J. Physiol. (1980), **304**, *pp.* 21–42
With 6 text-figures
Printed in Great Britain

INOTROPIC RESPONSES OF THE FROG VENTRICLE TO ADENOSINE TRIPHOSPHATE AND RELATED CHANGES IN ENDOGENOUS CYCLIC NUCLEOTIDES

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(*Received 31 May 1979*)

SUMMARY

1. A study has been made of a well documented but poorly understood response of the isolated frog ventricle to treatment with exogenous adenosine 5′ triphosphate (ATP). Measurements of membrane potential, isometric twitch tension and levels of endogenous 3′,5′-cyclic nucleotides have been made at various times during the ATP-induced response.

2. ATP elicits a characteristic *triphasic* response, which comprises an initial, abrupt increase in contractility, rising to a maximum within a few beats (*first* phase); followed by a period when the twitch amplitude falls, sometimes to below the control level (*second* phase); and superceded by a more slowly developing and longer-lasting increase in contractile force (*third* phase). The response is unaffected by atropine, propranolol or phentolamine. However, the prostaglandin synthetase inhibitor indomethacin depresses the *first* phase and entirely suppresses the *third* phase.

3. The inotropic effects of ATP are accompanied by changes in the shape of the action potential. These effects are dose-related. The duration of the action potential ($D_{-30\,mV}$) and its positive overshoot (O) are increased during all phases of the response, for $[ATP]_o$'s up to 10^{-5} M. However, at higher $[ATP]_o$'s, $D_{-30\,mV}$ and O are both reduced during the *second* phase (but not the *first* or *third* phase), when isometric twitch tension is also depressed. The relationship between action potential duration and twitch tension (P) for different $[ATP]_o$'s is linear for all three phases of the response, but the *slopes* of the curves ($\Delta P/\Delta D$) are markedly different, indicating that the sensitivity of the contractile system to membrane depolarization is not constant, but varies continuously throughout the response.

4. ATP has a potent stimulatory effect on the metabolism of endogenous 3′,5′-cyclic nucleotides. The time courses of the changes in adenosine 3′,5′-cyclic monophosphate (3′,5′-cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (3′,5′-cyclic GMP) are complex, but the accompanying change in isometric twitch tension is paralleled closely by corresponding changes in the ratio 3′,5′-cyclic AMP: 3′,5′-cyclic GMP.

5. It is concluded that ATP exerts a dual effect on the ventricle and that the contractile response is regulated by changes in the metabolism of 3′,5′-cyclic nucleo-

0022-3751/80/8820-0252 $07.50

tides. The effects of indomethacin indicate a possible involvement of prostaglandins in mediating the ATP response. It is suggested that the initial effect of ATP on the ventricle is to increase the permeability of the fibres to Ca^{2+}.

6. The relationship between 3′,5′cyclic nucleotide levels and ventricular contractility is discussed. It is postulated that the antagonistic effects of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP are expressed at the level of certain phosphoproteins which regulate both the *availability* of Ca^{2+} and the *sensitivity* of the contractile proteins to Ca^{2+}.

INTRODUCTION

In 1929, Drury & Szent-Györgyi showed that crude extracts of bullock heart exerted strong pharmacological effects on guinea-pig, rabbit and dog hearts, producing marked changes in cardiovascular performance. The active constituent in their extracts was identified as adenylic acid (adenosine monophosphate: AMP) and since then the actions on the heart of a wide range of purine and pyrimidine based nucleosides and their nucleotides have been investigated.

The effects of exogenous adenosine 5′triphosphate (ATP) on the heart are especially well documented, although its mode of action remains obscure. The confusion appears to be due, at least in part, to the fact that different regions of the heart respond to ATP in different ways (see Drury, 1936). Its chronotropic and inotropic effects are predominantly *negative* on isolated mammalian atria (*dog*: Emmelin & Feldberg, 1948; *cat*: Green & Stoner, 1950; Acierno, Burno, Burnstein & Di Palma, 1952; Bertelli, Bianchi & Beani, 1972; *rabbit*: Bielschowsky, Green & Stoner, 1946; Emmelin & Feldberg, 1948; Bertelli *et al.* 1972; *rat*: Hollander & Webb, 1957; Bertelli *et al.* 1972; Meinertz, Nawrath & Scholz, 1973), but *positive* on both mammalian (*rabbit*: Green & Stoner, 1950: Gillespie, 1934) and amphibian (Lindner & Rigler, 1931; Lichtneckert & Straub, 1949; Loewi, 1949; Marshall & Andrus, 1953; Szent Györgyi, 1953; Kanda, Sekiya & Inoue, 1954; Schenberg, 1956; Versprille, 1963, 1965; Boyd & Forrester, 1968) ventricles. Not surprisingly, then, the responses to ATP of isolated whole hearts, and those produced by injection into intact animals, are complex (*cats*: Bielschowsky *et al.* 1946; Emmelin & Feldberg, 1948; Green & Stoner, 1950; *dogs*: Emmelin & Feldberg, 1948; Angelakos & Glassman, 1961; *rabbit*: Sydow & Ahlquist, 1954; Buckley, Tsuboi & Zeig, 1961; *guinea-pig*: Rand, Stafford & Thorp, 1955; *rat*: Versprille & Van Duyn, 1966; *man*: Wayne, Goodwin & Stoner, 1949). They appear to be dominated by chronotropic effects which tend to obscure the no-less interesting inotropic effects. The interpretation of experiments with intact animals is further complicated because ATP is rapidly degraded *in vivo* by extrinsic 5′-nucleotidases which produce ADP, AMP and adenosine (Arch & Newsholme, 1978).

The experiments to be described in this paper are concerned with the inotropic effects of ATP on the isolated frog ventricle. The characteristic *triphasic* form of the contractile response evoked by ATP (Lindner & Rigler, 1931; Loewi, 1949; Lichtneckert & Straub, 1949; Kanda *et al.* 1954; Green & Stoner, 1950; Versprille, 1963), and the way in which it is affected by the 3′,5′-cyclic nucleotide phosphodiesterase inhibitor, theophylline, led us to postulate earlier (Flitney, Lamb & Singh, 1977) that ATP exerts a dual effect on the ventricle; that the two underlying

components of the response are antagonistic; and that they are probably mediated through changes in the levels of endogenous 3′,5′-cyclic nucleotides (Flitney, Lamb & Singh, 1978*a*).

The present work provides evidence in support of this hypothesis; it will be seen that ATP induces rapid and substantial changes in the levels of both 3′,5′-cyclic AMP and 3′,5′-cyclic GMP. Moreover, despite the complex nature of the contractile responses evoked by ATP, the results nevertheless reveal a striking correlation between changes in isometric twitch tension and changes in the ratio 3′,5′-cyclic AMP: 3′,5′-cyclic GMP. This latter relationship is considered to be especially significant, since it also applies to several other, superficially quite-different physiological responses (Singh, Flitney & Lamb, 1978; Flitney, Lamb & Singh, 1978*b*; Flitney & Singh, 1979*a*; Singh & Flitney, 1980).

Preliminary accounts of some aspects of this work were presented to the Physiological Society (Flitney *et al.* 1977, 1978*a*).

METHODS

The experiments to be described were made with isolated ventricles from male and female specimens of *Rana temporaria*. Details of the method of dissecting and superfusing the ventricle are to be found in the preceding paper (Flitney & Singh, 1980). In all these experiments, with the exception of those involving microelectrode recording of action potentials (below), preparations were superfused with Ringer solution at a flow rate of 100 ml. min^{-1} and stimulated at 0·5 Hz. This caused the preparation to become hypodynamic and sufficient time was allowed for a steady-state tension (generally around 25–30% of the initial value, after 80–100 min) to be attained before examining the effects of ATP. Hence, the levels of intracellular 3′,5-cyclic nucleotides and the contractile force produced by the hypodynamic ventricle served as control values against which the effects of ATP were compared. Care was taken to ensure, in all experiments, that the test half ventricle was allowed to become hypodynamic to *exactly the same extent* as the control before exposing it to ATP.

Micro-electrode recordings. Recordings of membrane potential were obtained using conventional 3 M-KCl-filled micro-electrodes, with tip resistances in the region 10–15 MΩ. The perfusion conditions were modified somewhat in order to avoid dislodging the microelectrodes; the flow rate was reduced from 100 ml. to only 20 ml. min^{-1} and narrow strips of tissue (approximately 1·0 × 1·5 × 4–5 mm) were used instead of half ventricles. Electrical and mechanical responses were recorded on a dual-beam storage oscilloscope (Tektronix, Type 5103N) and measurements made subsequently from enlarged photographic recordings of the traces.

Assays. 3′,5′-cyclic nucleotide and protein assays were made as described in the previous paper (Flitney & Singh, 1980). The appropriate control experiments were performed to assess the effect (if any) of ATP on the 3′,5′-cyclic nucleotide assays. Known quantities of authentic 3′,5′-cyclic AMP and 3′,5′-cyclic GMP were assayed in the presence and absence of differing concentrations of ATP. The results showed that ATP had no detectable effect on either the 3′,5′-cyclic AMP or 3′,5′cyclic GMP assay, even when present at a concentration of 10^{-3} M (the maximum concentration used in this study).

RESULTS

Inotropic effects of ATP

Characteristic triphasic form of the response to ATP. Fig. 1*A* shows the time course of the decline in peak isometric twitch tension during the development of the hypodynamic condition. The data are from sixteen preparations and each point represents the mean twitch tension ± S.E. In this series of half-ventricles

contractile force fell, during a period of some 75–80 min, to around 25% of its initial value. Fig. 1*B*, *C* shows the effect of superfusing a hypodynamic preparation with 10^{-5} M (*B*) and 10^{-3} M (*C*) ATP. There is an initial, abrupt increase in the force of contraction, rising to a maximum after only 3–5 beats (*first* phase), which is followed by a period when the twitch amplitude falls, sometimes to below the control (hypodynamic) level (*second* phase). This is then superceded by a slowly

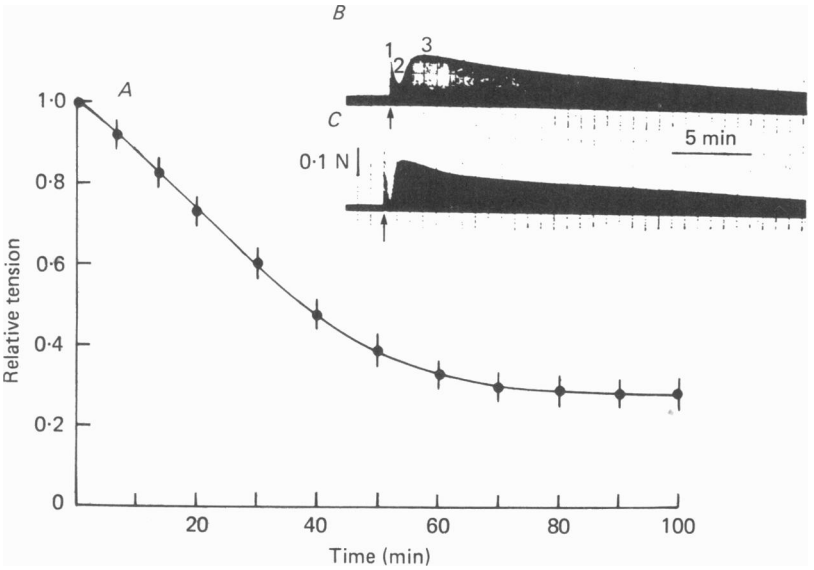

Fig. 1*A*, time course of the decline in contractility during superfusion with Ringer solution. Standard superfusion conditions: flow-rate, 100 ml. min^{-1}; stimulation frequency, 0·5 Hz; temperature, 18–19 °C. Each point = mean ± S.E.; n = 16. *B*, *C*, original chart recordings of responses produced by superfusing half-ventricles with 10^{-5} M (*B*) and 10^{-3} M (*C*) ATP. Time of addition of ATP denoted by vertical arrows. Both recordings illustrate the triphasic nature of the response; each shows an early, abrupt increase in contractile force (1); a transient reduction in twitch amplitude (2); and a long-lasting, secondary potentiation (3). Note that isometric tension during the *second* phase is greater with 10^{-5} M than with 10^{-3} M-ATP.

developing, secondary increase in contractile force (*third* phase), which is maximal after 1·5–2·0 min and persists above the control level for a further 80–100 min.

The effect of varying $[ATP]_o$'s (range: 10^{-10} to 10^{-3} M) on the form of the response was studied. Fig. 2*B* illustrates diagrammatically the three parameters which have been measured. The results are presented in Fig. 2*A*, where contractile force is expressed as a multiple of the pre-ATP, control (hypodynamic) level. These curves show several features of interest. The dose–response curve for the *third* phase (50th twitch) is sigmoidal, rising to a maximum value of 3·6 × control twitch at 10^{-3} M-ATP. The amplitude of the *first* phase (3rd twitch) increases in a similar way with increasing $[ATP]_o$, but with a slight downward trend at the higher concentrations ($> 10^{-4}$ M) used. The *second* phase of the response varies in a different manner. Its amplitude (18th twitch) increases for increasing $[ATP]_o$'s up to 10^{-7} M, but thereafter, becomes progressively reduced, falling below the control level at

around 10^{-5} M. In some preparations, $[ATP]_o$'s $\geqslant 10^{-4}$ M temporarily abolished the twitch altogether.

This latter curve is particularly significant, because it implies that the reduction in twitch amplitude seen during the *second* phase of the response cannot be due

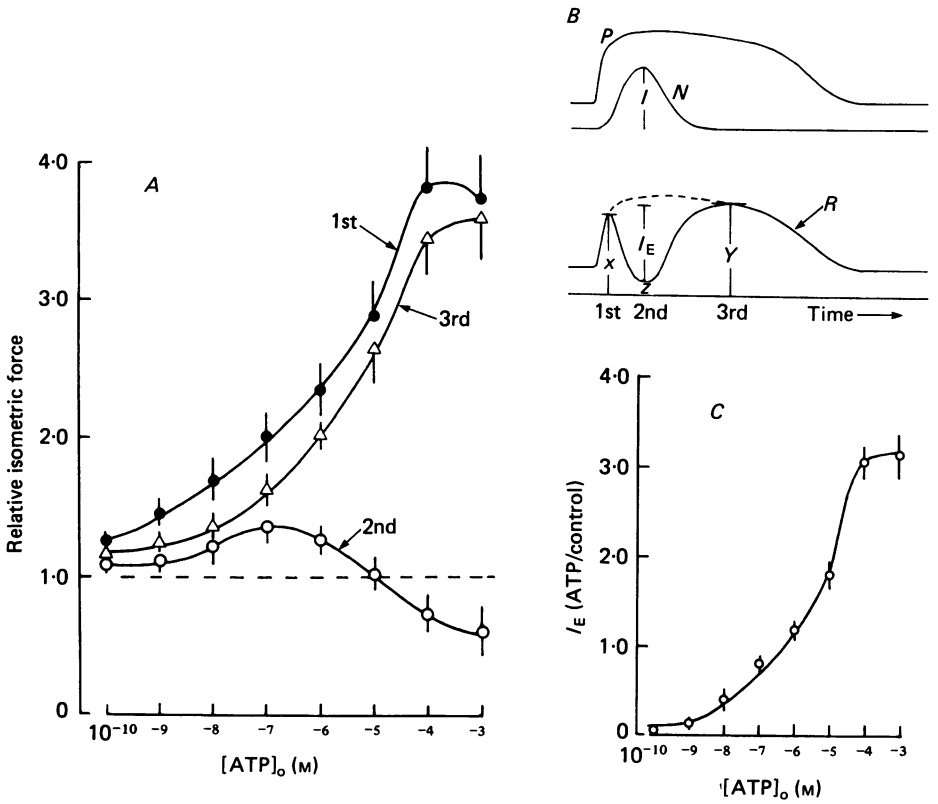

Fig. 2*A*, log dose–response curves showing the effect of differing $[ATP]_o$ on twitch amplitude measured at the peak of the *first*, *second* and *third* phases of the responses. Each point = mean ± S.E. (n = 10). Note that the twitch amplitude is depressed below the control level during the *second* phase, for $[ATP]_o$'s > 10^{-5} M. *B*, diagrammatic representation of the working hypothesis detailed in the text. The triphasic form of the ATP-induced response is assumed to arise as a result of two antagonistic influences, one potentiating the twitch (*P*) and the other exerting a negative inotropic influence (*N*). The amplitude of the latter is indicated by *I*. The resultant of the two curves is shown below (*R*). The dashed line indicates the shape of the response to be expected in the absence of any inhibitory component. x, z and y are the measured twitch amplitudes (used in constructing 2*A*); they correspond with twitch 3–5 (x), 18–20 (z) and 54–60 (y). I_E is the *estimated* amplitude of the inhibitory component, obtained as described in the text. *C*, log dose–response curves showing the progressive increase in I_E with increasing $[ATP]_o$'s, obtained using the method of analysis summarised in *B* and described in the text.

simply to the absence of a positive inotropic effect, since at higher concentrations (> 10^{-5} M) contractile force falls substantially below the control level. It shows, instead, that the *second* phase of the response is the outward expression of an underlying inhibitory effect of ATP.

It was this conclusion which led us to postulate earlier that ATP exerts a dual effect on the ventricle and that what is recorded represents the resultant of two antagonistic influences – a slightly delayed and transient *negative* inotropic effect, superimposed on a rapidly developing and longer-lasting *positive* inotropic effect

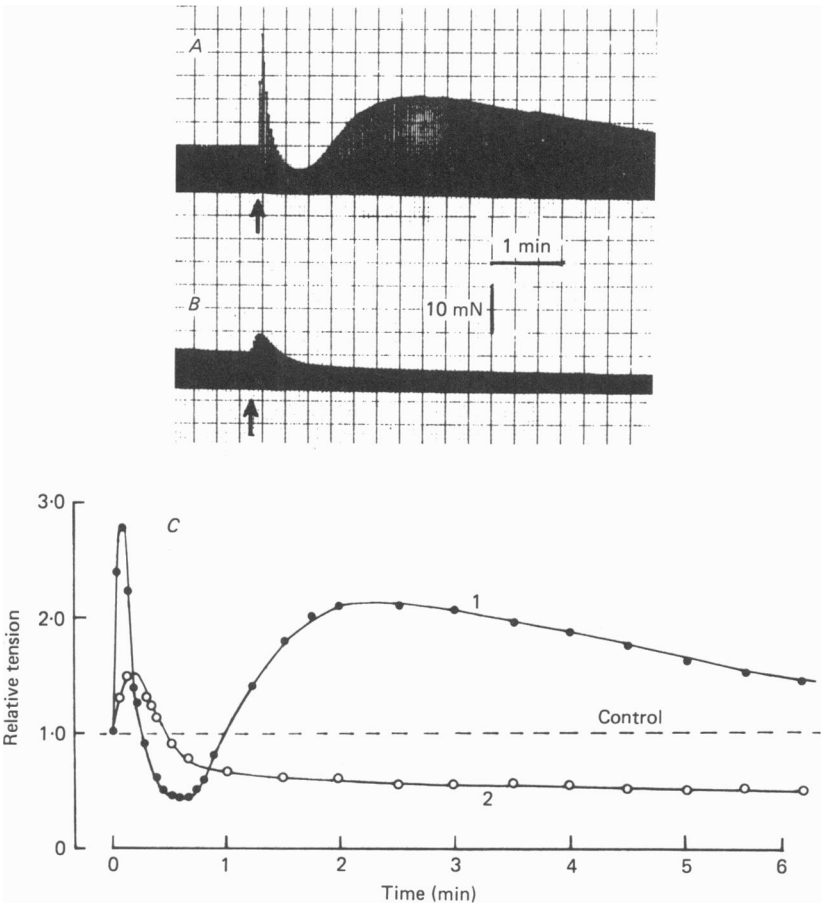

Fig. 3. Effects of indomethacin on the ATP-induced responses. *A*, control response, produced by 10^{-4} M-ATP. *B*, response to 10^{-4} M-ATP (same preparation) after superfusing the ventricle for 10 min in Ringer solution containing 10^{-5} M-indomethacin. Note reduction in amplitude of the *first* phase and the complete disappearance of the *third* phase. After the initial, small increase in contractile force, the twitch amplitude fell below the control level and remained depressed throughout the response. *C*, records *A* and *B* replotted, after scaling control (hypodynamic) twitch tension to 1·0. The magnitude of the (persistent) reduction of the twitch in the presence of indomethacin is comparable with that seen (transiently) during the control ATP response.

(Fig. 2*B*). If we assume for the present that this explanation is correct, then an *estimate* of the size of the inhibitory component (I_E) of the response at different $[ATP]_o$'s can be made by subtracting the isometric force developed during the *second* phase of the response (18th twitch; marked *z*) from the mean of the values observed at the peak of the *first* (3rd twitch, marked *x*) and *third* (50th twitch, marked *y*) phases, namely $I_E = [\frac{1}{2}(x+y)]-z$. This analysis will of course lead to

an underestimate of I, as indicated by the vertical line (Fig. 2*B*). The result of analysing records in this way, depicted in Fig. 2*C*, shows that the extent of the inhibition increases progressively with increasing $[ATP]_o$'s.

Effects of neurotransmitter antagonists and indomethacin on the ATP-induced responses. The possibility that ATP exerts its effects on the ventricle either by releasing endogenous neurotransmitters, or alternatively, by combining directly with their receptors, has been tested by recording responses in the presence of α and β-adrenoceptor (phentolamine, 10^{-6} M; propranolol, 10^{-6} M) and muscarinic (atropine, 10^{-6} M) antagonists. In each case, the dose of antagonist used was shown to be sufficient, in control experiments, to entirely inhibit the responses produced by maximal doses of their corresponding agonists. The effects of indomethacin, a prostaglandin synthetase inhibitor (Vane, 1971), were also examined, since it appears that some effects of ATP are mediated by prostaglandins (Brown, Burnstock & Cocks, 1979).

Neither phentolamine, propranolol nor atropine had any significant effect on the form of the ATP response, a result which precludes the possibility that it is mediated by the release of endogenous transmitters. It also seems unlikely (though not impossible; see later) that ATP interacts directly with the appropriate receptors. Indomethacin on the other hand, has a profound effect on the ATP response. Pre-treating the hypodynamic ventricle with 10^{-5} M-indomethacin for 10 min before the addition of 10^{-4} M-ATP entirely abolished the secondary increase in contractility (*third* phase) and greatly suppressed the *first* phase (Fig. 3). Interestingly, following the initial small rise in contractile force, the twitch amplitude fell well below the control level and remained there throughout the response (*C*). The results suggest that some of the effects induced by ATP may be mediated by prostaglandins.

Effects of ATP on the action potential

Intracellular micro-electrode recordings show that ATP has marked effects on the shape of the action potential. These changes are dose-dependent. At $[ATP]_o$'s $\leqslant 10^{-5}$ M, the positive overshoot potential (O) is increased and the time of onset of the rapid phase of depolarization is delayed, raising the 'plateau' towards more positive membrane potentials and prolonging the duration of the action potential (measured at the -30 mV level; denoted by $D_{-30\,mV}$). These changes correlate with the observed increase in isometric twitch tension. $D_{-30\,mV}$ and O are also increased during the *first* and *third* phases of the response for $[ATP]_o$'s $> 10^{-5}$ M, but both are decreased during the *second* phase, when isometric twitch tension is also depressed below the control (hypodynamic) level.

Fig. 4*A* shows superimposed oscilloscope recordings of twitch tension (B) and action potentials (A), before the addition of ATP (c), and at the peak of the *first* phase (1), during the *second* phase (2) and close to the peak of the *third* phase (3) of a response evoked by 10^{-3} M-ATP. Table 1 summarises the data obtained in ten such experiments. On average, $D_{-30\,mV}$ *increased* by 17% (520–608 msec) and by 36% (520–705 msec) during the *first* and *third* phases respectively, but *decreased* by 30% (520–368 msec) during the *second* phase of the response. Similarly, O increased by 11 mV during both *first* and *third* phases, but decreased by 6 mV during the *second* phase. ATP had no detectable effect on the diastolic membrane potential.

Effects of verapamil on action potential. These effects of ATP on the action potential and contractile response are almost entirely suppressed by verapamil (α-iso propyl-α-[(N-methyl-N-homoveratryl)-γ-aminopropyl]-3,4-dimethoxyphenyl-acetonitrile hydrochloride), an agent which is known to impede transmembrane Ca^{2+} movements (Kohlhardt, Bauer, Krause & Fleckenstein, 1972). Fig. 4*B* illustrates the results obtained in an experiment in which the effects of ATP (10^{-3} M) on both action

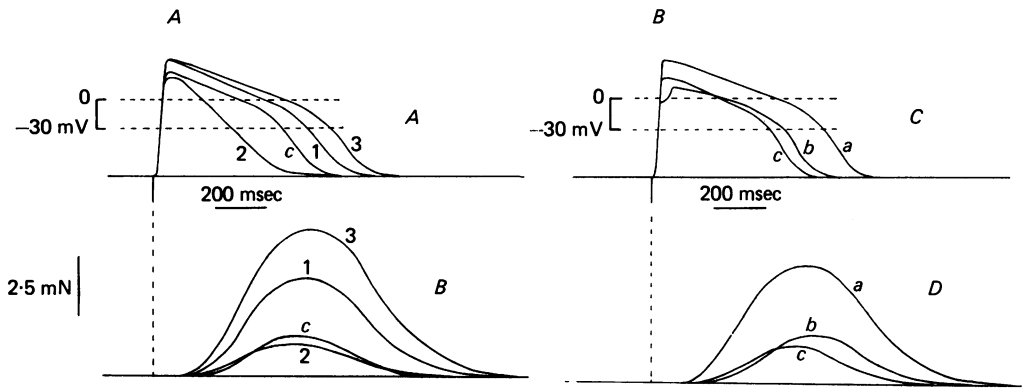

Fig. 4. *A*, *B*, effects of ATP (10^{-3} M) on action potential (*A*) and isometric twitch tension (*B*) recorded during the *first*, *second* and *third* phases of the ensuing response. Note that action potential overshoot and duration are increased during *first* and *third* phases, but reduced during the *second*. These changes correlate with the observed increases (*first* and *third* phases) and decreases (*second* phase) in twitch amplitude. *C*, *D*, effects of verapamil on changes in action potential and isometric twitch tension. *a*, effects of ATP (10^{-3} M) alone; *c*, control; *b*, effects of ATP (10^{-3} M) after superfusing with 10^{-5} M-verapamil for 10 min. Note that the changes elicited by ATP alone are almost completely suppressed by verapamil.

potential (*C*) and isometric twitch (*D*) were first measured and compared subsequently to the response obtained in the present 10^{-5} M-verapamil. The control (hypodynamic) action potential and twitch are labelled *c* and the responses recorded in the presence of ATP alone are labelled *a*. ATP alone increased the twitch amplitude by approximately 3×, and both $D_{-30\,mV}$ and *O* were increased too, the former by 207 msec and the latter by 10 mV. The preparation was then allowed to recover by superfusing it for 20 min with normal Ringer solution. It was then exposed for 10 min to 10^{-5} M-verapamil and the effect of ATP (10^{-3} M) examined in the continuing presence of verapamil. The recordings labelled *b* illustrate the results obtained. As can be seen, verapamil almost completely suppressed the changes normally elicited by ATP. Note that there is no positive overshoot during the rapid upstroke of the action potential (see 'notch' on *b*); that $D_{-30\,mV}$ is increased by only 60 msec, as compared to 207 msec; and that the twitch amplitude is only 1·27 × the control value.

Relationship between action potential duration and contractile force. The relationship between changes in action potential duration ($\Delta D_{-30\,mV}$) and isometric twitch tension (ΔP) for $[ATP]_0$'s ranging from 10^{-8} to 10^{-3} M are shown in Fig. 5. The results were obtained from eleven preparations and all three phases of the responses

are represented. In each case there is a linear relationship between $\Delta D_{-30\,mV}$ and ΔP, consistent with the idea that the action potential exerts some measure of control over the ensuing contraction. However, the slopes of the three lines ($\Delta P/\Delta D_{-30\,mV}$) are markedly different, decreasing in the order 1 > 3 > 2. These results are significant because they show that the *sensitivity* of the ventricle to membrane depolarization is not fixed, but varies throughout the response. This point is taken up again later.

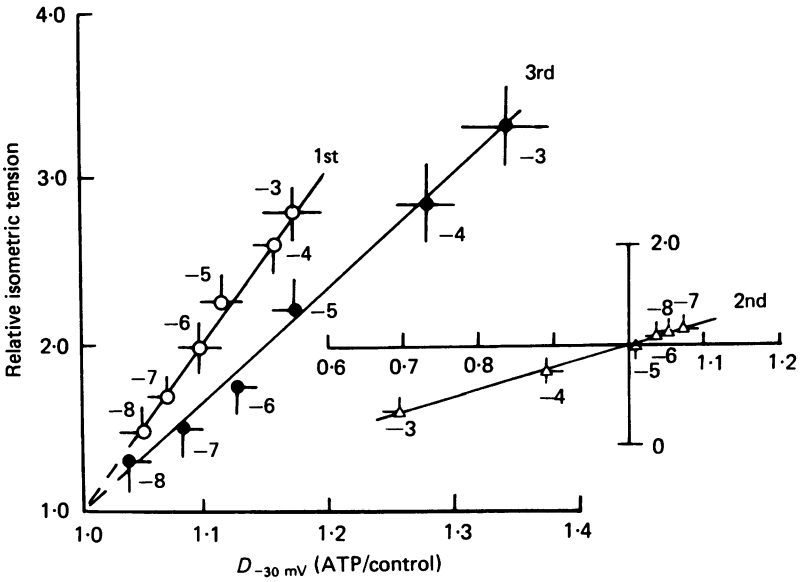

Fig. 5. Relationship between action potential duration ($D_{-30\,mV}$) and contractile force for different $[ATP]_o$'s. All three phases of the responses are represented. Numbers next to each point indicate $[ATP]_o$ used. All values are expressed as multiples of the control values. Each point = mean ± S.E.; n = 11. Data for second phase shown inset, with twofold reduction in the scales of both axes. The slopes of lines 1, 2 and 3 ($\Delta P/\Delta D_{-30\,mV}$) may be compared directly. Note that each set of data gives a linear correlation between $\Delta D_{-30\,mV}$ and ΔP, but that the slopes are different: 1 > 3 > 2. Values for *second* phase, lying in the lower left quadrant of inset figure, obtained for $[ATP]_o$'s > 10^{-5} M. See also Table 1.

Effects of ATP on 3',5'-cyclic nucleotide metabolism

The postulated negative and positive inotropic components of the ATP response can be thought of as being cholinergic – and β-adrenergic-*like* in nature, and this similarity prompted us to investigate the possibility that they may be associated with changes in endogenous 3',5'-cyclic nucleotide levels. There were two principal reasons for thinking that this might be so. First, it is well-established that the *positive* inotropic effects of several β-agonists are accompanied by elevated levels of 3',5'-cyclic AMP (reviewed recently by Tsien, 1977) and a number of authors (though not all; see e.g. Brooker, 1977; Diamond, Ten Eick & Trapani, 1977; and also p. 37) have reported that the negative inotropic responses to acetylcholine are associated with increased 3',5'-cyclic GMP levels (George, Polson, O'Toole & Goldberg, 1970; George, Wilkerson & Kadowitz, 1973; George, Ignarro, Paddock,

TABLE 1. Effects of ATP (10^{-3} M) on action potential

	Control values	First phase	Second phase	Third phase
'Diastolic' potential (mV)	78·36 ± 3·34 (14)	78·27 ± 3·60 (10)	78·14 ± 3·67 (10)	79·18 ± 3·86 (14)
Action potential height (mV)	104·76 ± 4·10 (14)	115·76 ± 4·67 (10)	98·50 ± 3·96 (10)	116·78 ± 5·57 (14)
Positive overshoot potential (mV)	26·40	37·49	20·50	37·60
Action potential duration ($D_{-30\,mV}$), mV	520·00 ± 12·90 (14)	608·40 ± 10·40 (10)	368 ± 16·85 (10)	705·00 ± 19·0 (14)
% age change in $D_{-30\,mV}$		+17·0	−29·24	+35·57

TABLE 2. Effects of ATP (10^{-3} M) on ventricular contractility and endogenous 3′5′-cyclic nucleotide levels

Time (sec)	Cyclic AMP* (ATP)	Cyclic AMP* (control)	Cyclic AMP ratio (ATP/control)	Cyclic GMP* (ATP)	Cyclic GMP* (control)	Cyclic GMP ratio (ATP/control)	R	P_R
4	27·48	9·50	2·89	2·26	1·25	1·81	1·59	1·57
6	28·14	6·28	4·48	1·76	0·85	2·07	2·10	2·30
24	4·68	6·35	0·76	1·39	0·50	2·78	0·26	0·23
24	5·30	7·06	0·75	0·96	0·35	2·74	0·27	0·29
38	5·76	8·61	0·67	0·43	0·27	1·57	0·42	0·42
50	13·33	9·55	1·40	1·50	1·04	1·44	0·96	0·94
75	17·40	8·72	2·00	1·00	1·08	0·93	2·13	2·28
84	22·55	9·38	2·40	2·10	1·26	1·66	1·44	1·44
106	19·20	9·05	2·20	0·73	1·11	0·65	3·25	3·22
152	30·29	9·47	3·19	1·23	1·45	0·84	3·77	3·86
152	21·76	6·29	3·45	0·60	0·50	1·20	2·86	2·76

* p-mole.mg protein^{-1}.

White & Kadowitz, 1975; Goldberg, Haddox, Nicol, Glass, Sandford, Kuehl & Estensen, 1975); secondly, pretreatment of the ventricle with a combination of the phosphodiesterase inhibitor theophylline, together with either 8 bromo-3′,5′-cyclic GMP or dibutyryl 3′,5′-cyclic AMP (in an attempt to elevate intracellular 3′5′-cyclic GMP and 3′,5′-cyclic AMP respectively) transforms the response in a way which is consistent with the idea that both 3′,5′-cyclic nucleotides are normally involved in mediating the inotropic effects of ATP (Flitney *et al.* 1978*a*; Singh, 1978).

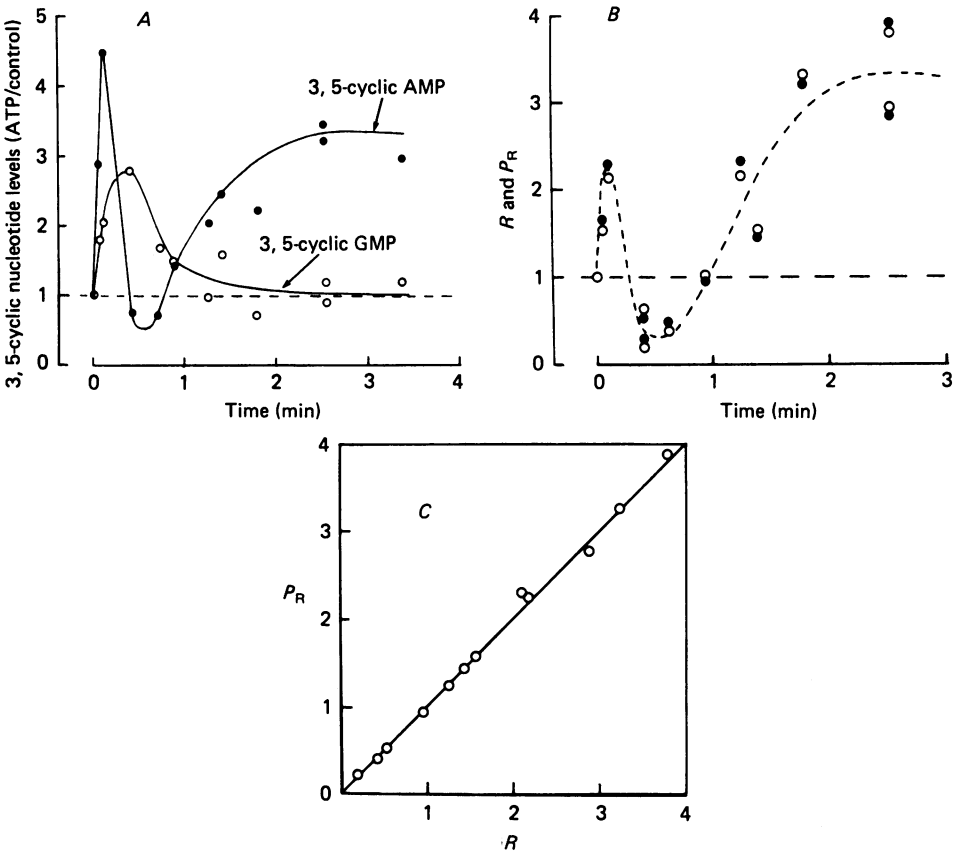

Fig. 6. *A*, time course of changes in 3′,5′-cyclic AMP (filled circles) and 3′5′-cyclic GMP (open circles) during superfusion with 10^{-3} M-ATP. Note (i) rapid increase and decrease in 3′5′-cyclic AMP levels; and (ii) slower, *transient* increase in 3′,5′-cyclic GMP levels. Each point is the result for one half-ventricle, expressed as a multiple of the value found in its corresponding control half-ventricle. *B*, changes in isometric force (filled circles) and 3′,5′cyclic nucleotide ratio *R* (defined in text; open circles) plotted against time after addition of ATP. The dashed line is drawn in to indicate the *approximate* shape of the ATP-induced response. Note that the change in twitch tension is paralleled closely by changes in *R*. See also Table 2, columns 8 and 9. *C*, relationship between *R* and P_R. Continuous line denotes a 1:1 correlation. All points in *A*, *B* and *C* normalised to corresponding control values, as described in the text.

The latter results were sufficiently encouraging to warrant making a series of experiments in which the levels of both 3′,5′-cyclic nucleotides were measured at different times throughout the course of the ATP-induced response. A concentration

of 10^{-3} M-ATP was chosen, since this produced the greatest degree of depression of the twitch during the *second* phase of the response and therefore maximised the possibility of detecting any change in 3′,5′-cyclic GMP levels.

Time course of the changes in 3′,5′-cyclic AMP and 3′,5′-cyclic GMP during the ATP-induced response. A series of eleven half-ventricles was allowed to become hypodynamic, each one was 'crush-frozen' and the levels of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP determined, as described previously (Flitney & Singh, 1980). Each of the eleven 'partner' half-ventricles was also rendered hypodynamic and then superfused with Ringer solution containing 10^{-3} M-ATP. Each one was subsequently crush-frozen at a different time during the ensuing response, the levels of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP were afterwards measured, and the values obtained compared to those found in the corresponding control half-ventricles.

The changes in 3′,5′-cyclic nucleotide levels, expressed as multiples of the control values, and plotted as a function of time are shown in Fig. 6*A*. ATP induced a large and rapid increase in intracellular 3′,5′-cyclic AMP (filled circles), rising to 4·5 × the control level within 5–8 sec, at a maximum velocity of 4·9 p-mole mg.protein^{-1}. sec^{-1}. This initial rise was followed by an abrupt fall, to around 0·6 × the control level after 30–40 sec. 3′,5′-cyclic AMP levels then rose again, but more slowly (maximum velocity: 0·4 p-mole.mg protein^{-1}.sec^{-1}), to reach a secondary maximum of 3·4 × the control value after 140–160 sec. Thereafter the levels of 3′,5′-cyclic AMP declined slowly towards the initial value (horizontal, dashed line).

The early rapid rise and fall in 3′,5′-cyclic AMP is accompanied by a somewhat slower, *transient* increase in 3′,5′-cyclic GMP levels (open circles). 3′,5′-cyclic GMP increased to around 2·8 × its control value after 20–25 sec at a maximum velocity of 0·1 p-mole.mg protein^{-1}.sec^{-1}. It then declined slowly, returning to the control level after 80–100 sec, and stayed there throughout the remainder of the response.

Attention should be drawn to three important features of these results. First, it is clear that ATP markedly stimulates the production of both 3′,5′-cyclic nucleotides. Second, the increase in 3′,5′-cyclic GMP peaks at a time which corresponds closely with (*a*) the time at which the twitch is depressed maximally; and (*b*) the time at which the *rate of fall* of 3′,5′-cyclic AMP is maximal. Third, the subsequent (secondary) increase in 3′,5′-cyclic AMP levels commences as 3′,5′-cyclic GMP approaches its control value.

Relationship between 3′,5′-cyclic nucleotide levels and contractile force. Table 2 summarizes the data obtained in this series of experiments. Columns 8 and 9 reveal a remarkably precise correlation between changes in the ratio of 3′,5′-cyclic AMP: 3′,5′-cyclic GMP in each 'test' half-ventricle, compared to its value in the corresponding control half-ventricle, namely:

$$R = \frac{\text{3′5′-cyclic AMP: 3′,5′cyclic GMP (ATP-treated half-ventricle)}}{\text{3′,5′-cyclic AMP: 3′,5′-cyclic GMP (control half-ventricle)}}$$

and the accompanying change in ventricular contractility, P_R:

$$P_R = \frac{P \text{ (ATP-treated}}{P \text{ (control)}}.$$

The time course of the changes in P_R (filled circles) and R (open circles) are shown graphically in Fig. 6*B*. It can be seen that the effect of ATP on the contractile response is almost exactly paralleled by corresponding changes in R. This relationship is more clearly illustrated by plotting R against P_R as shown in Fig. 6*C*. The data all lie close to the continuous line, drawn in with a slope of 1 to indicate a direct proportionality between these two parameters.

DISCUSSION

The results of the experiments described here imply that exogenous ATP exerts a *dual* effect on the frog ventricle and that the characteristic triphasic form of the contractile response is the outward expression of two antagonistic influences, a predominantly positive inotropic effect which is temporarily suppressed, shortly after its onset, by a transient negative inotropic effect. These effects of ATP on the ventricle are accompanied by changes in the action potential, and by marked alterations in the levels of intracellular 3′,5′-cyclic nucleotides.

In the discussion that follows we will focus attention on two main points arising from these observations. First, we discuss the probable site of action and initial effects of ATP on the ventricle; and secondly, we consider the biochemical implications of the apparent antagonistic effects of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP in regulating ventricular contractility.

Possible sites of action and primary effects of ATP on the ventricle

Does ATP modulate Ca^{2+} entry during the action potential? It is well established that part of the inwardly directed current during the plateau phase of the action potential is associated with the entry of Ca^{2+} into the fibres (frog ventricle: Niedergerke & Orkand, 1966; Morad & Orkand, 1971), and there is abundant evidence to show that this component plays a crucial role in linking membrane excitation with contraction (Morad & Orkand, 1971; see Morad & Goldman, 1973). The effect of a number of cardioactive agents, including several naturally occurring neurotransmitters, have been attributed, in part, to changes in the magnitude of this so-called slow Ca^{2+} current ($I_{Ca^{2+}}$). The positive inotropic effects of adrenaline, for example, are characterized by an increase in $I_{Ca^{2+}}$, a shift of the plateau toward more positive membrane potentials and an increase in action potential duration (*frog atria*: Vassort, Rougier, Garnier, Sauviat, Coraboeuf & Gargouil, 1969; *cow* and *cat*: Reuter & Scholz, 1977), whereas the negative inotropic responses to acetylcholine are accompanied by a decrease in $I_{Ca^{2+}}$, a lowered plateau and a reduction in the duration of the action potential (*frog atria*: Giles & Noble, 1976; Ikemoto & Goto, 1977). The changes in shape of the action potential following treatment with exogenous ATP bear a strong resemblance to those described above for adrenaline (*all* phases of the response, for $[ATP]_o$'s $< 10^{-5}$ M) and for acetylcholine (*second* phase only, for $[ATP]_o$'s $\geqslant 10^{-4}$ M) and it is tempting to speculate, on the basis of these similarities, that they too reflect changes in the Ca^{2+} permeability of the fibres. Goto, Yatani & Tsuda (1977) and Yatani, Goto & Tsuda (1978) reported a comparable effect of ATP on the action potential in isolated frog *atria*, and also recorded changes in $I_{Ca^{2+}}$ using a double glycerol-gap voltage-clamp technique.

The possibility that ATP modulates Ca^{2+} entry during the action potential has not been investigated explicitly in this study. It is an attractive hypothesis, but the supporting evidence is entirely circumstantial. It is based, first, on the ability of verapamil to block the changes normally evoked by ATP, and secondly, on certain similarities between our observations and those reported by other workers, who in addition employed voltage clamp techniques. It would therefore be unwise at the present time to place too much emphasis upon it. Clearly, it is premature to preclude the possibility that ATP affects membrane conductance to ions other than Ca^{2+} (e.g. Na^+, K^+).

Relationship between action potential and contractile force. Although the ionic basis for the effects of ATP on the action potential is not yet established, it is nonetheless clear that there is a close correlation between ventricular contractility and the duration of the action potential. The nature of this relationship is of considerable interest, for it implies that while the action potential exerts some degree of control over the ensuing contraction, its ability to do so is not fixed, but changes throughout the response (Fig. 5); in other words, the *sensitivity* of the mechanism linking membrane excitation with contraction *varies continuously* in the presence of ATP, being greatest during the *first* phase, least during the *second* and intermediate during the *third*.

Three possible explanations for this merit some consideration. First, ATP may modulate the quantity of Ca^{2+} entering the fibres during the action potential, a point we have already discussed above. Secondly, it is conceivable that the efficiency of coupling between Ca^{2+} entry and the release of additional activator Ca^{2+} from an internal store (e.g. sarcoplasmic reticulum, inner surface membrane) is affected by ATP, such that during the *third* phase of the response a given pulse of Ca^{2+} entering from the exterior during the action potential induces a larger release of internally bound Ca^{2+}, and hence results in a greater contraction, than that produced by a similar Ca^{2+} pulse, occurring either during the *first* or *second* phases. This can be thought of as an effect of ATP on the *availability* of Ca^{2+} to the contractile system. Third, ATP may influence the *sensitivity* of the contractile proteins to a given quantity of Ca^{2+}.

It should be emphasized that neither of the possible mechanisms listed above necessarily implies a direct involvement of ATP, in the sense that it must first enter the fibres and then induce these changes (see below); indeed, there is evidence to suppose (p. 33) that they could arise as a consequence of changes in endogenous 3′,5′-cyclic nucleotide levels.

Does ATP act intracellularly? The earliest component of the ATP response develops abruptly, isometric twitch tension increasing by as much as 4–6 times within a few seconds, and for this reason it seems probable that the initial effects of ATP occur at the fibre membrane. The equally abrupt onset of changes in the shape of the action potential tend to support this view. However, it is important to know whether ATP penetrates the fibres and exerts some of its effects intracellularly.

Evidence concerning the ability or otherwise of ATP to enter cells is contradictory. It was once thought that it could not do so (Boyle & Conway, 1941; Glynn, 1968) although there is now evidence to the contrary. Certainly, ATP appears to be able to leave cells (Paddle & Burnstock, 1974; Forrester & Hamilton, 1975; Forrester

& Williams, 1977) and evidence for its entry into cat soleus muscle is provided by the work of Chaudry & Gould (1970). Where there is reason to suppose that ATP can enter cells, its mode of entry is thought to be indirect, involving an enzymatic dephosphorylation step, with the production of adenosine and inorganic phosphate, entry through the membrane as adenosine, followed by rephosphorylation inside the fibres (Hoffman & Okita, 1963; Hatori, Miyazaki & Nakamura, 1969; Krause & Wollenberger, 1968). The influence of ATP on the contractile performance of the frog ventricle, however, does not seem to depend on such a mechanism because the inotropic effects of the enzymatically non-hydrolysable analogue of ATP, AMP-PNP (adenylyl-imidodiphosphate; Yount, Babcock, Ballantyne & Ojala, 1971), are not significantly different from those evoked by ATP itself (unpublished experiments). This has been shown to be the case for frog atrial tissue too (Yatani *et al.* 1978). On balance, it seems more likely that stimulation of 3′,5′-cyclic nucleotide metabolism by ATP arises as a consequence of changes in the properties of the surface membrane.

Does ATP stimulate specific ('purinergic') receptors? The inability of either α or β-adrenoceptor or cholinergic antagonists to block the ATP-induced responses would seem to rule out the possibility that ATP acts by releasing endogenous neurotransmitters. However, there are obvious similarities between its effects on the contractile response and on 3′,5′-cyclic nucleotide metabolism and those produced by either cholinergic or β-adrenergic stimulation. The responses to adrenaline and other β-agonists are known to be accompanied by elevated levels of 3′,5′-cyclic AMP (see Tsien, 1977 for a review), which we have seen also dominate the *first* and *third* components of the ATP responses; in contrast, those evoked by acetylcholine are characterized by elevated 3′,5′-cyclic GMP levels, which the present study suggests may be the basis for the observed decrease in isometric twitch tension during the *second* phase of the ATP response. We are left, then, with several possibilities; either ATP stimulates specific purinergic (Burnstock, 1978) receptors; or it interacts directly and simultaneously with β-adrenoceptors and cholinergic receptors; or it exerts a non-specific effect on the permeability of the cell membrane.

It is important to note that comparative studies on the effects of related nucleosides and nucleotides reveal some degree of stereochemical specificity, both in terms of their ability to influence the contractile response and their effects on the metabolism of 3′,5′cyclic nucleotides. Adenosine 5′-diphosphate, adenosine 5′-monophosphate and cytidine 5′-triphosphate have qualitatively similar effects to ATP on the contractile response (Boyd & Forrester, 1968; Singh, 1978) and they share certain common structural features; namely, the presence of a ribose-bound phosphate, together with an $-NH_2$ group located on the 4C (pyrimidine) or equivalent 6C (purine) ring position. The responses to inosine 5′-triphosphate, guanosine 5′-triphosphate and uridine 5′-triphosphate (UTP) differ in that they comprise a relatively slow, *monotonic* increase in contractility, and in these compounds, the 4C or 6 C–NH_2 group is replaced by a C = O group. The importance of the ribose-phosphate can be inferred from the fact that the nucleoside adenosine elicits a negative inotropic response only (Hollander & Webb, 1957; Meinertz *et al.* 1973; Singh & Flitney, 1980). Recent studies (Flitney & Singh, 1979; Singh & Flitney, 1980) have shown that UTP and adenosine are also potent stimulators of 3′,5′-cyclic nucleotide metabolism.

The significance of the relationship between 3',5'-cyclic nucleotide levels and isometric twitch tension

The results of the present study extends to five the list of apparently unrelated physiological responses that have been investigated recently which, it now transpires, share a common feature, namely *that changes in isometric twitch tension vary in direct proportion to changes in the ratio 3',5'-cyclic AMP: 3',5'-cyclic GMP.* The existence of this relationship is consistent with the following two conclusions; either that *both* 3',5'-cyclic nucleotides are essential components of a cellular regulatory system that controls force production by the ventricle; or alternatively, that the observed changes in contractility and in 3',5'-cyclic nucleotides occur simultaneously, as a result of another event.

The latter possibility, which means in effect that the changes in 3',5'-cyclic nucleotide levels may only be incidental to the accompanying changes in contractility, cannot be excluded altogether, although as argued in the previous paper (Flitney & Singh, 1980), it is not easy to reconcile this view with the results of those experiments concerned with the effects of exogenous 3',5'-cyclic nucleotide derivatives on the ventricle. Indeed, the *direction* of the changes in twitch tension evoked by these substances – 8 bromo 3',5'-cyclic GMP depressed the twitch, whereas dibutyryl 3',5'-cyclic AMP potentiated it – are entirely consistent with the first conclusion. Moreover, recent experiments (unpublished) in which changes in twitch tension and 3',5'-cyclic nucleotides have been measured following exposure of the ventricle to 8 bromo 3',5'-cyclic GMP and dibutyryl 3',5'-cyclic AMP, show that under these conditions too, the inotropic effects are again closely paralleled by quantitatively equivalent changes in the 3',5'-cyclic nucleotide ratio.

If we accept, on the basis of the above considerations, that the first possibility is more likely than the second, then it follows that the regulatory effects of 3',5'-cyclic AMP and 3',5'-cyclic GMP must be antagonistic, the former increasing contractility and the latter acting in a counter fashion. This is not a novel concept within the wider context of cellular regulation in general. The idea that perhaps all control mechanisms in which 3',5'-cyclic nucleotides are implicated might be modulated in a bidirectional fashion by 3',5'-cyclic AMP and 3',5'-cyclic GMP acting antagonistically was first proposed by Goldberg and associates (Goldberg *et al.* 1975), in their so-called 'Yin-Yang' hypothesis. Briefly stated, this postulates that 3',5'-cyclic GMP also functions as a second messenger, but that its effects on cellular function oppose those of 3',5'-cyclic AMP.

The results of previous studies concerning the role of 3',5'-cyclic nucleotides in regulating myocardial contractility lend some support to this view. It has been shown that the effects of several β-agonists are mediated by changes in 3',5'-cyclic AMP (see Tsien (1977) for a recent review) and a similar correlation between the negative inotropic responses evoked by acetylcholine and increased 3',5'-cyclic GMP levels has been established (George *et al.* 1970, 1973, 1975; Goldberg *et al.* 1975). However, other studies have yielded more contentious results. Thus, a number of cardioactive agents elicit positive inotropic effects but appear to have no effect on 3',5'-cyclic AMP production (see table 1, p. 409, in Tsien's (1977) review for

original references); similarly, two recent studies concerning the effects of acetylcholine on the heart show that negative inotropic responses may be elicited without any appreciable change in intracellular 3′,5′-cyclic GMP (Diamond *et al.* 1977; Brooker, 1977). Not all of these so-called 'dissociation' experiments are appropriate, in so far as in some instances the levels of only one (and not *both*) 3′,5′-cyclic nucleotide was measured; whereas in others, although both 3′,5′-cyclic AMP and 3′,5′-cyclic GMP levels were monitored, measurements were made at only one (or a few) time points during the ensuing responses.

The design of such experiments can be criticised on two grounds. *First*, in terms of Goldberg's hypothesis, there is clearly a need to measure both 3′,5′-cyclic AMP and 3′,5′-cyclic GMP, since it states quite explicitly that 'if cyclic AMP and cyclic GMP act in opposition to one another then...the relative proportion of one to the other under certain circumstances may be more important that the absolute change in the concentration of only one of the components'. *Secondly*, the time courses of the underlying changes in 3′,5′-cyclic nucleotide levels are often complex; in two responses studied recently (*isoprenaline*; Singh *et al.* 1978; and *uridine 5′-triphosphate*: Flitney & Singh, 1979) we recorded early, rapid *decreases* in 3′,5′-cyclic GMP levels, prior to any significant effects on 3′,5′-cyclic AMP levels, followed later by a return to the control level, and then later still by significant *increases*. It is conceivable, then, that misleading conclusions might be drawn if the time at which 3′,5′-cyclic nucleotide levels were measured happened, by chance, to coincide with that at which the control levels were re-established.

The above criticisms cannot be levelled at the more recent studies of Brooker (1977) and Diamond *et al.* (1977), who concluded that their results were not compatible with the idea that 3′,5′-cyclic GMP mediates the negative inotropic effects of cholinergic agonists. Briefly, Brooker's experiments (with carbachol and acetylcholine, using guinea-pig, rabbit and rat atria; and rat and frog ventricles) showed that approximately 100 times more carbachol is needed to elevate intracellular 3′,5′-cyclic GMP levels than is required to produce a 90% reduction in twitch amplitude; conversely, Diamond *et al.* (working with cat atria) were able to increase intracellular 3′,5′-cyclic GMP 17-fold (with sodium nitroprusside) and actually recorded a small increase in contractile force. The latter authors also examined the effects of acetylcholine (but only made measurements of 3′,5′-cyclic GMP at 15 and 60 sec after adding the drug); although 3′,5′-cyclic GMP rose, it returned to the control level at a time when the twitch was still depressed. In neither of these studies was there any detectable change in intracellular 3′,5′-cyclic AMP levels.

These results, then, are at variance with the idea that 3′,5′-cyclic GMP is involved in regulating contractility. However, it should be noted in passing that they also conflict with the results of a recent study by ourselves (unpublished experiments) on the effect of acetylcholine on the frog ventricle. Acetylcholine (10^{-7} M) produced a rise in intracellular 3′,5′-cyclic GMP and also depressed 3′,5′-cyclic AMP levels. Again, the time course of the depressant effect on the twitch was paralleled by a corresponding reduction in the ratio 3′,5′-cyclic AMP: 3′,5′-cyclic GMP. There is no obvious explanation for these disparate results, and more work is required to clarify the situation.

Effects of 3′,5′-cyclic AMP on cellular proteins involved in regulating force production. The evidence presently available largely supports the idea that 3′,5′-cyclic AMP and 3′,5′-cyclic GMP are involved in regulating contractility, but the nature of the underlying mechanism is not yet understood. 3′,5′-cyclic AMP exerts many of its effects (though not all; see Singh & Flitney, 1980) by stimulating a class of phosphorylating enzymes called 3′,5′-cyclic AMP-dependent (or type a_1) protein kinases, and several phosphoprotein substrates for these enzymes have been shown to be important in regulating myocardial contractility. These include: troponin I (TN-I), a subunit of the regulatory protein complex (Cole & Perry, 1975; England, 1975, 1976; Solaro, Moir & Perry, 1976); 'phospholamban', a 22,000 dalton constituent of the sarcoplasmic reticulum (Katz, Tada & Kirchberger, 1975; Tada, Yamamoto & Tonomura, 1978); and a surface membrane bound protein (SMBP) which is thought to be a structural component of the slow inward (Ca) current channel (Wollenberger & Will, 1978).

Phospholamban and the SMBP are thought to regulate the distribution of Ca^{2+}, between the sarcoplasmic reticulum and myoplasm, and also between the fibre interior and the extracellular fluid, and so could influence contraction by controlling the *availability* of Ca^{2+} to the contractile proteins. The role of TN-I is less clear. It is, of course, well established that it prevents the interaction of actin and myosin in a resulting muscle, and that this inhibitory effect is suppressed when TN-C interacts with Ca^{2+} following stimulation, but there are also indications that its effects may be more subtle than this. It has been suggested that it may influence the *sensitivity* of the contractile system to Ca^{2+}, although the evidence concerning this point is somewhat contradictory: Rubio, Bailey & Villar-Palasi (1975) found that phosphorylation of TN-I from guinea-pig cardiac muscle increased the sensitivity of actomyosin ATPase to Ca^{2+}, but other work, using bovine and rat cardiac muscle, has shown a decrease in Ca^{2+} sensitivity (Ray & England, 1976; Cole, Frearson, Moir, Perry & Solaro, 1977). This discrepancy has not so far been explained. It is possible that species differences and, or, differences in experimental techniques may be important, but this must await clarification. Whatever the reason, it has been clearly shown (England, 1976; Solaro *et al.* 1976) that increased myocardial contractility is sometimes associated with increased phosphorylation of TN-I *in vivo*, and this correlation may be significant in a causal sense.

Possible sites of action of the antagonistic effects of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP

Does 3′,5′-cyclic GMP stimulate protein phosphatase activity? There is evidence to suppose that the state of phosphorylation of TN-I may be governed by the relative amounts of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP present in the fibres. This conclusion is based on a comparison of the the present results, on *amphibian* ventricle, showing parallel changes in the 3′,5′-cyclic nucleotide ratio and isometric twitch tension, and those obtained by England (1975; 1976) and Solaro *et al.* (1976), working with mammalian hearts, who found a similar parallelism between myocardial contractility and the incorporation of $^{32}P_i$ into TN-I. If this inference turns out to be correct (and it should be emphasised that the definitive experiments, involving measuring contractile response, 3′,5′-cyclic nucleotide levels and the

phosphorylation of TN-I in the same species, or preferably in the same heart, have not yet been made), then it provides a strong clue concerning the site of action of 3′,5′-cyclic GMP. By analogy with the effects of 3′,5′-cyclic AMP, 3′,5′-cyclic GMP is thought to act principally by stimulating 3′,5′-cyclic GMP-dependent (type a_2) protein kinases, known to be present in the heart (Kuo, 1974). The inferred antagonistic action of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP, implied by the nature of the relationship between ventricular contractility and the 3′,5′-cyclic nucleotide ratio, suggests that 3′,5′-cyclic GMP may promote the dephosphorylation of regulatory proteins by stimulating protein *phosphatase* activity. The potential significance of this conclusion is that a similar mechanism could also determine the state of phosphorylation of a wide range of phosphoproteins, including those involved in regulating energy metabolism.

Does 3′,5′-cyclic GMP regulate 3′,5′-cyclic AMP metabolism? The changes in 3′,5′-cyclic AMP and 3′,5′-cyclic GMP levels in response to treatment of the frog heart with ATP show an interesting feature, also seen during the development of hypodynamic depression (Flitney *et al.* 1978*b*) and in response to treatment with adenosine (Singh & Flitney, 1980); namely, that when 3′,5′-cyclic GMP levels increase, the levels of 3′,5′-cyclic AMP decrease. This is shown in a striking way by the results of Fig. 6*A*, where the initial rapid increase in 3′,5′-cyclic AMP is quickly reversed, coincident with the increase in 3′,5′-cyclic GMP levels. Attention was also drawn earlier (p. 32) to the fact that the maximum rate of fall of 3′,5′-cyclic AMP coincides with the peak increase of 3′,5′-cyclic GMP. These observations suggest that 3′,5′-cyclic GMP may constitute part of cellular mechanism which is involved in controlling the levels of 3′,5′-cyclic AMP, thereby determining the activity of 3′,5′-cyclic AMP-dependent protein kinase and so regulating the *synthesis* of phosphoproteins. This function may be complementary to its postulated role in stimulating dephosphorylation reactions (above), affording two possible ways in which 3′,5′-cyclic GMP may act to effect a change in the contractile status of the ventricle.

The authors thank the British Heart Foundation, Medical Research Council and Wellcome Trust for financial support. F.W.F. is currently in receipt of a Wellcome Trust Research Fellowship. The expert technical assistance provided by Mr R. Adam and Mr J. Eastwood is gratefully acknowledged.

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