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EFFECTS OF EXOGENOUS NEUROTRANSMITTERS ON CONTRACTILITY AND CYCLIC NUCLEOTIDE METABOLISM IN THE ISOLATED FROG VENTRICLE

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Abstract—A study has been made of the effects of acetylcholine and adrenaline on the metabolism of 3′,5′-cyclic nucleotides by the isolated frog ventricle. Measurements of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) were made: (a) at various times during responses produced by a given concn of acetylcholine (10^{-7} M) or adrenaline (10^{-6} M); and (b) at fixed times following exposure to different concns of the two agonists. The negative inotropic effect of acetylcholine is accompanied by reciprocal changes in the two cyclic nucleotides: cyclic GMP levels rise and cyclic AMP falls. Both effects are dose-related. A more complex pattern of changes occurs during responses produced by adrenaline. The initial increase in twitch tension is accompanied by a transient reduction in cyclic GMP and by a large increase in cyclic AMP. The rate at which cyclic AMP accumulates is greatest at the time when cyclic GMP levels are maximally depressed. An increase in cyclic GMP, seen later, coincides with a gradual fall in both twitch tension and cyclic AMP. The increments in both cyclic AMP and cyclic GMP, measured when the twitch is maximally potentiated, are dose-related. The magnitude of the decrease in peak twitch tension produced by acetylcholine and of the increase produced by adrenaline correlate closely with corresponding changes in the relative proportion of cyclic AMP:cyclic GMP present in the fibres. The possible significance of the changes in cyclic AMP and cyclic GMP in modulating the response of the ventricle is discussed.

The autonomic innervation of the heart modulates both the rate and force of contraction, and, in the frog, responses to stimulation of its sympathetic and parasympathetic components are mediated by adrenaline and acetylcholine respectively [1, 2]. In this paper we examine the effects of both neurotransmitters on cyclic nucleotide metabolism and relate this to their ability to modify the contractile performance of the ventricle. Previous studies, using a wide range of different cardioactive agents, have established a clear correlation between changes in the relative proportion of cyclic AMP:cyclic GMP and changes in isometric twitch tension [3–9]. The experiments now to be described will show that a similar relationship exists during responses produced by acetylcholine and adrenaline.

METHODS

Isolated ventricles (from specimens of *Rana temporaria*) were divided into two halves, one of which served as a control for the other. The method of Lamb and McGuigan [10] was used to superfuse each half-ventricle with Ringer's solution [composition (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1; NaH_2PO_4, 0.85; Na_2HPO_4, 2.15; glucose, 5.6; pH 7.2] at a flow-rate of 100 ml·min^{-1}. The total volume of recirculating fluid was 1 litre. Preparations were stimulated electrically through silver wire electrodes (10 V amplitude, 5 msec duration) at a frequency of 0.5 Hz. Isometric tension was recorded using a strain gauge (Devices type 4157) fitted with a metal extension and hook (compliance 40 μg^{-1}). The output from the tension transducer was recorded continuously on a chart recorder. The optimum length, giving a maximal twitch, was established for each half-ventricle at the commencement of the experiment.

Under these circumstances, the twitch tension decreases, leading to a relatively stable but depressed condition termed the hypodynamic state [11]. This process is itself characterised by changes in both cyclic AMP and cyclic GMP levels [12], and so care was taken to make certain that the test preparation was allowed to become hypodynamic to exactly the same degree as the control before exposing it to acetylcholine or adrenaline.

The procedure was as follows. First, both preparations were superfused as described earlier, until the developed twitch tension declined to around 30% of its initial value. The 'test' preparation was then exposed to either acetylcholine or adrenaline, while the control half-ventricle continued to be superfused with Ringer's solution alone. At a predetermined time during the response, both preparations were rapidly frozen by compressing the tissue between forceps which had been cooled previously in liquid nitrogen. The frozen tissue was then pulverised in a stainless steel mortar and extracted with acidified ethanol (1 ml 1 N HCl, 100 ml ethanol). The solvent was blown-off in a stream of nitrogen and the resulting residue taken up in Tris–EDTA buffer (0.05 M

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Tris, pH 7.5; 4 mM EDTA). Cyclic AMP and cyclic GMP levels were then measured using the Radiochemical Centre assay kits [TRK 432 and TRK 500, respectively (Radiochemical Centre, Amersham, U.K.)]. Total tissue protein was measured using the biuret method [13]. Details of the extraction, cyclic nucleotide and protein assays procedures, including appropriate control and recovery experiments, are described elsewhere [12].

RESULTS

Acetylcholine responses

The decrease in peak twitch tension during exposure of the ventricle to 10^{-7} M acetylcholine is shown in Fig. 1A. Contractile force fell rapidly initially, and then more slowly, approaching a steady-state level of around 0.2 times its control value. The changes in cyclic nucleotide concns in 13 different preparations, frozen at various times during similar responses, are shown in Fig. 1B. Each point is the result for one half-ventricle, and is expressed as a multiple of the concn found in its control (partner) half-ventricle. The time course of the decline in twitch tension (solid triangles) is also shown for comparison.

The decrease in twitch tension was accompanied by a reduction in endogenous cyclic AMP levels (filled circles) and by an increase in the concn of cyclic GMP (open circles). Both changes were virtually complete after only 40 sec by which time the twitch had also attained its new steady-state level.

The changes in cyclic AMP and cyclic GMP in another series of experiments with 15 half-ventricles, each of which was frozen after 2–3 min exposure to different concns of acetylcholine (10^{-10}–10^{-6} M), are shown in Fig. 2. There are two points to emphasise here. First, the accumulation of cyclic GMP (open circles) and the associated reduction in cyclic AMP (filled circles) levels are both dose-related. The concns of acetylcholine producing a half-maximal effect on cyclic AMP and cyclic GMP levels were

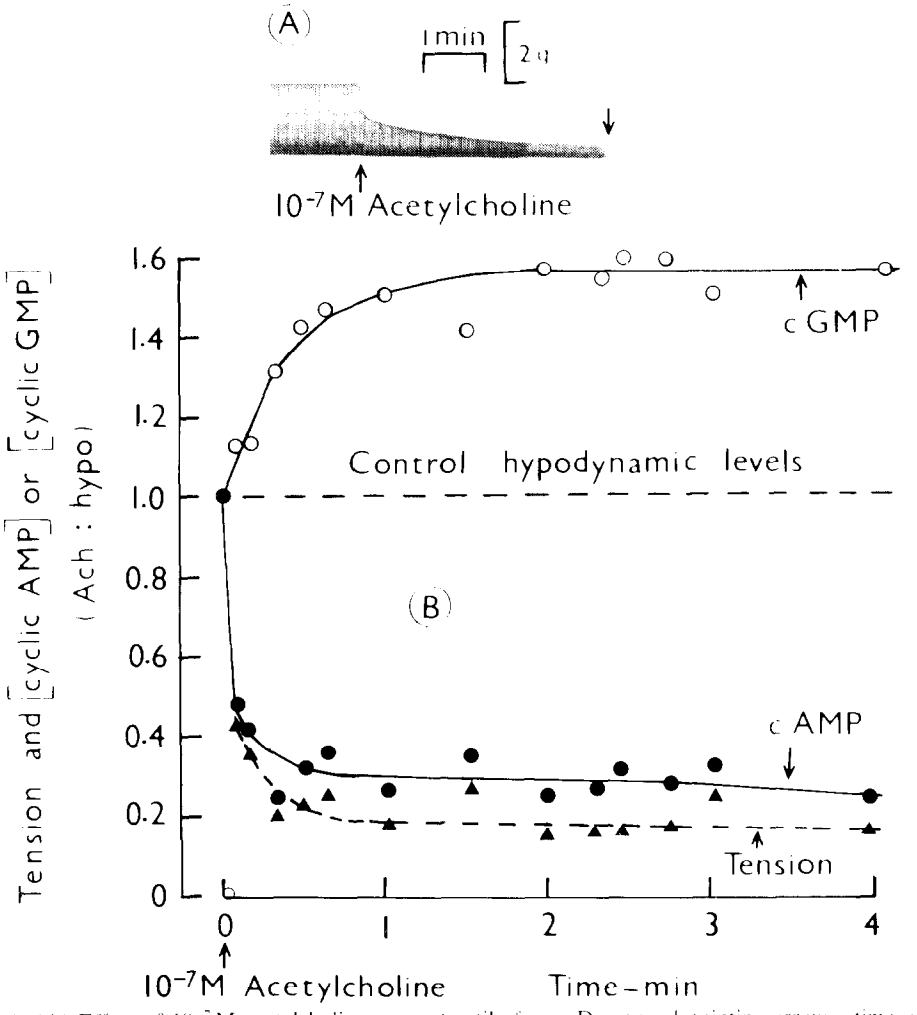

Fig. 1. (A) Effect of 10^{-7} M acetylcholine on contractile force. Downward pointing arrow = time of freezing preparation. (B) Time course of changes in cyclic AMP (solid circles), cyclic GMP (open circles) and isometric twitch tension (solid triangles) during superfusion with 10^{-7} M acetylcholine. Each point is the result of one half-ventricle, expressed as a multiple of the value found in its 'partner' (control) half-ventricle. The levels of cyclic AMP and cyclic AMP in control preparations were 8.39 ± 0.11 and 1.16 ± 0.05 pmoles·(mg protein)$^{-1}$, respectively (N = 13).

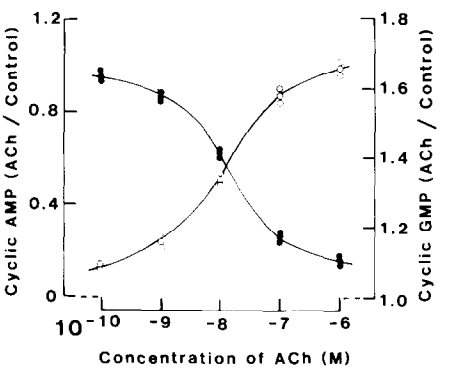

Fig. 2. Dose–response curves showing the effects of acetylcholine (range 10^{-10}–10^{-6} M) on cyclic GMP (open circles) and cyclic AMP (solid circles) levels measured at the times (2–3) of freezing the preparation. Cyclic nucleotide levels expressed as multiples of control values. The levels of cyclic AMP and cyclic GMP in control preparations were 8.33 ± 0.10 and 1.25 ± 0.04 pmoles·(mg protein)$^{-1}$, respectively (N = 15).

2.1×10^{-8} and 7.2×10^{-9} M, respectively (≡ PD_2 values 7.68 and 8.14). Second, the ventricle is extremely sensitive to acetylcholine, significant changes in the levels of both cyclic nucleotides showing with doses as low as 10^{-10} M.

Adrenaline responses

The effect of 10^{-6} M adrenaline on peak twitch tension is shown in Fig. 3A. The twitch increased rapidly, reaching a maximum value of 4 times the control after only 40 sec. Thereafter, it declined gradually settling to around 2.5 times its initial value after 3 min.

The pattern of changes in cyclic AMP (filled circles) and cyclic GMP (empty circles) produced by adrenaline is more complex than that seen following treatment with acetylcholine. Fig. 3B shows that the early increase in twitch tension (solid triangles) was associated with a transient reduction in cyclic GMP levels and by a large increase in cyclic AMP. The rate of increase of cyclic AMP was found to be greatest [1.6 pmoles·(mg protein)$^{-1}$·sec^{-1}] at the time when cyclic GMP levels were maximally

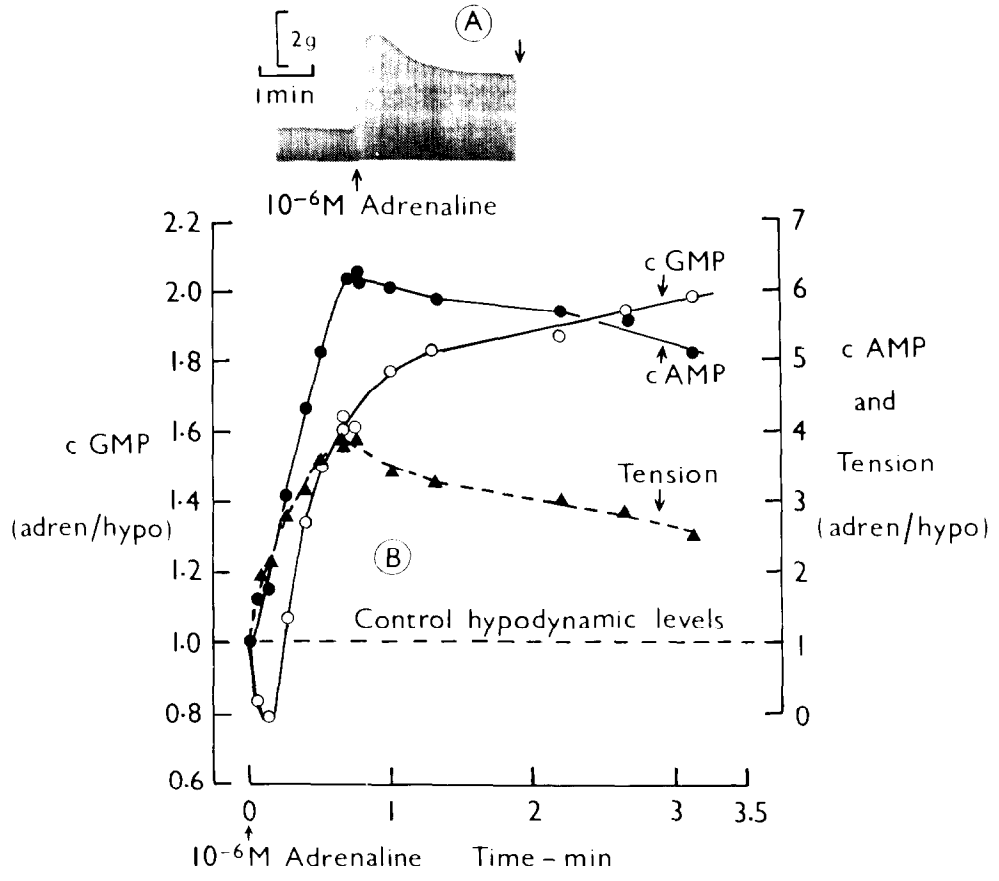

Fig. 3. (A) An original chart recording showing the response of the ventricle to 10^{-6} M adrenaline. The upward and downward pointing arrows indicate the time of application of adrenaline and of freezing the preparation, respectively. (B) Time course of changes in isometric twitch tension (solid triangles), cyclic AMP (solid circles) and cyclic GMP (open circles) levels produced by 10^{-6} M adrenaline. Cyclic nucleotides and force expressed as multiples of control values. Control levels (means ± S.E.) of cyclic AMP and cyclic GMP were 7.9 ± 0.11 and 1.14 ± 0.02 pmoles·(mg protein)$^{-1}$, respectively (N = 13).

depressed (after 6 sec). The subsequent increase in the concn of cyclic GMP, which was rapid initially and slower later, was accompanied at first by a decreased rate of cyclic AMP accumulation and then (after 40 sec) by a reversal. These changes are qualitatively similar to those seen during responses produced by isoprenaline [3] or uridine 5′-triphosphate [8].

The levels of cyclic AMP and cyclic GMP following treatment of the ventricle (18 preparations) with different concns of adrenaline are shown in Fig. 4. These preparations were frozen 40–60 sec after commencing treatment. The twitch was by this time maximally potentiated, and both cyclic AMP and cyclic GMP levels were elevated above the control concns. Again, the extent to which the two cyclic nucleotides increased was dose-dependent: the effects on cyclic AMP and cyclic GMP accumulation were half-maximal at concns of 5×10^{-8} and 2.7×10^{-7} M, respectively ($\equiv PD_2$ values 6.30 and 6.57).

Relationship between changes in ventricular contractility and cyclic nucleotide levels

Previous work (see Refs 3–9) has established a correlation between changes in the relative proportion of cyclic AMP : cyclic GMP present in the fibres and changes in peak developed tension. The results obtained in the present study show that responses evoked by acetylcholine and adrenaline are also paralleled by corresponding changes in the ratio cyclic AMP : cyclic GMP.

Acetylcholine responses. Fig. 5A and B show changes in isometric tension (filled symbols) recorded at different times during a series of responses produced by 10^{-7} M acetylcholine (A) and after 2–3 min exposure to different concns (10^{-10}–10^{-6} M) of acetylcholine (B). The corresponding changes in the relative proportion (R) of cyclic AMP : cyclic GMP (open symbols):

$$R = \frac{\text{cyclic AMP : cyclic GMP (test ventricle)}}{\text{cyclic AMP : cyclic GMP (control ventricle)}}$$

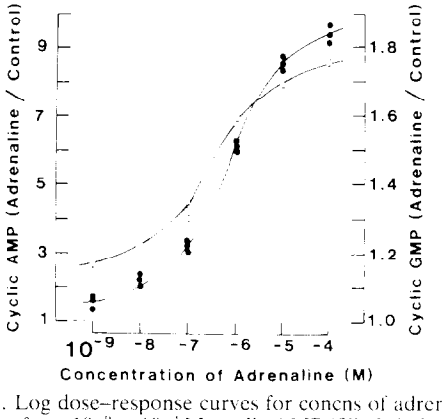

Fig. 4. Log dose–response curves for concns of adrenaline ranging from 10^{-9} to 10^{-4} M: cyclic AMP (filled circles) and cyclic GMP (open circles). Preparation superfused for approximately 40–60 sec prior to freezing. Both parameters expressed as multiples of control values. Mean (± S.E.) control levels of tissue cyclic AMP and cyclic GMP concns were 7.48 ± 0.15 and 1.28 ± 0.06 pmoles·(mg protein)$^{-1}$ respectively (N = 18).

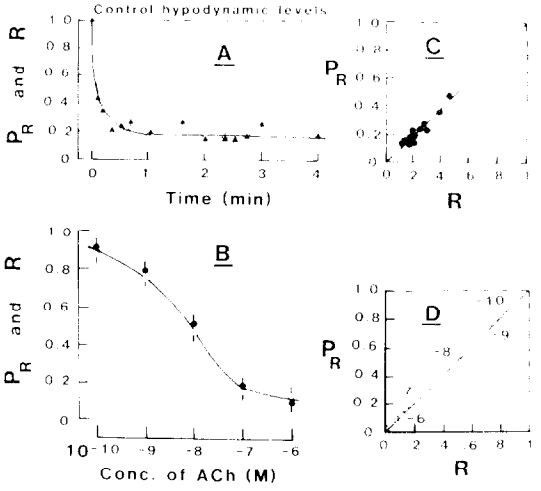

Fig. 5. (A) Time-dependent changes in isometric force (solid triangles) and cyclic nucleotide ratio (open triangles) during responses to 10^{-7} M acetylcholine (ACh). Cyclic nucleotide ratio (R) (defined in text) and contractile force (P_R) (defined in text) expressed as multiples of control values. Note (C) that the change in twitch tension is directly proportional to changes in R (correlation coefficient ± S.E. 0.99 ± 0.06, N = 13, $P < 0.001$). (B) Effects of different concns of acetylcholine (range 10^{-10}–10^{-6} M) on steady-state twitch tension (solid circles) and cyclic nucleotide ratio (open circles). Each point is mean ± S.E. for three experiments. (D) Force and cyclic nucleotide ratio (correlation coefficient ± S.E. 0.99 ± 0.08, N = 15, $P < 0.001$).

are also shown for comparison. In Fig. 5A and B the decrease in the value of R parallels closely the decrease in the peak tension (P_R). This relationship is shown graphically in Fig. 5C and D, where the lines are drawn in with a slope of unity to indicate a direct proportionality between the two parameters.

Adrenaline responses. The results presented in Fig. 6A–D show that the increase in twitch tension produced by adrenaline is also paralleled by corresponding increases in R. The data are presented in a similar way to those shown in Fig. 5.

DISCUSSION

These experiments have shown that acetylcholine and adrenaline, the two autonomic neurotransmitters present in frog heart, induce changes in the metabolism of both cyclic AMP and cyclic GMP. These changes are such that the effect on the twitch is paralleled by a corresponding change in the relative proportion of cyclic AMP : cyclic GMP. This is a feature which is common to many responses, produced by a range of pharmacologically-distinct agents (see Refs 3–9), and so it seems probable that it has some physiological significance.

It is important to emphasise, however, that the generality of the relationship does not in itself constitute proof of a causal connection between changes in cyclic nucleotide levels and in contractility—the two events may occur simultaneously, as the result of another (unidentified) change in the excitation–contraction process. However, for the present (but

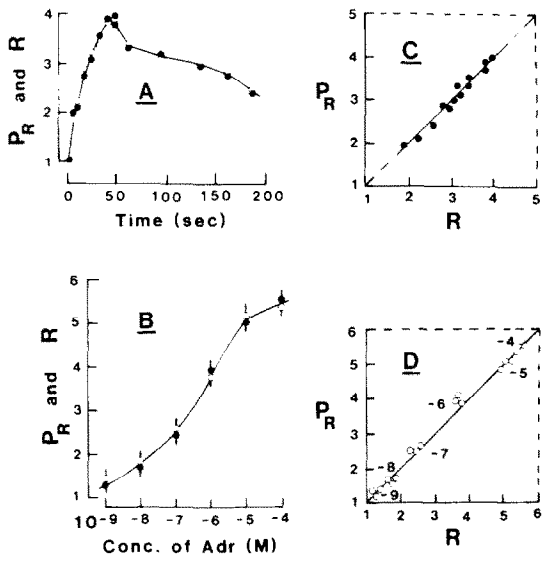

Fig. 6. Time course of changes in the ratio cyclic AMP : cyclic GMP (open circles) and contractile force (solid circles) measured at various times during exposure of ventricles to 10^{-6} M adrenaline (Adr). Cyclic nucleotide ratio (R) and contractile force (P_R) expressed as multiples of control values. (B) Effects of adrenaline ranging from 10^{-9} to 10^{-4} M on contractile force (solid circles) and cyclic nucleotide ratio (open circles). Each point is mean ± S.E., N = 3. (C) Relationship between cyclic nucleotide ratio and isometric force (correlation coefficient ± S.E. 0.99 = 0.08, N = 13, $P < 0.001$). (D) Relation between isometric force and cyclic nucleotide ratio (correlation coefficient ± S.E. 0.99 ± 0.08, N = 18, $P < 0.001$).

see later) we will assume that both cyclic AMP and cyclic GMP are involved in regulating contractility and then enquire into the nature of the biochemical basis for such control. The first conclusion to be drawn is that since peak force is related to the ratio cyclic AMP : cyclic GMP, then cyclic AMP must act to potentiate these processes which generate tension, whereas cyclic GMP exerts an antagonistic effect, tending to depress the twitch. The ability of cyclic AMP (acting through cyclic AMP dependent protein kinase) to stimulate the phosphorylation of proteins known to be involved in regulating contraction is well-documented [14] and the physiological properties of three such proteins [the inhibitory subunit of troponin (TN-I); phospholamban, a small mol. wt protein component of the sarcoplasmic reticulum (SR); and a surface membrane-bound protein, thought to be involved in gating Ca^{2+} entry] have been shown to be altered as a result. Thus, the Ca^{2+}-sensitivity of cardiac myofibrillar ATPase activity is decreased by phosphorylation of TN-I [15]; the rate of calcium sequestration and storage capacity of the SR is enhanced by phosphorylation of phospholamban [16, 17] and it is suggested (on the basis of rather less evidence) that phosphorylation of an 11,500 mol. wt component of the sarcolemma may be involved in the process of Ca^{2+} gating [18].

The nature of the involvement of cyclic GMP is much less clear. It has been known for some years [19–21] that the effect of acetylcholine on mammalian heart is accompanied by elevated cyclic GMP levels, but subsequent studies [21, 22] have raised some doubts concerning the interpretation of these earlier experiments. Brooker's work (using various mammalian atria and rat and frog ventricles) showed that 100 times more carbachol is required to elevate intracellular cyclic GMP levels than that needed to substantially depress the twitch; and Diamond *et al* [23] were able to increase cyclic GMP levels 17-fold in cat atria using sodium nitroprusside, but actually recorded a small increase in the force of contraction. The effect of sodium nitroprusside on the amphibian ventricle is quite different; it evokes a powerful negative inotropic response and the degree of depression of the twitch is again closely correlated with changes in the ratio cyclic AMP:cyclic GMP [7]. Although the involvement of cyclic GMP in modulating cardiac contractility remains contentious, it has been established that a cyclic GMP dependent form of protein kinase is present in the heart [24, 25]. However, to date there have been no reports of a physiologically-relevant protein substrate for this enzyme having been found.

It was emphasised earlier that the existence of a correlation between altered cyclic nucleotide levels and changes in the performance of the ventricle does not necessarily indicate a causal connection. It is possible that both effects have a common cause, and in this context it is important to consider the involvement of Ca^{2+} in regulating contraction. It is clear that changes in the availability of this ion to the myofilaments will significantly affect the force of contraction, at least in the range of concns where the contractile system is not fully saturated. There is also reason to suppose that such changes could alter the metabolism of both cyclic nucleotides, acting through the Ca^{2+}-dependent modulator protein, calmodulin [26, 27]. Calmodulin is a calcium-binding protein that functions as an allosteric effector of a cyclic nucleotide phosphodiesterase isozyme. When activated by Ca^{2+}, calmodulin stimulates the rate of hydrolysis of cyclic AMP and cyclic GMP, but to differing degrees: the rate of hydrolysis of cyclic GMP is accelerated to a greater extent than that of cyclic AMP [28], which would result in an increase in the relative proportion of cyclic AMP:cyclic GMP. Conversely, under conditions where Ca^{2+} availability is reduced, and calmodulin is deactivated, the relative proportion of cyclic AMP:cyclic GMP would fall. The cardioactive agents studied to date all influence the duration of the action potential, in a way that suggests that they affect transmembrane Ca^{2+} entry into the fibres. Thus, the negative inotropic agents we have studied (acetylcholine, adenosine, sodium nitroprusside and 8-bromo cyclic GMP) shorten the action potential duration, whereas those producing positive inotropic responses (including adrenaline, isoprenaline, uridine triphosphate, adenosine triphosphate and dibutyryl cyclic AMP) tend to prolong it. It remains to be seen whether in all these instances the change in the action potential precedes changes in cyclic nucleotide levels, or if it is instead delayed. In the case of two substances studied recently, namely, sodium nitroprusside [29] and trifluoperazine [30], there is clear evidence that significant changes in twitch tension and cyclic

nucleotide levels occur *before* there is any detectable change in the shape of the action potential. This question needs to be investigated more extensively before any general conclusion can be reached. Meantime, it is important to emphasise that changes in Ca^{2+} entry during the action potential could arise *secondarily*, as the result of drug-induced changes in cyclic nucleotide levels.

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