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Contractile responses to endothelin-1 are regulated by PKC phosphorylation of cardiac myosin binding protein-C in rat ventricular myocytes

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Manuscript Number: JMCC8881R3

Title: Contractile responses to endothelin-1 are regulated by PKC phosphorylation of cardiac myosin binding protein-C in rat ventricular myocytes

Article Type: Resubmission of Rejected Manuscript

Keywords: endothelin; PKC; InsP3; Inositol 1,4,5-trisphosphate; Inotropy; ECC; heart, myofilaments

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Manuscript Region of Origin: BELGIUM

Abstract: The shortening of sarcomeres that co-ordinates the pump function of the heart is stimulated by electrically-mediated increases in $[Ca^{2+}]$. This process of excitation contraction coupling (ECC) is subject to modulation by neurohormonal mediators that tune the output of the heart to meet the needs of the organism. Endothelin 1 (ET-1) is a potent modulator of cardiac function with effects on contraction amplitude, chronotropy and automaticity. The actions of ET-1 are evident during normal adaptive physiological responses and increased under pathophysiological conditions, such as following myocardial infarction and during heart failure, where ET-1 levels are elevated. In myocytes, ET-1 acts through ETA- or ETB-G protein-coupled receptors (GPCRs). Although well studied in atrial myocytes, the influence and mechanisms of action of ET-1 upon ECC in ventricular myocytes are not fully resolved. We show in rat ventricular myocytes that ET 1 elicits a biphasic effect on fractional shortening (initial transient negative and sustained positive inotropy) and increases the peak amplitude of systolic Ca^{2+} transients in adult rat ventricular myocytes. The negative inotropic phase was ETB receptor dependent, whereas the positive inotropic response and increase in peak amplitude of systolic Ca^{2+} transients required ETA receptor engagement. Both effects of ET 1 required phospholipase C (PLC)-activity, although distinct signalling pathways downstream of PLC elicited the effects of each ET receptor. The negative inotropic response involved inositol 1,4,5-trisphosphate (InsP3) signalling and protein kinase C epsilon (PKC ϵ). The positive inotropic action and the enhancement in Ca^{2+} transient amplitude induced by ET-1 were independent of InsP3 signalling, but suppressed by PKC ϵ . Serine 302 in cardiac myosin binding protein-C was identified as a PKC ϵ substrate that when phosphorylated contributed to the suppression of contraction and Ca^{2+}

transients by PKC ϵ following ET-1 stimulation. Thus, our data provide a new role and mechanism of action for InsP3 and PKC ϵ in mediating the negative inotropic response and in restraining the positive inotropy and enhancement in Ca²⁺ transients following ET-1 stimulation.

llewelyn.roderick@kuleuven.be

01/02/2018

RE MS: JMCC8881

Dear Prof Solaro,

Please find our response to the referees' comments relating to our manuscript: '**Contractile responses to endothelin-1 are regulated by PKC phosphorylation of cardiac myosin binding protein-C in rat ventricular myocytes**'.

We were pleased that the reviewers recognised that we have addressed many issues raised and in fact satisfied a second of the three original reviewers. Since our original submission, we have added considerable new data and have gone to great lengths to satisfy the referees. We have now addressed the remaining major comments. Specifically, the design and content of the cartoon and how it was different between the graphical abstract and Fig 7. The differences were originally present for the sake of conveying the major message in a simple manner in the graphical abstract. We have now modified both as requested by the reviewer. Importantly, we have also provided additional immunoblots that show considerable overexpression of cMyBP-C and its non phosphorylatable mutant in cardiac myocytes. Based on this level of overexpression, and supported by our physiology data, it is clear that these overexpressed proteins are playing the dominant MyBP function in cardiac myocytes, enabling the mutant to elicit a dominant-negative effect. Indeed, ET-1 responses in myocytes expressing the mutated cMyBP-C are similar to those in myocytes expressing DN-PKC ϵ or that have been treated with a PKC epsilon inhibitory peptide. cMyBP-C mutant expressing myocytes also elicit the same potentiation of the second phase of the ET-1 response (contraction and Ca²⁺ transient amplitude) as observed in myocytes exposed to the PKC inhibitors Chelerythrine and Bisindolylmaleimide 1. Our data therefore provides strong evidence that PKC ϵ phosphorylation of cMyBP-C at S302 following ET-1 exposure acts to restrain the increase in contraction and Ca²⁺ transient amplitude induced by ET-1.

A full response to the reviewers' comments is provided in a separate document.

We thank you for considering our manuscript and our responses to the reviewers.

Your sincerely

Prof. Dr. Llewelyn Roderick

The shortening of sarcomeres that co-ordinates the pump function of the heart is stimulated by electrically-mediated increases in $[Ca^{2+}]$. This process of excitation-contraction coupling (ECC) is subject to modulation by neurohormonal mediators that tune the output of the heart to meet the needs of the organism. Endothelin-1 (ET-1) is a potent modulator of cardiac function with effects on contraction amplitude, chronotropy and automaticity. The actions of ET-1 are evident during normal adaptive physiological responses and increased under pathophysiological conditions, such as following myocardial infarction and during heart failure, where ET-1 levels are elevated. In myocytes, ET-1 acts through ET_A- or ET_B-G protein-coupled receptors (GPCRs). Although well studied in atrial myocytes, the influence and mechanisms of action of ET-1 upon ECC in ventricular myocytes are not fully resolved. We show in rat ventricular myocytes that ET-1 elicits a biphasic effect on fractional shortening (initial transient negative and sustained positive inotropy) and increases the peak amplitude of systolic Ca^{2+} transients in adult rat ventricular myocytes. The negative inotropic phase was ET_B receptor-dependent, whereas the positive inotropic response and increase in peak amplitude of systolic Ca^{2+} transients required ET_A receptor engagement. Both effects of ET-1 required phospholipase C (PLC)-activity, although distinct signalling pathways downstream of PLC elicited the effects of each ET receptor. The negative inotropic response involved inositol 1,4,5-trisphosphate (InsP₃) signalling and protein kinase C epsilon (PKCε). The positive inotropic action and the enhancement in Ca^{2+} transient amplitude induced by ET-1 were independent of InsP₃ signalling, but suppressed by PKCε. Serine 302 in cardiac myosin binding protein-C was identified as a PKCε substrate that when phosphorylated contributed to the suppression of contraction and Ca^{2+} transients by PKCε following ET-1 stimulation. Thus, our data provide a new role and mechanism of action for InsP₃ and PKCε in mediating the negative inotropic response and in restraining the positive inotropy and enhancement in Ca^{2+} transients following ET-1 stimulation.

RE: JMCC8881R2

Dear Dr. Roderick:

Your manuscript, "Contractile responses to endothelin-1 are regulated by PKC phosphorylation of cardiac myosin binding protein-C in rat ventricular myocytes," submitted for publication in the Journal of Molecular and Cellular Cardiology, has been read by 2 expert reviewers. While reviewer was favorable in the review, the other had some worthwhile suggestions. In looking over the comments, I have come to the decision that, in its present form the manuscript is not acceptable for publication.

We would be willing to reconsider the manuscript after it has undergone a revision that takes into account the criticisms of the reviewer. Your revised manuscript will be subject to re-evaluation, with no assurance of acceptance.

If you elect to submit a revised manuscript addressing the concerns of the reviewer(s), please prepare a letter detailing the changes that you have made in response to their comments. These should be keyed to the general and specific concerns. Changes in the manuscript must be identified by page and paragraph and noted by underlining or italics in the text. Please submit your revised manuscript and response online at <https://ees.elsevier.com/jmcc/> within 90 days of receiving this decision. Please be sure that the text is uploaded as a Microsoft Word or WordPerfect file and the figures are uploaded individually in the original source file format (JPG, TIFF, etc.). Also be sure to label each illustration with its corresponding figure number. Please also be sure that these files are not saved as read-only.

If the JMCC editorial office does not receive the revision by Jan 27, 2018, we will classify it as a de novo submission if and when we do receive it. Also, we are currently allowing a maximum of 2 revisions.

PLEASE NOTE: Journal of Molecular and Cellular Cardiology would like to enrich its relevant online articles by displaying 3D molecular models that allow the reader to interactively explore the underlying research data. Hence, if applicable, we would like to invite you to upload with your manuscript 3D models (in PDB, PSE or MOL/MOL2 format) as supplementary material to our online submission system. Elsevier will generate interactive viewer for those models and include it with the online article on ScienceDirect.

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Response to Reviewers (mandatory)

This should be a separate file labeled "Response to Reviewers" that carefully addresses, point-by-point, the issues raised in the comments appended below. You should also include a

suitable rebuttal to any specific request for change that you have not made. Mention the page, paragraph, and line number of any revisions that are made.

Manuscript and Figure Source Files (mandatory)

We cannot accommodate PDF manuscript files for production purposes. We also ask that when submitting your revision you follow the journal formatting guidelines. Figures and tables may be embedded within the source file for the submission as long as they are of sufficient resolution for Production. For any figure that cannot be embedded within the source file (such as *.PSD Photoshop files), the original figure needs to be uploaded separately. Refer to the Guide for Authors for additional information.

<http://www.elsevier.com/journals/journal-of-molecular-and-cellular-cardiology/0022-2828/guide-for-authors>

Highlights (mandatory)

Highlights consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See the following website for more information

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Graphical Abstract (optional)

Graphical Abstracts should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Refer to the following website for more information: <http://www.elsevier.com/graphicalabstracts>

Please note that this journal offers a new, free service called AudioSlides: brief, webcast-style presentations that are shown next to published articles on ScienceDirect (see also <http://www.elsevier.com/audioslides>). If your paper is accepted for publication, you will automatically receive an invitation to create an AudioSlides presentation.

The revised version of your submission is due by Jan 27, 2018.

On behalf of the International Society for Heart Research, I thank you for submitting this manuscript to the JMCC. I look forward to seeing your revision.

Sincerely,

R. John Solaro
Editor-in-Chief

<https://ees.elsevier.com/jmcc/>

COMMENTS TO AUTHORS:

Reviewer #1: The major concerns raised during the review of the R1 version of the manuscript remain significant issues. Although the authors responded to these concerns in their comments for the reviewers, they remain concerns in the revised manuscript.

Major concerns

1. Signaling pathway in Figure 7. The concern about Figure 7 may not be a problem if the graphical abstract figure is used and then minimally modified instead of the current Figure 7. If so, then one change that should be considered is to add arrows between the Ca^{2+} transient responses and the increase in contraction. A second change is to show that IP3 influences the negative response without a change in peak Ca^{2+} (this is inconsistent between the graphical abstract and Figure 7).

If Figure 7 was intended to differ from the graphical abstract, then the following issues should be considered. Based on Figures 1 and 2, the Ca^{2+} pathway should be removed from the left side of Figure 7. If the authors conclude (see p. 23) that IP3 mediates the early phase response (Figure 2) via a significant change in the rate of rise of Ca^{2+} (Figures 1, 2), then the arrow between PLC and IP3 on the left pathway should remain, but the arrow from IP3 to Ca^{2+} plus the arrow from Ca^{2+} to reduced function on the left side should be removed from this figure. In summary, the data do not support the idea that the "IP3-dependent" decrease in peak shortening during the first phase (2 min) is mediated by a change in the Ca^{2+} transient.

2. Alternate mechanisms/pathways to explain observations which do not fit with the pathways shown in Figure 7 should be discussed. Alternatively, there could be an acknowledgement that these observations do not fit with the proposed pathway due to the complexity of the signaling.

A. What mechanism could explain endothelin-1-induced increases SR Ca^{2+} fractional release if PKC inhibition further increases the response (Figure 4)? If the IP3 pathway is responsible for increasing this release, there should be an experiment showing that IP3 inhibition prevents the increase. If not mediated via the IP3 pathway, what other potential pathways should be considered in future work.

B. The data showing PKCε-mediated attenuation of the initial negative inotropic response is described using EAVSLKPT and dnPKCε, which each partially attenuate the increase in peak Ca^{2+} during the 2nd phase response while chelerythrine completely blocks this response (Figures 3, 5). What PKC isoforms are likely to contribute to the increased peak Ca^{2+} response during the 2nd phase? A sentence acknowledging a role for other PKCs could be incorporated into the Discussion on p. 41.

2. Role of cMyBP-C Ser302. The previously requested experiments to validate the cMyBP-C S302A work in Figure 6B are necessary to show that there is sufficient cMyBP-C S302A expression to inhibit S302 phosphorylation in Figure 6B. Earlier work by other investigators showed the 20 MOI used in this experiment produces adequate expression in null myocytes, but a higher MOI is likely to be needed for rapid cMyBP-C mutant integration into the sarcomeres of myocytes expressing wildtype cMyBP-C. Thus, an experiment to quantitate replacement (e.g. approaches such as mass spec analysis with AQUA peptides) or a set of experiments showing reduced S302 phosphorylation in myocytes expressing cMyBP-CS302A after the endothelin-1 treatment are necessary to validate the interpretation of Figure 6B results. Without these experiments, the impact of the adenoviral-mediated gene transfer of cMyBP-CS302A on the Ca^{2+} transient also remains a significant issue.

There is also an issue about the use of the term "over-expression" related to these experiments. On pp. 34 and 43, the gene transfer experiments with cMyBP-C indicate that there was "over expression" (presumably meaning protein over-expression). If there is over-

expression, this should be documented by Western analysis and this finding would significantly impacts the interpretation of the data. More than likely, there is stoichiometric replacement of endogenous cMyBP-C rather than protein over-expression, and this alternative possibility also should be validated in experiments.

Minor concerns:

1. Some statements should be modified because they mis-represent the experiments and/or the results.

A. The results shown in Figure S4 for the 5'Phos do not support the conclusion that the 5'Phos prevented the negative inotropic response (p. 25 last paragraph). Unlike the 2-APB, the 5-Phos experiments does not indicate there is a significant difference and this statement should be modified.

B. The description of the dn-PKCe results is vague and should be edited. As written, the description of these results (p. 32) could be interpreted to indicate that dn-PKCe potentiated the ET-1-induced increase in the rate of rise of the contraction and Ca^{2+} signal during the first phase. Instead, the rate of rise of shortening and Ca^{2+} are only influenced during the 2nd phase.

C. The Discussion about the impact of IP3 on the SR Ca^{2+} release (pp. 37-38) should indicate that this refers to the 2nd phase response.

D. The representative blots shown in Figure 6AIII do not support the quantitative figures. A more representative blot for the S302 phosphorylation would improve this figure.

2. The previous recommendation to change the statement about IP3 (IP3 affects the kinetics of CICR) in the Highlights was not modified in this revision. This "highlight" is vague and not helpful for the reader.

02/02/2018

llewelyn.roderick@kuleuven.be

RE MS: JMCC8881

Dear Prof Solaro,

Please find our response to the referees' comments relating to our manuscript: '**Contractile responses to endothelin-1 are regulated by PKC phosphorylation of cardiac myosin binding protein-C in rat ventricular myocytes**'.

We were pleased that the reviewers recognised that we have addressed many issues raised and in fact satisfied a second of the three original reviewers. Since our original submission, we have added considerable new data and have gone to great lengths to satisfy the referees. We have now addressed the remaining major comments – specifically, the design and content of the cartoon and how it was different between the graphical abstract and Fig 7. The differences were originally present for the sake of conveying the major message in a simple manner in the graphical abstract. We have now modified both as requested by the reviewer. We have also provided additional immunoblots that show considerable overexpression of cMyBP-C and its non phosphorylatable mutant in cardiac myocytes. Based on this level of overexpression, and supported by our physiology data, it is clear that these overexpressed proteins are playing the dominant MyBP function in cardiac myocytes, enabling the mutant to elicit a dominant negative effect. Indeed, myocytes expressing the mutated cMyBP-C respond similarly to ET-1 as do myocytes expressing DN-PKCε or treated with a PKCε inhibitory peptide. Mutant expressing myocytes also elicit the same potentiation of the second phase of the ET-1 response (contraction and Ca²⁺ transient amplitude) as myocytes exposed to the PKC inhibitors Chelerythrine and Bisindolylmaleimide I. Our data therefore provides strong evidence that PKCε phosphorylation of cMyBP-C at S302 following ET-1 exposure acts to restrain the increase in contraction and Ca²⁺ transient amplitude induced by ET-1.

A full response to the reviewers' comments is provided below:

Reviewer #1: The major concerns raised during the review of the R1 version of the manuscript remain significant issues. Although the authors responded to these concerns in their comments for the reviewers, they remain concerns in the revised manuscript.

Major concerns

Signaling pathway in Figure 7. The concern about Figure 7 may not be a problem if the graphical abstract figure is used and then minimally modified instead of the current Figure 7. If so, then one change that should be considered is to add arrows between the Ca²⁺ transient responses and the increase in contraction. A second change is to show that IP3 influences the negative response without a change in peak Ca²⁺ (this is inconsistent between the graphical abstract and Figure 7).

The graphical abstract has been modified to include an arrow between the altered Ca²⁺ and contractile responses in the second phase of the response to ET-1.

During the acute phase, an arrow between the effect on IP₃ and the negative inotropic phase has been drawn and the 'Ca²⁺' present in the pathway between IP₃ and contraction in the early phase removed. Ca²⁺ was originally included since, we could not propose another mechanism by which IP₃ operates in cells.

If Figure 7 was intended to differ from the graphical abstract, then the following issues should be considered. Based on Figures 1 and 2, the Ca²⁺ pathway should be removed from the left side of Figure 7. If the authors conclude (see p. 23) that IP₃ mediates the early phase response (Figure 2) via a significant change in the rate of rise of Ca²⁺ (Figures 1, 2), then the arrow between PLC and IP₃ on the left pathway should remain, but the arrow from IP₃ to Ca²⁺ plus the arrow from Ca²⁺ to reduced function on the left side should be removed from this figure. In summary, the data do not support the idea that the "IP₃-dependent" decrease in peak shortening during the first phase (2 min) is mediated by a change in the Ca²⁺ transient.

We have now addressed this issue in the answer to the first comments on the graphical abstract. The graphical abstract and Fig. 7 are now identical and include the modifications suggested by the reviewer.

2. Alternate mechanisms/pathways to explain observations which do not fit with the pathways shown in Figure 7 should be discussed. Alternatively, there could be an acknowledgement that these observations do not fit with the proposed pathway due to the complexity of the signaling.

The discussion already includes some alternative mechanisms including the role of Rho and other PKC isoforms (page 44). Others were removed in the previous revision of the MS to reduce the length of the discussion. We have now however included an explicit statement that other signalling pathways likely contribute to the effects of ET-1 (Page 45, line 23).

What mechanism could explain endothelin-1-induced increases SR Ca²⁺ fractional release if PKC inhibition further increases the response (Figure 4)? If the IP₃ pathway is responsible for increasing this release, there should be an experiment showing that IP₃ inhibition prevents the increase. If not mediated via the IP₃ pathway, what other potential pathways should be considered in future work.

The data do indeed show an increase in fractional Ca²⁺ release (Fig. 4B). We do not see an effect of inhibition of IP₃ signalling on the amplitude of Ca²⁺ transients following ET-1 stimulation (Fig 2B). Fractional Ca²⁺ release is a measure of the amplitude of the cytosolic Ca²⁺ transient relative to the caffeine-induced Ca²⁺ transient. In the absence of an effect directly on release, Ca²⁺ entry, NCX current or SERCA activity (Fig. 4), the effect on Ca²⁺ transient amplitude could be brought about via altered Ca²⁺ association with the contractile machinery – as shown previously for Tnl. This was discussed in the previous response to referees:

'Specifically, we would agree that the second phase of the contractile response to ET-1 is Ca²⁺ dependent since contraction and Ca²⁺ cannot be fully dissociated - Ca²⁺ is required for contraction. However, the effects of ET-1 and PKC inhibition on contraction are more pronounced than on Ca²⁺. We have not suggested that changes

in Ca^{2+} do not contribute to the ability of PKC (or PKC inhibition) to elicit its effects in this study although we show that the alterations in Ca^{2+} dynamics are not due to changes in NCX, SERCA activity, SR Ca^{2+} load or LTCC. Notably, altered Ca^{2+} dynamics are also observed in ET-1 stimulated myocytes that express a cMyBP-C-S302A that cannot be phosphorylated by PKC. This is reminiscent of the altered Ca^{2+} transients in myocytes expressing a mutant Tnl in which sites phosphorylated by PKC downstream of ET-1 are mutated. It is intriguing however that we observe some differences between the effects of general PKC inhibition and PKC ϵ specific inhibition on Ca^{2+} and contraction but the effects of PKC ϵ inhibition reflect those observed in myocytes expressing the cMyBP-C-S302A that harbours a mutation in a sequence for PKC phosphorylation.'

This possibility was also highlighted in the discussion: 'Intriguingly, expression of mutated Tnl that cannot be phosphorylated PKC downstream of ET-1 also results in altered Ca^{2+} transient amplitude and kinetics [1] [1].' (page 44, Line 12)

B. The data showing PKC ϵ -mediated attenuation of the initial negative inotropic response is described using EAVSLKPT and dnPKC ϵ , which each partially attenuate the increase in peak Ca^{2+} during the 2nd phase response while chelerythrine completely blocks this response (Figures 3, 5). What PKC isoforms are likely to contribute to the increased peak Ca^{2+} response during the 2nd phase? A sentence acknowledging a role for other PKCs could be incorporated into the Discussion on p. 41.

During the second phase of the response to ET-1, PKC inhibition by EAVSLKPT or DN-PKC, as for Chel, results in a potentiation of the Ca transient amplitude, not its suppression (Fig. 5). These data would indicate that PKC activated downstream of ET-1 acts to restrain the Ca^{2+} transient. During this second phase of the ET-1 response, the effect of Chel and EAVSLKPT and DN-PKC overlap completely, leaving little room for other PKC isoforms to potentiate the signal.

During the initial negative inotropic response to ET-1, the EAVSKLPT peptide and DN-PKC ϵ prevent the negative inotropic response, whereas Chel does not. Here, other PKC isoforms insensitive to Chel could act to counter the effects of PKC epsilon[2]. The lower potency of Chel estimated for PKC epsilon by the manufacturer (Sigma UK) could contribute to this effect. Cells expressing the cMyBP-C-S302A mutant also exhibit similar responses to cells in which PKC ϵ is expressed strongly supporting our conclusion that this isoform is the dominant player in both the initial negative inotropic response and the second phase response. This was indicated in the previous response to referees.

The statement: 'Incomplete inhibition of PKC ϵ by Chel or Bis may also explain the differences between the PKC inhibitors employed.' Has now been added to the discussion (Page 41, Line 19).

Also, please see the last paragraph of the discussion where we state: 'Notably, in order for PKC ϵ inhibition to elicit an inotropic effect, ET-1 stimulation was required, indicating that other pathways downstream of the ET receptor including other PKC isoforms are required' (Page 45).

2. Role of cMyBP-C Ser302. The previously requested experiments to validate the cMyBP-C S302A work in Figure 6B are necessary to show that there is sufficient cMyBP-C

S302A expression to inhibit S302 phosphorylation in Figure 6B. Earlier work by other investigators showed the 20 MOI used in this experiment produces adequate expression in null myocytes, but a higher MOI is likely to be needed for rapid cMyBP-C mutant integration into the sarcomeres of myocytes expressing wildtype cMyBP-C. Thus, an experiment to quantitate replacement (e.g. approaches such as mass spec analysis with AQUA peptides) or a set of experiments **showing reduced S302 phosphorylation in myocytes** expressing cMyBP-CS302A after the endothelin-1 treatment are necessary to validate the interpretation of Figure 6B results. Without these experiments, the impact of the adenoviral-mediated gene transfer of cMyBP-CS302A on the Ca^{2+} transient also remains a significant issue.

There is also an issue about the use of the term "over-expression" related to these experiments. On pp. 34 and 43, the gene transfer experiments with cMyBP-C indicate that there was "over expression" (presumably meaning protein over-expression). If there is over-expression, this should be documented by Western analysis and this finding would significantly impacts the interpretation of the data. More than likely, there is stoichiometric replacement of endogenous cMyBP-C rather than protein over-expression, and this alternative possibility also should be validated in experiments.

We have previously discussed our rationale regarding expression of mutated cMyBP-C in the myocytes analysed. The capacity of the viral vectors to drive high level expression of cMyBP-C is shown in MyBP null HeLa cells. The viral vector employed to drive cMyBP-C or cMyBP-C-S302A expression also expressed RFP. Ca^{2+} and contraction was only analysed in RFP +ve myocytes. While adenovirally-mediated expression of WT cMyBP-C did not affect Ca^{2+} or contraction at baseline nor following ET-1 stimulation (Supplementary Fig. 10 and 12), expression of cMyBP-C-S302A resulted in increased inotropic responses to ET-1, greater amplitude of Ca^{2+} transient during the second phase of the ET-1 response and a loss of the negative inotropic response to ET-1. These effects phenocopy those for inhibition of PKC ϵ . These data provide strong evidence for expression of mutated MyBP. Moreover, this Dominant negative effect of cMyBP-C-S302A strongly supports our conclusion that this mutated form of cMyBP-C replaced the endogenous wild type protein.

Here, in this response, we provide additional immunoblot data that demonstrates adenovirally-mediated overexpression of cMyBP-C or cMyBP-C-S302A protein in cardiac myocytes. Specifically, we demonstrate ~6 fold increase in cMyBP-C/cMyBP-C-S302A protein in virally transduced cells (now in Fig. 6B). Given this level of expression, we would predict that the overexpressed protein is primarily responsible for the function of cMyBP-C in these cells. Notably while WT cMyBP-C overexpression does not alter Ca^{2+} and contractile responses, expression of cMyBP-C-S302A at the same level substantially modifies these responses, phenocopying the effects of DN-PKC ϵ expression. We would therefore conclude that based on this high level expression of the mutated cMyBP-C, a dominant negative effect is achieved. As previously stated, the cMyBP-C constructs employed were not modified to include a tag and thus migrated at the same molecular weight as the endogenous protein.

Using PdBU to maximally activate PKCs, we have assessed whether phosphorylation of cMyBP-C is affected by the presence of the mutated cMyBP-C (Supplementary Fig. 12III). While our data indicate that the presence of the mutated protein does not prevent the phosphorylation of the endogenous protein, the substantially greater abundance of the overexpressed protein would result in its function dominating over that of the endogenous. This dominant effect of the mutated protein is supported by

the physiological function of the ectopically expressed mutated cMyBP-C, which phenocopies the effect of PKC inhibition.

As we have now demonstrated cMyBP-C overexpression, I feel that our description of cMyBP-C as being overexpressed is valid and should remain. If you consider otherwise, we can alter.

Minor concerns:

1. Some statements should be modified because they mis-represent the experiments and/or the results.

A. The results shown in Figure S4 for the 5'Phos do not support the conclusion that the 5'Phos prevented the negative inotropic response (p. 25 last paragraph). Unlike the 2-APB, the 5-Phos experiments does not indicate there is a significant difference and this statement should be modified.

We agree with the referee on this point as the data was presented. However, and I must apologise for this, while we had correctly stated the data in the text previously and this was also shown in the full time-course of the data presented in Fig S4 IX, in generating the figure S4 I, the correct data was not included. This has been corrected and now reflects the full time course data.

B. The description of the dn-PKCe results is vague and should be edited. As written, the description of these results (p. 32) could be interpreted to indicate that dn-PKCe potentiated the ET-1-induced increase in the rate of rise of the contraction and Ca²⁺ signal during the first phase. Instead, the rate of rise of shortening and Ca²⁺ are only influenced during the 2nd phase.

This section has been reworded.

C. The Discussion about the impact of IP3 on the SR Ca²⁺ release (pp. 37-38) should indicate that this refers to the 2nd phase response.

This has been modified as requested.

D. The representative blots shown in Figure 6AIII do not support the quantitative figures. A more representative blot for the S302 phosphorylation would improve this figure.

This blot shows an increase in S302 phosphorylation following ET-1 stimulation as per the histogram. Perhaps this did not reproduce in the MS viewed. The increase in S302 phosphorylation shown in the histogram is not substantial and thus we would not anticipate substantial differences in the band intensity in the blot. We have however modified the figure. We hope this better conveys the changes reported.

2. The previous recommendation to change the statement about IP3 (IP3 affects the kinetics of CICR) in the Highlights was not modified in this revision. This "highlight" is vague and not helpful for the reader.

This has been reworded to: 'InsP₃-mediated Ca²⁺ release contributes to the increase in rate of rise of the Ca²⁺ transient during the second inotropic phase of the response to ET-1' (Page 47).

Yours sincerely,

A handwritten signature in blue ink, appearing to read 'H Roderick'.

Prof. Dr. Llewelyn Roderick

References

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Highlights

- ET_B receptor activation mediates the transient ET-1-induced negative inotropic response in ARVMs. ET_A receptor activation underlies positive inotropic response and increase in systolic Ca^{2+} transient amplitude resulting from prolonged ET-1 stimulation in ARVMs.
- $InsP_3R$ activity contributes to the negative inotropic response to ET-1.
- $InsP_3$ -mediated Ca^{2+} release contributes to the increase in rate of rise of the Ca^{2+} transient during the second inotropic phase of the response to ET-1.
- Enhanced systolic Ca^{2+} transient amplitude induced by prolonged exposure to ET-1 in ARVMs is independent of $InsP_3R$ -mediated signalling.
- $PKC\epsilon$ is required for the negative inotropic responses to ET-1 and restricts the subsequent enhanced contractility and increased amplitude of systolic Ca^{2+} transients.
- PKC elicits its effects on contraction through phosphorylation of cMyBP-C on S302.
- It can be concluded that $PKC\epsilon$ protects ET-1-stimulated myocytes from hypercontraction and Ca^{2+} overload.

Contractile responses to endothelin-1 are regulated by PKC phosphorylation of cardiac myosin binding protein-C in rat ventricular myocytes

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Abbreviations

ECC: Excitation-Contraction Coupling

CICR: Ca^{2+} -induced Ca^{2+} release

ET-1: Endothelin-1

SR: Sarcoplasmic Reticulum

SERCA: SR/ER Ca^{2+} ATPase

NCX: $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger

NHE: $\text{Na}^{+}/\text{H}^{+}$ exchanger

$\text{InsP}_3(\text{R})$: Inositol 1,4,5-trisphosphate (receptor)

PLC: Phospholipase C

DAG: Diacylglycerol

DN-PKC ϵ : Dominant-Negative PKC ϵ

ET_A/ET_B: Endothelin receptor subtype (A or B)

ARVMs: Adult Rat Ventricular Myocytes

FCR: Fractional Ca^{2+} Release

5'Phos: Adenoviral-mediated expression of the type 1 InsP_3 5'phosphatase

Chel: Chelerythrine

BimI/V: Bisindolylmaleimide I/V

Iso: Isoproterenol

cMyBP-C: Cardiac Myosin Binding Protein-C

Abstract

The shortening of sarcomeres that co-ordinates the pump function of the heart is stimulated by electrically-mediated increases in $[Ca^{2+}]$. This process of excitation-contraction coupling (ECC) is subject to modulation by neurohormonal mediators that tune the output of the heart to meet the needs of the organism. Endothelin-1 (ET-1) is a potent modulator of cardiac function with effects on contraction amplitude, chronotropy and automaticity. The actions of ET-1 are evident during normal adaptive physiological responses and increased under pathophysiological conditions, such as following myocardial infarction and during heart failure, where ET-1 levels are elevated. In myocytes, ET-1 acts through ET_A - or ET_B -G protein-coupled receptors (GPCRs). Although well studied in atrial myocytes, the influence and mechanisms of action of ET-1 upon ECC in ventricular myocytes are not fully resolved. We show in rat ventricular myocytes that ET-1 elicits a biphasic effect on fractional shortening (initial transient negative and sustained positive inotropy) and increases the peak amplitude of systolic Ca^{2+} transients in adult rat ventricular myocytes. The negative inotropic phase was ET_B receptor-dependent, whereas the positive inotropic response and increase in peak amplitude of systolic Ca^{2+} transients required ET_A receptor engagement. Both effects of ET-1 required phospholipase C (PLC)-activity, although distinct signalling pathways downstream of PLC elicited the effects of each ET receptor. The negative inotropic response involved inositol 1,4,5-trisphosphate ($InsP_3$) signalling and protein kinase C epsilon ($PKC\epsilon$). The positive inotropic action and the enhancement in Ca^{2+} transient amplitude induced by ET-1 were independent of $InsP_3$ signalling, but suppressed by $PKC\epsilon$. Serine 302 in cardiac myosin binding protein-C was identified as a $PKC\epsilon$ substrate that when phosphorylated contributed to the suppression of contraction and

Ca²⁺ transients by PKCε following ET-1 stimulation. Thus, our data provide a new role and mechanism of action for InsP₃ and PKCε in mediating the negative inotropic response and in restraining the positive inotropy and enhancement in Ca²⁺ transients following ET-1 stimulation.

1. Introduction

Cardiac myocyte contraction is mediated by the process of excitation-contraction coupling (ECC). ECC is initiated by a propagating action potential that depolarises the cardiomyocyte sarcolemma, leading to opening of L-type voltage-gated Ca^{2+} channels allowing Ca^{2+} entry into the cell. Through Ca^{2+} -induced Ca^{2+} release (CICR) [1], this Ca^{2+} influx stimulates the opening of ryanodine receptor channels (RyRs), inducing Ca^{2+} release from the sarcoplasmic reticulum (SR) intracellular Ca^{2+} store. The Ca^{2+} signal is thus amplified generating a global cellular Ca^{2+} signal that engages the contractile apparatus, inducing myocyte contraction [2,3]. Myocyte relaxation during diastole is brought about by Ca^{2+} sequestration into the SR via the actions of the SR/ER Ca^{2+} ATPase (SERCA) pump and, to a lesser extent, by extrusion from the cell by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) [3,4].

ECC is modified to increase cardiac output during stress or disease through alterations in Ca^{2+} dynamics and myofilament Ca^{2+} sensitivity [3]. Acute increase in cardiac output is induced via the actions of circulating or locally released mediators that act on myocytes of the heart, as well as the pacemaker and conduction system. Although, catecholamines play a central role in this process, cardiac contractility is also regulated by neurohumoral regulators, such as ET-1 and angiotensin. ET-1 is an autocrine/paracrine messenger synthesised by cardiac myocytes [3], cardiac fibroblasts and endothelial cells [5,6] that modifies many aspects of cardiac physiology, exerting both beneficial and detrimental effects in the heart [7,8]. Acutely, ET-1 modulates myocyte contraction and can cause arrhythmias [6,9,10], whereas the long-term effects of ET-1 have been associated with myocyte growth,

maladaptive hypertrophy, and pro-fibrotic remodelling and progression to heart failure [8,11,12].

ET-1 elicits its actions through ET_A and ET_B receptor subtypes, the expression of which vary across the heart, with ET_A receptor being most abundant in ventricular and atrial myocytes [13,14]. ET receptors are localised on t-tubular membranes of ventricular myocytes where they are optimally localised to signal to RyRs [15], L-type Ca²⁺ channels [16] and NCX [17] and regulate ECC. ET-1 has a very high affinity for its receptor that lies in the nanomolar range (EC₅₀ <0.1 nM), which has been termed ‘quasi’ irreversible [18]. Thus, irrespective of its concentration, maximal occupation and activation of a significant proportion of receptors is eventually achieved [19]. As a consequence, the low circulating levels of ET-1 are able to exert a physiological effect on cardiac myocytes. Indeed, in common with other studies [20-22], we and others have observed comparable effects of ET-1 on contraction and Ca²⁺ in rat ventricular myocytes over a concentration range spanning 1 and 100 nM [9,23]. During pathology however, ET-1 abundance is increased, eliciting greater effects on target tissues, including the induction of cardiac arrhythmias [12,24-26].

Both receptors engage the G protein G_{q/11}, causing activation of PLC, which, in turn, hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP₂) to generate InsP₃ and diacylglycerol (DAG) [27]. InsP₃ diffuses to its cognate receptor (InsP₃R) located on the ER inducing Ca²⁺ release, whereas DAG remains at the sarcolemma, where it mediates its signalling effects via activation of protein kinase C (PKC) [28].

InsP₃ and PKC play important and varied roles in cardiac myocytes [29-31]. InsP₃Rs are expressed in the heart where they mediate Ca²⁺ release from intracellular stores and participate in cardiac hypertrophic remodelling [9,12,32-34]. Notably, InsP₃R

expression is increased in the hypertrophic heart, contributing to the ectopic Ca^{2+} signals associated with pathology [20,35,36]. These effects of InsP_3Rs are surprising, given their substantially lower expression compared to RyRs [12,32,37]. Concurrently, PKC signalling is implicated in the regulation of Ca^{2+} handling and contraction, as well as chronic effects in heart failure, cardiac hypertrophy and ischaemia-reperfusion injury [38-40]. In mediating its effects on myocyte contraction, PKC modifies the activity of many targets including the sodium/hydrogen exchanger (NHE) [41], sodium calcium exchanger (NCX) [42,43], Ca^{2+} current (I_{CaL}) [44], delayed rectifier K^+ current [45], as well as altering the Ca^{2+} sensitivity of myofilaments [46]. Despite these identified targets, inconsistent effects of PKC isozymes on cardiac contraction and Ca^{2+} handling have been reported [47-50]. A complete picture of the role of PKCs in GPCR regulation of contraction and Ca^{2+} handling in rat ventricular myocytes therefore remains to be established.

Here, we investigated the signalling pathways involved in mediating the inotropic response of adult rat ventricular myocytes (ARVMs) to ET-1. Consistent with previous studies [10], the effect of ET-1 on contractility was biphasic, consisting of an initial transient negative inotropic response, followed by a positive inotropic response. The positive inotropic response was accompanied by an augmentation in the amplitude and rate of rise of the electrically-evoked Ca^{2+} transients. The stimulatory effects of ET-1 on contraction and Ca^{2+} transients were ET_A receptor-dependent, whereas the negative inotropic response was mediated by the ET_B receptor. The effects of ET-1 on Ca^{2+} transients and contraction both required PLC activity. Signalling via InsP_3Rs contributed to the negative inotropic effect of ET-1 stimulation. Activation of PKC, in particular the novel $\text{PKC}\epsilon$ isoform, specifically suppressed the positive effects of ET-1 on inotropy and Ca^{2+} transients without

affecting SR Ca^{2+} load. We identified that the effects of PKC were selectively mediated through its phosphorylation of cardiac myosin binding protein-C (cMyBP-C). In this way, PKC and its phosphorylation of cMyBP-C may act together to prevent overactivation of cardiac myocytes following agonist stimulation, thereby protecting them from pathological consequences. Taken together, our findings provide new insights into the molecular events that dictate ET-1-mediated effects on cardiac contractility and Ca^{2+} signalling.

2. Materials and Methods

2.1 Materials

Collagenase type II was purchased from Worthington Biochemical Corporation. ET-1, isoproterenol, and pharmacological inhibitors were obtained from Calbiochem. Salts for extracellular solutions were from Sigma-Aldrich or BDH, and indo-1, AM was from Invitrogen. Rats were obtained from Harlan, Charles River and Janvier.

2.2 Myocyte isolation

ARVMs were isolated as described previously [10]. Briefly, 6-7-week-old male Wistar rats were anaesthetised in a CO₂ chamber and subsequently sacrificed by cervical dislocation. The heart was exposed, aorta cannulated and the heart perfused with oxygenated, ice-cold modified Tyrode solution (in mM: NaCl 135, KCl 5, HEPES 10, glucose 10, MgCl₂ 0.4 and CaCl₂ 1; pH adjusted to 7.35 with NaOH). The heart was quickly dissected from the thorax and suspended on a modified Langendorff apparatus. It was perfused in a retrograde manner with oxygenated HEPES-Ca²⁺ buffer at 37 °C, followed by perfusion with oxygenated, low-Ca²⁺ buffer (in mM NaCl 120, KCl 5, HEPES 10, glucose 20, taurine 20, MgSO₄ 5, NTA 5.5, sodium pyruvate 5, CaCl₂ 0.08; pH adjusted to 6.96 with NaOH). The heart was digested by perfusion with oxygenated collagenase-containing buffer (in mM: NaCl 120, KCl 5, HEPES 10, glucose 20, taurine 20, MgSO₄ 5, sodium pyruvate 5, CaCl₂ 0.035; pH adjusted to 7.4 with NaOH and containing 319 U/ml Collagenase type II) for 10 min. Following dissociation, myocytes were allowed to settle after which Ca²⁺ was reintroduced in an incremental manner to avoid intracellular Ca²⁺ overload and hyper-contraction. Myocytes were allowed to recover for a minimum of 2 h before seeding onto 16 mm laminin-coated coverslips or 6 well tissue culture dishes. All

experiments were performed in accordance with the guidelines from the code of practice for humane killing under Schedule 1 of the Animals (Scientific Procedures) Act 1986 and in accordance with the European Directive 2010/63/EU and approved by either the Ethical Committee for Animal Experiments of KU Leuven (Belgium) or Norwegian National Animal Research Authority (FOTS 3284).

2.3 Adenoviral infection of cardiac myocytes

Myocyte expression of DN-PKC ϵ , InsP₃ 5'-phosphatase (5'Phos) and cMyBP-C was achieved using adenovirus as previously described [12]. The adenoviral DN-PKC ϵ construct was kindly provided by R. Heads (King's College London) and has been previously described [51]. The GFP control and GFP-tagged InsP₃ 5'-phosphatase adenoviral constructs were as previously described [12]. Myocytes to be infected with adenovirus were resuspended in culture medium (M199 media containing 0.2% BSA, 100 units penicillin, 100 μ g streptomycin and 0.25 μ g amphotericin B/ml) and plated onto 16 mm laminin-coated coverslips (for imaging) or 6-well culture dishes (for immunoblotting) and transferred to a tissue culture incubator at 37 °C and 5% CO₂. After allowing a period of 1 h for myocytes to adhere, cells were gently washed with culture medium to remove debris and dead cells. The number of viral particles necessary to achieve >90% infection was added to the medium overlying the cells and incubated for 16 h (MOI of 50 for DN-PKC ϵ , 20 for 5'Phos, cMyBP-C-WT and cMyBP-C-S302A). Cultured myocytes exhibited similar peak amplitude of Ca²⁺ transients and fractional shortening at baseline compared to cells that were used in experiments performed on the day of isolation (Supplementary Figure 10).

2.4 Generation of adenoviral constructs to express wild-type and mutated

cMyBP-C

Mutagenesis of cMyBP-C was performed using the QuikChange II Site-directed Mutagenesis kit (Agilent), following the manufacturer's protocol. The mouse cMyBP-C cDNA cloned in the pCR-Blunt II-TOPO vector was obtained from MRC Gene Service. Mutagenic primers were designed for conversion of Serine 302 to Alanine (S302A) (5' CCTGCTGAAGAAGAGAGACGCTTCCGGAGGGACTCAAAG 3' and 5' CTTTGAGTCCCTCCGGAAAGCGTCTCTCTTCTTCAGCAGG 3'). The presence of the mutation was confirmed by Sanger sequencing (Supplementary Figure 12; I). Wild-type (WT) and mutated cMyBP-C cDNAs were subcloned into the pAd-RFP shuttle vector (Addgene #12520). In addition to the cassette for expression of the gene of interest under the control of a CMV promoter, the shuttle plasmid pAd-RFP has a separate cassette for expression of red fluorescent protein (RFP) to allow identification of infected cells. The adenoviral constructs were generated using the AdEasy Adenoviral Vector System, following the manufacturer's instructions (Agilent). Briefly, viral recombinants were generated in BJ5183 bacterial cells, and viral particles were produced and amplified in HEK293 cells. Viral purification was performed using the Vivapure[®] kit (Sartorius), following the manufacturer's protocol. Adenoviral mediated expression of the cMyBP-C constructs and RFP was confirmed in HeLa cells by immunoblot (Supplementary Figure 12; II)

2.5 Analysis of myocyte intracellular Ca²⁺ and contraction

Intracellular Ca²⁺ levels and contraction were simultaneously assessed by single-cell photometry of indo-1 fluorescence and by edge detection respectively as previously described [52]. Data was processed and analysed using Origin Pro (Silverdale Scientific, UK) and Sigma Plot (Systat Software, Inc.) software packages and plotted with PRISM (Graphpad). Photometry was performed using a D104C dual channel

photometer (PTI) attached to an Olympus IX71 inverted microscope. To prevent bleaching of the indicator, cells were sampled discontinuously (30 sec illumination/recording, 30 sec pause). Myocytes were field-stimulated throughout the recording in a homemade imaging chamber with integrated platinum electrodes (0.33 Hz, 100 V). For Ca^{2+} recordings, myocytes adhered to laminin-coated 16 mm coverslips were loaded with indo-1 AM (3 μM) for 30 min at room temperature. The cells were subsequently washed with imaging buffer (modified Tyrode buffer containing 1 mM Ca^{2+}) and incubated for a further 30 min prior to imaging for dye de-esterification. To ensure equivalent loading of indo-1, thus avoiding potential effects on functional responses to ET-1, a strict timing regimen for dye loading and de-esterification was followed. Coverslips were mounted in the imaging chamber, and a solenoid-controlled perfusion system was positioned appropriately to allow continuous perfusion and rapid switching of solutions (control or agonist/antagonist solutions diluted in imaging buffer). In experiments where myocytes were exposed to pharmacological agents, these were applied 5 (2-APB), 20 (Chel, BimI and BimV) or 30 min (U73122, H89, EAVSLKPT peptide conjugated to a cell-permeant 10 amino acid peptide from the HIV TAT protein [53]) prior to the start of recording during dye de-esterification and then maintained throughout the duration of the recording. Ca^{2+} dependent changes in indo-1 emission, excited at 360 nm, were sampled at 405 nm and 490 nm using two independent photomultiplier detectors. Photomultiplier signals were digitized using an analogue to digital converter controlled by Felix software (Photon Technology International). After background subtraction (non cell background after the end of recording) from each channel, the 405:490 emission ratio (R) was derived and smoothed using the adjacent-averaging method. From these data, the amplitude (R at systole – R at diastole) and kinetics of the Ca^{2+} transients

averaged over each 30 s recording period were determined and normalised to the point of agonist/control buffer addition. The rate of rise of the Ca^{2+} transient was calculated by dividing the transient amplitude by the time-to-peak. The rate of decay and time constant were calculated after fitting Ca^{2+} transients with a mono-exponential decay curve using a Levenberg-Marquardt non-linear fitting algorithm. Full time courses and summary time courses that include point of addition, agonist/control + 2 min and agonist/control + 20 min are plotted. For presentation of Ca^{2+} traces comparable with the normalised time course data, baseline was subtracted from the 405:490 ratio and transients normalised with the peak ratio at the point of agonist addition set at 1 and diastolic ratio at 0. Where myocytes were pre-incubated with a drug or transduced with virus, the effect of these manoeuvres on baseline Ca^{2+} transient amplitude was determined by comparison of background subtracted 405:490 ratio between conditions (Supplementary Figure 10).

Contraction (fractional shortening) was measured simultaneously with Ca^{2+} and determined by edge-detection analysis (VED-205, Crescent Electronics) of bright field images of myocyte obtained by illumination with light at ≥ 650 nm and recorded using Felix software (PTI). The amplitude of contraction was calculated as the difference between the maximum cell length at the end of the diastolic phase and the minimum cell length during each pacing cycle. To calculate fractional shortening of each myocyte, peak amplitude of contraction was divided by the maximum cell length at the beginning of each experiment. Fractional shortening was averaged for each 30 s. recording period and was normalised to levels of contraction at the point immediately prior to agent/buffer addition. Where myocytes were pre-incubated with a drug or were transduced with virus, the effect of these manoeuvres on baseline contraction was determined by comparison of baseline fractional shortening data

between conditions (not normalised for temporal analysis and without agonist added) (Supplementary Fig 10). The rate of contraction was calculated by dividing the amplitude of contraction by the time required to reach peak contraction.

Cells were stimulated with 100 nM ET-1, which is a concentration widely employed in physiological studies [20-22]. While this may be a relatively high concentration of ET-1, owing to the very high-affinity interaction of ET-1 for its receptor (being considered quasi-irreversible), the off-rate constant (k_{off}) of ligand is effectively discounted for purposes of these experiments, and it is only the time taken to reach full receptor occupancy that is affected by agonist concentration [18]. Indeed, stimulation of cardiac myocytes with ET-1 at a lower concentration (10 nM) has similar effects on cell contractility and Ca^{2+} signalling, albeit at delayed time points [9]. As maintaining cell health and viability for the duration of the imaging experiments was a priority, particularly in experiments where cells were cultured overnight, ET-1 was used at 100 nM in all conditions.

As in our previous studies, recordings were performed at room temperature (20-22 °C). Under these conditions, in addition to improved cell viability, we have found that dye loading is maintained without compartmentalisation through the time course of the experiment, thereby ensuring a good signal-to-noise ratio [54]. Apart from a more rapid onset of response to agonist, we have also shown no qualitative difference in results between experiments carried out at 37 °C and room temperature [9]. Prior to the start of recording, cells were electrically paced (10-20 depolarisations) to establish a steady-state Ca^{2+} response. During this period, a 'positive staircase' was observed indicative of Ca^{2+} loading of the SR [55]. Only myocytes with no signs of structural deterioration, with no spontaneous activity at rest

that responded to field stimulation and that exhibited changes in indo-1 emission following electrical stimulation, were selected for analysis.

2.6 Fractional Ca^{2+} release (FCR) and SR Ca^{2+} load

To calculate fractional Ca^{2+} release from the SR (FCR) and the total releasable Ca^{2+} in the SR, an experimental protocol originally published by Kockskamper et al. was followed [29]. Briefly, Ca^{2+} release from the SR following application of caffeine (20 mM), which rapidly and exhaustively depletes both SR and perinuclear Ca^{2+} stores, was measured [56]. Ventricular myocytes were electrically paced, superfused with control imaging buffer and allowed to reach a steady state. Electrical stimulation was paused and caffeine rapidly applied. The amplitude of the caffeine-induced Ca^{2+} transient in the cytoplasm was taken as a measure of the maximum releasable Ca^{2+} . FCR was calculated as the ratio of the amplitude of the electrically stimulated cytoplasmic Ca^{2+} transient to the caffeine-evoked Ca^{2+} transient. Caffeine was subsequently washed out and electrical stimulation resumed. Myocytes were exposed to imaging buffer containing ET-1 (100 nM) for 20 min before a second application of caffeine to determine the effect of ET-1 on FCR.

Indo-1 has a K_d of 844 nM in the cytosol of cardiac myocytes, which allows quantitative measurement of electrically-stimulated Ca^{2+} transients [57]. Previous studies using indo-1 have shown that the caffeine-evoked Ca^{2+} transients reach a peak significantly greater than that of Ca^{2+} transients induced by electrical stimulation, which is further exceeded during calibration procedures [57]. Experiments were therefore carried out to establish R_{max} in our system and to demonstrate that the dynamic range of indo-1 was sufficient to detect changes in Ca^{2+} transient amplitude. For calculation of R_{max} , myocytes were electrically paced to establish a steady-state

Ca²⁺ response. After recording Ca²⁺ transients for 1 min, caffeine (20 mM) was applied, causing complete depletion of SR Ca²⁺. After caffeine was washed out and myocyte contraction returned to the levels at the beginning of the experiment, R_{max} was achieved by addition of a solution containing 2 μM of the non-fluorescent Ca²⁺ ionophore bromo-A23187 together with the metabolic uncouplers CCCP (5 μM) and rotenone (2 μM) [52,58]. R_{max} was reached 6 min after application of ionophore.

2.7 Patch clamp electrophysiology

Whole cell patch clamp electrophysiological recordings in ventricular myocytes were performed as previously described [59]. Myocytes were voltage clamped using an Axoclamp 2B amplifier and pCLAMP software (both Axon Instruments, Foster City, CA, USA). Patch pipettes (1.7-2.5 MΩ) were filled with internal solution (in mM: CsCl 115, TEACl 20, HEPES 10, MgATP 5, Na₂-Phosphocreatinine 5, and EGTA 0.04; pH 7.2 by CsOH). Myocytes were patched in modified Tyrode solution containing (in mM: NaCl 140, HEPES 5, KCl 5.0, CaCl₂ 1.0, MgCl₂ 0.5, D-glucose 5.5, and NaH₂PO₄ 0.4; pH adjusted to 7.4 with NaOH). When whole cell access was reached, superfusion was changed to a solution containing (in mM) NaCl 125, CsCl 20, D-glucose 5, MgCl₂ 1, CaCl₂ 1.8, HEPES 10, 4-aminopyridine 5, and probenecid 2; pH adjusted to 7.4 with NaOH. 100 nM ET-1 and/or 1 μM Chel were added to the superfusate. Myocytes were exposed to 100 nM ET-1 and/or 1 μM Chel for 20 min before the start of recordings.

After whole cell access was obtained, the myocytes were dialyzed for 3 min. Measurements of L-type Ca²⁺ currents were then obtained. To this end, a holding potential was used (45 mV) and L-type Ca²⁺ currents triggered by a 100 ms square voltage step from -45 to 0 mV at 0.125 Hz. SR Ca²⁺ load was determined from the

integral of the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (NCX) current (I_{NCX}) during caffeine-induced release of Ca^{2+} from SR stores. I_{NCX} was measured using a protocol in which myocytes were held at -45 mV and inward current measured during application of 10 mM caffeine (Sigma-Aldrich) added to the superfusate. Peaks and integrals of ion currents were analyzed by relating the currents to the tail current [59]. All analyses were performed in ClampFit.

2.8 Analysis of protein expression

Protein lysates were prepared from ARVMs by homogenisation in lysis buffer (in mM: EGTA 1, DTT 1, Tris-HCl 10, NaCl 140; pH 7.6 adjusted with NaOH and supplemented with 1x phosphatase inhibitor cocktail and 1x protease inhibitor cocktail (both from SIGMA)) and subsequent incubation on ice for 30 min. For immunoblotting of cMyBP-C, cells were lysed in a modified RIPA buffer [in mM: NaCl 150, Tris-HCl 20, EDTA 10, DTT 1 and 1% each of sodium deoxycholate and TRITON X-100 and 0.1 % SDS; pH 7.6 adjusted with NaOH and supplemented with 1x phosphatase inhibitor cocktail and 1x protease inhibitor cocktail (both from SIGMA)]. Cell lysates were collected after centrifugation at 25,000 g for 15 min at 4 °C. Equivalent amounts of protein, as determined by BCA protein assay (Pierce), were separated on a 4-12% BIS-TRIS, SDS-PAGE (NuPage, NOVEX), and analysed by standard immunoblotting techniques [Enhanced Chemiluminescence using HRP conjugated secondary antibodies (Figures 3, 5 and Supplementary Figures 6 and 12; II) and fluorescence detection using fluorescently labelled secondary antibodies and imaged on a Licor Odyssey CLx imaging system (Figure 6 and Supplementary Figure 12; III) as previously described [60]. The following primary antibodies were used: anti pan-phosphorylated PKC (1:1000; pan pPKC, Cell Signalling Technology #9379)

that recognizes active classical, novel and atypical PKC isoforms, α PKC ϵ (1:1000; Cell Signalling Technology #2683), anti phosphorylated PKC ϵ (1:1000; pPKC ϵ , Millipore #06-821-I), anti total cMyBP-C (1:20,000; kind gift of Prof. M. Gautel, King's College London or a monoclonal anti cMyBP-C from Santa Cruz #137180 diluted 1:1000), p-cMyBP-C on S273 and S302 (1:20,000; [61]), anti phospho-(Ser) PKC Substrate Antibody (1:500; Cell Signalling Technology #2261), and anti α -actinin (1:1000; SIGMA #A7732).

2.9 Alkaline phosphatase treatment of protein lysates

20 μ g of protein lysate together with 10 μ l of calf intestinal alkaline phosphatase (0.5 U/ μ l of protein) (AP; Roche) and 10 μ l AP buffer was incubated in a final volume of 100 μ l for 30 min at 37 °C. Control treatment was performed by replacement of alkaline phosphatase with lysis buffer. The reaction was terminated by addition of 5X SDS sample buffer.

2.10 Immunofluorescence

Immunofluorescence was performed, as previously described, with minor modifications [15,35]. Briefly, myocytes were permeabilised with PBS containing 0.2% Triton X-100 and were subsequently incubated with blocking buffer (PBS containing 0.1% Triton X-100, 5% normal goat serum and 10% BSA). Cells were stained with antibodies against ET_AR (clone 53D at 1:50 dilution in blocking buffer; a generous gift from Dr. A. Davenport, University of Cambridge, Cambridge), ET_BR (clone 51D at 1:50 dilution in blocking buffer; a generous gift from Dr. A. Davenport, University of Cambridge), NCX (1:100 dilution in blocking buffer; generous gift from Prof. K. Philipson, UCLA, California, USA). Appropriate secondary antibodies,

conjugated with either Alexa Fluor 488 or Alexa Fluor 568, were used at a dilution of 1:500. Cells were mounted in Vectashield containing DAPI on glass slides, and images were acquired using a Nikon A1R confocal microscope using a Plan Fluor DIC H N 40x oil immersion objective (N.A. = 1.3).

2.11 Immunoprecipitation and mass spectrometry analysis

Immunoprecipitation (IP) was performed on 2 mg of ARVM total cell lysate prepared as described above. Prior to IP with specific antibodies, samples were first pre-cleared to avoid nonspecific protein isolation. 5 µg of normal IgG antibody were then pre-conjugated with 25 µl protein A/G sepharose beads (Santa Cruz) for 2 h at 4 °C on a rocking platform. Excess antibody was washed off, and the beads-IgG antibody complex was incubated with appropriate amount of lysate for 3 h at 4 °C on a rotating platform. Beads were removed by centrifugation at 10,000 g for 5 min at 4 °C, and pre-cleared lysates were transferred to fresh tubes. One-tenth of each lysate sample was retained for use as input samples, and the remainder was incubated with the respective primary antibody (anti pPKC substrates antibody at 1:25 dilution or anti total polyclonal cMyBP-C used at 1 µg/mg protein) and 25 µl protein A/G sepharose beads (Santa Cruz, California) overnight at 4 °C on an over-end rotator. Following centrifugation to remove the supernatants, beads were extensively washed three times with lysis buffer (10,000 g, 30 s). Following a final wash with 100 mM Tris-HCl, pH 7.6, beads were resuspended in 50 µl loading buffer and heated to 90 °C for 5 min. Samples were centrifuged at 16,000 g to pellet the beads, and supernatants were subjected to electrophoresis on a 4-12% gradient gel (NuPAGE, NOVEX). For detection of co-IP'd proteins, gels were removed from the tank and assembled in a transfer cassette. Membranes were subjected to immunoblotting as described

previously. For mass spectrometry, gels were stained with excess Instant Coomassie Blue stain (Invitrogen) for 1 h on a rocking platform. Bands of interest were excised from the gel, subjected to trypsin digestion and analysed by mass spectrometry as previously described (Babraham Research Campus, Cambridge) (Supplementary Figure 11) [62].

2.12 Data analysis and statistics

For each photometry experiment, data were collected from n_{cells} from N_{rats} with these numbers indicated in the figure legends. Unless indicated otherwise, cells per animal were pooled and the effect of intervention calculated from an average mean of all animals. Results are expressed as mean $\pm$ standard error of the mean (SEM).

Statistical analysis of the differences between treatments over time for each group of data was carried out using Two-way Repeated-Measures ANOVA in SPSS (IBM).

Post-hoc Bonferroni tests were carried out to analyse differences between treatments at each time point. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1 ET-1 stimulates a biphasic response that is dependent on activation of ET_A and ET_B receptors

Consistent with previous reports [10], ET-1 caused an initial reduction in fractional shortening (2 min), followed by a gradually increasing positive inotropy, which plateaued after 20 min (Figure 1; AI and AII). The rate of contraction was not altered from baseline at 2 min, but it was significantly greater at the peak of the inotropic response (Figure 1; AIII). In parallel with measuring fractional shortening, the associated electrically-evoked Ca²⁺ transients following ET-1 stimulation were also analysed. As we have previously shown [10,52], at 2 min, where a negative inotropic response to ET-1 was observed, no alteration in the amplitude of the associated Ca²⁺ transients was seen; however, the amplitude of the Ca²⁺ transients was significantly elevated when measured at the peak of the inotropic response (20 min) (Figure 1; BI and BII). The rate of rise of the Ca²⁺ transient, which may better reflect Ca²⁺ flux from the SR independent of sequestration and buffering mechanisms was also measured [54]. A significant increase in the rate of rise of Ca²⁺ transients was observed after 20 min of ET-1 stimulation (Figure 1; BIII).

ET-1 acts via ET_A and ET_B receptors, which are both expressed in cardiomyocytes. The contribution of each receptor subtype to the actions of ET-1 was therefore determined. Inhibition of ET_A receptors with BQ-123 (1 µM) abrogated the positive inotropic effect and suppressed the enhancement in Ca²⁺ transient amplitude elicited by ET-1, without affecting the negative inotropy (Figure 1; CI and CIII and Supplementary Figure 1). BQ-123 also attenuated the increases in the rate of rise of contraction and of Ca²⁺ transient generation (Figure 1; CII and CIV). In contrast,

inhibition of ET_B receptors with BQ-788 (200 nM) prevented the negative inotropic action of ET-1 without affecting the positive inotropic phase of the response to ET-1 or the ET-1-mediated increase in the rate of contraction (Figure 1; DI and DIII and Supplementary Figure 1). BQ-788 application did not affect the amplitude of the Ca²⁺ response to ET-1, but it did prevent the increased rate of rise of the Ca²⁺ response (Figure 1; DII and DIV).

Pre-incubation of myocytes with BQ-123 or BQ-788 for 30 min prior to the start of recordings did not affect fractional shortening or Ca²⁺ transients at baseline or throughout the experimental time course in which ET-1 was not added (Supplementary Figures 1 and 10).

The expression of both ET receptor isoforms in cardiac myocytes was confirmed by immunofluorescence analysis. Confocal imaging of immunostained cells revealed that both receptor isoforms were distributed on the sarcolemma and along the t-tubular membranes in ARVMs. This distribution of ET receptors overlapped with that of the Na⁺/Ca²⁺ exchanger (NCX), which is an established marker of the surface and t-tubular membranes of ARVMs (Figure 1; EI and EII) [17]. These data are consistent with our previous findings demonstrating t-tubular-localisation of ET_A receptors and their close proximity to RyRs on the SR [15], further supporting the role of ET receptors in regulating the machinery underlying ECC.

Taken together, these data show discrete effects of both ET receptor isoforms in regulation of myocyte contractility. Signalling downstream of the ET_A receptor underlies the positive inotropic effect and increased Ca²⁺ transient amplitude elicited by ET-1, whereas signalling from the ET_B receptor mediates the negative inotropic response to ET-1.

3.2 PLC activity is required for the effects of ET-1 on ventricular inotropy and Ca^{2+} signalling

The contrasting physiological responses of ARVMs to ET_A and ET_B receptor activation [13-15] are consistent with their coupling to different signal transduction cascades [8,14,27]. The signalling pathways involved in mediating the effects of ET receptor activation in these responses were therefore investigated. Given the importance and hierarchical position of phospholipase C (PLC) in signalling the acute responses to G protein-coupled receptors (GPCR) in cardiomyocytes [27], we focused on dissecting its role in mediating the effects of ET-1. PLC inhibition with U73122 (10 μM) abrogated changes in contractility and Ca^{2+} handling (amplitude and rate of rise) elicited by ET-1 (Figure 2A and Supplementary Figure 2). Pre-incubation with U73122 did not alter fractional shortening or Ca^{2+} transients at baseline or throughout an experimental time course without addition of ET-1 (Supplementary Figures 2 and 10).

Based on these data, we hypothesised that the products of PLC-mediated hydrolysis of PIP_2 , InsP_3 and DAG acting via PKC mediate the effects of ET-1 on contraction and Ca^{2+} signalling.

3.3 InsP_3R signalling affects the kinetics of electrically-evoked Ca^{2+} transients.

We, and others, have shown that InsP_3Rs are functionally expressed in ventricular myocytes [9,10,12,33,35]. However, although direct activation of InsP_3Rs with cell-permeant InsP_3 increases the amplitude of electrically-evoked Ca^{2+} transients in diseased ARVMs [36], the contribution of this pathway to the regulation of contraction by ET-1 in healthy rat ventricular myocytes is not clear.

To determine whether InsP_3 signalling contributes to the activity of ET-1, this pathway was manipulated using two previously validated complementary approaches [12,36,63]. Specifically, InsP_3 Rs were inhibited with 2-APB [63] or InsP_3 signalling to the receptor suppressed via ectopic expression of a GFP-tagged form of type 1 InsP_3 5'phosphatase (5'Phos), which converts InsP_3 to inactive InsP_2 .

Inhibition of InsP_3 Rs with 2-APB (2 μM) did not affect the positive inotropic response following ET-1 application, whereas its application abrogated the transient negative inotropic phase despite no Ca^{2+} change during this phase of the ET-1 response (Figure 2B and Supplementary Figure 3). The significantly increased rate of contraction in ET-1 stimulated myocytes observed during the positive inotropic phase of the response was also absent when 2-APB was co-applied. (Figure 2; CI). Notably, inhibition of InsP_3 signalling did not affect the increase in amplitude of electrically-evoked Ca^{2+} transients detected 20 min after ET-1 stimulation. The ET-1 dependent increased rate of rise of Ca^{2+} transients at this time point was however also absent in myocytes in which 2-APB was co-applied (Figure 2; CII). As previously shown [63], application of 2-APB did not affect fractional shortening or Ca^{2+} transients at baseline or over an experimental time course without ET-1 stimulation (Supplementary Figures 3 and 10).

Consistent with the results obtained with 2-APB, overexpression of 5'Phos did not affect the positive inotropic phase and enhancement in Ca^{2+} dynamics elicited by ET-1 (Supplementary Figure 4; I to IV). As with 2-APB, expression of 5'Phos prevented negative inotropic response after ET-1 stimulation, when compared to GFP-expressing myocytes (Supplementary Figure 4; I and III). Expression of GFP or 5'Phos did not affect contraction and Ca^{2+} transients during an experimental time

course without ET-1 stimulation (Supplementary Figure 4). In addition, expression of 5'Phos did not affect baseline contraction or electrically evoked Ca^{2+} transients (Supplementary Figure 10).

Together, these data show distinct roles of InsP_3 signalling during the positive and negative inotropic phases of the ET-1 response. InsP_3 is required for the negative inotropic effect but not the positive inotropic effect of ET-1. Whilst InsP_3 signalling contributes to the enhanced rate of rise of Ca^{2+} transients in ET-1 stimulated myocytes, it is not required for the associated increase in amplitude of Ca^{2+} transients.

3.4 ET-1-mediated inotropy is regulated by PKC

Although PLC activity was required for both negative and positive inotropic effects of ET-1, our data indicate that metabolites of its activity, other than InsP_3 , are involved in regulating positive inotropy. We therefore assessed the role of PKC activated downstream of DAG. Immunoblotting of lysates prepared from ET-1-stimulated myocytes using a pan-specific antibody that recognises classical and novel PKC isoform families in their active phosphorylated form revealed an increase in activated PKCs (ϵ , α , β , γ , η , and θ). This effect of ET-1 was prevented by application of the PKC inhibitor chelerythrine (Chel) (Figure 3; AI). The loss of immunoreactivity of this antibody following alkaline phosphatase treatment of the protein lysates demonstrated its specificity for the phosphorylated forms of the various PKC isoforms (Figure 3; AII).

Application of Chel had a pronounced impact on the effects of ET-1 on fractional shortening and amplitude of Ca^{2+} transients (Figure 3; BI to BIV and Supplementary Figure 5). Specifically, Chel application did not affect the negative inotropic phase of the contractile response to ET-1 but it caused substantial increases in fractional

shortening and Ca^{2+} transient amplitude during the positive inotropic phase of the ET-1 response. The rates of cell shortening and of Ca^{2+} transient increase at 2 and 20 min after ET-1 stimulation were not affected by Chel (Figure 3; BV and BVI). Addition of Chel in the absence of ET-1 did not have any effect on fractional shortening, indicating that the inhibitors were acting on signals downstream of ET-1 (Supplementary Figure 5). Notably, application of Chel alone induced an increase in Ca^{2+} transient amplitude in the absence of ET-1 stimulation (Supplementary Figure 5), although these effects were significantly smaller than those observed when Chel was applied in the presence of ET-1 (Figure 3B and Supplementary Figure 5).

The effects of Chel on the contractile response to ET-1 were mirrored by Bisindolylmaleimide I (BimI) that prevents PKC signalling via a mechanism distinct from that of Chel (Supplementary Figure 6); Chel interacts with the PKC catalytic domain and prevents translocation of PKC to the sarcolemma necessary for activation, whereas BimI competitively binds to the ATP binding site of PKC, inhibiting its activity. Application of Bisindolylmaleimide V (BimV; 5 μM), an inactive analogue of BimI, did not impact upon the effects of ET-1 on contraction or Ca^{2+} transients (Supplementary Figure 6). Like Chel, BimI also prevented ET-1-stimulated increase in PKC phosphorylation (Supplementary Figure 6; I). In addition, pre-incubation with Chel or BimI did not alter fractional shortening or amplitude of Ca^{2+} transients at baseline (Supplementary Figure 10). These results collectively indicate that PKC is activated downstream of ET-1, contributing to the associated inotropy and increased Ca^{2+} transient amplitude.

To determine whether PKC selectively contributed to the ET-1-induced effects on contraction and Ca^{2+} handling, or whether it was a generic mediator of cardiotropic

agents, we also examined the consequences of its inhibition upon the inotropic response to beta-adrenergic receptor stimulation with isoproterenol (Iso). To allow comparison of the effects of PKC inhibition between inotropy induced by Iso and ET-1, Iso was applied at a concentration that induced a response similar in magnitude to that of ET-1 [10]. Application of Iso (10 nM) induced the expected inotropic response and enhancement in Ca^{2+} transient amplitude. These effects were insensitive to PKC inhibition (Supplementary Figure 7; I and II) but consistent with the established role of PKA in mediating the effects of beta-adrenergic receptor activation, were abrogated by inhibition of PKA with H89 (an inhibitor of the adenosine triphosphate site on the PKA catalytic subunit) (Supplementary Figure 7; III and IV). Notably, the effects of ET-1 on contraction and Ca^{2+} transients were not affected by H89 (Supplementary Figure 7; V to VIII).

Taken together, these data demonstrate that PKC is selectively engaged by ET-1 but not by isoproterenol, to regulate myocyte contraction and Ca^{2+} transients. In particular, PKC activation restricts the enhancement of Ca^{2+} transients and inotropy elicited by ET-1.

3.5 PKC inhibition increases cytosolic Ca^{2+} transient amplitude independent of SR Ca^{2+} load

We next considered whether increase in Ca^{2+} transient amplitude following PKC inhibition was brought about through greater Ca^{2+} release from SR stores. To this end, we first assessed the magnitude of the electrically-evoked Ca^{2+} transients as a fraction of SR Ca^{2+} load (fractional Ca^{2+} release; FCR). FCR was determined according to the protocol outlined in Figure 4A and calculated as the ratio of the amplitude of electrically-evoked Ca^{2+} transient to the amplitude of the Ca^{2+} transient elicited by

rapid perfusion of caffeine, which releases all Ca^{2+} from the store [29]. Consistent with previous reports, ET-1 significantly increased FCR (Figure 4B) [29]. FCR was further increased in ET-1-stimulated myocytes by PKC inhibition with Chel (1 μM), which was applied 20 min prior to the start and then also present throughout the experiment (Figure 4B). The amplitude of the caffeine-stimulated Ca^{2+} transient was not affected by ET-1 and/or inhibition of PKC (Figure 4C), indicating that the effect of PKC inhibition did not result from altering the pool of releasable Ca^{2+} . Importantly, indo-1 was not saturated by Ca^{2+} released by caffeine, as revealed by a greater amplitude of the Ca^{2+} transient induced by the Ca^{2+} ionophore 8-Br-A23187, thus validating this assay for measuring caffeine-stimulated Ca^{2+} transients (Supplementary Figure 8; I).

As a further measure of released Ca^{2+} , we also analysed the rate of decay of the caffeine-stimulated Ca^{2+} transient [64]. Under these conditions, NCX is the principal contributor to cytosolic Ca^{2+} clearance allowing the rate of decay (K) to be employed as an index of released Ca^{2+} . We did not observe an effect of ET-1 or Chel on the rate of decay, which is suggestive of no change in NCX-mediated extrusion of Ca^{2+} under those conditions (Figure 4D). Moreover, no effect of these interventions on the rate constant of decay of electrically-evoked Ca^{2+} transients, where SERCA is primarily responsible for Ca^{2+} clearance, was observed suggesting no effect of ET-1 or PKC on SERCA activity (Supplementary Figure 8; II)

The above data indicate an effect of PKC inhibition on FCR, but no impact on the maximal releasable Ca^{2+} . However, the amplitude of caffeine-induced Ca^{2+} transients reported by indo-1 may not accurately reflect the total SR content, which may obscure an effect of ET-1 and Chel on store loading [65]. To more accurately analyse SR store

load, electrophysiological analysis of NCX current (I_{NCX}) following caffeine-evoked mobilisation of SR Ca^{2+} [65], was performed as previously described [59]. Under these conditions, the SR does not contribute to Ca^{2+} clearance, and the NCX current (I_{NCX}) reflects SR Ca^{2+} load. Using this approach, no effect of ET-1 or Chel on the integral of I_{NCX} was detected, indicating that store loading was neither influenced by ET-1 nor by PKC inhibition (Figure 4E). Since GPCR signalling has been shown to influence L-type Ca^{2+} channel activity [66], we also examined whether Ca^{2+} current (I_{CaL}) was altered following application of ET-1 and/or Chel. However, no significant effects were observed (Supplementary Figure 8; III)

Taken together, it can be concluded from these data that the effect of PKC inhibition on Ca^{2+} transient amplitude was the result of an enhancement of electrically-evoked Ca^{2+} release amplitude (fractional Ca^{2+} release) and not an alteration in store loading, NCX activity or I_{CaL} .

3.6 PKC ϵ restricts ET-1 enhancement of ECC-associated inotropy and Ca^{2+} transient amplitude

The effects of PKC inhibition described above suggest that the inotropic response and increase in Ca^{2+} transient amplitude induced by ET-1 stimulation are restricted by concurrently activated PKC. The data presented in Figure 3A shows that ET-1 stimulation increases the abundance of active phosphorylated forms of PKC (as two bands) in ARVMs, which was prevented by PKC inhibition. Based on their known migration in SDS-PAGE and established antibody profile, the upper band would be predicted to correspond to PKC ϵ (Figure 3A; upper band) and the lower band to other classical and novel PKC isoforms (Figure 3A; lower band). Owing to the prominence of the upper band and the established roles of PKC ϵ in cardiac physiology [67], we

focused on confirming the identity of PKC ϵ and, subsequently, its role in myocytes during ET-1 stimulation. Consistent with our hypothesis, a protein that migrated equivalently to the upper band detected using the pan pPKC antibody was also detected using antibodies that recognised phosphorylated PKC ϵ or total PKC ϵ (Figure 5; AI: lanes 1, 2 and 3, respectively). This pPKC ϵ antibody-reactive band was lost following dephosphorylation of proteins in the cell lysates with alkaline phosphatase (Figure 5; AII). Alkaline phosphatase treatment did not prevent detection using an antibody that recognises PKC ϵ , irrespective of its phosphorylation state (Figure 5; AII). Therefore, these data indicate that PKC ϵ is a prominent target of ET-1 in ARVMs.

Next, we investigated the role of PKC ϵ in mediating the effects of ET-1 on contraction and Ca²⁺ transients. To this end, two complementary approaches were employed to selectively inhibit PKC ϵ : 1) application of a cell-permeant peptide EAVSLKPT, which specifically inhibits the translocation of PKC ϵ to membranes, thereby preventing activation [68], and 2) adenoviral-mediated expression of a dominant-negative mutant of PKC ϵ (DN-PKC ϵ) [69].

In experiments with cell permeant EAVSLKPT peptide, cells were pre-incubated with the peptide (50 μ M) for 30 min before the start of recording. Neither cell contraction nor Ca²⁺ transient amplitude at baseline was affected by pre-incubation with the peptide (Supplementary Figure 10). Consistent with the effects of Chel or BimI, application of the peptide potentiated ET-1-stimulated inotropy and increase in Ca²⁺ transient amplitude (Figure 5B and Supplementary Figure 9; I and II). In the absence of ET-1, the peptide did not affect electrically evoked contraction or Ca²⁺ transient amplitude (Supplementary Figure 9; III and IV). Notably, in contrast to the effects of

non-selective pharmacological inhibition of PKC, application of the inhibitory peptide also abrogated the negative inotropic effect of ET-1 and induced a more rapid onset of positive inotropy (Figure 5; BI and BIII).

Expression of DN-PKC ϵ was achieved by adenoviral infection of myocytes maintained in culture for 16 h prior to experimental procedures. Immunoblotting of lysates using a PKC ϵ -specific antibody confirmed increased PKC ϵ expression (Figure 5; CI). Since DN-PKC ϵ has the same molecular weight as the endogenous wild-type protein [69], no additional immunoreactive band was detected. Importantly, as revealed by probing the same lysates with a pan-specific phospho-PKC antibody, DN-PKC ϵ expression did not affect the abundance of the activated forms of other classical and novel PKC isoforms, the most prominent of which is a band below that of PKC ϵ (α , β , γ , η or θ) (Figure 5; CI).

In line with the findings using other strategies to inhibit PKC, expression of DN-PKC ϵ resulted in increases in the inotropic response and amplitude of Ca²⁺ transient during the second phase of the response to ET-1 (Figure 5; CII to CV and Supplementary Figure 9; V and VI). The rates of rise of contraction and of the Ca²⁺ signal were also increased during this second phase of the ET-1 response in myocytes expressing DN-PKC ϵ (Figure 5; CVI and CVII). The initial negative inotropic phase after ET-1 stimulation was however prevented by DN-PKC ϵ expression (Figure 5; CVI and CVII).

Baseline fractional shortening and amplitude of Ca²⁺ transients were not affected by expression of DN-PKC ϵ when compared to cells infected with a β -gal control virus (Supplementary Figure 10). Moreover, in the absence of ET-1, DN-PKC ϵ expression

did not affect contraction or amplitude of Ca^{2+} transients (Supplementary Figure 9; VII and VIII).

Taken together, these data identify a role for PKC ϵ in the regulation of contraction and amplitude of Ca^{2+} transients by ET-1 in ARVMs. In particular, PKC ϵ activated following ET-1 stimulation acts to restrict the positive inotropic effects and enhancement of Ca^{2+} transient amplitude elicited by this agonist. PKC ϵ may also play a part in mediating the initial negative inotropic effect of ET-1.

3.7 PKC restricts ET-1-dependent increases in fractional shortening and amplitude of Ca^{2+} transients through phosphorylation of cMyBP-C

We aimed to identify a PKC effector protein that underlies the activities of ET-1 described above. Mass spectrometric analysis (MS) was employed to identify proteins that exhibited an increase in PKC-dependent phosphorylation upon ET-1 stimulation. Proteins phosphorylated by PKC following ET-1 stimulation were isolated by immunoprecipitation using an antibody that recognises phosphorylated PKC substrates. Control samples were prepared using non-specific (NS) IgG. Immunoprecipitated proteins were resolved by SDS-PAGE, and bands corresponding to abundant proteins identified by Coomassie staining were excised and subjected to trypsin digestion and MS analysis [62]. We identified cMyBP-C as a ~150 kDa protein that exhibited a PKC-dependent increase (i.e., Chel-sensitive) in phosphorylation upon ET-1 stimulation (Figure 6; AI and Supplementary Figure 11). Confirming the identification of cMyBP-C by mass spectrometry, cMyBP-C was also detected by immunoblotting of proteins immunoprecipitated with the pPKC substrates antibody from protein lysates of myocytes stimulated with ET-1, with and without PKC inhibition (Figure 6; AII).

To confirm our hypothesis that PKC, following ET-1 stimulation, phosphorylates cMyBP-C, we stimulated myocytes with ET-1 and immunoblotted for levels of phosphorylation at S273 and S302 of cMyBP-C, residues which have been previously shown to be phosphorylated by PKC [70]. We observed an ET-1-mediated increase in cMyBP-C phosphorylation on S302 but not of S273 (Figure 6; AIII).

To assess the contribution of PKC-mediated phosphorylation of cMyBP-C on S302 to the effects of ET-1, we generated adenoviruses to overexpress WT and a mutated form of cMyBP-C that could not be phosphorylated on S302 due to mutation of this residue to alanine (AdV.cMyBP-C-WT and AdV.cMyBP-C-S302A, respectively; Supplementary Figure 12; I and II). Transduced cells were identified by red fluorescent protein expressed via a separate promoter in the adenoviral vector. Immunoblotting of protein lysates prepared from ARVMs transduced with the cMyBP-C adenoviral vectors (WT and S302A) showed a 6-fold overexpression of cMyBP-C-WT and cMyBP-C-S302A compared to ARVMs transduced with control adenoviruses (Figure 6B). Given that the WT or mutated cMyBP-C were expressed at a 6-fold excess over endogenous levels of cMyBP-C, it would be expected that these proteins would dominate in the action of cMyBP-C in these cells. Moreover, using Pdbu to maximally activate PKCs, we assessed whether phosphorylation of cMyBP-C is affected by the presence of the mutated cMyBP-C. We show in Supplementary Figure 12; III that the presence of the mutated cMyBP-C does not prevent the phosphorylation of the endogenous protein. Taken together, we expect the function of the mutated protein over the endogenous to mediate the effects described here.

We subsequently assessed the effect of ET-1 on contraction and amplitude of Ca^{2+} transients in myocytes transduced with cMyBP-C-WT or cMyBP-C-S302A

adenoviruses. Overexpression of cMyBP-C-WT did not affect baseline Ca^{2+} transients or inotropic responses to ET-1 when compared to responses in cells infected with control RFP adenovirus (Supplementary Figure 12; IV and V). As observed with either non-PKC isoform-specific- (with Chel) or PKC ϵ -specific (after DN-PKC ϵ overexpression or with EAVSLKPT peptide) inhibition, ET-1-stimulated inotropy was substantially enhanced in myocytes expressing cMyBP-C-S302A, when compared to myocytes overexpressing cMyBP-C-WT (Figure 6; CI and CIII and Supplementary Figure 12; VI). Consistent with the effects of PKC ϵ -specific inhibition, the negative inotropic response to ET-1 was also diminished in cMyBP-C-S302A expressing cells with a more rapid onset of positive inotropy observed. The amplitude of Ca^{2+} transients in ET-1 stimulated myocytes was also significantly increased upon cMyBP-C-S302A expression (Figure 6; CII and CIV and Supplementary Figure 12; VII).

Together, these data support a model in which PKC-mediated phosphorylation of cMyBP-C on S302 restricts ET-1-mediated effects on fractional shortening and amplitude of Ca^{2+} transients.

4. Discussion

Here, we established that ET-1-stimulated increases in Ca^{2+} transient amplitude and contraction in ARVMs are subject to PKC regulation that involves phosphorylation of cMyBP-C (Figure 7). Unlike in atrial myocytes, where InsP_3 signalling contributes to the pro-arrhythmic and inotropic effects of ET-1 [9,10], we also show that InsP_3 does not play a part in inotropy or increase in Ca^{2+} transient amplitude following ET-1 stimulation in ventricular myocytes. However, InsP_3R -induced Ca^{2+} signalling is required for the negative inotropic actions of ET-1 and enhances the rate of rise of the Ca^{2+} transient following ET-1 stimulation.

In cardiac myocytes and tissues of rats and larger mammals, ET-1 elicits a biphasic response consisting of an initial transient negative inotropy response followed by sustained positive inotropy [23]. Mouse myocytes are an exception that only exhibit a negative inotropic response [71]. In agreement with previous studies, we found that these dichotomous effects are in part mediated by the action of the two ET-1 receptor subtypes (Figure 1) [27]. ET_A receptor signalling is primarily responsible for mediating the inotropic effects of ET-1 [12,29,72], whereas the ET_B receptor may contribute to the initial negative inotropy observed following ET-1 stimulation [71]. Different actions of ET_A and ET_B receptors have also been described in the vasculature. Specifically, ET_B dependent increased NO production in endothelial cells induces vasodilation whereas ET_A dependent inhibition of NO production in smooth muscle cells promotes vasoconstriction [73,74]. The effects of selective inhibition of ET_A and ET_B receptor on contraction suggest a functional interaction between signalling pathways activated downstream of these receptors - the balance of which is determined by the strength of signals from these two receptor isoforms. Indeed, the

negative inotropic effect of ET-1 was longer in duration when the ET_A receptor was inhibited and the positive inotropic response developed more rapidly when ET_B receptors were inhibited (Supplementary Figure 1). Notably, while ET-1 elicited a biphasic effect on contraction that involved both ET receptor subtypes, Ca²⁺ transient amplitude and kinetics were only altered during the second positive inotropic phase of the ET-1 response. Moreover, the magnitude of the increase in amplitude of the Ca²⁺ transient did not reflect that observed for contraction. The more robust effects of ET-1 in ventricular myocytes on contraction and regulation of myofilament function than Ca²⁺ regulatory mechanisms suggested that these two effects were not directly correlated.

ET_A and ET_B receptor signalling via phospholipase C engages multiple pathways in cardiac myocytes with effects on contractility and gene expression. Adding to our previous description of a contribution of PLC activation to the enhanced amplitude of Ca²⁺ transients during the second phase of ET-1 response [10], we report that PLC was also required for generating the ET_B-mediated negative and ET_A-mediated positive inotropic response to ET-1 (Figure 2). Whether signalling via InsP₃/Ca²⁺ or the DAG/PKC pathways were responsible for the actions of PLC were therefore determined.

InsP₃ stimulated Ca²⁺ release from the sarcoplasmic reticulum contributes to altered Ca²⁺ handling and to signalling hypertrophic gene expression during pathology of ventricular cardiac myocytes [12,32-34,36,75]. Here, consistent with our previous observations in ventricular myocytes from healthy rat [52], a limited contribution of InsP₃ signalling to the effects of ET-1 was observed. Notably, while a change in [Ca²⁺]_i is a primary readout of increased InsP₃ signalling, inhibition of InsP₃ action

did not impact on the amplitude of the Ca^{2+} transient during the first or second phase of the response to ET-1. These data contrast with findings in rabbit where ET-1 promotes a significant InsP_3 -dependent increase in the amplitude of electrically evoked Ca^{2+} transients. InsP_3 dependent increases in Ca^{2+} transient amplitude have also been observed in rat and rabbit myocytes exposed to cell permeant InsP_3 [10,76]. These conditions may however be associated with a greater intracellular level of InsP_3 than the low levels generated in myocytes following exposure to ET-1 or other GPCR agonists [77]. Differences between effects of InsP_3 in rabbit and rat may also arise due to varying contributions of the SR to the Ca^{2+} transient during ECC. Indeed, while Ca^{2+} release from the SR accounts for 90 % of the Ca^{2+} underlying the Ca^{2+} transient in rats, it is responsible for 70 % in rabbit [78,79]. An InsP_3 dependent increase in rate of rise of the Ca^{2+} transient was however observed during the second phase of the response to ET-1 (figure 2; CII). This effect is consistent with previous observations from our laboratory and elsewhere, and has been proposed to be a consequence of Ca^{2+} release via InsP_3 Rs sensitising neighbouring dyadic RyRs that enhance and synchronise CICR during ECC [36,80,81]. Given that the rate of rise of the Ca^{2+} transient provides a better measure of Ca^{2+} release flux from the SR than analysis of amplitude, which is influenced by Ca^{2+} buffering and clearance mechanisms, these effects on the Ca^{2+} transient may also indicate enhanced Ca^{2+} movement during ECC [54]. As a consequence, contraction may be influenced.

While inconsistent effects of InsP_3 signalling on Ca^{2+} handling are described in healthy ventricular myocytes [10,52,76], less is known regarding effects of InsP_3 signalling on myocyte contraction. Here, suppression of InsP_3 signalling prevented the negative inotropic response to ET-1. This effect is surprising given that Ca^{2+} transients were not altered in their amplitude or kinetics during this phase of the

response. We cannot rule out that these effects were due to off target actions of 2-APB on other ET-1 signalling effectors [82]. However, a similar loss of the negative inotropic response was observed when InsP₃ signalling was inhibited through overexpression of the InsP₃ 5'phosphatase, which metabolises InsP₃ to inactive InsP₂. The mechanism by which InsP₃/InsP₃R activity generated this negative inotropic effect remains to be determined. In our previous studies in rat ventricular myocytes, we reported changes in contraction in ET-1 stimulated myocytes but did not detect an inotropic response in myocytes exposed to InsP₃ despite augmented Ca²⁺ transients. Notably, a study on rabbit ventricular myocytes by Domeier et al., [76] described a robust InsP₃ dependent increase in Ca²⁺ transient amplitude, but without assessing myocyte contraction. Together, the poor correlation between the effects of InsP₃ signalling and contraction downstream of ET-1, would suggest that the predominant mechanism of action of ET-1 in healthy rat ventricular myocytes is via signalling downstream of DAG, which is also produced during PLC hydrolysis of PIP₂.

The minimal engagement of InsP₃R signalling by ET receptors in healthy rat ventricle stand in contrast to those in atria as well as in diseased ventricle where ET receptor activation stimulates a more robust InsP₃ dependent enhancement of Ca²⁺ transient amplitude as well as a substantial increase in the frequency of extra systolic Ca²⁺ transients [10,20,54,83]. A greater abundance of InsP₃Rs, ET-1 and ET_A receptors likely underlie these regional and disease related differences in cardiac InsP₃ signalling [15,34,52,84].

While InsP₃ signalling did not substantially contribute to regulation of contraction by ET-1, PKC was found to potently govern myocyte contraction, especially during the positive inotropic phase. Multiple PKC isoforms are expressed in the heart, although

considerable species differences exist [85-87], and their expression levels are closely associated with normal cardiac development and/or pathology [88,89]. Despite a plethora of studies on the role of PKC in the heart, and multiple targets identified including L-type Ca^{2+} current, Na^+/H^+ exchanger (NHE), NCX and myofilaments [42,43,49,50,90], the underlying molecular mechanism of its actions, particularly in the context of GPCR regulation of contraction, is not fully determined. Moreover, the particular PKC isoform responsible for triggering pathophysiological conditions remains to be fully established [47-50]. Using isoform-specific inhibitors and biochemical assays, we determined that PKC ϵ substantially contributed to the actions of ET-1 on Ca^{2+} handling and contraction ventricular myocytes. Specifically, PKC ϵ mediated the negative inotropy and was responsible for suppressing the positive inotropic response and increase in Ca^{2+} transient amplitude induced by ET-1 (Figures 3 and 5). While non-selective PKC inhibitors also resulted in an increase in the positive inotropic phase of the ET-1 response and increase in Ca^{2+} transient amplitude, these did not abrogate the negative inotropic phase. These differences between the effects on negative inotropic phase between the specific and non-selective PKC inhibitors are not readily explained but could be due to a differential potency on the opposing actions of ET $_A$ and ET $_B$ receptors on contractility. Incomplete inhibition of PKC ϵ by Chel or Bis may also explain the differences between the PKC inhibitors employed. Our data on PKC ϵ acting to restrict the positive inotropic effect of ET-1 are, however, consistent with earlier studies, which showed a reversal of the positive inotropic response to ET-1 by PKC activating phorbol esters [23]. A positive inotropic action of PKC ϵ in ET-1-stimulated rat ventricular myocytes has also been described [91]. While PKC was inhibited by overexpression of DN-PKC ϵ in our study as well as in the study by Kang et al., the

extended period of culture post-adenoviral infection in the latter study could have resulted in a substantially higher level of expression. As such, fidelity of targeting and activity of the DN-PKC ϵ may have been altered. Extended duration of culture also impacts on contractility and ET-1 responses [92], in part through a loss of T-tubules.

PKC has been shown to modulate myocyte contraction through changes in Ca²⁺ handling and altered myofilament properties [42,44,46]. Indeed, PKC phosphorylation of NCX is reported to increase NCX activity [43], which would result in a decrease in cytosolic and SR Ca²⁺, leading to a negative inotropic response. Inhibition of PKC regulation of NCX could thus lead to an enhancement in intracellular Ca²⁺ and contraction in ET-1-stimulated myocytes as described here. However, analysis of SR Ca²⁺ load and the decay phase of the caffeine-induced Ca²⁺ transient (a reporter of NCX activity [65]) showed no ET-1- or PKC- dependent regulation of NCX (Figure 4). Altered L-Type Ca²⁺ current could also contribute to the effects of ET-1. However, no interaction between ET-1 or PKC on L-type Ca²⁺ current was detected. Further supporting the absence of a contribution of store loading or RyR-mediated Ca²⁺ release to the inotropic action of ET-1, PKC inhibition did not affect the inotropic response to β -adrenergic stimulation, which elicits its effects primarily via cAMP and PKA-mediated modification of the Ca²⁺ handling and contractile machinery (Supplementary Figure 7) [3]. Moreover, PKA inhibition did not affect the inotropic action or enhanced Ca²⁺ transients induced by ET-1 (Supplementary Figure 7).

The inotropic effect of PKC inhibition on ET-1-stimulated cells was however more apparent than the increase in Ca²⁺ transient amplitude, suggesting that the dominant action of PKC ϵ was on the myofilaments. Indeed, a desensitisation of the

myofilaments to Ca^{2+} has been reported to mediate the negative inotropic action of ET-1 in mouse myocytes [71]. While alkalisation of the cytosol via PKC-mediated NHE activation is proposed to mediate myofilament sensitisation [93], it was reported that NHE activation only contributed towards approximately half of the inotropic response, indicating that pH is not solely responsible for the effects of ET-1. Although we did not study pH directly, the lack of an effect of ET-1/Chel on the activity of NCX (Figure 4D) is indicative of unaltered Na^+ levels and, subsequently, NHE activity. Neither our data nor the findings from other groups support this possibility (Figure 4) [91].

By mass spectrometric analysis of PKC-phosphorylated proteins, we identified cMyBP-C to be a downstream target of ET-1 [94,95] (Figure 6). cMyBP-C is a protein that influences both myofilament orientation and contractile mechanics. It is phosphorylated by a number of protein kinases, such as PKA, PKC, PKD and CaMKII at multiple residues (S273, S282, S302, S307), affecting the cross bridge cycle and, ultimately, cardiac contractility [70]. As such, it may be regarded as an integrator of signalling activity in the myocyte [96]. Increased PKC-dependent phosphorylation of cMyBP-C on S273 and S302, has been reported in heart failure suggesting a role for alterations of cMyBP-C function in disease [97]. We found that cMyBP-C phosphorylation on S302 contributed to the actions of PKC activated downstream of ET-1 on contraction and Ca^{2+} transient amplitude. Indeed, the effects of expression of a non-phosphorylatable (S302A) mutant of cMyBP-C on ET-1 stimulated changes in contraction and Ca^{2+} transients mirrored those in which PKC was inhibited (Figures 3, 5 and 6). Phosphorylation of cMyBP-C on S302 has recently also been shown to enhance myocyte contraction during β -adrenergic stimulation [98]. While our analysis did not examine contraction of myocytes expressing cMyBP-

C S302A when stimulated with β agonist, PKC inhibition did not influence the inotropic action of isoproterenol, unlike that observed for ET-1. Further supporting the specificity of this PKC regulation of cMyBP-C to the actions of ET-1, no effects of PKC inhibition or of cMyBP-C S302A overexpression were observed in the absence of ET-1 application. This would suggest that ET-1 creates the context, possibly involving modification of other targets, for PKC modification of cMyBP-S302 to restrict inotropy. Although an effect of S302A mutation in cMyBP-C on contraction was predicted based on previous studies [70,99,100], it was intriguing that expression of this mutant also affected the amplitude of Ca^{2+} transients. Indeed, no such effect of cMyBP-C on Ca^{2+} transients has been previously described. How altered cMyBP-C phosphorylation affects cytosolic Ca^{2+} transient amplitude is not clear. Intriguingly, expression of mutated TnI that cannot be phosphorylated PKC downstream of ET-1 also results in altered Ca^{2+} transient amplitude and kinetics [101]. These observations suggesting that ET-1 activates PKC, which then phosphorylates cMyBP-C on S302 to restrict ET-1 enhancement of ECC-associated inotropy and Ca^{2+} transients in myocytes, are consistent with previous reports of a decrease in myofilament sensitivity to Ca^{2+} following PKC phosphorylation of cMyBP-C [102]. PKC also alters myofilament sensitivity to Ca^{2+} through phosphorylation of troponin I. While phosphorylation of TnI on serines 23 and 24, amino acids also phosphorylated by PKA [103], reduces myofilament Ca^{2+} sensitivity, an opposing, and temporally delayed effect is elicited by PKC phosphorylation of troponin I on other sites [101,104,105]. ET-1 stimulated PKC also influences contractility via RhoA and regulation of myosin phosphorylation [101,106]. Further studies are required to dissect the relative contributions of these phosphorylation events on myofilament proteins. However, while we cannot fully

exclude the possibility that overexpression of MyBP-C S302A elicits certain indirect effects downstream of ET-1 stimulation, the data presented here support a significant role of cMyBP-C phosphorylation by PKC in mediating the effect of ET-1 on contraction and Ca^{2+} handling.

Targeting the PKC/cMyBP-C axis may be of therapeutic value. Enhancement of contraction, as well as $[\text{Ca}^{2+}]_i$ and SR Ca^{2+} release, as observed in our study in response to PKC inhibition, without the enhancement of luminal Ca^{2+} , suggests a suitable target for intervention during heart failure, where Ca^{2+} handling may be reduced and contraction suppressed. In this way, PKC ϵ inhibition could complement PKC α activity in heart failure, which enhances cardiac contractility through sensing intracellular $[\text{Ca}^{2+}]_i$ and signal transduction events that underlie the progression to heart failure [40,107]. Our data may also suggest a mechanism for the increased PKC ϵ in myocardial infarction and the protective effect elicited by PKC ϵ in ischaemic preconditioning [108]. Specifically, we propose that PKC ϵ activation downstream of ET-1, which is also increased in disease, represses Ca^{2+} transients and contraction, thereby preserving normal function and preventing hypercontracture, Ca^{2+} toxicity and death.

Here, we established that the ET_A-dependent positive and ET_B-mediated negative inotropic effects of ET-1 both require PLC activity and are regulated by InsP_3 and PKC ϵ (Figure 2). However, the mediators downstream of PLC that counteract PKC ϵ and underlie the positive phase of the ET-1-mediated effect on fractional shortening remain to be fully identified. Notably, in order for PKC ϵ inhibition to elicit an inotropic effect, ET-1 stimulation was required, indicating that other pathways downstream of the ET receptor including other PKC isoforms are also required. By

understanding how ET-1 couples to downstream effectors, we can better develop strategies to selectively manipulate both beneficial and pathological aspects of ET-1 signalling.

Highlights

- ET_B receptor activation mediates the transient ET-1-induced negative inotropic response in ARVMs. ET_A receptor activation underlies positive inotropic response and increase in systolic Ca^{2+} transient amplitude resulting from prolonged ET-1 stimulation in ARVMs.
- $InsP_3R$ activity contributes to the negative inotropic response to ET-1.
- $InsP_3$ -mediated Ca^{2+} release contributes to the increase in rate of rise of the Ca^{2+} transient during the second inotropic phase of the response to ET-1.
- Enhanced systolic Ca^{2+} transient amplitude induced by prolonged exposure to ET-1 in ARVMs is independent of $InsP_3R$ -mediated signalling.
- $PKC\epsilon$ is required for the negative inotropic responses to ET-1 and restricts the subsequent enhanced contractility and increased amplitude of systolic Ca^{2+} transients.
- PKC elicits its effects on contraction through phosphorylation of cMyBP-C on S302.
- It can be concluded that $PKC\epsilon$ protects ET-1-stimulated myocytes from hypercontraction and Ca^{2+} overload.

5. Figure Legends

Figure 1

ET-1 stimulates a biphasic response that is dependent upon activation of the ET_A and ET_B receptors. **A.** (I) Normalised inotropic response to ET-1 stimulation over a 20-minute period. (II) Representative normalised traces of fractional shortening at the time points indicated after ET-1 stimulation (100 nM). (III) Normalised rate of rise for fractional shortening at the time points indicated after ET-1 stimulation. For comparison, single traces have been time-shifted. **B.** As in A, on peak amplitude of Ca²⁺ transients. **C.** (I) Effect of inhibition of ET_A receptor with BQ-123 (1 µM) upon ET-1-induced changes in myocyte contractility. (II) Effect of inhibition of ET_A receptors with BQ-123 upon ET-1-induced changes in the rate of rise of fractional shortening. (III and IV) As in (I) and (II), on peak amplitude of Ca²⁺ transients. * in CIII signifies difference between 0 and 20 min time points in the ET-1-treated cells. No significant increase between 0 and 20 min in amplitude of Ca²⁺ transients is observed in ET+BQ123-treated myocytes. **D.** As in C, on inhibition of ET_B receptors. n_{cells} = 14; N_{rats} = 3 for control and n_{cells} = 12; N_{rats} = 5 for ET-1, n_{cells} = 6; N_{rats} = 3 for ET-1 and n_{cells} = 11; N_{rats} = 5 for ET-1 + BQ-123, n_{cells} = 7; N_{rats} = 3 for ET-1 and n_{cells} = 8; N_{rats} = 3 for ET-1 + BQ-788. **E.** Confocal images of ARVMs immunostained using antibodies against ET_AR (I) or ET_BR (II) (both in green). In both cases, myocytes were co-immunostained with an antibody that recognises NCX (in red). For both sets of immunostainings (ET_A and ET_B receptor), an image of the whole myocyte, a zoomed image of a region defined by a white box, and an intensity profile of staining of ET receptor and NCX along the white line indicated (to show overlapping distributions) are shown. In both I and II, nuclei are stained in blue with DAPI. Scale bars in the images of the whole myocyte are 50 µ and the zoomed

images 5 μ . * signifies differences between 0 and 2 or 2 and 20 min time points in the same conditions, and # denotes comparison between different experimental conditions, $P < 0.5$; Two-way repeated-measures ANOVA.

Figure 2

Signalling via InsP_3 receptors affects ET-1-induced negative inotropy and kinetics of InsP_3 -induced Ca^{2+} release. **A.** Representative normalised traces and normalised data of the effect of inhibition of PLC with U73122 (10 μM) on ET-1-stimulated (100 nM) changes in fractional shortening (I, III) and Ca^{2+} transient amplitude (II, IV) at the time points indicated. **B.** Representative normalised traces and normalised data of the effect of inhibition of InsP_3R with 2-APB (2 μM) on ET-1-stimulated (100 nM) changes in fractional shortening (I, III) and Ca^{2+} transient amplitude (II, IV) at the time points indicated. **C.** Normalised rate of rise for fractional shortening (I) and peak amplitude of Ca^{2+} transients (II) at the time points indicated after ET-1 stimulation and inhibition of InsP_3R with 2-APB. For comparison, single traces have been time-shifted. $n_{\text{cells}} = 11$; $N_{\text{rats}} = 5$ for ET-1 and $n_{\text{cells}} = 9$; $N_{\text{rats}} = 3$ for ET-1 + U73122, $n_{\text{cells}} = 6$; $N_{\text{rats}} = 3$ for ET-1 and $n_{\text{cells}} = 7$; $N_{\text{rats}} = 3$ for ET-1 + 2-APB. * signifies differences between 0 and 2 or 2 and 20 min time points in the same conditions and # denotes comparison between different experimental conditions, $P < 0.5$; Two-way repeated-measures ANOVA.

Figure 3

ET-1-mediated inotropy is dependent upon PKC activity. **A.** Detection of PKC activation downstream of ET-1 stimulation in ARVMs by immunoblotting using an antibody that recognises the activated phosphorylated forms of the ϵ , α , β , γ , η and θ PKC isoforms (pan pPKC). (I) Effect of pre-incubation of myocytes with the non-

isoform-specific PKC inhibitor chelerythrine (Chel; 1 μ M) on ET-1-mediated PKC activation (II). Effect of incubation of protein lysates prepared from ARVM with alkaline phosphatase on detection of active phosphorylated PKC. **B.** Representative normalised traces and normalised data of the effect of inhibition of PKC with Chel (1 μ M) on ET-1-stimulated (100 nM) changes in fractional shortening (I, III) and Ca^{2+} transient amplitude (II, IV) at the time points indicated. For comparison, single traces have been time-shifted. Normalised rate of rise for fractional shortening (V) and peak amplitude of Ca^{2+} transients (VI) at the time points indicated after ET-1 $\pm$ Chel. $n_{\text{cells}} = 8$; $N_{\text{rats}} = 3$ for ET-1 and $n_{\text{cells}} = 10$; $N_{\text{rats}} = 3$ for ET-1 + Chel. * signifies differences between 0 and 2 or 2 and 20 min time points in the same conditions, and # denotes comparisons between different experimental conditions, $P < 0.5$; Two-way repeated-measures ANOVA.

Figure 4

ET-1 increases fractional Ca^{2+} release (FCR) from the SR in ARVMs, which is potentiated by inhibition of PKC without affecting store loading. **A.** Electrically evoked and caffeine-induced (20 mM) Ca^{2+} transients before and 20 min after application of ET-1 (100 nM). **B.** Average values of FCR before and following application of ET-1 in control myocytes or in myocytes in which PKC was inhibited by pre-incubation with Chel (1 μ M). **C.** Amplitudes of caffeine-induced Ca^{2+} transients in control myocytes or in myocytes in which PKC was inhibited by pre-incubation with Chel (1 μ M). $n_{\text{cells}} = 6-8$; $N_{\text{rats}} = 4$ **D.** Rate of decay of caffeine-induced Ca^{2+} transients in ET-1 stimulated or control myocytes or in myocytes in which PKC was inhibited by pre-incubation with Chel (1 μ M) **E.** Integrated I_{NCX} measurements taken during caffeine stimulation (20 mM) in ARVM treated according

to the conditions shown. $n_{\text{cells}} = 7-8$; $N_{\text{rats}} = 4$. *, $P < 0.5$ and **, $P < 0.001$; Student's *t*-test.

Figure 5

PKC ϵ restricts the ET-1 enhancement of ECC-associated inotropy and Ca²⁺

transients A. (I) Immunoblotting of lysates prepared from ARVM with pan-specific pPKC and PKC ϵ -specific antibodies. (II) Effect of alkaline phosphatase treatment of ARVM lysates upon detection of pPKC ϵ and PKC ϵ . **B.** Representative normalised traces and normalised data of the effect of inhibition of PKC ϵ with EAVSLKPT (50 μM) on ET-1-stimulated (100 nM) changes in fractional shortening (I, III) and Ca²⁺ transient amplitude (II, IV) at the time points indicated. **C.** (I) Immunoblotting of lysates prepared from control ARVM and ARVM overexpressing DN-PKC ϵ with PKC ϵ -specific and pan pPKC antibody. (II to V) As in B, for cells expressing DN-PKC ϵ or β -gal control. (VI and VII) Normalised rate of rise for fractional shortening (VI) and peak amplitude of Ca²⁺ transients (VII) at the time points indicated in ARVM expressing DN-PKC ϵ or β -gal control. For comparison, single traces have been time-shifted. $n_{\text{cells}} = 8$; $N_{\text{rats}} = 3$ for ET-1 and $n_{\text{cells}} = 12$; $N_{\text{rats}} = 3$ for ET-1+EAVSLKPT, $n_{\text{cells}} = 6$; $N_{\text{rats}} = 4$ for ET-1 (β gal) and $n_{\text{cells}} = 7$; $N_{\text{rats}} = 4$ for ET-1 (DN-PKC ϵ). * signifies differences between 0 and 2 or 2 and 20 min time points in the same conditions, and # denotes comparisons between different experimental conditions. $P < 0.5$; Two-way repeated-measures ANOVA.

Figure 6

A. I Coomassie-stained gel of pPKC substrate proteins immunoprecipitated from ARVM stimulated with ET-1 (100 nM) with and without PKC inhibition with Chel (1

μM). Samples immunoprecipitated with non-specific IgG (NS) under the same conditions and an annotated tandem mass spectrum identifying a cMyBP-C tryptic peptide are also shown.

II. Immunoblot detection of cMyBP-C immunoprecipitated from ARVM lysate with the pPKC substrate protein antibody. Lysates were prepared from ARVM treated according to the conditions shown. **III.** Representative immunoblots and histograms of average data showing levels of cMyBP-C phosphorylation on S273 and S302 following ET-1 stimulation. $n = 3$ *, $P < 0.5$; Student's *t*-test. **B.** Representative immunoblots and histogram of average data showing levels of cMyBP-C following overexpression of WT and mutated cMyBP-C (S302A) compared to control. $n = 3$ *, $P < 0.05$ and **, $P < 0.01$; Student's *t*-test. **C.** Representative normalised traces and normalised data of the effect of expression of mutated cMyBP-C (S302A) on ET-1-induced changes in fractional shortening (I, III) and Ca^{2+} transient amplitude (II, IV) at the time points indicated. For comparison, single traces have been time-shifted. $n_{\text{cells}} = 9$; $N_{\text{rats}} = 4$ for cells overexpressing AdV.cMyBP-C-WT and $n_{\text{cells}} = 11$; $N_{\text{rats}} = 4$ for cells overexpressing AdV.cMyBP-C-S302A. * signifies differences between 0 and 2 or 2 and 20 min time points in the same conditions, and # denotes comparison between different experimental conditions, $P < 0.5$; Two-way repeated-measures ANOVA.

Figure 7

Schematic of identified pathways involved in mediating the effects of ET-1 on contraction and Ca^{2+} handling in rat ventricular myocytes. Pathways that mediate the -ve inotropic effects of ET_B receptor are in red (and downward pointing red arrow) and those mediating the +ve inotropic effects (upward pointing green arrow) and

enhancement in Ca^{2+} transient amplitude (upward pointing green arrow) and rate of rise (leftward pointing green arrow) by ET_A receptor activation are in green. Cartoons of Ca^{2+} transients before (black) and after ET-1 (green) are shown. The dashed vertical line indicates the temporal separation of the negative and positive inotropic responses.

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6. Disclosures

None

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Supplementary Figures

Fig 2 SI

I)

II)

III)

IV)

Fig 4 SI

Fig 5 SI

Fig 6 S1

Fig 7 S

Fig 9 S1

Fig 10 SI

cMyBP-C

MPEPGKRVPVSAFTKKPRSVETAGSAAVFEAETERSGLKVQWQRDGS DIAANDKYGLAAE
GKRHTLTVRDVGPDDQGSYAVIAGSSKVKFDLKVTEPAPPEKAESA VAPTSMEAPETPKE
VPALATQLEGNVSSPEGSVSVTQDGSVAGSQQGAPDDPIGLFLMRPQDGEVTVGGSIVFSA
RVAGASLLKPPVVKWFKGKWVDLSSKVGQHLQLHDSYDRASKVYLFELHITDAQATSAGG
YRCEVSTKDKFDSCNFNLT VHEAIGSGDLDLRSAFRRTSLAGTGRRTS DSHEDAGTLD FS
SLLKKSSFRRDSKLEAPAEEDVWEILRQAPPSEYERIAFQHGVTDLRGMLKRLKGMKHDE
KKSTAFQKKLEPAYQVNKGHKIRLTVELADPD AEVKWLKNGQEIQMSGRYIFESIGAKRT
LTISQCSLADDAAYQCVVGGEKCESTELFVKEPPVLITRSLEDQLVMVGQRVEFECEVSEE
GAQVKWLKDGVELTREETF KYRFFKKGDRKHHLIINEATLEDAGHYAVRTSGGQALAE LIV
QEKKLEVYQSIADLAVGAKDQAVFKCEVSDENVRGVWLKNGKELVPDNRIKVSHIGRVHK
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VVAGNKLRLDVPISGDPAPT VIWQKTITQGKKASAGPPPGAPEDAGADEEWVFDKLLCE
TEGRVRVETTKDRSVFTVEGA EKEDEGVYTVTVKNPVGEDQVNLT VKVIDVPDAPAAPKI
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AYEMRVYAVNAVGM SRPSPASQPFMPIGPPGEPTHLTVEDVSDTTVSLKWRPPERVGAGG
LDGYSVEYCEGECSEWVTALQGLTERTSLLVKDLPTGARLLFRVRAHN VAGPGGPITKE
PVTVQEILQRPRQLPRHLRQT IQKKVGEPVNLLIPFQGKPRPQVTWTKEGQPLAGEEVS
IRNSPTDTILFIRAAHRTHSGTYQVTVRIENMEDKATLV LQIVDKPSPPLDIRVVETWGF
SVALEWKPPQDDGNTEIWGYTVQKADKKTMEWFTVLEHYRQTHCVVSELIIGNGY YFRVF
SHNMVGSSDRAAATKEPIFIPRPGITYEPPKYKALDFSEAPSFTQPLTNRSIIAGYNAIL
CCAVRGSPKPKISWFKNGLDLGEDARFRMFCKQGVLTLEIRKPCPYDGGVYVCRATNLQG
EAQCECRLEVRVPQ

I)

II)

III)

IV)

V)

VI)

VII)