced CPT:Ca^{2+} ratio and this can only be explained in terms of a post-receptor defect. This loss of response to cAMP agonists has been shown before in this model [31] as well as in failing human myocytes [32] and could imply down-regulation of signal transduction distal to cAMP generation, perhaps at the level of SERCA2a or L-type Ca^{2+} channels. An alternative explanation is that failing myocytes and NA treated cells are more sensitive to the induction of arrhythmias which occur at a lower inotropic threshold, effectively limiting the maximum response obtainable in these cells [32].

5.2. CPT and extracellular Ca^{2+} normalised the AFR in NA cells

Supplementing the basal cAMP levels with CPT effectively normalised the slope of the AFR in noradrenaline-treated cells. The concentration of CPT (10 μM) chosen was a threshold one which did not increase contraction amplitude at low stimulation frequencies (see Fig. 3).

CPT was also effective in increasing the slope of the AFR in cells from control animals suggesting a submaximal frequency response under basal conditions in non-desensitised animals. This is consistent with other models in which dobutamine has been used to increase the slope of the force–frequency response in animals without heart failure [16]. High Ca^{2+} also normalised the AFR and the AFRs in CPT and high Ca^{2+} were superimposable (compare Fig. 2a and b). Other investigators have shown in heart failure that a variety of inotropic agents acting through different mechanisms can all increase the slope of the force–frequency relationship-calcium, low dose isoprenaline, ouabain [13], forskolin [14] and the Na^{+} channel opener, BDF 9148 [33] are all effective in muscle strips.

5.3. Rp-cAMPS and Rp-8-CPT-cAMPS

The positive control experiments demonstrated the effectiveness of the two antagonists in blocking the response to isoprenaline and to the cAMP analogue CPT-cAMP. Other investigators [34] have shown significant cAMP-dependent chronotropic but not inotropic responses in guinea pig muscle strips using Rp-cAMPS. In view of the dramatic and consistent effect on β-agonist induced contraction of Rp-cAMPS in our series it may be that previous negative results might be due to poor tissue penetration of protein kinase A blockers. In this series the full effect of blockade took an average of 30 min to develop. Other studies have shown a similar time-course for the action of this compound, which is supposedly readily membrane permeant, in isolated myocytes [35]. It is therefore likely that in muscle strips penetration of protein kinase A blockers would take considerably longer than this.

The slow onset of action made the use of time-matched controls essential because there was a small but significant reduction of contraction amplitude with time (but not of the slope of the AFR). A direct cytotoxic effect of Rp-cAMPS on the myocytes is unlikely in view of the ready reversibility of the blockade on washing off the antagonist.

Rp-cAMPS had no effect on cellular responses at 2 mM and high calcium concentrations suggesting that it is unlikely to be altering excitation–contraction coupling. Both antagonists and the agonist CPT are analogues of cAMP. The consistent results with both antagonists in blocking isoprenaline and CPT induced contraction without affecting calcium responses support the proposed site of action at the level of protein kinase A.

5.4. The effect of PKA blockade on AFR and beat duration

Neither the slope of the AFR, nor times-to-peak contraction or relaxation, were affected by incubation of SHAM and NA myocytes with the cAMP antagonists. This is strong evidence that basal cAMP does not tonically enhance the contraction or relaxation of guinea pig myocytes and that reduction of basal cAMP alone is not sufficient to reduce the AFR. It supports the conclusion that the ability of CPT to normalise AFR in the NA guinea pigs was not related to a reversal of low basal cAMP.

In contrast to the lack of effect of the PKA blockade, both the previous in vivo NA treatment and thapsigargin significantly increased the time-to-peak contraction in addition to reducing the slope of the AFR.

5.5. Thapsigargin and the AFR

Thapsigargin completely suppressed the increase in contraction amplitude at higher stimulation frequencies. The incubation of beating myocytes with 3 μM thapsigargin for 10 min would have been sufficient to empty completely the SR Ca^{2+} stores, so that contraction was occurring under SR independent conditions [10]. Guinea pig myocytes can continue to function under these circumstances because of a strong contribution to Ca^{2+} entry for contraction (which increases on removal of SR activity) and to Na^{+}/Ca^{2+} exchange for relaxation [36]. Thapsigargin mimicked the effect of NA treatment on the AFR of guinea pig myocytes, suggesting that a decrease in SERCA2a activity might underlie the depression on frequency response. We have shown similar effects in myocytes from human ventricle, where thapsigargin flattens the positive AFR in cells from non-failing heart until the curve is superimposable on that of cells from failing myocardium [10]. There is good evidence that a reduction in SERCA2a activity underlies the poor AFR in failing human heart [9,11,37].

We suggest that there is a depression of SR function in the NA-treated guinea pig, and that inotropic interventions such as CPT or increasing Ca^{2+} are able to stimulate the remaining function sufficiently to normalise AFR. Measurement of SR Ca^{2+}-uptake and SERCA2a activity/protein level will be necessary to characterise this model fully. We would also suggest that the effect of isoprenaline [13] or forskolin [14] to restore the AFR or relaxation rate [38] in failing human myocardium may be as a result of stimulation of residual SERCA2a function, rather that a restoration of basal cAMP as has been suggested [16]. It is notable that interventions which raise cAMP were very effective in myocardium from patients with mitral valve disease, in which function was only moderately compromised, but were unable to fully normalise the AFR in severely failing myocardium [13].

5.6. Conclusions

Prolonged exposure to noradrenaline results in depression of the AFR at the single myocyte level. This observation may provide a link between the chronic sympathetic activation in human heart failure and the depression of force–frequency response in failing myocardium. However, the effect on AFR does not appear to be directly related to the low cAMP levels in the hearts of NA-treated guinea pigs, since there was no evidence for tonic support of contraction or relaxation by basal cAMP in untreated animals. It is more likely that a reduction in SR function underlies the changes observed.

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