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SHORT COMMUNICATION

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# Cyclic AMP but not phosphorylation of phospholamban contributes to the slow inotropic response to stretch in ferret papillary muscle

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**Abstract** cAMP has been suggested to mediate the increased intracellular $Ca^{2+}$ transient and contraction seen during the slow response to stretch in cardiac muscle. We measured cAMP in ferret papillary muscles stretched from 80–85% to 98% of their length at which maximum active tension is produced ($L_{max}$) for 15 min. cAMP was significantly ($P<0.05$) increased by 53% in muscles at the longer length which showed the slow response compared with controls. By contrast, in a population of muscles that were stretched but did not show the slow response, cAMP was not significantly different from that in muscles at the short length. Although cAMP can increase sarcoplasmic reticulum (SR) $Ca^{2+}$ uptake by phosphorylation of phospholamban, we found no significant effect of stretch on phosphorylation of phospholamban at either $Ser^{16}$ or $Thr^{17}$. Further support for the hypothesis that cAMP is a mediator of the slow response was obtained by exposure of some muscles to the cell-permeable cAMP antagonist *8-bromo, adenosine 3′,5′-cyclic monophosphorothioate, Rp isomer* (Rp-8-Br-cAMPS, (2.5–10 μM). The slow response was reduced by 30% ($P<0.05$) in the presence of this antagonist. Our results not only provide evidence for the mediation of the slow response to stretch by cAMP, they also suggest that cAMP may rise in an intracellular compartment inaccessible to the SR.

**Key words** cAMP · Cardiac · Phospholamban · Phosphorylation · Stretch

## Introduction

When cardiac muscle is stretched a biphasic increase in the force of contraction is observed which is characterised by an immediate increase in force, followed by a slower maintained increase in force which occurs over 10–15 min. The rapid effects of stretch have been ascribed primarily to an increase in myofibrillar $Ca^{2+}$ sensitivity [7]. By contrast, the slow increase in contractility can be accounted for by a corresponding increase in the amplitude of the intracellular $Ca^{2+}$ transient ($[Ca^{2+}]_i$) [1, 7], yet the exact mechanism underlying the increase in $[Ca^{2+}]_i$ remains elusive. Neither blockade of L-type $Ca^{2+}$ channels, nor inhibition of sarcoplasmic reticular (SR) function abolishes the slow response [5, 7], suggesting that increased $[Ca^{2+}]_i$ does not originate entirely from $Ca^{2+}$ entry through L-type $Ca^{2+}$ channels or from the SR.

Although the mechanism for increased $[Ca^{2+}]_i$ is unknown, several lines of investigation have pointed to the involvement of the intracellular second messenger cAMP in the slow response. Both Kentish et al. [8] and Todaka et al. [10] have demonstrated a reversal of the slow response in the presence of the β-agonist isoprenaline, suggesting that the maintained elevation of cAMP produced by β-stimulation could be obscuring a smaller stretch-induced increase in cAMP. Increased myocardial cAMP has been observed following stretch of isolated frog ventricle [9], in pressure-overloaded Langendorff-perfused rat hearts [11], and in canine heart in situ following left ventricular stretch [10]. Furthermore, in non-cardiac preparations the membrane-bound enzyme adenyl cyclase has been shown to be sensitive to membrane stretch [12], thereby providing a mechanism by which intracellular cAMP could increase following membrane deformation.

However, although increased myocardial cAMP has been demonstrated following stretch [9, 10, 11], it has yet to be shown that it is cAMP which mediates the positive inotropic response to stretch. Furthermore, the mechanism by which cAMP may act to increase the force of contraction has not been resolved. cAMP increases phosphorylation of the SR protein phospholamban at $Ser^{16}$ through stimulation of cAMP-dependent protein kinase. The resulting potentiation of $Ca^{2+}$ uptake by the SR increases SR $Ca^{2+}$ load and consequently the amplitude of the intracellular $Ca^{2+}$ transient. However, although phosphorylation of phospholamban has been shown to be the main mechanism by which the positive inotropic effect of β agonists is mediated, SR function has been shown not to be a prerequisite for the slow response to stretch [7]. The aim of the present study, therefore, was to test the hypothesis that the slow response is mediated through an increase in myocardial cAMP but not through an increase in the cAMP-dependent phosphorylation of phospholamban.

## Materials and methods

Hearts were removed from ferrets (either sex; 700–900 g) which had been deeply anaesthetised with an intraperitoneal injection of pentobarbitone (150 $mg \cdot kg^{-1}$). Papillary muscles were excised from the heart and mounted in a muscle bath between a fixed hook and a lever system (Series 300B, Cambridge Technology) which acted as a force transducer and was also capable of altering muscle length. The muscle was perfused with Tyrode solution of the following composition (mM): NaCl 93; KCl 5; $CaCl_2$ 2; $NaHCO_3$ 20; $Na_2HPO_4$ 1; $MgSO_4 \cdot 7H_2O$ 1; glucose 10; Na acetate 20; insulin 5 $U \cdot l^{-1}$ equilibrated with 95% $O_2$/5% $CO_2$ (pH 7.4). Muscles were

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maintained at 30°C and stimulated at 0.33 Hz with 2-ms pulses at a voltage 10% above threshold. The relationship between length and force of contraction was determined and, following a period of equilibration, each muscle was left at a short length (80–85% $L_{max}$ where $L_{max}$ is the length at which maximum active tension is produced) for 1 h. After this time some muscles were stretched to 98% $L_{max}$ for 15 min and others were left at 80–85% $L_{max}$ for15 min (controls). At the end of the experiment, all muscles were quickly removed from the muscle bath and frozen in liquid $N_2$.

cAMP was measured in papillary muscles using a [$^{125}$I]-cAMP scintillation proximity assay (Amersham, Bucks, UK) with acetylation of samples to enhance sensitivity. Site-specific phosphorylation of phospholamban was measured quantitatively according to the method of Calaghan et al. [3, 4]. Briefly, myocardial proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes by semi-dry blotting. Membranes were probed with primary antibodies which are specific for the Ser$^{16}$ (PS-16; 1:10,000) and Thr$^{17}$ (PT-17; 1:5,000) phosphorylated forms of phospholamban. Immunoreactivity was visualised using a peroxidase-based chemiluminescent substrate kit (Amersham).

In a separate series of experiments, the effect of the cAMP antagonist 8-bromo, adenosine 3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-8-Br-cAMPS; Calbiochem, UK) on the slow response to stretch was investigated. In each muscle, the concentration of Rp-8-Br-cAMPS required to reduce the inotropic response to $8\times10^{-10}$ M isoprenaline by at least 25% was determined. The effect of this concentration of Rp-8-Br-cAMPS on the slow response to stretch was then tested. All muscles were incubated with Rp-8-Br-cAMPS for 15 min before the addition of isoprenaline, or stretch.

## Results and discussion

Figure 1A shows superimposed fast time-base recordings of active tension recorded from a representative papillary muscle at short length (82% $L_{max}$), at 1 min following stretch to 98% $L_{max}$, and at 15 min following stretch to 98% $L_{max}$. At 1 min following stretch, both the amplitude of the contraction and the duration of the twitch were increased compared with that seen before stretch. At 15 min following stretch, twitch amplitude was increased further but there was little corresponding change in twitch duration. Mean data obtained from 15 muscles showing the effect of stretch on the magnitude of contraction at the three time points selected is summarised in Fig. 1B. At 1 min after stretch, the force of contraction had increased 4.5 times compared with that at 80–85% $L_{max}$. The slow response represented a further 41% increase in force over that seen at 1 min following stretch.

Figure 1C shows levels of cAMP and phosphorylated phospholamban measured at 15 min following stretch in papillary muscles that showed the slow response, and in controls. cAMP in muscles maintained at the short length was 12.0±1.0 pmol·mg protein$^{-1}$ (mean ±SEM, $n$=8). In muscles that were stretched and showed the slow response, cAMP was 53% higher ($P$<0.05). A proportion of muscles that we stretched did not show a secondary slow increase in force. A similar finding has been reported both for single cardiac myocytes [13] and isolated papillary muscles [1]. We cannot, at present, explain why some muscles did not show a slow response. In these muscles, the immediate increase in force upon stretch was comparable with that seen in muscles that did show the slow response, suggesting that they were not functionally compromised. cAMP in muscles that did not show the slow response was similar to that observed in control muscles (10.9±1.5 pmol·mg protein$^{-1}$; $n$=5) and significantly lower ($P$<0.05) than that seen in stretched muscles that showed the slow response.

The degree of phosphorylation at Ser$^{16}$ and Thr$^{17}$ of phospholamban in muscles at the short length was similar (30±6 and 35±5 pmol·mg protein$^{-1}$ respectively; $n$=7). Although cAMP was increased in muscles that showed the slow response to stretch, phosphorylation of phospholamban at

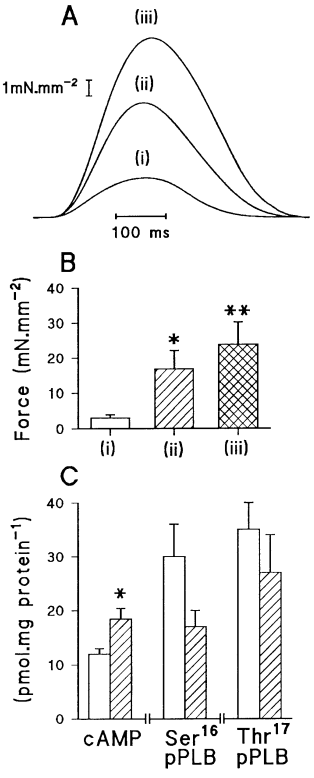


**Fig. 1A–C** The rapid and slow effects of stretch on contraction, cAMP and the phosphorylation of phospholamban in ferret papillary muscles. **A** Fast time-base recordings averaged from ten consecutive contractions at a short length of 82% $L_{max}$ (where $L_{max}$ is the length at which maximum active tension is obtained) (*i*), at 1 min following stretch to 98% $L_{max}$ (*ii*), and at 15 min following stretch to 98% $L_{max}$ (*iii*) in a representative muscle. Resting tension was subtracted from contractions following stretch for ease of comparison of active tension. **B** Mean data showing the effect of stretch on force of contraction in 15 muscles (mean ±SEM, $^{*}P$<0.05 compared with *i*, $^{**}P$<0.01 compared with *ii*. **C** Myocardial cAMP ($n$=8) and phosphorylated phospholamban (*pPLB*; $n$=7) in muscles at 80–85% $L_{max}$ (*open bars*) and in those stretched to 98% $L_{max}$ (*hatched bars*) for 15 min. $^{*}P$<0.05 with respective control group. All results were analysed using repeated measures ANOVA followed by Student's *t*-test with Bonferroni correction for multiple comparisons

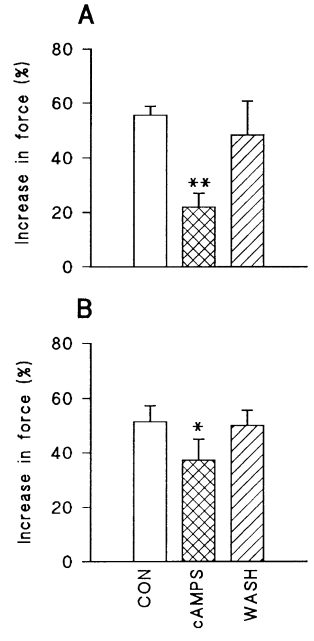


**Fig. 2A, B** The effect of Rp-8-Br-cAMPS on the response of ferret papillary muscle to isoprenaline and to stretch. **A** Increase in force in response to $8\times10^{-10}$ M isoprenaline. **B** Increase in force during the slow response to stretch. [*CON* In the absence of Rp-8-Br-cAMPS, *cAMPS* in the presence of Rp-8-Br-cAMPS (3.8±1.3 μM), *WASH* at 30 min following washout of Rp-8-Br-cAMPS.] All results are mean ±SEM of six observations. $^{*}P$<0.05; $^{**}P$<0.01 compared with respective control group (paired Student's *t*-test)

both Ser$^{16}$ and Thr$^{17}$ was reduced, although not significantly ($P$>0.05) (see Fig. 1C). By comparison, in a study using isoprenaline-stimulated rat ventricular myocytes [4], an increase in cAMP of a similar magnitude to that observed following stretch (around 50%) has been shown to increase phosphorylation of phospholamban at Ser$^{16}$ over twofold. In order to show that we were able to stimulate phosphorylation of phospholamban under similar conditions to those that we used to perform the stretch experiments, we investigated the effect of the β agonist isoprenaline ($10^{-6}$ M) on papillary muscles maintained at 95% $L_{max}$. Following 5 min of exposure to this concentration of isoprenaline, the force of contraction was in-

creased from 4.5±1.5 mN·mm$^{-2}$ to 10.1±2.5 mN·mm$^{-2}$ ($n$=5). There was a corresponding sevenfold increase in phosphorylation at Ser$^{16}$ of phospholamban (from 54±38 to 400±72 pmol·mg protein$^{-1}$; $n$=5; $P$<0.01), although phosphorylation at Thr$^{17}$ did not increase significantly (16.8±16.8 versus 32.6±13.7 pmol·mg protein$^{-1}$; $n$=5; $P$>0.05). Because we were able to stimulate phosphorylation of phospholamban at Ser$^{16}$ with isoprenaline under these conditions, we feel that if there was an increase in phospholamban phosphorylation during the slow response we would be able to detect it.

The effect of the cell-permeable cAMP antagonist Rp-8-Br-cAMPS(3.8±1.3 μM) on the response of papillary muscles to isoprenaline and the slow response is shown in Fig. 2. Only muscles which showed the slow response to stretch were used in this series of experiments. The increase in force of contraction in response to 8×10$^{-10}$ M isoprenaline was significantly reduced ($P$<0.01) by 59±10% ($n$=6) in muscles exposed to Rp-8-Br-cAMPS. At 30 min following washout of the antagonist, the increase in force returned to around 90% of control levels (Fig. 2A). The slow response was also significantly reduced ($P$<0.05) by 30±11% ($n$=6) in the presence of Rp-8-Br-cAMPS. At 30 min following washout of the antagonist, the slow response was 97% of control levels (Fig. 2B).

Although increased myocardial cAMP has been demonstrated previously following stretch [9, 10, 11], only one study to date has measured simultaneous changes in cAMP and contractility [10]. The present study is the first to demonstrate the functional significance of the increase in cAMP by presenting four pieces of information which support the hypothesis that cAMP mediates, in part at least, the slow response to stretch. Firstly, in muscles that showed the slow response cAMP was significantly higher than in control muscles. Secondly, in muscles that failed to show a slow increase in contraction, cAMP was not different from controls. Thirdly, given that the concentration of the β agonist isoprenaline that gives a maximum inotropic response increases myocardial cAMP by 100% [4], the 53% increase in cAMP that we measured during the slow response to stretch in the present study is likely to be of physiological significance. Finally, the slow response was attenuated in the presence of concentrations of the cAMP antagonist Rp-8-Br-cAMPS which reduce the inotropic response to isoprenaline.

Although cAMP was significantly elevated in muscles showing the slow response, we saw no increase in phosphorylation of phospholamban. This observation clarifies the seemingly contradictory findings of involvement of cAMP in the slow response, yet lack of importance of a functional SR[ 7, 8]. One interpretation of our results is that cAMP may be raised in a cellular compartment that is inaccessible to the SR.

cAMP-dependent protein kinase phosphorylates various sites within the cardiac cell besides Ser$^{16}$ of phospholamban, including troponin I of the myofilaments and the L-type $Ca^{2+}$ channel. However, evidence argues against the involvement of these mechanisms in the slow response to stretch [5, 7]. It has been suggested that the slow response arises because of a length-dependent change in cellular $Ca^{2+}$ loading during systole [7]. More specifically, a recent study using an ionic model of the cardiac myocyte has shown that the slow response is consistent with increased $Ca^{2+}$ entry through the $Na^{+}$-$Ca^{2+}$ exchanger secondary to a length-induced change in sarcolemmal $Na^{+}$ fluxes [2]. However, a study using single rat ventricular myocytes has failed to show a stretch-induced increase in intracellular $Na^{+}$ [6].

To summarise, this study has documented for the first time that myocardial cAMP increases in cardiac muscle that is stretched and shows the slow response, but does not change in stretched muscle that does not show the slow response. Further evidence for an involvement of cAMP is provided by experiments that show that the slow response is attenuated in the presence of an antagonist to cAMP. We have also shown that the slow response cannot be accounted for by increased phosphorylation of phospholamban. However, there are some issues that are, as yet, unresolved. For example, the differential effect of Rp-8-Br-cAMPS on the magnitude of the fall in inotropic response to isoprenaline compared with that to stretch suggests that more than one mechanism may underlie the slow response to stretch. In addition, whilst our results do not exclude the possibility that the observed increase in cAMP in intact muscles occurs within non-myocyte cells (e.g. fibroblasts), this seems unlikely considering that the slow response has been seen in single cardiac myocytes [6, 13]. Our results do suggest that cAMP increases in a compartment that is inaccessible to the SR. The target protein which may be phosphorylated by stimulation of the cAMP-dependent signalling pathway has yet to be identified.

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