Abstract
Concentration-dependent changes in cyclic AMP (cAMP), site-specific phosphorylation of phospholamban, the intracellular calcium ([Ca^{2+}]_i) transient and contraction were measured in isolated rat ventricular myocytes exposed to the β-adrenoceptor agonist isoprenaline. Cyclic AMP was measured by [125I]-cAMP scintillation proximity assay, phosphorylation of phospholamban at Ser16 and Thr17 was assessed using a pair of site-specific polyclonal antibodies, and [Ca^{2+}]_i was monitored with the fluorescent dye fura 2. Cyclic AMP rose to twice basal levels in the presence of 10^{-6} M isoprenaline. The maximum increase in phosphorylation at Ser16 and Thr17 of phospholamban was seen at 10^{-7} M isoprenaline. At this stage Ser16 phosphorylation was six times higher, and Thr17 phosphorylation was three times higher than that recorded in the absence of isoprenaline. Phosphorylation at Ser16 correlated more closely with changes in the [Ca^{2+}]_i transient and contraction than did phosphorylation at Thr17. This is the first study of its kind to measure simultaneous changes in cAMP, the phosphorylation of phospholamban, the [Ca^{2+}]_i transient and contraction over a range of concentrations of β-agonist. The results suggest that phosphorylation of phospholamban at Thr17 is of lesser physiological relevance to the effects of β-adrenergic stimulation on the heart than phosphorylation at Ser16.

Key words Cardiac myocyte · Isoprenaline · [Ca^{2+}]_i transient · Phospholamban · Phosphorylation · Cyclic AMP

Introduction
β-adrenergic stimulation increases the force of contraction and the rate of relaxation of the heart. These responses are mediated through co-ordinated changes in cyclic nucleotides, intracellular Ca^{2+} ([Ca^{2+}]_i) and protein phosphorylation. Sites that are phosphorylated in response to β-adrenergic stimulation include the myofibrillar proteins troponin I and C protein [8, 26], the sarcolemmal protein phospholemman [25], and the sarcoplasmic reticular protein phospholamban [18]. The ultimate effects of β-adrenergic stimulation are mediated by both decreased myofilament sensitivity and increased Ca^{2+} signalling, but the effects on Ca^{2+} signalling are of greater significance [7].

Phospholamban is a small homopentameric protein and, because of the pivotal role of the sarcoplasmic reticulum in controlling [Ca^{2+}]_i, might be considered as an important mediator of the effects of β-adrenergic stimulation. Indeed, phospholamban is one of the main proteins in the heart that is phosphorylated in response to β agonists [35], and on the basis of studies using phospholamban-deficient mice which do not respond to these agonists, it has been shown to be the most important mediator of the effects of β-adrenergic stimulation [19].

Phospholamban, in its unphosphorylated state, suppresses the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase [5]. Phosphorylation removes this inhibitory effect, thereby increasing Ca^{2+} uptake and release [27]. In vivo, phospholamban is phosphorylated at two distinct sites: Ser16 by cAMP-dependent protein kinase, and Thr17 by Ca^{2+}/calmodulin-dependent protein kinase [5]. β-adrenergic stimulation increases phosphorylation at both sites of phospholamban [35].

There are two issues, as yet unresolved, relating to these two adjacent phosphorylation sites on phospholamban. The first concerns the interaction between phosphorylation at the two sites. In vitro, phosphorylation occurs independently at Ser16 and Thr17 in response to cAMP or Ca^{2+}/calmodulin respectively [2,15]. However, under conditions of β-adrenergic stimulation in vivo...
there is evidence that phosphorylation at Ser\(^{16}\) is a prerequisite for phosphorylation at Thr\(^{17}\) [20, 30, 35].

The second issue concerns the contribution of phosphorylation at the two distinct sites of phospholamban to changes in cellular Ca\(^{2+}\) handling, and ultimately in the mechanical function of the heart; this is of particular interest following \(\beta\)-adrenergic stimulation when both Ser\(^{16}\) and Thr\(^{17}\) are phosphorylated. Although phosphorylation at each site has been shown to increase Ca\(^{2+}\) uptake by the sarcoplasmic reticulum in vitro, there is disagreement as to whether phosphorylation of the second residue within phospholamban confers additional stimulatory effects [14, 16]. In addition, Mattiazzi et al. [21] demonstrated functionally distinct effects of inhibition of cAMP-dependent and Ca\(^{2+}\)/calmodulin-dependent protein kinases, which resulted in increased Ca\(^{2+}\) affinity and decreased maximum velocity of the Ca\(^{2+}\)-ATPase respectively, whereas others have found that phosphorylation at both Ser\(^{16}\) and Thr\(^{17}\) results only in a shift in Ca\(^{2+}\) affinity of the ATPase [23].

In terms of the contribution of site-specific phosphorylation to the mechanical function of the heart following \(\beta\)-adrenergic stimulation, in the isolated guinea-pig heart it is dephosphorylation at Ser\(^{16}\), but not at Thr\(^{17}\), that mirrors reversal of inotropic and lusitropic effects following termination of an infusion of isoprenaline [30]. Using transgenic technology, the importance of the Ser\(^{16}\) site has been demonstrated by a recent study in which Ser\(^{16}\) Ala-mutated phospholamban was inserted into phospholamban-knockout mice [20]. A role for phosphorylation at Thr\(^{17}\) in the response to \(\beta\)-adrenergic stimulation has not been excluded; experiments using the specific calmodulin antagonist W7 in the isolated rat heart have suggested that phosphorylation at Thr\(^{17}\) is involved in part in mediating the lusitropic but not inotropic effects of \(\beta\)-adrenergic stimulation [33]. The mediation of lusitropic effects by Thr\(^{17}\) phosphorylation has also been observed following exposure of isoprenaline-perfused rat hearts to conditions of acidosis [34].

The aim of the present study was to clarify and extend our knowledge of the co-ordinated changes in cAMP, the site-specific phosphorylation of phospholamban and [Ca\(^{2+}\)], and to relate these changes to the ultimate effects of \(\beta\)-adrenergic stimulation on the heart. Many workers in this field use intact hearts, but our study was performed using isolated ventricular myocytes, which afforded a degree of control over a number of variables that may influence function in the intact heart (e.g. endogenous catecholamine release). Our investigation was further refined by the use of a pair of polyclonal antibodies which recognize phospholamban phosphorylated at either Ser\(^{16}\) or Thr\(^{17}\) [6] and a method which enables quantification of the levels of phosphorylated phospholamban [3].

### Materials and methods

#### Cell isolation

Single rat ventricular myocytes were isolated according to the method of Frampton et al. [10]. Briefly, male Wistar rats (200–250 g) were killed by cervical dislocation following stunning, and their hearts were removed quickly and mounted on a Langendorff apparatus. Hearts were perfused retrogradely at a flow rate of 11 ml min\(^{-1}\) with a N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES)-based isolation solution of the following composition (mM): NaCl 130; KCl 5.4; NaH\(_2\)PO\(_4\) 0.4; MgCl\(_2\) 6H\(_2\)O 1.4; CaCl\(_2\) 0.75; HEPES 10; glucose 10; taurine 20; creatine 10 (pH 7.3). When the coronary circulation had cleared of blood, perfusion of the hearts was achieved with Ca\(^{2+}\)-free isolation solution in which 0.75 mM CaCl\(_2\) had been replaced with 0.1 mM ethyleneglycolbis[\(\beta\)-aminooxyethyl] ether, dried at 60°C under nitrogen, and reconstituted in 0.05 M NaCl and 0.1 mg ml\(^{-1}\) protease (type XIV; Sigma). After this time, the ventricles were excised from the heart, minced, and gently shaken at 37°C in collagenase-containing isolation solution supplemented with 1% bovine serum albumin. Cells were filtered from this solution at 5 min intervals and resuspended in isolation solution containing 0.75 mM Ca\(^{2+}\).

#### Preparation of cells for biochemical analysis

All experiments were performed at 22°C. After isolation, cells were resuspended in a HEPES-based bathing solution of the following composition (mM): NaCl 113; KCl 5; NaH\(_2\)PO\(_4\) 1; MgSO\(_4\) \(\cdot\)7H\(_2\)O 1; CaCl\(_2\) 1; HEPES 5; glucose 10; sodium acetate 20; and insulin (5 U l\(^{-1}\)) at pH 7.4. The [Ca\(^{2+}\)] in this solution was 1 mM, which is close to the value of ionised extracellular Ca\(^{2+}\) concentration in the rat [9]. The ratio of rod-shaped cells to rounded cells was 1:1; this ratio was confirmed for each suspension (n=8). Aliquots of cells were placed in the wells of a Perkin-Elmer cell suspension was divided into two aliquots which were centrifuged at 2,000 g for 15 min at 4°C, the supernatants decanted and stored at –70°C until analysis. For measurement of cAMP, the resulting cell suspension was divided into two aliquots which were centrifuged at 2,000 g for 12 s. For measurement of cAMP, the resulting cell pellet was immediately frozen in liquid nitrogen and stored at –70°C until analysis. For measurement of phospholamban, the cell pellet was dispersed in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% sodium dodecylsulphate (SDS), and 0.1% bromophenol blue and stored at –20°C until analysis. Values of cAMP and phosphorylated phospholamban are expressed per 1000 rod-shaped cells. The contribution from rounded cells to both basal and 10–6 M isoprenaline-stimulated cAMP and phosphorylated phospholamban were assessed by measuring cAMP and phosphorylated phospholamban in two populations of cells: one containing mostly rod-shaped cells and one containing mostly rounded cells (see Results).

#### cAMP assay

For extraction of cAMP, cell pellets were homogenised for 1 min with 0.5 ml of ice-cold 6% trichloroacetic acid using a pestle disperser (Anachem, Bedfordshire, UK). Homogenates were centrifuged at 2,000 g for 15 min at 4°C, the supernatants decanted, and the pellets retained for protein assay. Supernatants were washed four times with five volumes of water-saturated diethyl ether, dried at 60°C under nitrogen, and reconstituted in 0.05 M