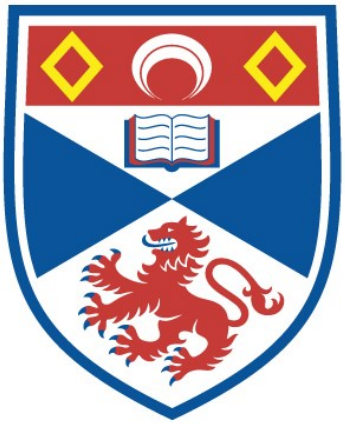
EVIDENCE FOR THE INVOLVEMENT OF INTRACELLULAR CYCLIC 3', 5' - NUCLEOTIDES IN REGULATING MIOCARDIAL CONTRACTILITY

Gordon D. Kennovin

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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EVIDENCE FOR THE INVOLVEMENT OF INTRACELLULAR CYCLIC 3',5'- NUCLEOTIDES IN REGULATING MYOCARDIAL CONTRACTILITY.

by

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Abstract

An investigation was made into one aspect of the molecular control of cardiac contractility, namely, the putative role of the two endogenous cyclic 3',5'-nucleotides, adenosine cyclic 3',5' monophosphate (cyclic AMP) and guanosine cyclic 3',5' monophosphate (cyclic GMP) in regulating the capacity of the heart to produce force. It began as a study of a poorly understood but well documented effect of exogenous adenosine 5'-triphosphate (ATP) on the electrical and mechanical properties of the isolated frog ventricle. In common with several related purine and pyrimidine nucleotides, treatment of the ventricle with ATP elicits a characteristic triphasic response which is not blocked by either atropine or propranolol.

Preliminary experiments with ATP led to the hypothesis that it exerts a dual effect on the heart. The two effects were attributed tentatively to the induction of elevated levels of intracellular cyclic AMP and cyclic GMP respectively. Indirect evidence for the involvement of cyclic nucleotides in mediating the ATP-induced response comes from studies with agents which are known to influence cyclic 3',5'-nucleotide metabolism on the form of the ATP-induced response.

Measurements of endogenous cyclic 3',5'-nucleotides during the development of the hypodynamic state is associated by a gradual decline in intracellular cyclic AMP and a progressive increase in cyclic GMP. This gradual loss in contractility is also accompanied by the release of prostaglandins E_1 and E_2 and one or more prostaglandin-related substances into the perfusate. Subsequent experiments with exogenous

ATP elicits profound changes in cyclic 3',5'-nucleotides. Both cyclic AMP and cyclic GMP are affected as had been postulated. A third series of experiments with the β-agonist isoprenaline (isoproterenol) was undertaken to investigate the generality of the relationship between intracellular cyclic nucleotide levels and contractility, and this produced similar results.

The most significant feature to emerge from the results was the finding that in all three responses, which on the face of it appear to be physiologically distinct and quite unrelated, changes in the ratio of intracellular cyclic AMP / cyclic GMP almost exactly parallel the observed changes in contractile force. This observation has led to the formulation of a hypothesis which postulates that both endogenous cyclic 3',5'-nucleotides are integral components of an intracellular control mechanism which is responsible for regulating the contractility of the amphibian ventricle.

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A C K N O W L E D G E M E N T S

It is a pleasure to express my gratitude, thanks and indebtedness to my supervisors, Professor J.F. Lamb and especially Dr. F.W. Flitney for their invaluable help, insight, advice and inspiration throughout the course of this study.

I have also benefitted considerably from valuable help and discussions with Dr. I. Johnston and other members of the teaching and technical staff of the department.

I am deeply indebted to my wife, Aileen, who supported me during the course of this work. No written acknowledgement will be enough to express my gratitude for her help, patience, tolerance and understanding throughout my study.

Finally, my thanks to Donna P. Sharma for her careful typing of this thesis, and to Mr. Robert Adam for his invaluable photographic assistance.

D E C L A R A T I O N

This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy, in the University of St. Andrews, entitled 'Evidence for the involvement of intracellular cyclic 3', 5'- nucleotides in regulating myocardial contractility', is my own composition and is the result of work done mainly by me during the period of matriculation for the above degree. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology and Pharmacology, United College of St. Salvator and St. Leonard, University of St. Andrews under the supervision of Professor J.F. Lamb and Dr. F.W. Flitney.

A C A D E M I C R E C O R D

I first matriculated at the University of Guyana in September 1966 and graduated with a B.Sc. (General degree) in Biology in November 1971. I also graduated with a Postgraduate Diploma in Education from the University of Guyana in November 1972. I matriculated as a Postgraduate research student of the Department of Physiology and Pharmacology, University of St. Andrews in October 1974.

C E R T I F I C A T E

We hereby certify that Jaipaul Singh has spent nine terms engaged in research work under our direction, and that he has fulfilled the conditions of General Ordinance No.12 (Resolution of the University Court No.1 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Dr. F.W. Flitney

Professor J.F. Lamb

This was difficult ... to write because the subject refused to sit still. It was not simply that it behaved like a naughty child at the photographer's, for that would be expected in any viable field of scientific research. Rather, it has seemed to us more like an imaginary child who, in the course of having his picture taken, suddenly grew to adult proportions and then left the studio badly in need of a shave.

G. Alan Robison

Reginald W. Butcher

Earl W. Sutherland

In: Cyclic AMP, 1971.

C O N T E N T S

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Abbreviations

The following abbreviations are used in the text and for labelling figures:

ATP	Adenosine 5'- triphosphate.
ADP	Adenosine 5'- diphosphate.
AMP	Adenosine 5'- monophosphate.
8 Br cyclic GMP	8 Bromo guanosine 3', 5' monophosphate.
Ca^{2+}	Ionised calcium.
Cyclic AMP	Adenosine 3', 5' cyclic monophosphate.
Cyclic GMP	Guanosine 3', 5' cyclic monophosphate.
CTP	Cytidine 5'- triphosphate.
DB cyclic AMP	Dibutyryl adenosine 3', 5' cyclic monophosphate.
GTP	Guanosine 5'- triphosphate.
H^{+}	Ionised hydrogen.
ITP	Inosine 5'- triphosphate.
K^{+}	Ionised potassium.
Mg^{2+}	Ionised magnesium.
Mn^{2+}	Ionised manganese.
Na^{+}	Ionised sodium.
OH^{+}	Ionised hydroxide.

P	Isometric tension.
Pi	Inorganic phosphate.
PG	Prostaglandin.
PLS	Prostaglandin-like substance.
PRS	Prostaglandin-related substance.
SR	Sarcoplasmic reticulum
$[\,]_o$	Concentration i.e. $[ATP]_o$ ATP concentration

SUMMARY

A. A study has been made of the decline in contractility and associated metabolic changes which accompany the development of the hypodynamic condition in the isolated superfused frog ventricle.

B. Evidence is presented to show that prostaglandins E_1 and E_2, together with an unidentified prostaglandin-related substance, are released from the ventricle during the development of the hypodynamic depression. There is a close correlation between the extent of the depression and the quantity of prostaglandins released into the superfusate. Agents which interfere with prostaglandin biosynthesis (arachidonic acid and indomethacin) affect the rate at which the hypodynamic state develops and the final steady-state twitch tension attained.

C. Measurements of endogenous cyclic 3', 5'- nucleotide levels reveal a time-dependent decrease in intracellular

adenosine 3', 5'- cyclic monophosphate (cyclic AMP) and a corresponding increase in guanosine 3', 5'- cyclic monophosphate (cyclic GMP) levels. These data show that the changes in contractility are almost exactly paralleled by an equivalent change in the ratio $\left[\text{cyclic AMP}\right] : \left[\text{cyclic GMP}\right]$.

D. Superfusion of isolated ventricles with Ringer solution containing exogenous lipid-soluble derivatives of cyclic AMP and cyclic GMP affect both the time course of the decline in twitch tension and the final steady-state level attained in a dose-dependent fashion: 8-Bromo cyclic GMP accelerates the decline in contractility and depresses further the steady-state level, whereas dibutyryl cyclic AMP delays the development of the hypodynamic condition and elevates the final steady-state level.

E. It is concluded that the leakage of prostaglandins and/or a prostaglandin-related substance from the fibres initiates changes in intracellular cyclic 3', 5'- nucleotide levels and that this may be responsible for the loss of contractility which characterises the hypodynamic state.

F. A study has also been made of the response of the superfused (hypodynamic) frog ventricle to treatment

with exogenous adenosine triphosphate (ATP) and other related compounds. Measurements of membrane potential, isometric twitch tension and the levels of endogenous cyclic 3', 5'- nucleotides have been made at various times during the ATP-induced response.

G. Exogenous ATP elicits a characteristic triphasic response. This comprises an initial, rapidly-developing positive inotropic response (first phase), followed by a period when the twitch is reduced sometimes to below the pre-ATP (control hypodynamic) level (second phase), and superseded by a slowly-developing and long-lasting increase in contractility (third phase).

H. The duration of the action potential and the magnitude of the overshoot are increased during the first and third components, but reduced during the second. Verapamil (α - Irosproyl - α- [(N - methyl - N - homoveratryl) - γ - aminopropyl] - 3, 4 - dimethoxyphenyl - acetonitrile hydrogen chloride) reduces these changes and also decreases the twitch amplitude. The observations are consistent with an effect on the slow inward (calcium) current.

I. The ATP-induced response is not blocked by either

atropine or propranolol. The observations exclude the possibility that ATP exerts its effects by releasing endogenous neurotransmitters or by stimulating directly cholinergic or β- adrenergic receptors.

J. Pretreatment of the ventricle with theophylline (10^{-4}M) enhances the second and third components of the ATP-induced response, but depresses the first. ATP produces a monotonic positive inotropic effect in the continuing presence of exogenous 8-Bromo cyclic GMP and theophylline and an inhibitory response only in the continuing presence of dibutyryl cyclic AMP and theophylline.

K. ATP elicits changes in endogenous cyclic 3', 5'-nucleotide levels. Cyclic AMP and cyclic GMP are both affected. The changes induced by ATP are such that the time course of the contractile response is paralleled closely by the time-dependent changes in the ratio of the two cyclic nucleotides (cyclic AMP: cyclic GMP).

L. It is concluded that the ATP-induced inotropic responses are mediated through changes in metabolism of of cyclic 3', 5'- nucleotides. It is postulated that the primary effect of ATP is to modulate the slow inward calcium current.

M. The mechanism of action of ATP, some related purine and pyrimidine nucleotides and adenosine has been investigated on ventricular contractility in an attempt to relate molecular structure to the differing pharmacological effects. ATP, ADP, CTP and AMP (type A agents) exert a characteristic triphasic positive inotropic response, whereas UTP, ITP and GTP (type B agents) produce a slowly-developing positive inotropic effect which corresponds to the peak of the third component of type A agents. Adenosine elicits a negative inotropic effect. The inotropic actions of these compounds are not affected by pretreatment of the ventricle with either atropine or propranolol.

N. Finally, a study has been made of the time course and dose-dependent changes in contractile force and intracellular cyclic 3', 5'- nucleotide levels following exposure of the hypodynamic ventricle to isoprenaline.

O. The β -agonist elicits changes in contractility and the levels of endogenous cyclic AMP and cyclic GMP. A striking correlation exists between the changes in isometric force and the ratio of cyclic AMP: cyclic GMP for both the time course and dose-dependent experiments.

P. The present observation led to the formulation of a hypothesis which postulates that both endogenous cyclic 3', 5'- nucleotides are integral components of an intracellular control mechanism that regulates the contractile state of the heart.

CHAPTER I

INTRODUCTION

SCOPE OF THE PRESENT STUDY

The majority of multicellular animals have evolved a complex circulatory system to circumvent the problem which would otherwise arise of distributing essential nutrients and metabolites to each and every cell of the body. In most higher animals, this comprises a system of tubes through which blood is propelled by the rhythmic pumping of the heart. The heart itself is essentially a muscular bag, although to describe it as such is an over simplification and obscures the fact that it is a remarkable organ. In a resting adult, for example, it pumps on average 5 litres of blood every minute, amounting to an incredible 2×10^8 litres during an average life span! Even more remarkable is the fact that its performance can be greatly altered to meet the everchanging demands which are made upon it, as for example, during severe exercise, when its output may increase in a matter of seconds to around 5 - 8 times its resting level. This is achieved in part by an increase in the frequency of beating and partly by an increase in the volume of blood ejected with each stroke, both of which are controlled ultimately by neuronal and/or hormonal influences.

Clearly, the various factors which act together to regulate the performance of the heart are of great importance and one must look ultimately to the molecular level in order

to understand how its contractile properties are modulated. The work to be described in this thesis is concerned with one aspect of the molecular control of cardiac contractility; namely, the putative role of the two endogenous cyclic 3', 5' - nucleotides, adenosine cyclic 3', 5' monophosphate (cyclic AMP) and guanosine cyclic 3', 5' - monophosphate (cyclic GMP), in regulating the capacity of the heart to produce force. It began as a study of a poorly-understood but well-documented effect (Part II, Introduction) of exogenous adenosine triphosphate (ATP) on the electrical and mechanical properties of the isolated frog ventricle. In common with several related purine and pyrimidine nucleosides and their nucleotide derivatives, treatment of mammalian and amphibian hearts with ATP elicits a characteristic triphasic response, comprising an initial, rapidly - developing positive inotropic effect, reaching a peak within a few beats, followed by a period when the twitch amplitude is markedly reduced, sometimes to below its control level, and superseded by a slowly-developing increase in contractile force, persisting for up to 100 minutes. Preliminary experiments, concerned with the effect of superfusing isolated frog ventricular strips with varying concentrations of ATP on the size of the response, and with the effect on its form of the phosphodiesterase inhibitor, theophylline, led to the hypothesis that ATP exerts a dual effect on the heart - an immediate

and relatively long - lasting positive inotropic effect, and, superimposed upon it, a somewhat delayed and transient negative inotropic effect — and that what is actually observed is simply the resultant of the two opposing influences. For reasons which are described in detail later (Chapter VII), the two effects were attributed tentatively to the induction of elevated levels of intracellular cyclic AMP and cyclic GMP respectively. This same series of experiments established clearly that the precise form of the contractile response is a function of time during which time the preparation is superfused with physiological saline prior to treatment with ATP. It is well known that the force of contraction of the isolated, perfused heart diminishes progressively with time, to reach ultimately a reduced but relatively stable level, a condition which Clark (1913) called the hypodynamic state, and, in keeping with the idea that changes in cyclic AMP and cylic GMP levels are involved in mediating the ATP — induced response, it was logical to examine the possibility that the hypodynamic state might itself be associated with altered levels of intracellular cyclic nucleotides. This turned out to be the case; it was found that the loss of contractility is accompanied by a gradual decline in intracellular cyclic AMP and a progressive increase in cyclic GMP. Subsequent experiments then affirmed that ATP does indeed have a profound effect on the metabolism of both cyclic nucleotides, as had been

postulated. A third series of experiments, with the β-agonist isoprenaline (isoproterenol), was undertaken to investigate the generality of the relationship between intracellular cyclic nucleotide levels and contractility, and this produced similar results; however, in contrast with previously published findings on mammalian hearts (Robison, Butcher, Øye & Sutherland, 1965; Cheung & Williamson, 1965; Schümann, Endoh & Brodde, 1975), it was found that both cyclic AMP and cyclic GMP levels are affected.

The most significant feature to emerge from the results was the finding that in all three responses, which on the face of it appear to be physiologically distinct and quite unrelated, changes in the ratio of intracellular cyclic AMP/cyclic GMP almost exactly parallel the observed changes in contractile force. It therefore appears likely that both cyclic 3',5' - nucleotides together constitute part of an intricate cellular control mechanism which is responsible for regulating the contractility of the amphibian ventricle.

The remainder of this introduction is devoted to a review of cardiac contractility, with particular emphasis being given to the role of calcium in excitation-contraction coupling and to the involvement of endogenous cyclic 3', 5' - nucleotides in regulating the contractile performance of the heart. For convenience, the two other principal

areas covered in this work - namely, the various factors which influence the development of the hypodynamic condition and the previously-known effects of ATP and related substances on the electrical and mechanical properties of the heart - are reviewed separately, and preceed the relevant experimental chapters.

CONTROL OF CARDIAC CONTRACTILITY.

Calcium and the cardiac action potential.

The myocardial cell has a high resting membrane potential (-80 mV to - 90 mV) which is determined largely by the transmembrane gradient of potassium ions to which the sarcolemma is selectively permeable; and in this respect it resembles skeletal muscle and nerve. However, the ionic basis for the action potential is quite different. In skeletal muscle, the action potential is of short duration, comparable with that of, say, the squid axon, and it preceeds the mechanical response; whereas in a cardiac muscle fibre, the action potential persists for several hundred milliseconds, well into the contractile period. The form of the action potential is quite different too. It comprises an initial rapid upstroke, due to a selective increase in sodium permeability, followed by a maintained plateau of depolarisation, and terminated by a rapid repola-

risation. The most significant development in recent years has been the recognition that the plateau phase of the cardiac action potential is maintained by an inwardly-directed calcium current (Reuter, 1967) which is activated when the membrane potential reaches about -45 mV. This slow inward current plays a crucial role in initiating and modulating the contractile response of the heart (Reuter, 1967); Hagiwara & Nakajima, 1966; Lüttgau, 1966, Niedergerke & Orkand, 1966). Experiments using voltage-clamp techniques have established a clear correlation between the 'steady-state' contractile force and the magnitude of the slow inward current (Morad & Trautwein, 1968; Reuter & Beeler, 1969; Beeler & Reuter, 1970a, b, c; Noble, 1975). Moreover, it is known that a variety of cardioactive agents exert their inotropic effects by modulating this current. The catecholamines increase its size (Natham & Beeler, 1975), producing an elevation and prolongation of the plateau phase of the action potential, whereas the effects of acetylcholine are accompanied by a reduction in its size and an abbreviation of the action potential (Giles & Tsien, 1975; Giles & Noble, 1976).

The central importance of calcium in regulating myocardial contractility has been known since the end of the last century. Ringer (1883) observed that the frog heart stops beating when the extracellular medium is deficient in calcium. Locke & Rosenheim (1907) and Mines (1913) later

demonstrated that the electrical excitablility of the heart is not markedly impaired by this procedure, a finding which clearly implicated calcium in the process of coupling excitation with contraction. The situation is different in skeletal muscle. Frog skeletal muscle gives many contractions in a calcium deficient medium (Frank, 1960; Jenden & Reger, 1962,; Luttgau, 1963) and eventually fails to contract only because its membrane becomes inexcitable (Edman & Grieve, 1964). This is not to say that calcium is unimportant in activating skeletal muscle; it has been shown repeatedly that it is the only physiologically occurring ion able to elicit a contraction when micro-injected into skeletal muscle fibres (Heilbrunn & Wiercinski, 1947; Niedergerke, 1955; Caldwell & Walster, 1963). The fundamental difference between the two is that the calcium required to initiate a contraction in the case of the skeletal muscle fibre is derived from an internal store, and not from the extracellular fluid. There are theoretical grounds too for concluding that this must be so. Hill's (1948, 1949) consideration of the time required for diffusion of substances into skeletal muscle fibres led him to conclude that the interval between excitation and full activation of the contractile machinery is much too short to be accounted for on the basis of diffusion alone. However, similar calculations for cardiac muscle fibres, which are generally of much smaller diameter, do not preclude diffusion of an activator substance such

as calcium from the extracellular fluid as being the principal step in excitation-contraction coupling.

The sarcoplasmic reticulum and T-system; Paradoxically, much of our understanding of muscle activation comes from studies of the relaxation process. Biochemical studies of fractionated muscle revealed the existence of a 'relaxing factor' (Marsh, 1951) capable of reversing the ATP-induced synergesis of actomyosin and of relaxing contracted glycerinated muscle (Bendal, 1953). Kumagai, Ebashi & Takeda (1955) later demonstrated that Marsh's relaxing factor was particulate in nature and they recognised that it was identical to the so-called Kielley-Mayerhof granular ATP-ase fraction (Kielley & Mayerhof, 1948a, 1948b). It was later postulated that the relaxing factor in reality consisted of fragmented and resealed intracellular membranes (Ebashi & Lipman, 1962).

A great deal of subsequent biochemical studies of isolated muscle membrane (specifically, those of the sarcoplasmic reticulum; see below) showed that these fragments have the ability to accumulate and store calcium. The membranes contain a Ca^{2+} sensitive ATP-ase, capable of transporting 2 moles of Ca^{2+} per mole of ATP hydrolysed (Hasselbach & Makinose, 1961; Makinose & Hasselbach, 1971; Tada, Yamamoto and Tonomura, 1978) at a rate which is sufficient to account for the rapidity of the relaxation process (Ohnishi & Ebashi, 1963; Weber, 1971a, b). Comparable preparations from cardiac muscle have similar properties

(Carsten, 1964; Carsten & Reedy, 1971) although it is recognised that there are some important differences, considered later in this chapter (pages 33 - 35), which permit the rate of Ca^{2+} pumping to be modified under certain conditions. The Ca^{2+} pumping and storage capability of the SR maintains the level of free Ca^{2+} below around 10^{-7} M in a resting muscle and maximum stimulation of the contractile system is achieved when this is made to rise, during activation, to around 10^{-6} to 10^{-5} M (Portzehl, Caldwell & Ruegg, 1964; Ebashi & Endo, 1968: Hellam & Podolsky, 1966).

The importance of the intracellular membranes of muscle fibres first became apparent in the 1950's when internal membrane systems in general were generating a great deal of interest, largely as a result of an expansion in electron microscopic studies of cells which occurred at this time. Pioneer studies were made by Porter & Palade (1957), who investigated the structure of membrane system in amphibian and mammalian skeletal muscle, as well as in mammalian heart. These authors described a complex system of membranes, apparently homologous with the smooth endoplasmic reticulum of other cell types, which they accordingly called the sarcoplasmic reticulum (SR). Characteristic three-component structures, termed triads, were described, consisting of a central element, flanked by two lateral elements, Triads were found in the interfibrillar space, located

opposite the Z - line in amphibian skeletal and mammalian cardiac muscle, and opposite the A - I boundary in the mammalian skeletal muscle. Andersson-Cedergren (1959) later made a careful three dimensional reconstruction of the internal membrane system found in mouse skeletal muscle and she provided the first evidence for a second system of tubules, distinct from the SR, which ran transversely across the fibres and formed a continuous network. She called this system the transverse tubular or T-system. Subsequent studies by Franzini - Armstrong & Porter (1964) using improved fixation methods, obtained direct evidence for continuity of the wall of the T-system with the surface membrane in fish myotomal muscle, and Huxley (1964) and D.K. Hill (1964) obtained indirect evidence for continuity of the lumen of the T-system with the extracellular fluid using externally-applied protein markers. Huxley's experiments with the iron-containing protein ferritin established that the central element of the triad represented cross sectional profiles of T-tubules; ferritin particles were found in central elements but not in the lateral cisternae. Thus it was established that the SR is a closed-off, internal system membranes, which makes close contact with the outer wall of the T-system in the region of the triads.

The notion that the T-system and SR are together involved in the process of excitation-contraction coupling came

as a result of experiments carried out by A.F. Huxley et al (1958, 1964) who applied local depolarising current pulses through small micropipettes applied to the outer surface of a muscle fibre. Early studies with frog skeletal muscle (Huxley & Taylor, 1955, 1958; Huxley & Straub 1958) showed that local stimulation of small areas of sarcolemma located directly over the Z-lines induced a contractile response in each half-sarcomere on either side of the Z-line in question. The contractile response propagated transversely (but not longitudinally) in a graded fashion with increasing amplitude of current applied, and on the basis of these results it was concluded that the Z-line itself constitutes an internal conducting pathway transmitting a signal from the cell surface to its interior. However, experiments with crab skeletal muscle produced a different result (Huxley & Peachey, 1964). In crab muscle responsive areas of sarcolemma were located opposite the A-I boundary, and stimulation of areas overlying the Z-line did not elicit a local contraction. It therefore appeared likely that some other structure was involved in coupling, at least in the case of crab skeletal muscle, and subsequent electron microsopic work established that the triads are the only features common to both kinds of muscle fibre. Thus in frog skeletal muscle the triads are found at the Z-line level, whereas in crab muscle they are located close to the A-I boundary, as in mammalian skeletal muscle.

The fine structural details of cardiac muscle fibres differ in two important respects from skeletal muscle fibres. First, the SR is generally less abundant than in skeletal muscle fibres (Fawcett, 1961) and it comes into close apposition with the inner surface of the cell membrane (Fawcett & McNutt, 1969), as well as with the outer wall of the T-system. Secondly, T tubules, when present, are generally of larger diameter; there are frequent longitudinal extensions running parallel to the myofibrills, a feature rarely seen in skeletal muscle (Peachey, Schild, 1968); and their inner surface is coated with an amorphous basement-membrane material. Comparative studies of the ultrastructure of cardiac muscle have revealed striking differences between mammalian and amphibian hearts. Frog ventricular fibres lack a distinct T-system (Chapman 1971) and the SR is only sparsely developed (Page & Niedergerke, 1972). The fibres themselves are of relatively small diameter as compared to those of mammalian hearts (Fawcett & McNutt, 1969) and the ventricular wall has a less compact, spongy texture, being made up of long strands (trabeculae) of fibres of about 20 -28μ in diameter (Lamb & McGuigan, 1966).

Extracellular calcium and excitation-contraction coupling in the heart: On the basis of these structural differences it was surmised that some at least of the calcium required to activate a contraction must come either from

the exterior, or alternatively, from a rapidly-exchanging, superficial store (Staley & Benson, 1968). Certainly, studies of ^{45}Ca uptake by cardiac muscle fibres (Winegrad & Shanes, 1962) have shown that with repeated stimulation the intra-cellular pool is increased, and this observation, together with the marked dependence of force of contraction on extracellular calcium (Willbrandt & Koller, 1948; Chapman & Niedergerke, 1970a, 1970b), adds weight to the view that calcium entry during the action potential may either activate the contractile proteins directly, or alternatively, stimulate the release of bound calcium from a store within the fibres (Ford & Podolsky, 1970; Endo, Takana & Ogawa, 1970).

The result of elevating the level of free calcium within the sarcoplasm is to overcome the inhibitory effect of troponin I, a component of the regulatory protein complex, which in a resting muscle prevents the interaction of actin and myosin. Repression of the inhibitory function of troponin I is indirect, and results from the binding of calcium to troponin C, a second component of the regulatory protein complex. It is clear that the quantity of calcium entering during the action potential is inadequate to saturate all the troponin C known to be present within the fibres - it is estimated that calcium entry into the frog heart amounts to around 1 nmole per gram per beat under normal conditions (Niedergerke 1963;

Niedergerke, Page & Talbot, 1969) as compared with a figure of 100 nmoles of troponin C per gram of muscle (Perry, 1974) -clear evidence that its critical role in influencing the contractile response is of an indirect nature. Evidence is now accumulating which indicates that its effects may be linked to changes in intracellular cyclic nucleotide levels and this is considered in more detail in the general discussion (Chapter X).

Involvement of intracellular cyclic 3', 5' - nucleotides in regulating myocardial contractility.

The contractility of the heart is modulated by a variety of neuronal and hormonal agents and there is a growing belief that these actions are mediated through changes in the slow calcium current and in the levels of intracellular cyclic 3', 5' - nucleotides (Tsien, 1977). The role of cyclic AMP as a regulator of metabolic function has been the subject of intensive investigation since its discovery in the late 1950's by Sutherland and Rall (1958). These studies have led to the view that it functions as a 'second messenger' in mediating the actions of a wide range of hormones, including certain cardioactive neurotransmitters, on cellular metabolism (Robison, Butcher & Sutherland, 1968; Tsien, 1977). Far less is known about the role of the other naturally occurring cyclic

3', 5' - nucleotide, cyclic GMP, although its ubiquitous occurrence in cells, including those of the heart (George, Polson, O'Toole & Goldberg, 1970; George, Wilkerson & Kadowitz, 1973; Goldberg, Haddox, Nicol, Glass, Sanford, Kuehl & Estensen, 1975; George, Busuttil, Paddock, White & Ignarro, 1975: Watanabe & Besch, 1975; England, 1976; Gardner & Allen, 1976a, b, 1977), testifies to its potential importance in cellular metabolism. Indeed, the principal conclusion arising from the present work is that cyclic GMP is equally as important as cyclic AMP in regulating contractile performance.

Metabolism of cyclic AMP; Cyclic AMP is formed from intracellular ATP by the action of an enzyme called adenylate cyclase. Adenylate cyclase is located chiefly in the plasma membranes of the heart (Øye & Sutherland, 1966; Drummond & Duncan, 1970; Wollenberger & Schultz, 1976) and in the membranes of the sarcoplasmic reticulum (Entman, Levey & Epstein 1969; Katz, Tada, Repke, Iorio & Kirchberger, 1974). Particulate adenylate cyclase preparations from the hearts of several species have been shown to be stimulated by several hormones and neurohumors (Drummond & Severson, 1974). The conversion of ATP to cyclic AMP (Fig.1.1) requires Mg^{2+}, and it is likely therefore that the substrate for the reaction is the Mg^{2+} - ATP complex (Rall & Sutherland, 1962). Membrane - bound adenylate cyclase preparations are stimulated by a wide range of

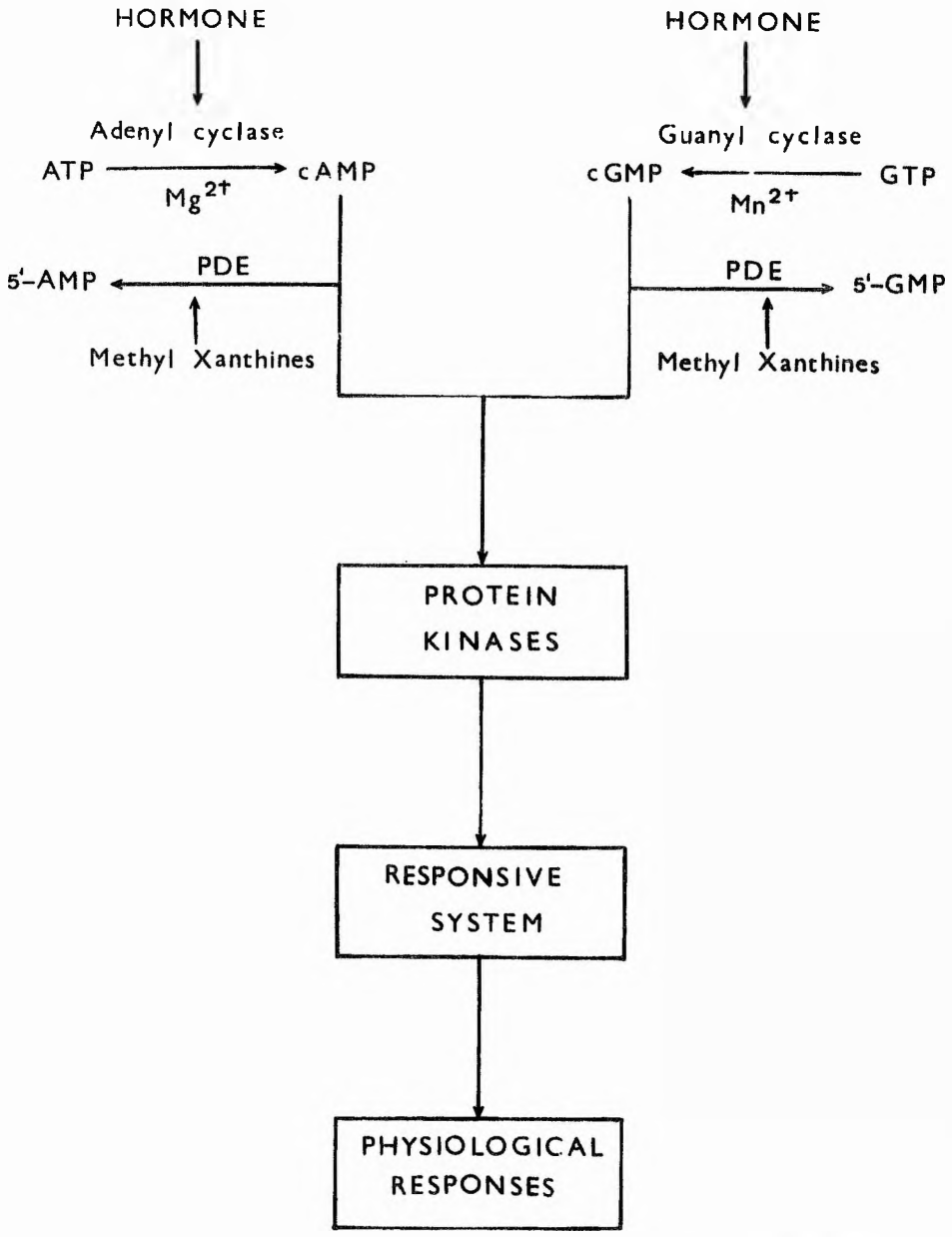


Fig.1.1 The metabolism of cyclic 3',5'-nucleotides and their mode of action.

cardioactive agents, including the catecholamines (Murad, Chi, Rall & Sutherland, 1962; Levey & Epstein, 1969b; La Raia and Reddy, 1969; Cheung & Williamson, 1965; Robison et al, 1965; Namm & Mayer, 1968; Mayer, 1972); glucagon (Morad & Vaughn, 1969; Levey & Epstein, 1969a); thyroxine (Levey & Epstein, 1969b); histamine (Klein & Levey, 1971a; McNeil & Muchet, 1972) and the prostaglandins (Sobel & Robison, 1969; Klein & Levey, 1971b). The stimulatory action of the catecholamines is inhibited by β- adrenergic antagonists (Murad et al, 1962; Mayer, 1972) but these do not prevent the actions of either glucagon (Lucchesi, 1968; Glick, Parmley, Wechsler & Sonnenblick, 1968) or the prostaglandins (Klein and Levey, 1971b).

In addition, many workers have demonstrated that treatment of intact cells with catecholamines elevates intracellular levels of cyclic AMP, presumably by stimulating adenylate cyclase activity (Sutherland et al, 1968; Epstein, Levey and Skeleton, 1971; Robison, Butcher & Sutherland, 1971; Kukovetz & Pöch, 1972; Brooker, 1973; Katz & Repke, 1973; Wollenberger & Krause, 1973; Morkin & LaRaia, 1974; Osnes and Øye, 1975; Wollenberger, 1975; Tsien, 1977) suggesting that cyclic AMP is in some way responsible for the accompanying inotropic effects. The original basis for this hypothesis was the finding that the inotropic effect of adrenaline on rat heart is preceeded and accompanied by a rapid rise in cyclic AMP Levels (Øye, Butcher, Morgan

& Sutherland, 1964; Cheung & Williamson, 1965: Robison et al, 1965). Comparable results have been obtained with preparations from different species and under a wide range of experimental conditions (Drummond, Duncan & Herlzman, 1966; LaRaia, Craig & Reddy, 1968; Namm & Mayer, 1968; Brooker, 1971; Kukovetz, Pöch & Wurm, 1973; McNeil & Verma, 1973; Osnes, Christofferson & Øye, 1973, Venter, Ross & Kaplan, 1975; Endoh, Brodde & Schümann, 1975; Schümann et al, 1975; Osnes & Øye, 1975; Dobson, Ross & Mayer, 1976; Martinez & McNeil, 1977). Nevertheless, it is clear that the underlying mechanism is more complex. A number of authors have reported that the inotropic effects of catecholamines, as well as other agents which stimulate the heart, are not invariably associated with elevated levels of cyclic AMP (Shanfeld, Frazer & Hess, 1969: Benfey, 1971; Øye & Langslet, 1972; Benfey, Kunos & Nickerson, 1973; Osnes et al, 1973; Osnes & Øye, 1975; Schümann et al, 1975). These so called 'dissociation experiments' suggest that in some cases an alternative pathway is implicated, which is not related to the production of cyclic AMP (Shanfeld et al, 1969; Osnes Øye, 1975), and it has been suggested that these involve stimulation of α - receptors (Benfey et al, 1973; Osnes et al, 1973; Schümann et al, 1975).

Glucagon has biochemical and physiological effects on the heart which superficially resemble those produced by the

catecholamines although its inotropic action, first described by Farah and Tuttle (1960), is not blocked by the β-antagonist propranolol (Lee, Kuo & Greengard, 1971). It stimulates cyclic AMP formation in ventricular slices (Lee et al, 1971), in broken cell preparations (Entman, 1974) and it has been reported to do so in the intact myocardium too (Mayer, Namm & Rice, 1970; Robison et al, 1971; England, 1976; Gardner & Allen, 1977), although Mayer et al (1970) noted that the increase in cyclic AMP levels lagged behind the increase in contractility. England (1976) observed a parallel increase in the production of cyclic AMP and isometric force on exposure to glucagon, but only after 30 seconds had elapsed. The positive inotropic response to glucagon is also seen on treatment of guinea-pig hearts, but there is little or no effect on intracellular cyclic AMP levels (Henry, Dobson & Sobel, 1975).

The mechanism of action of the prostaglandins is difficult to assess. There is some evidence that the A & E series increase cyclic AMP production by membrane preparations of guinea-pig heart (Sobel & Robison, 1969; Klein & Levey, 1971b; Levey & Klein, 1973) whereas the F series are active only on solubilized membrane preparations (Levey & Klein, 1973). On the other hand, prolonged perfusion of PGE_2 in the case of the rat heart produces no effect, either on contractile force or on cyclic AMP levels (Sen, Sunahara

& Talesnik, 1976). It has been shown that the positive inotropic actions of PGE_1 are associated with increased uptake of calcium in the case of the guinea-pig heart (Sabatini-Smith, 1972); an increase in the duration of the action potential (Smejkal, Rougier & Gardnier, 1973); an increase in slow inward and outward currents in the case of the frog heart (Mironneau & Grosset, 1976); and that these actions are not blocked by propranolol (Gudbjarnason, 1975).

Effects of phosphodiesterase inhibitors on contractility and on cyclic AMP levels: Cyclic AMP is converted to inactive 5' - AMP by the enzyme phosphodiesterase. Indirect evidence for a causal relation between the intracellular levels of cyclic AMP and contractile force comes from experiments with agents such as theophylline, and related methylxanthines, which inhibit phosphodiesterase activity. These may either increase contractility directly, or alternatively, augment the effects of other inotropic agents. Theophylline, for example, potentiates the glucagon - induced response of intact cat papillary muscle (Marcus, Skeleton, Prindle & Epstein, 1970); the response of rat atria to noradrenaline (Rall & West, 1963; the response of isolated guinea-pig, rabbit, rat (Kukovetz & Pöch, 1970) and cat (Skeleton, Karch, Hougen, Marcus & Epstein, 1971) hearts to exogenous dibutyryl cyclic AMP and noradrenaline. In addition, it augments the effects

of small doses of noradrenaline and histamine on both the inotropic response and intracellular phosphorylase activity of the guinea-pig heart (Kukovetz & Pöch, 1972).

The inotropic effects of theophylline alone on cardiac contractility (Blinks, Olson, Jewell & Bravenly, 1972) have been attributed to elevation of cyclic AMP levels (Sutherland et al, 1968; Kukovetz & Pöch, 1972) although in the case of frog ventricle (Massingham & Nasmyth, 1972) and rat atrium (Martinez & McNeil, 1977) there is little or no effect on cyclic AMP and in these instances, the positive inotropic effect is attibuted to a change in calcium metabolism. The positive inotropic effects of theophylline are not affected by either propranolol or phentolamine and they are therefore unlikely to be due indirectly to catecholamine release (Massingham & Nasmyth, 1972).

Papaverine, a more potent inhibitor of phosphodiesterase activity (Amer & Kreighbaum, 1975), enhances the accumulation of cyclic AMP and potentiates the inotropic effect of rabbit papillary muscle resulting from treatment with the β - agonist isoprenaline (Schümann et al, 1975; Endoh et al, 1975) but, it does not influence the effects attributed to increased Ca^{2+} or to α - mediated responses. Papaverine has been shown to elevate cyclic AMP levels in the heart by several authors (Henry & Sobel, 1972;

Kukovetz et al, 1973; Endoh et al, 1975: Schümann et al, 1975; Henry et al, 1975; Dobson et al, 1976) but there is still some controversy concerning the relationship between cyclic AMP and contractility under these conditions. Klaus, Krebs and Seitz (1970) and Endoh and Schümann (1975) reported a positive inotropic effect associated with elevated cyclic AMP levels, whereas Henry et al (1975) and Dobson et al (1976) reported elevated levels of cyclic AMP in the absence of any inotropic effect. Henry et al (1975) concluded that their results provide evidence against the existence of a cause - and - effect relation between cyclic AMP and myocardial contractility.

Effects of exogenous cyclic AMP and its derivatives on cardiac contractility: Exogenous, membrane - soluble derivatives of cyclic AMP (chiefly dibutyryl cyclic AMP) have been used extensively in the search for a causal relationship between intracellular levels of cyclic AMP and the contractile state of the heart. Cyclic AMP itself is not suitable, because of its poor membrane permeability (Rall & West, 1963) and because intracellular phosphodiesterase activity is sufficiently great to degrade the small amounts that may successfully penetrate the cell membrane (Robison et al, 1965). Low temperature (Langslet & Øye, 1970) and pretreatment with dimethylsulphoxide (Kjekshus, Henry & Sobel, 1971) both enhance the permeability of the membrane to cyclic AMP, but have no signi-

ficant inotropic effect, even though preparations treated in this way respond normally to subsequent exposure to β - agonist. However, N^6, 2' - O - dibutyryl cyclic AMP (DB cyclic AMP), a derivative which penetrates the cell membrane readily (Falbriard, Posternak & Sutherland, 1967) and which is more resistant to phosphodiesterase degradation (Posternak, Sutherland & Henion, 1962) produces a positive inotropic effect (Kukovetz & Pöch, 1970; Skeleton, Karch & Epstein, 1970: Skeleton, Levey & Epstein, 1970; Kobayashi, Nakayama & Kimura, 1971; Ahren, Hjalmarson & Isakasson, 1971; Boder & Johnson, 1972; Meinertz , Nawrath & Scholz, 1973a; Bertelli, Bianchi & Beani, 1972; Drummond & Hemmings, 1972; Crass, 1973; Cutiletta, Lin, Thilenius & Arulla 1973) which is not blocked by propranolol (Kukovetz & Pöch, 1970; Skeleton et al, 1970) although it develops rather slowly (taking 10 - 30 minutes) and requires high concentrations of DB cyclic AMP. Similar results are obtained with monobutyryl derivative (MB cyclic AMP; Meinertz et al, 1973a).

The inotropic effects of externally administered DB cyclic AMP are strikingly similar to those of adrenaline with respect to both the maximum tension reached and the rate of rise of tension (Morad & Rollet, 1972; Skeleton et al 1970), and also with respect to the influence of extracellular calcium concentration (Meinertz et al, 1973b). It is especially significant that both DB cyclic AMP and MB cyclic AMP shorten the time to peak tension and accelerate

the rate of relaxation (Meinertz Nawrath & Scholz, 1975). Furthermore, DB cyclic AMP increases the slow inward calcium current (Tsien, Giles & Greengard 1972) and enhances the rate of calcium entry (Meinertz et al, 1973b). The effects of DB cyclic AMP on the slow calcium current are strikingly illustrated by its ability to restore excitability to hearts depolarised by potassium rich solution (Watanabe & Besch, 1974; 1975; Tritthart, Weiss & Walter, 1975) and to cultured heart cells previously arrested with high potassium solution (Warbanow, Will-Shahab & Wollenberger, 1975).

Finally, the direct introduction of cyclic AMP or its derivatives into cells, a procedure which avoids any surface membrane effects, elicits responses which are similar to those of the catecholamines on intact cells. Tsien (1973) applied cyclic AMP by iontophoretic injection and observed adrenaline - like responses in Purkinje fibres and comparable results were obtained by Ikemoto (1977) using bull-frog atrial muscle. Yamasaki, Fugiwara and Toda (1974) reported chronotropic effects on S - A node cells and Tsein and Winegart (1976) obtained an increase in twitch amplitude in cut ventricular preparations. Skinned heart cells (Fabiato & Fabiato 1975) also respond to exogenous cyclic AMP in a way that closely resembles the action of the catecholamines on intact preparations.

The results of experiments involving the direct introduction of cyclic AMP in cells provide compelling evidence in support of the idea that cyclic AMP *per se* has the ability to increase the force of contraction.

Involvement of cyclic AMP - dependent protein kinases in regulating contractile performance: Cyclic AMP exerts its regulatory actions indirectly, by stimulating a class of protein - phosphorylating enzymes, termed cyclic AMP - dependent protein kinases (Greengard & Kuo, 1970). These enzymes comprise a cyclic AMP binding, or regulatory (R), sub-unit and a cyclic AMP - independent catalytic (C) sub-unit (Krebs, 1972). Cyclic AMP stimulates protein kinase activity by combining with the regulator moiety and causing dissociation of the catalytic sub-unit (Brostrom, Corbin, King & Krebs, 1971);

$$2cAMP + R_2 - C_2 \rightleftharpoons R_2 - cAMP + 2C$$

Cyclic AMP-dependent protein kinase was first described in heart muscle by Brostrom, Reimann, Walsh and Krebs (1970) and by Kuo, Kruger, Sanes and Greengard (1970). It exists in several different locations, including the cytosol (Corbin, Keeley, Soderling & Park, 1975) and also associated with a variety of sub-cellular organelles; the myofibrils (Reddy, Ballard, Giri & Schwartz, 1973), microsomal fractions enriched with SR (Katz & Repke, 1973; Wray, Gray

& Olson, 1973) and the sarcolemmal membranes (Krause, Will, Schirpke & Wollenberger, 1975). Phosphorylation of a protein substrate by cyclic AMP-dependent protein kinase involves the transfer of the terminal phosphate group of ATP to either a serine or threonine residue, to induce a change in its physiological function.

Several phosphoprotein substrates for cyclic AMP-dependent protein kinase have been implicated in regulating myocardial contractility, including: troponin - I, a sub-unit of the regulatory protein complex (Perry, 1975; England, 1976); phospholamban, a 22,000 dalton component of cardiac SR (Kirchberger, Tada & Katz, 1975); and a surface-membrane bound protein, thought to be a structural component of the slow inward (calcium) current channels (Wollenberger, Will & Krause, 1975). Essentially, then, there are two principal aspects of cyclic AMP - dependent protein kinase activity which are relevant to a consideration of the regulation of cardiac contractility; first, an effect on calcium movements, between the exterior and the interior of the cell on the one hand, and between the interior of the sarcoplasmic reticulum and the cytoplasm on the other; secondly, an effect on the sensitivity of the regulatory protein system to calcium. These two aspects are of critical importance to the main theme of this thesis and are considered below.

Effects of cyclic AMP on phosphoprotein substrates involved in regulating calcium movements: Movement of calcium across the surface membrane is of paramount importance in regulating the contractile response of the heart (Morad & Goldman, 1973). We have seen (page 13) that the positive inotropic effect of the sympathomimetic amines is accompanied by an increase in calcium influx during the action potential (Grossman & Furchgott, 1964; Reuter, 1965) and that this correlates well with the observed increase in twitch amplitude. A number of authors have shown that this is associated with an increase in the state of phosphorylation of a membrane-bound protein, regulated by a cyclic AMP-dependent protein kinase (Krause et al, 1975; Hui, Drummond & Drummond, 1976; Sulakhe, Leung & St. Louis, 1976, Will, Schirpke & Wollenberger, 1976) and these findings lend support to the view that the phosphoprotein in question may be a structural component of the slow inward calcium channel (Rasmussen, 1970: Tsien, 1973; Wollenberger et al, 1975; Sperelakis & Schneider, 1976). In vitro studies of isolated membrane preparations which comprise an endogenous (bound) protein kinase, together with a 24,000 dalton protein substrate, have shown that very low levels of cyclic AMP markedly increase the level of phosphorylation of the phosphoprotein and that this is accompanied by an increase in the calcium binding properties of the membrane fragments. Krause et al (1975) reported that cyclic AMP - dependent protein kinase-sti-

mulated phosphorylation of the 24,000 dalton component increased its affinity for calcium, but did not affect the number of calcium binding sites. Will et al (1976) found that calcium binding was inhibited by 120 mM - NaCl and suggested that the binding sites are located at the inner surface of the membrane and that they may be involved in a calcium sodium exchange mechanism. This notion receives support from studies by Lüllmann, Peters, Druemer and Reuther (1975) who found that phosphorylation of 'inside out' vesicles (reconstituted chiefly from broken sarcolemmal membranes) accumulate calcium in the presence of ATP and oxalate at a rate which is greater than that seen with control vesicles. Hence, the results suggest that phosphorylation of the surface phosphoprotein may have a role in controlling calcium efflux as well as calcium influx. It is possible that this is related to the 'relaxant' effect of catecholamines on the heart (Reuter, 1965; Morad & Rollet, 1972), although there is considerable evidence now that phosphorylation of a component of the SR is also involved by enhancing calcium uptake intracellularly.

This aspect of cyclic AMP-dependent protein kinase activity has been investigated extensively in recent years. A number of authors have demonstrated that incubation of cardiac SR vesicles in the presence of cyclic AMP-dependent protein kinase and γ-^{32}P ATP results in phosphorylation of a 22,000 dalton constituent of the SR (LaRaia & Morkin,

1974; Tada, Kirchberger & Katz) 1975; Schwartz, Entman, Kanube, Lane, van-Wilke & Bornet, 1976) termed 'phospholamban' (phosphate receiver; Tada, Kirchberger, Irorio & Katz, 1973) and that the extent of phosphorylation is closely correlated with an increase in calcium transport rate (Kirchberger & Chu, 1976). In addition, it has been shown that pretreatment of hearts with catecholamines increase the level of phosphorylation of phospholamban and simultaneously enhances calcium transport by isolated SR fragments (Kirchberger, Tada, Repke & Katz, 1972; 1974; Wray et al, 1973; LaRaia & Morkin, 1974; Tada, Kirchberger, Repke & Katz, 1974; Tada et al, 1975; Schwartz et al, 1976; Kirchberger & Tada, 1976).

Dephosphorylation of phospholamban is catalysed by intrinsic (LaRaia & Morkin, 1974; Tada, Kirchberger & Li, 1975) and soluble (Tada et al, 1975) phosphoprotein phosphatases. This is accompanied by a decrease in the rate of calcium transport by isolated cardiac SR membranes (Kirchberger & Raffo, 1977). The physiological significance of phosphorylation - dephosphorylation of phospholamban is as yet unclear, but it has been implicated as a possible factor contributing to the abbreviation of systole which is seen following treatment of hearts with catecholamines (Tada et al, 1974).

Effects of cyclic AMP on the regulatory protein complex: The

regulatory protein complex comprises three well characterised components (Perry, 1975); a calcium binding sub-unit, troponin C (TN - C); an inhibitory sub-unit, troponin I (TN - I) which prevents interaction of actin and myosin in the resting muscle; and a tropomyosin binding sub-unit, troponin T (TN - T). Both TN - I and TN - T are phosphorylated in vitro by phosphorylase kinase and cyclic AMP - dependent protein kinase in skeletal muscle (Bailey & Villar - Palasi, 1971; Stull, Brostrom & Krebs, 1972; England, Stull, Huang & Krebs, 1973; Perry & Cole, 1973) and cardiac muscle (Reddy et al, 1973; Cole & Perry, 1975; Rubio, Bailey & Villar - Palasi, 1975). Phosphorylation of cardiac TN - I is not impaired by TN - C, as has been found to be the case in skeletal muscle (Perry & Cole, 1973), and cardiac TN - I contains more covalently bound phosphates (approximately 2 moles phosphate /1 mole TN - I) than its skeletal counterpart (0.5 moles phosphate /1 mole TN - I). Moreover, it differs from skeletal TN - I in its association with large amounts of endogenous cyclic AMP - dependent protein kinase (Reddy et al, 1973; Reddy & Schwartz, 1974; Perry, 1975). The rate of phosphorylation of cardiac TN - I is also much greater (approximately 30 x) than that of skeletal TN - I.

There is now considerable amount of evidence to show that increased contractility in response to adrenergic stimulation of the heart is accompanied by elevated levels of

phosphorylation of TN - I (England, 1975, 1976; Solaro, Moir & Perry, 1976). England (1975, 1976) studied phosphorylation of TN - I in rat hearts perfused with varying concentrations of either adrenaline or isoprenaline, in the presence of ^{32}Pi to label the terminal phosphate of intracellular ATP. Both agents were found to induce a time-dependent increase in the state of phosphorylation of TN - I which almost exactly paralleled the time course of the resulting contractile response. Solaro et al (1976) extended these results using chemical methods to determine the total phosphate content of TN - I in perfused rabbit heart treated with adrenaline; they also found that the increase in phosphate incorporation into TN - I is closely associated with the increase in isometric force, and that this parallelism holds for differing concentrations of adrenaline.

Cyclic GMP Metabolism and the control of myocardial contractility.

Ashman, Lipton, Melicow and Price (1963) first discovered cyclic GMP in mammalian urine and since then, it has been found in virtually all cell types. Its obvious structural similarity to cyclic AMP has given rise to the view that it may have a comparable role, serving as a second messenger in mediating the action of certain hormones on cellular

functions. A number of years elapsed before a relationship between myocardial cyclic GMP levels and hormonal-induced changes in cardiac performance was established. George et al, (1970) first demonstrated an acetylcholine-induced rise in myocardial cyclic GMP levels in rat heart, a finding which has since been confirmed by a number of authors (George et al, 1973; Goldberg, Haddox, Hartle & Hadden, 1973; Goldberg et al, 1975; George et al, 1975). The effects of acetylcholine on the contractile performance of the heart and on cyclic GMP levels are blocked by atropine (Lee, Kuo & Greengard, 1972; Goldberg et al, 1973; George et al, 1975; Gardner & Allen, 1977). Significant increases in tissue cyclic GMP are also observed in quiescent ventricular slices following treatment with acetylcholine (Lee et al, 1972; Kuo, Lee, Reyes, Walton, Donnelly & Greengard, 1972). In guinea-pig, acetylcholine increases cyclic GMP levels but has a negligible effect on the contractile performance of the heart (Watanabe & Besch,1975).

A number of authors have established that exogenous cyclic GMP, or one of its more lipid - soluble derivatives, mimics the chronotropic and inotropic effects of acetylcholine. Cyclic GMP itself has little effect on mammalian ventricular or atrial preparations (Nawrath, 1976) but cellular fragments respond with a decrease in force and frequency of beating (Trautwein & Trube, 1977). Dibutyryl cyclic GMP (DB cyclic GMP) mimics the chronotropic effects of carbachol on spon-

taneously beating cultured cells (Krause, Hale & Wollenberger, 1972) and induces a concentration - dependent antagonism of the positive inotropic response to isoprenaline on the guinea-pig heart (Watanabe & Besch, 1975). In addition, monobutyryl cyclic GMP (MB cyclic GMP) antagonises the positive inotropic effect due to exogenously-administered DB cyclic AMP in cat papillary muscle (Wilkerson, Paddock & George, 1973). More recently, the 8 - Bromo derivative of cyclic GMP (8 - Br cyclic GMP) has been employed. This exerts a negative inotropic action on cat papillary muscle and rat atria (Nawrath, 1976; 1977) and negative inotropic and chronotropic actions on spontaneously beating cultured cells (Trautwein & Trube, 1977). These actions are not affected by atropine (Nawrath, 1976). Apart from diminishing contractile force, 8 - Br cyclic GMP induces a shortening of the action potential and an accompanying decrease in calcium uptake, with little or no effect on either sodium influx or potassium efflux (Nawrath, 1977). These results taken together suggest that cyclic GMP exerts effects both on force production and on transmembrane calcium movements.

Metabolism of cyclic GMP: The formation of cyclic GMP from GTP is catalysed by the enzyme guanylate cyclase. In contrast to the membrane-bound nature of adenylate cyclase, guanylate cyclase is present in both particulate and soluble forms, each of which has its own distinct properties (Kimura & Murad, 1974). Myocardial guanylate cyclase has an ab-

solute requirement for Mn^{2+} and there may be a dependence on Mn^{2+}/Ca^{2+} ratio (Krause & Wollenberger, 1977).

The reported effects of neurohumoral agents on guanylate cyclase activity are contradictory. Acetylcholine has been found to stimulate its activity in broken cell preparations (White, Ignarro & George, 1973; Sulakhe, Sulakhe & Leung, 1975), whereas others (Kimura & Murad, 1974; Limbird & Lefkowitz, 1975) were unable to confirm these findings. White et al (1973) found that elevated calcium concentration increased guanylate cyclase activity and that the stimulation induced by acetylcholine could be abolished by atropine.

Cyclic GMP - dependent protein kinases and cardiac contractility: Kuo (1974) demonstrated the existence of a cyclic GMP - dependent protein kinase in guinea-pig heart although to date there have been no reports of any naturally occurring substrates for this enzyme in the myocardium. However, recent investigations have demonstrated the presence of endogenous substrates for cyclic GMP - dependent protein kinases in smooth muscle (Casnellie & Greengard, 1974), intestinal brush border epithelium (De Jonge, 1976) and the rabbit cerebellum (Schlichter, Casnellie & Greengard, 1978). By anology with cyclic AMP - dependent protein kinase, it is assumed that the enzyme functions by altering the state of phosphorylation of phosphoprotein substrates

within the fibres (Kuo, 1975). The question of the precise role of cyclic GMP in regulating myocardial contractility is a major consideration in the light of the results to be presented, and it will be useful to mention briefly at this time that these results are consistent with the idea that cyclic GMP acts indirectly, through cyclic GMP-dependent protein kinase, to stimulate phosphoprotein phosphatase activity in such a way that it opposes the action of cyclic AMP-dependent protein kinases.

Inter-relationships between cyclic 3', 5' nucleotide levels, calcium entry and contractility.

It has been suggested (Goldberg et al, 1975) that cyclic AMP and cyclic GMP have opposing roles in regulating a number of biological systems, a hypothesis which receives support from this study and from previously published work by Nawrath (1976). It has been known for several years that synchronised oscillations in the levels of both cyclic AMP and cyclic GMP occur throughout a single heart beat (Brooker, 1973; Wollenberger, Babski, Krause, Genz, Blohm & Bogdonova, 1973). These have been attributed to pulse synchronised discharges of cardiac sympathetic and parasympathetic transmitters and/or to oscillations in calcium entry to which both adenylate and guanylate cyclases are sensitive. Cyclic AMP fluctuations are absent

in animals previously treated with 6-hydroxydopamine, which causes depletion of adrenaline stores, and after pretreatment with sotalol, a β- adrenergic blocker. Similarly the transient changes in cyclic GMP levels are diminished in the presence of atropine (Krause, Janiszewski, Bartel, Bogdanova, Karczewski & Wollenberger, (1976).

There is also evidence that the intracellular changes in the two cyclic nucleotides are interactive. Thus, addition of acetylcholine results in a significant rise in cyclic GMP which preceeds a small reduction in cyclic AMP levels (George et al, 1970, 1973, 1975: Lee et al, 1971; Goldberg et al, 1973; Gardner & Allen, 1976a, b). Moreover, acetylcholine produces a decline in cyclic AMP levels which have previously been raised by treatment with either isoprenaline (Kuo et al, 1972) or adrenaline (Gardner & Allen, 1976b) and this is accompanied by a significant rise in tissue cyclic GMP. Conversely, isoprenaline (George et al, 1975) and isoprenaline in the presence of theophylline (George et al, 1970; Goldberg et al, 1973) elevate cyclic AMP levels and produce a slight decrease in cyclic GMP Levels. These effects are blocked by propranolol (George et al, 1975). Finally, the acetylcholine induced rise in cyclic GMP is decreased by isoprenaline (Kuo et al, 1972) and this too is associated with a rise in cyclic AMP level. However, it should be emphasised that other workers have questioned this relationship and the situation

is by no means clear. Several authors observed acetylcholine-induced increases in cyclic GMP levels without any change in cyclic AMP levels (Watanabe & Besch, 1975; England, 1976; Gardner & Allen, 1977) and it has been reported (Gardner & Allen, 1976b) that adrenaline elevates cyclic AMP without simultaneously affecting cyclic GMP levels.

The calcium sensitive modulator protein: The present work focuses attention on the significance of the ratio of cyclic AMP: cyclic GMP concentrations, rather than on the level of each cyclic nucleotide separately. It is thought likely that the recently discovered calcium sensitive modulator protein (Wang, 1977) is of importance in controlling the levels of both cyclic AMP and cyclic GMP. This protein has stimulatory effects on cyclic GMP phosphodiesterase activity and on the activity of adenylate cyclase. Thus, increased activity of the modulator protein, caused by an enhanced influx and/or release of calcium from the SR, would act to lower cyclic GMP levels and simultaneously elevate cyclic AMP levels, leading to an increase in the ratio of cyclic AMP: cyclic GMP. Conversely, a reduced calcium entry would tend to lower this ratio. Evidence is presented later to show that the time course of the action potential is markedly affected by treatment with exogenous ATP. It is prolonged during the initial and secondary phases of the response, and reduced during the

intervening period when the contractile response is seen to be depressed. These observations afford the basis for the view that one important role for the entry into the fibres of extracellular calcium during the action potential is to provide a link between the effects of cardioactive agents at the cell surface and altered levels of cyclic 3', 5' - nucleotides within the fibres, and that the calcium sensitive modulator protein is involved in this process.

CHAPTER II

GENERAL METHODS AND MATERIALS.

CHAPTER II

METHODS.

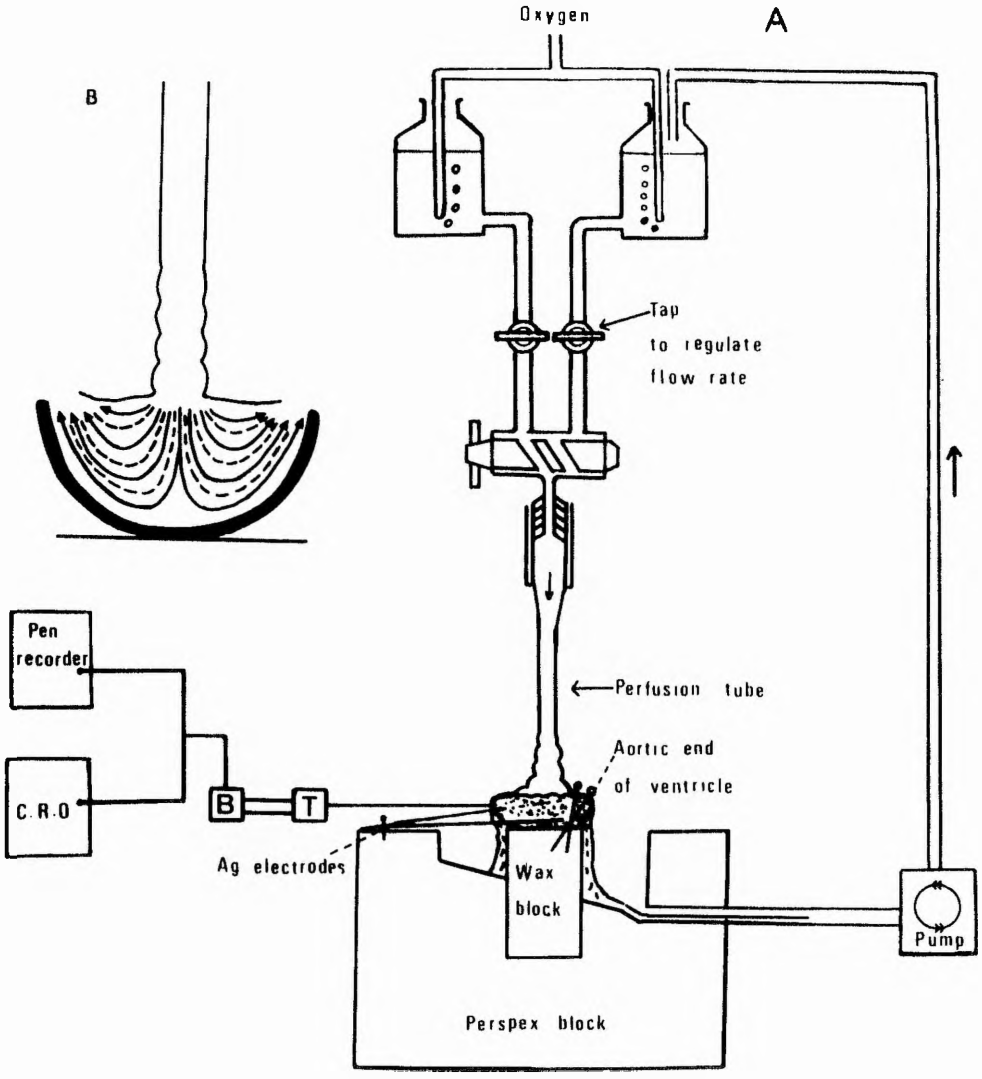
All experiments were made with isolated ventricular preparations from the frog. In general, adult male and female from the species *Rana temporaria* were used, but in a few cases when these were not available, specimens of *R. pipiens* or *R. escuelenta* were employed instead. Animals were stored at $4^{o}C$ prior to use. They were killed with a blow to the head and then pithed. Experiments were conducted at room temperature (18 - $19^{o}C$).

Dissection and mounting procedure: Hearts were rapidly excised and the auricles, containing pacemaker tissue, were removed with a single incision. The remaining single ventricle was then cut into posterior and anterior halves, providing two cup-shaped pieces of tissue. The preparation was washed free of blood in physiological salt solution (below) and mounted on a wax block, as shown in Fig. 2.1. The apex of the ventricle was attached to a stainless steel wire (diameter 0.2mm) connected to a strain guage. The base of the half ventricle was secured to the wax block using two entomological pins.

Figure 2.1.

(A) Perfusion and recording apparatus. Flow of perfusion fluid is from one of two storage Mariotte bottles via a two-way tap and perfusion tube through ventricle tissue, and thence by 'spilling over' and suction from the wax block to which the half ventricle is pinned down, to either waste or a peristaltic pump which recirculates the fluid. Tension recording is by means of an isometric (force) transducer (T) connected to the tip of the ventricle. The output of the transducer is fed, via a bridge (B), to the inputs of the cathode ray oscilloscope (C.R.O.) and pen recorder.

(B) Suggested route of perfusion fluid through ventricular tissue. Note the reflected waves on the perfusing fluid.


Oxygen
A
B
Tap
to regulate
flow rate
Pen
recorder
C.R.O
B
T
Perfusion tube
Aortic end
of ventricle
Ag electrodes
Wax
block
Pump
Perspex block

Electrical and mechanical experiments.

Superfusion procedure: The upturned, inner surface of the ventricle was superfused with Ringer solution (composition (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.0; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85; glucose, 5.55; pH, 7.2) through a vertical glass tube (internal diameter 2mm) positioned 1.5 - 2cm above its centre. A flow rate of 100ml min^{-1} was used in the majority of experiments. The rational for selecting this particular value is discussed later (page 71). It was found to be sufficiently great to keep the ventricle 'inflated' (Lamb & McGuigan, 1966) thereby ensuring that the trabeculae are in an 'opened' condition, providing rapid equilibration of solutions with the fibres. The superfusing fluid spilled over the edge of the preparation and was returned to a reservoir (capacity: generally 1 litre; see later, page 71), with a Watson-Marlowe peristaltic pump. The superfusing solutions were kept oxygenated throughout the experiments. Changes of solution were achieved by means of a two-way tap. The dead space clearance time was in the order of 100 - 150 msec. Solutions were stored in Mariotte bottles positioned approximately 60cm above the preparation. At the end of each experiment, the ventricles were gently blotted on tissue paper and weighed. Weights of the order 20 - 30mg were typical.

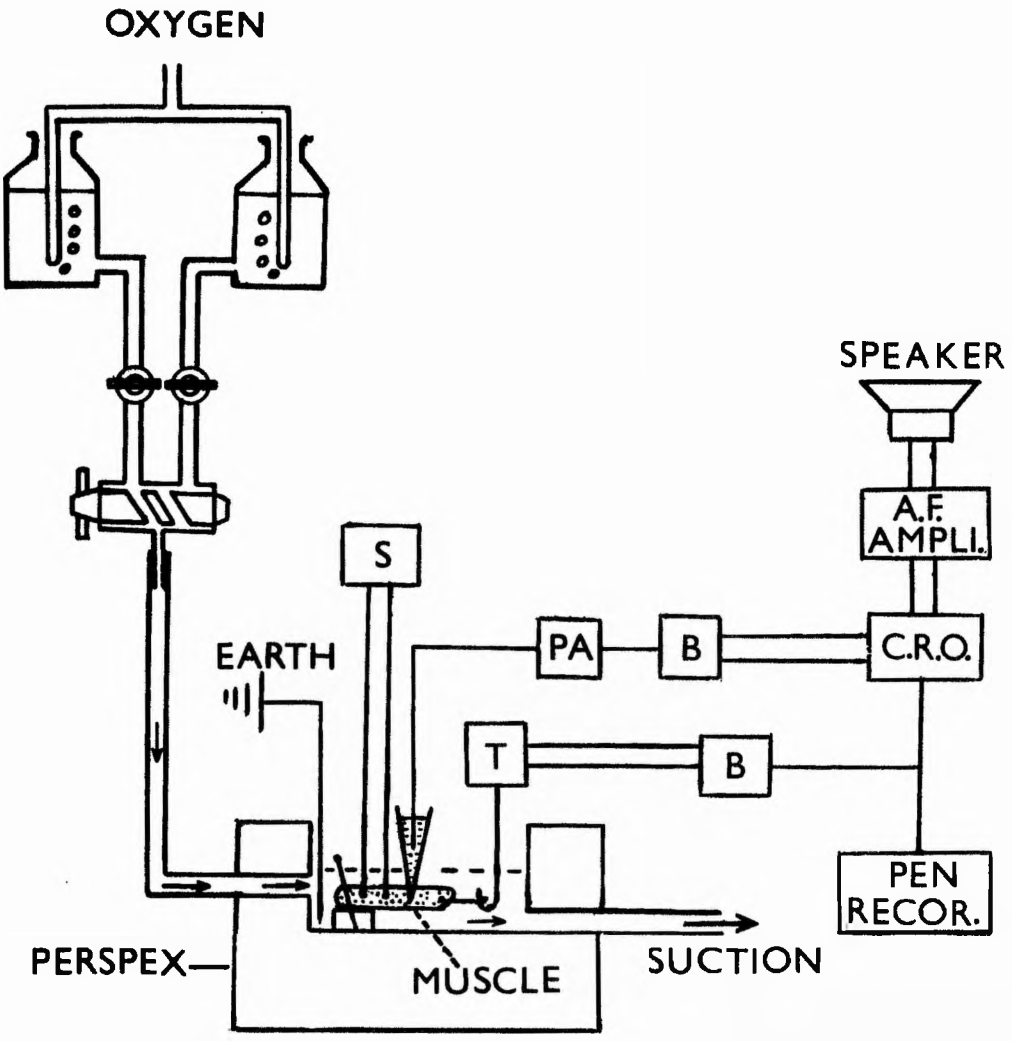
Stimulation: The principal interest was in the inotropic effects of various agents studied, and to minimise complications arising from any accompanying chronotropic effects, the preparations were paced by stimulating through two silver wire electrodes located on either side of the ventricle. Square pulses of 5 msec duration and amplitude of 10 volts were employed at frequency of 30 min^{-1}, unless stated otherwise.

Tension recording: A device strain guage (Type 4151 - 100 gm) was used to record the contractile response. The force transducer was mounted on a micro-manipulator and at the start of each experiment the optimal length giving the maximum contractile response was established. The compliance of the transducer and that of the connecting stainless steel wire is very low, but considerable local shortening of the preparation could be seen and so conditions were only nominally isometric. The transducer was calibrated at the end of each experiment using a series of known weights. Tension responses were recorded on either a devices chart recorder (Type DC. 2C No.M2 R2) or on a D13 dual beam, storage oscilloscope (Tektronix, Type 5103N).

Action potential recordings: The experimental conditions were modified somewhat for making membrane potential recordings. Smaller ventricular strips were used (approximate dimensions

Figure 2.2.

Schematic diagram of experimental arrangement for measuring membrane potentials. Flow of perfusion fluid (as shown by arrows) is from one of two Mariotte bottles via a two-way tap and perfusion tube over the muscle through a perfusing channel of a perspex chamber and then to waste (suction). One end of the muscle is pinned down to a wax block embedded into the base of the perfusing channel and the other end to an isometric (force) transducer (T). The output of the transducer is fed, via a bridge (B) to the inputs of a pen recorder and a Tektronix Storage Oscilloscope (C.R.O.) Stimulation is produced by an isolated stimulator(s) via two silver wire electrodes set into the sides of the perfusing channel. Membrane potentials are measured with conventional glass microelectrode filled with 3MKCl and coupled via an Ag - AgCl wire to the high impedience inputs of a preamplifier (P.A.). The output of the preamplifier is fed, via a bridge (B) to a Tektronix Storage Oscilloscope (C.R.O.).


OXYGEN
SPEAKER
A.F.
AMPLI.
S
EARTH
PA
B
C.R.O.
T
B
PEN
RECOR.
PERSPEX
MUSCLE
SUCTION

1.5mm wide, 1mm thick and 4mm long). The apparatus is shown in Fig. 2.2. The perfusion rate was reduced to around 20ml min^{-1} in order to avoid dislodging the micro-electrodes. Stimulation was by means of two silver strips located on either side of the preparation. Square pulses (5msec duration, 25 volts amplitude) were used at a rate of 30 beats min^{-1}. Resting and action potentials were recorded by means of conventional intracellular micro-electrodes filled with 3 M-KCl. Microelectrodes were pulled from glass capillary tubes using a Narishige electrode puller. They were coupled to a preamplifier with high input impedience through silver - silver chloride wires. Electrode tip resistances were typically of the order of 5 - 15 MΩ. Electrical and mechanical responses were displayed simultaneously on a dual beam oscilloscope and recorded photographically. Twitch and action potential parameters were measured subsequently from photographic enlargements.

General procedure: In the majority of cases the frog ventricle was superfused with frog Ringer until it had become fully hypodynamic prior to the addition of the test agents. At a flow rate of 100ml min^{-1}, stimulation frequency of 30 min^{-1} and with a circulatory volume of 1 litre, the steady-state twitch tension reached was around 25-35% of the

initial response, attained within a period of between 60 to 80 minutes. This condition served as a control and twitch tensions obtained on subsequent treatment were expressed as a multiple of this value. In those experiments involving cyclic nucleotide assays (see below), the observed levels of cyclic AMP and cyclic GMP in the fully hypodynamic ventricle were used as controls and test values were also expressed as multiples of these control levels.

Experiments involving the measurements of intracellular cyclic nucleotide levels.

Procedure: Two kinds of experiments are described; in the first the time course of the changes in cyclic AMP and cyclic GMP were followed during three different responses (development of the hypodynamic state and during treatment with either isoprenaline or ATP); in the second, dose-dependent changes in cyclic nucleotide levels were measured at a specific time, usually at the peak of the response. In all experiments two half-ventricles taken from the same heart were used for test and control preparations.

Freezing procedure: At a predetermined time, the ventricles were 'crush-frozen' by compression between flattened forceps,

previously cooled by immersion for 15 minutes in liquid nitrogen. This was done with the preparation *in situ* to avoid unnecessary delays. The frozen, crushed cells were then quickly transferred to liquid nitrogen and stored prior to extracting for cyclic nucleotides.

Extraction procedure: The frozen, crushed ventricle was pulverized with a stainless steel mortar and pestle and then extracted with 2ml acidic ethanol (1ml 1NHCl: 100ml ethanol). It was vortex-mixed for 10 seconds, allowed to stand for a further 5 minutes in an ice-cold water-bath and then centrifuged at 3000 rpm (5 minutes; $0^{o}C$). The supernatant was removed, the pellet resuspended in 1ml of ice-cold acidic ethanol and the extraction/centrifugation procedure repeated. The combined supernatants from the two extractions were evaporated to dryness at room temperature using a stream of nitrogen gas. The residue from the extraction procedure was dissolved in 1ml of distilled water and the total protein content estimated using the Biuret method (details given in appendix II A). The dried, extract was dissolved in Tris-EDTA buffer (0.05M Tris, pH, 7.5, containing 4 mM EDTA). The resulting solution was used for cyclic nucleotide assays.

Cyclic nucleotide assays: Cyclic AMP and cyclic GMP were

estimated using the Radiochemical Centre Kits (TRK 420; TRK 500). The cyclic AMP assay is based on a competitive protein binding method. Tissue cyclic AMP in a sample of the extract displaces previously bound tritiated cyclic AMP from the protein and the radioactivity remaining associated with the protein is estimated by scintillation counting. Cyclic GMP was assayed using a specific radioimmunoassay method; again tissue cyclic GMP displaces previously bound tritium labelled cyclic GMP from the antibody and the radioactivity remaining associated with the protein is measured (details of both methods are given in appendix II B,C).

The specificity of the assays is high. Cyclic AMP does not interfere with cyclic GMP assay but there is some interference of cyclic GMP in the assay for cyclic AMP. This is not appreciable until the concentration of cyclic GMP exceeds that of cyclic AMP by a factor of about 1000 (see Radiochemical Centre Booklets Nos.TRK 420; TRK 500). Generally, tissue levels of cyclic AMP and cyclic GMP were in the region of 8.623 ± 0.410 pmoles mg^{-1} protein and 1.193 ± 0.088 pmoles mg^{-1} protein respectively, in control (hypodynamic) ventricles. In fresh ventricles, the levels were in the region of 15.770 ± 0.309 pmoles mg^{-1} protein and 0.552 ± 0.038 pmoles mg^{-1} protein respectively.

The lower limits of detection of each method were: 0.05 pmoles of cyclic AMP and 0.04 pmoles of cyclic GMP. Linear calibration curves were obtained in the ranges of 1 to 16 pmoles of cyclic AMP and 0.5 to 8 pmoles of cyclic GMP.

R E S U L T S

P A R T I

THE HYPODYNAMIC STATE.

****

INTRODUCTION

It has been known for many years that the force of contraction of an isolated, perfused heart declines with time. Clark (1913) referred to this enfeebled condition as the hypodynamic state. It has been demonstrated that a wide variety of factors can influence both the rate of development of the hypodynamic condition and the final steady-state attained. It is the purpose of this Introduction to review briefly some of the more important conclusions which have been drawn.

Whole blood and serum components: A number of authors have demonstrated that the hypodynamic condition can be reversed, at least temporarily, by the addition of either whole blood or serum to the perfusing solution (Kronecker, 1897 ; Ringer, 1885; Schnecking, 1901; Lussana, 1908; Clark, 1913; Szent-Gyorgyi, 1953). Walden (1899) attributed this beneficial action to the presence of serum protein, since the effect was abolished by prolonged boiling. Schnecking's (1901) studies showed that perfusion *per se* is important; an isolated, *not* perfused, heart survives for prolonged periods (up to 5 days) but when perfused, survival is limited to around 24 hours. Clark (1913) made extensive studies of the mechanism of the hypodynamic effect. He showed that the volume of the perfusing solution was important; survival was markedly improved when small volumes of solution were circulated through the heart

as compared to large volumes. Moreover, he confirmed that the addition of small ammounts (1%) of whole blood or serum greatly stimulated the hypodynamic (but not fresh) heart and concluded that the beneficial effects were due to an alcohol-soluble constituent, rather than to serum protein.

Inorganic ions: Clark (1913) also demonstrated that the hypodynamic heart is more sensitive than the fresh heart to changes in extracellular inorganic ions. He found that elevating the ratio of Ca^{2+}: Na^{+} or Ca^{2+}: K^{+} increased the force of contraction, whereas changes in the ratio of H^{+}: OH^{-} had little or no effect. He concluded that the hypodynamic state is characterised by a decrease in the sensitivity of the fibres to extracellular Ca^{2+}. Loss of Ca^{2+} from the heart during the development of the hypodynamic state was observed by Boehm (1914) and Lieb and Loewi (1918). More recently, Chapman & Niedergerke (1970a, b) showed that the hypodynamic condition is accompanied by a decrease in calcium influx and they concluded that this was due to the loss of a hypothetical calcium binding site, normally located in the surface membrane.

Ca^{2+} and membrane bound lipids: Another potentially important observation of Clark's (1913) was his finding that the addition of "lipoids" (soaps of aromatic fatty acids) to the perfusate had a marked restorative effect. Moreover, addition of a small volume of solution which

had previously been used to perfuse a heart reversed the hypodynamic effect. His studies led him to conclude that the contractile state of the heart depends upon the semi-permeability of its fibres to electrolytes, and that this is regulated by the presence of membrane lipids and bound Ca^{2+}. The development of the hypodynamic state, in his view, is due to the loss of membrane lipids and a consequent increase in permeability to electrolytes. He believed that the function of calcium was to modify the 'colloidal' nature of membrane- associated lipids. Similar conclusions were reached later by Szent-Gyorgyi (1953). The more modern studies of Chapman & Niedergerke (1970a, b) broadly reaffirm Clarks's hypothesis. In their view the lipid factor lost from the heart during perfusion is in some way involved in the entry of calcium during the action potential. Its loss results in a reduced calcium influx with the resulting decline in contractile force.

Metabolic changes: A number of metabolites have been reported which either delay or, alternatively, accelerate the rate of development of the hypodynamic state. Anoxia has been shown to accelerate it (Lindner & Rigler, 1931; Lundin & Strom, 1948). In other studies, the time course of the effect has been shown to be closely paralleled by a fall in high - energy metabolites, including endogenous ATP (Greiner, 1952) and creatine phosphate (Vassort & Ventura-Clapier, 1977). Conversely, perfusion with solu-

tions containing metabolites, including glycogen(Clark, 1913), ATP (Lichtneckert & Straub, 1949; Marshall & Andrus, 1953), digitalis (Lundin & Strom, 1948; Sciarini, Ackerman & Slater, 1948; Greiner, 1952), creatine phosphate (Saks, Rosenshtraukh, Undrovinas, Smirnov & Chazov, 1976) and creatine, oxygen or pyruvate in the presence of polyvinyl pyrolidone (Vassort & Ventura-Clapier, 1977) all delay the onset and/or reverse the hypodynamic condition. The last mentioned authors have suggested that the hypodynamic state is due primarily to an energetic deficiency.

Prostaglandins: A number of authors have demonstrated that prostaglandins are released from the heart at rest and during stimulation (Block & Vane, 1973; Minkes, Douglas & Needleham, 1973; Junstad & Wennmalm, 1974; Block, Feinberg, Herbaczynska-Cedro & Vane, 1975). These observations are of great significance, since precursor fatty acids involved in prostaglandin biosynthesis are now known to be located in the cell membrane (Gudbjarnason, 1975). Moreover, prostaglandins E_1, E_2 and 2, 3 (trans) dehydro E, exert a positive inotropic effect on the hypodynamic frog heart (Vergroesen & De Boer, 1971). Others have demonstrated a marked positive inotropic effect of prostaglandins E_1, E_2 and $F_{1\alpha}$ (Berti, Lentati & Usardi, 1965; Bergstrom, Carlson & Weekes, 1968; Sabatini-Smith, 1970; Nakano, 1971; Smejkal, Rougier & Gardnier, 1973; Mironneau & Grosset, 1976) on intact hearts. In addition, the con-

tractile responses of both rat and frog hearts which have been deliberately depressed either by elevating the K^+: Ca^{2+} ratio or treating with the beta-blocker propranolol, with barbituates or with excess Mg^{2+} (De Boer, Houtsmuller & Vergroessen, 1973), can be restored to normal by prostaglandin E_1.

The mechanism of action of the prostaglandins is not fully understood. Their interest in the context of the present work arises from the fact that some prostaglandins (for example the E series) have been shown to stimulate cyclic AMP production in several tissues, including the heart (Robison, Butcher & Sutherland, 1971; Klein & Levey, 1971b).

Present Study: The work reported here was undertaken to investigate some of the physical factors (rate of perfusion, volume of perfusates, stimulation frequency) which affect the rate of development of the hypodynamic state, and to examine the possibility that Clark's lipid factor (and Chapman & Niedergerke's postulated calcium binding lipid) might in reality be a prostaglandin or a prostaglandin precursor which is lost from the heart during perfusion. These aspects are dealt with in Chapter III. Chapter IV is concerned with changes in the levels of cyclic AMP and cyclic GMP during the development of the hypodynamic condition.

A preliminary account of some aspects of this work was presented to the Physiological Society (Flitney, Lamb & Singh, 1978; Flitney & Singh, 1978).

C H A P T E R III

FACTORS AFFECTING THE DEVELOPMENT OF THE HYPODYNAMIC STATE: EVIDENCE FOR THE INVOLVEMENT OF A PROSTAGLANDIN AND/OR A PROSTAGLANDIN-RELATED SUBSTANCE.

I N T R O D U C T I O N

This series of experiments was undertaken with two aims; first, to characterise more precisely the physical conditions which affect the rate of development and extent of the hypodynamic depression; and secondly, to investigate the mechanism of the effect.

Physical factors influencing the rate and extent of the hypodynamic depression.

Clark's (1913) contention that the leakage of a lipid soluble factor from the heart leads to the observed loss in contractility forms the starting point for these experiments. Three parameters have been studied; perfusion rate, volume of circulating perfusate and stimulation frequency. All three variables have been found to affect the rate at which the ventricle becomes hypodynamic and/or the degree to which contractile force is reduced.

Perfusion rate: In these experiments both stimulation frequency and total volume of circulating perfusates were held constant (30 ml min^{-1} and 1 litre, respectively). Fig. 3.1A shows the time course of the decline in contractile force at perfusion rates of 50, 100 and 200 ml min^{-1}. Each point represents mean values $\pm$ S.E.'s for 6 preparations.

It can be seen that both the rate and extent of the depres-

Figure 3.1.

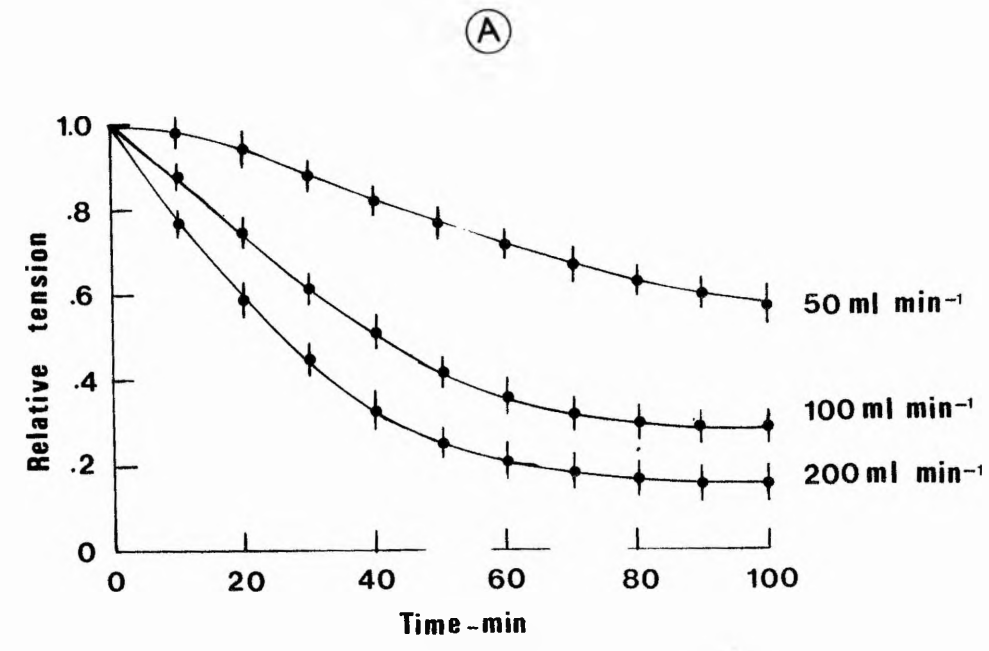
(A) Time course of the decline in contractile force at perfusion rates of 50, 100 and 200 ml. min^{-1}. The steady-state tension after 100 minutes is markedly depressed with increasing flow rates. Abscissa; Time (min). Ordinate; Relative tension.

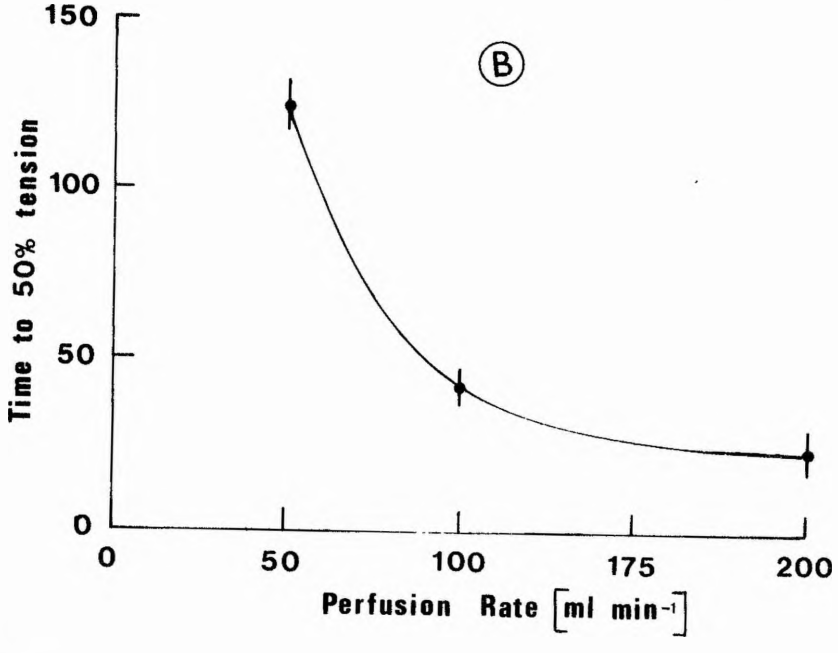
(B) Relationship between the time taken for isometric tension to decline to 50% of the initial value and varying perfusion rates. The rate of development of the hypodynamic condition increases with increasing flow rates. Abscissa; Perfusion rates (ml. min^{-1}) Ordinate; Time to 50% fall in tension (min).

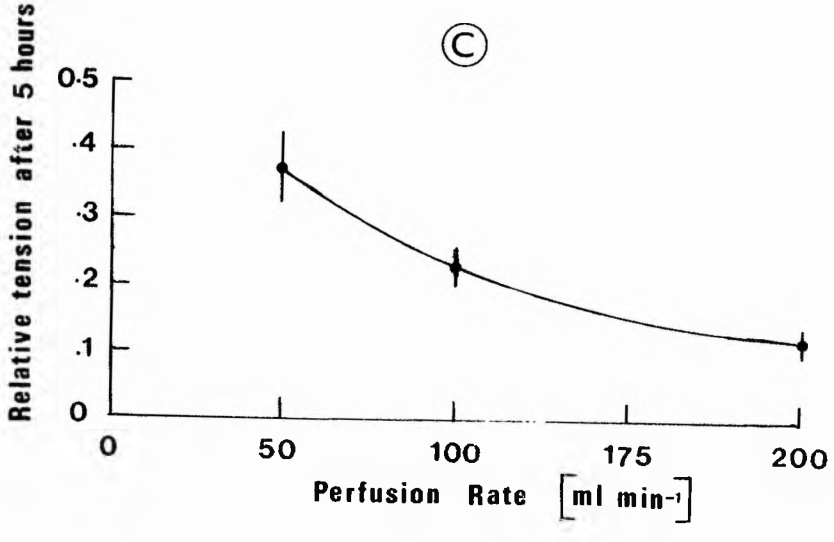
(C) The effects of the 3 different flow rates on the final steady-state tension reached after 5 hours of perfusion. Even after several hours perfusion, the ventricle is becoming more hypodynamic.

Each point is the mean $\pm$ S.E. taken from 6 preparations. Tension values are expressed as multiples of the initial (control) levels. Stimulation frequency and perfusate volume were kept constant at 30 min^{-1} and 1 litre respectively.

Stimulation parameters: Pulse width: 5 msec; amplitude, 10V. Temp, 18.5^{o}C.


A
1.0
.8
.6
.4
.2
0
Relative tension
50 ml min⁻¹
100 ml min⁻¹
200 ml min⁻¹
0
20
40
60
80
100
Time - min


B
150
100
50
0
Time to 50% tension
0
50
100
175
200
Perfusion Rate [ml min⁻¹]


C
0·5
·4
·3
·2
·1
0
Relative tension after 5 hours
0
50
100
175
200
Perfusion Rate [ml min⁻¹]

sion increase with increasing rates of perfusion. After 100 minutes, the 'steady-state' twitch tension, expressed as a fraction of the initial force developed, amounted to 0.57, 0.29 and 0.16, and the times taken for the contractile force to fall to 50% (Fig.3.1B) of its initial value were 125, 43 and 24 minutes, at perfusion rates of 50, 100 and 200 ml min^{-1}, respectively. The marked differences between the apparent steady-state tension reached at the different perfusion rates after 100 min perfusion are considerably reduced when preparations are superfused for longer periods of time (Fig.3.1C), indicating a further, more gradual decrease in contractility.

These results are broadly consistent with the idea that an essential factor is lost from the heart during the superfusion procedure, the argument being that the rate at which it is lost would be expected to increase with the increasing rates of perfusion, leading to a more rapid onset of the hypodynamic state, as is observed experimentally. However, the differences remaining between the steady-state levels attained, even after several hours perfusion, are not entirely consistent with the hypothesis, since this would not be expected to show any dependence on flow rate. The only explanation which can be advanced at this time to account for this result is the possibility that even after 5 hours the ventricle is still not fully hypodynamic.

Figure 3.2.

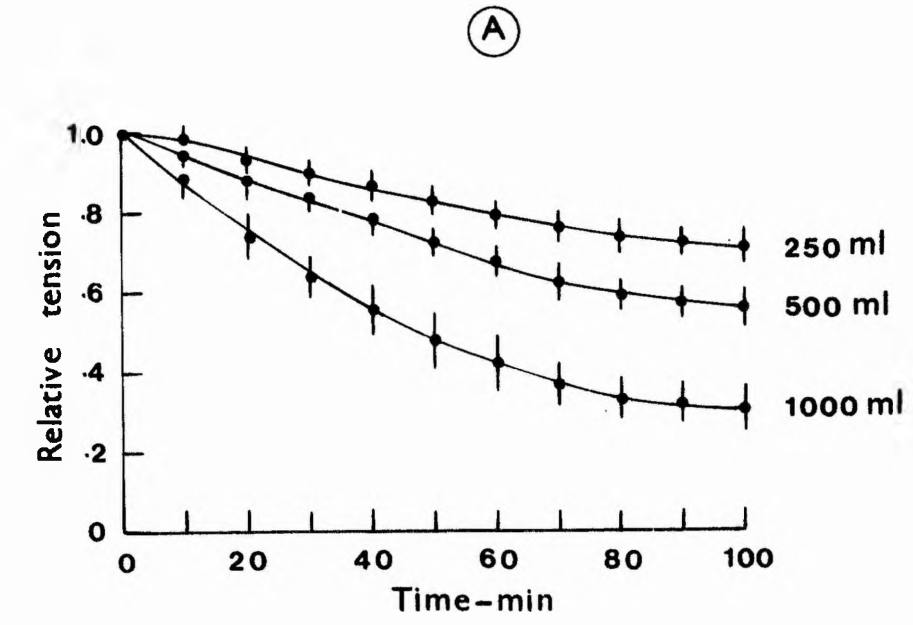
(A) Time course of decline in isometric force following superfusion with 250, 500 and 1000 ml. of circulating perfusates. Abscissa; Time (min). Ordinate; Relative tension.

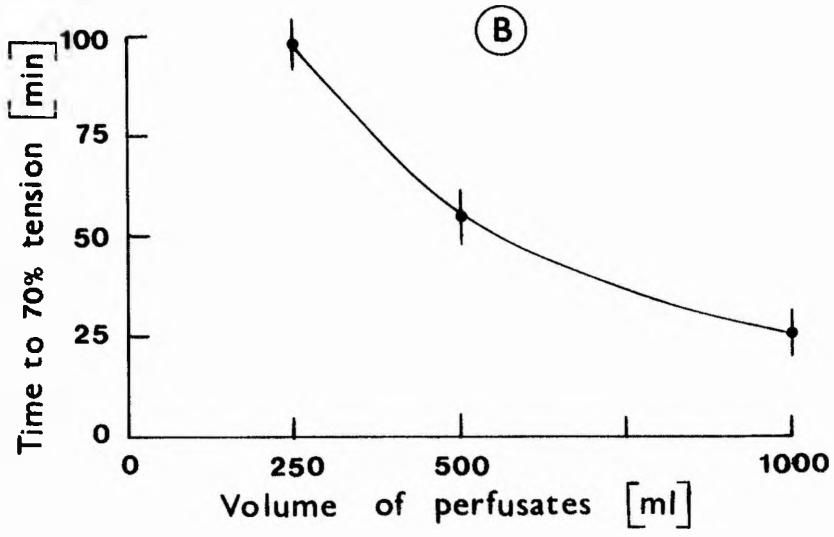
(B) Rate of development of the hypodynamic condition at the three perfusate volumes. Abscissa; Volume of perfusates. Ordinate; Time to 70% fall in isometric tension (min).

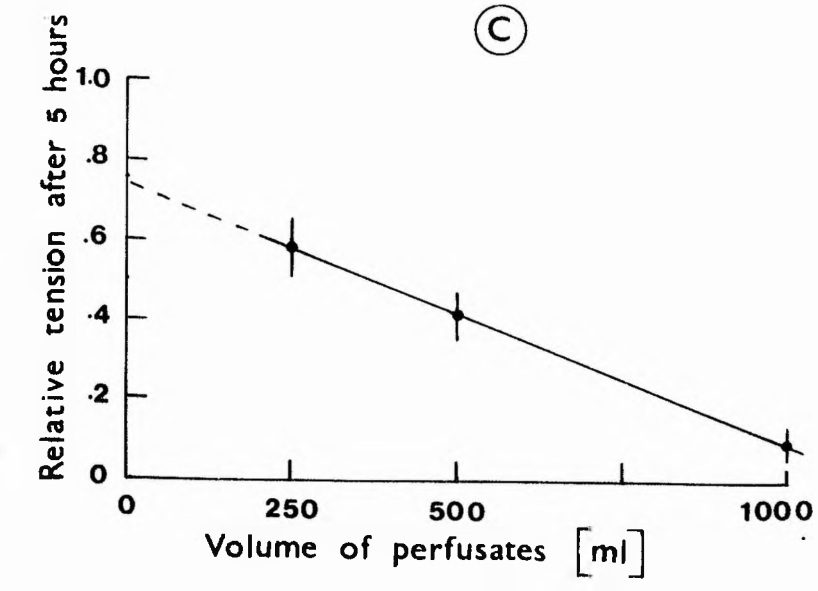
(C) The extent of the hypodynamic depression after 5 hours of perfusion. The data show a linear relationship between the steady-state tension and the volume of perfusates. The curve (dashed line) is extrapolated intercepting the ordinate at a value approximately 75% of the control level. Abscissa; Volume of perfusates. Ordinate; Relative tension after 5 hours.

Each point is the mean $\pm$ S.E. taken from 6 preparations. Tension is expressed as a multiple of the initial (control) level. Stimulation frequency and flow rate were kept constant at 30 min^{-1} and 100 ml. min^{-1}, respectively.

Stimulation parameters: Pulse width, 5 msec; amplitude, 10V; Temp, 18.5^{o}C. R. pipiens.


A
1.0
.8
.6
.4
.2
0
Relative tension
250 ml
500 ml
1000 ml
0
20
40
60
80
100
Time-min


B
100
75
50
25
0
Time to 70% tension [min]
0
250
500
1000
Volume of perfusates [ml]


C
1.0
.8
.6
.4
.2
0
Relative tension after 5 hours
0
250
500
1000
Volume of perfusates [ml]

Volume of circulating perfusates: In these experiments, stimulation frequency and perfusion rates were kept constant (30 beats min^{-1} and 100 ml min^{-1}, respectively) and the time course of the decline in contractile force was measured using circulating volumes of 250 ml, 500 ml and 1000 ml.

The results for 6 experiments (means $\pm$ S.E.'s) are shown in Fig. 3.2A. In these experiments, both the rate of development of the hypodynamic condition (Fig. 3.2B) and the extent (Fig. 3.2C) of the depression (after 5 hours of perfusion) increase with increasing volumes of perfusates. The data in Fig. 3.2C show a linear relationship between the steady-state tension and the volume of the perfusates, the extrapolated portion of the curve (dashed line) intercepting the ordinate at a value ca. 75% of the control level.

These results too lend weight to Clark's hypothesis. In this case, the argument is that the factor lost from the heart becomes more diluted when larger volumes of perfusates are used. The relationship between steady-state contractile force and perfusate volume shown in fig. 3.2C is particularly interesting; the relatively high values indicated by the broken line suggest that the condition of the heart may be substantially improved by perfusing the preparation with very small circulating volumes of fluid, but that even under these conditions some reduction ca. 25% in contractile force remains.

Figure 3.3.

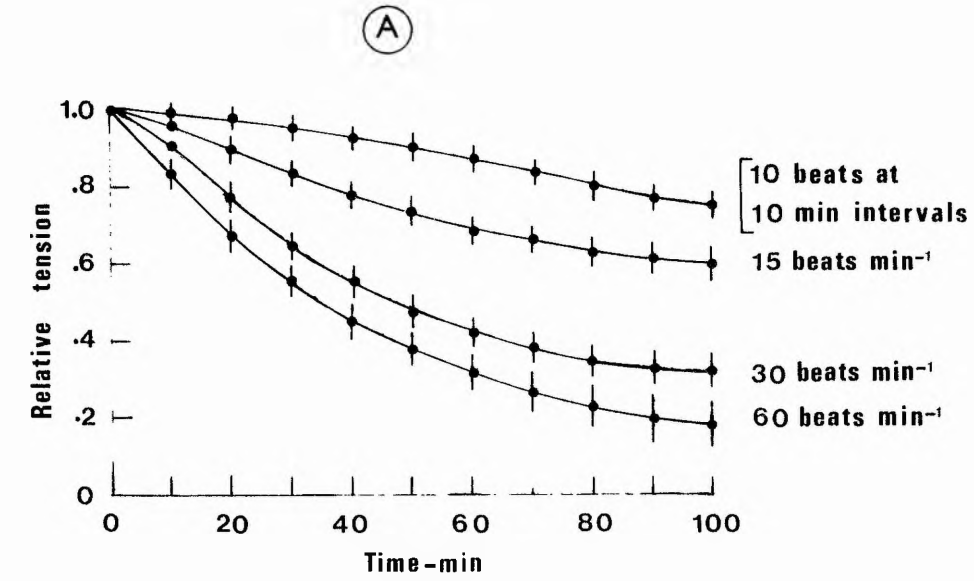
(A) Time course of the development of the hypodynamic state at varying rates of stimulation (from effectively '0' (10 beats at 10 min interval) through 15, 30 and 60 beats min^{-1}). Abscissa; Time (min). Ordinate; Relative tension.

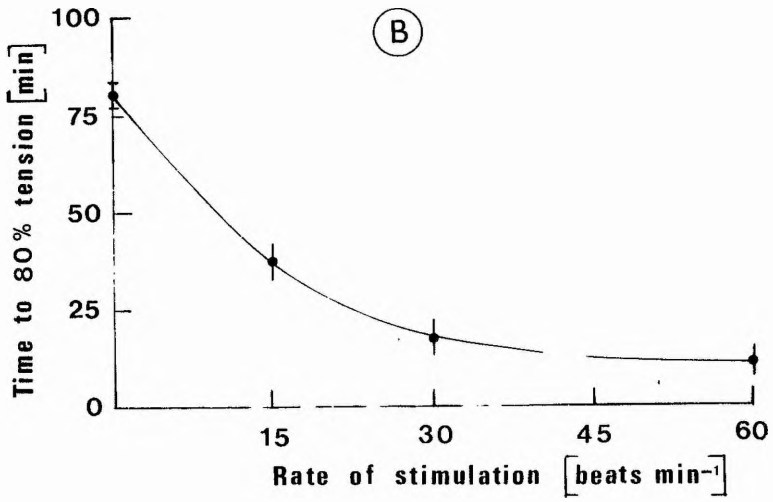
(B) Rate of development of the hypodynamic depression at 4 different rates of stimulation. Abscissa; Rate of stimulation (beats min^{-1}). Ordinate; Time to 80% of the initial tension value (min).

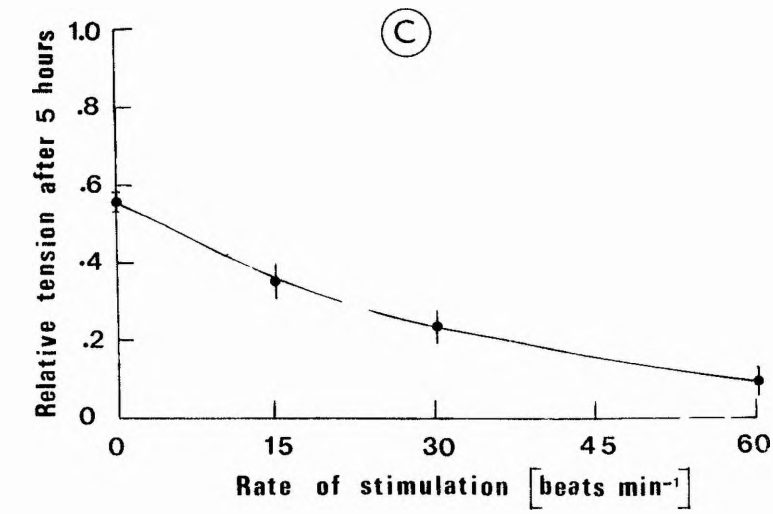
(C) Relation between the steady-state tension reached after 5 hours of perfusion at the 4 different rates of stimulation. Contractility reduced to about 55 - 60% of the control level at very low frequency (approximately to zero). Abscissa; Rate of stimulation (beats min^{-1}). Ordinate; Relative tension after 5 hours.

Each point is the mean $\pm$ S.E. taken from 6 preparations. Tension is expressed as a multiple of the initial (control) level. Flow rates and circulating perfusates were kept constant at 100 ml. min^{-1} and 1 litre respectively.

Stimulation parameters; Pulse width, 5 msec; amplitude, 10V. Temp, 18.5°C. R. pipiens.


A
1.0
.8
.6
.4
.2
0
Relative tension
0
20
40
60
80
100
Time-min
10 beats at
10 min intervals
15 beats min⁻¹
30 beats min⁻¹
60 beats min⁻¹


B
100
75
50
25
0
Time to 80% tension [min]
15
30
45
60
Rate of stimulation [beats min⁻¹]


C
1.0
.8
.6
.4
.2
0
Relative tension after 5 hours
0
15
30
45
60
Rate of stimulation [beats min⁻¹]

Stimulation frequency: The effects of varying the frequency of stimulation (from effectively '0' through 15, 30 and 60 min^{-1}) have been studied at a perfusion rate 100 ml min^{-1} and with total circulating volume of 1 litre.

The results in Fig. 3.3A clearly show that increasing the stimulation frequency leads to a more rapid onset of the hypodynamic condition, and that it also depresses the steady-state amplitude finally attained. These data also reveal stimulation frequency - dependent and independent components in the mechanism of the hypodynamic effect. Fig. 3.3C shows that the contractility of the superfused ventricle is substantially reduced (down to 55 - 60% of the control level) even in the absence of any stimulation (i.e. stimulation at a frequency of 10 beats delivered every 10 minutes in order to assess the potential for force development). Since considerable shortening of the preparation could be discerned by eye (i.e. the tension recordings were only nominally isometric) this result may indicate work - dependent and work - independent components in the development of the hypodynamic state. This is an area of considerable interest which deserves further study.

Is Clark's lipid factor a prostaglandin or prostaglandin - related substance?

The results presented above support Clark's hypothesis

that the hypodynamic depression results from the loss of an essential factor/s from the heart. His conclusion that this is a lipid substance raises the interesting possibility that a prostaglandin (PG) or prostaglandin - like substance (PLS) may be involved. The experiments described below were made in order to test this hypothesis.

Indirect evidence for the release of a PG or PLS from the ventricles: Effects of agents which interfere with PG biosynthesis: The most prevalent precursor in PG biosynthesis is arachidonic acid, giving rise to PGE_2 and $PGF_{2\alpha}$ (Samuelsson, 1972). Prostaglandins A, B and C arise from PGE by dehydration and isomerization. The synthesis is achieved by a complex of microsomal enzymes which are collectively termed 'prostaglandin synthetases'. The conversion of arachidonic acid to PGE_2 or $PGF_{2\alpha}$ is inhibited by certain anti-inflammatory agents, such as aspirin and indomethacin (Vane, 1971). In these experiments, the effects of indomethacin and arachidonic acid on the rate of development and the extent of the hypodynamic depression were investigated.

Fig. 3.4A shows the time course of the decline in contractility for 7 preparations (control, solid squares). The effects of varying concentrations of arachidonic acid (open circles) and indomethacin (solid circles) are shown for comparison. Indomethacin produces a dose - dependent increase in the rate at which the perfused ventricle becomes

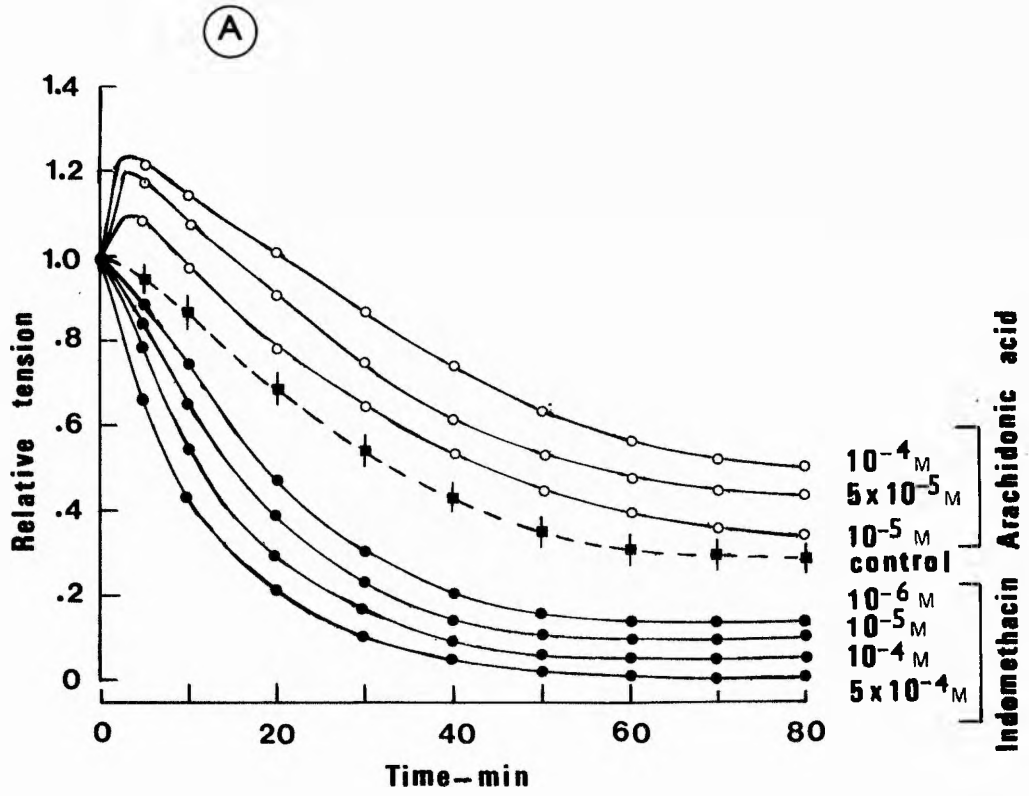
Figure 3.4.

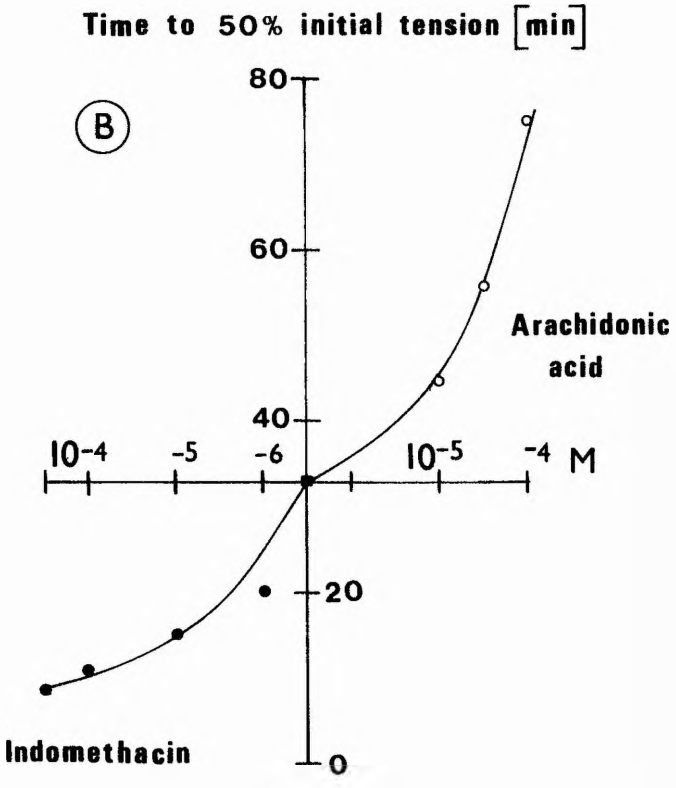
(A) Influence of varying concentrations (range 10^{-4} to 10^{-5}M) of arachidonic acid (open circles) and (range 10^{-4} - 10^{-6}M) indomethacin (solid circles) on the time course of the development of the hypodynamic state. The control response (means $\pm$ S.E's for 7 preparations) is illustrated by the broken line (solid squares) for comparison. Arachidonic acid elevates the final steady-state tension reached while indomethacin depresses it. Abscissa; Time (min). Ordinate, Relative tension (expressed as a multiple of the control level).

(B) The effects of varying concentrations (10^{-4} - 10^{-5}M) of arachidonic acid (open circles) and (10^{-4} - 10^{-6}M) indomethacin (solid circles) on the rate of development of the hypodynamic condition. The control is shown by the solid square where the two axes intersect. Indomethacin accelerates the rate at which the perfused ventricle becomes hypodynamic whereas exogenous arachidonic acid delays it.

The data for each curve, following stimulation with either arachidonic acid or indomethacin, were obtained from one preparation.

Stimulation parameters: Pulse width, 5 msec; amplitude, 10V. Frequency, 30 min^{-1}. Flow rate, 100 ml. min^{-1}, Temp, 18.5^{o}C.


A
1.4
1.2
1.0
.8
.6
.4
.2
0
Relative tension
0
20
40
60
80
Time–min
10⁻⁴ M
5x10⁻⁵ M
10⁻⁵ M
control
10⁻⁶ M
10⁻⁵ M
10⁻⁴ M
5x10⁻⁴ M
Arachidonic acid
Indomethacin


Time to 50% initial tension [min]
B
80
60
40
20
0
10⁻⁴
-5
-6
10⁻⁵
-4 M
Arachidonic acid
Indomethacin

hypodynamic, and also it depresses the steady-state levels finally attained, whereas increasing concentrations of exogenous arachidonic acid have the reverse effects. The data in Fig. 3.4B emphasise the effects of these agents on the time course of the reponses.

The results clearly show that inhibition of prostaglandin biosynthesis with indomethacin greatly accelerates the rate at which the ventricle becomes hypodynamic, while the converse effect is seen when a supply of exogenous PG precursor, in the form of arachidonic acid is provided.

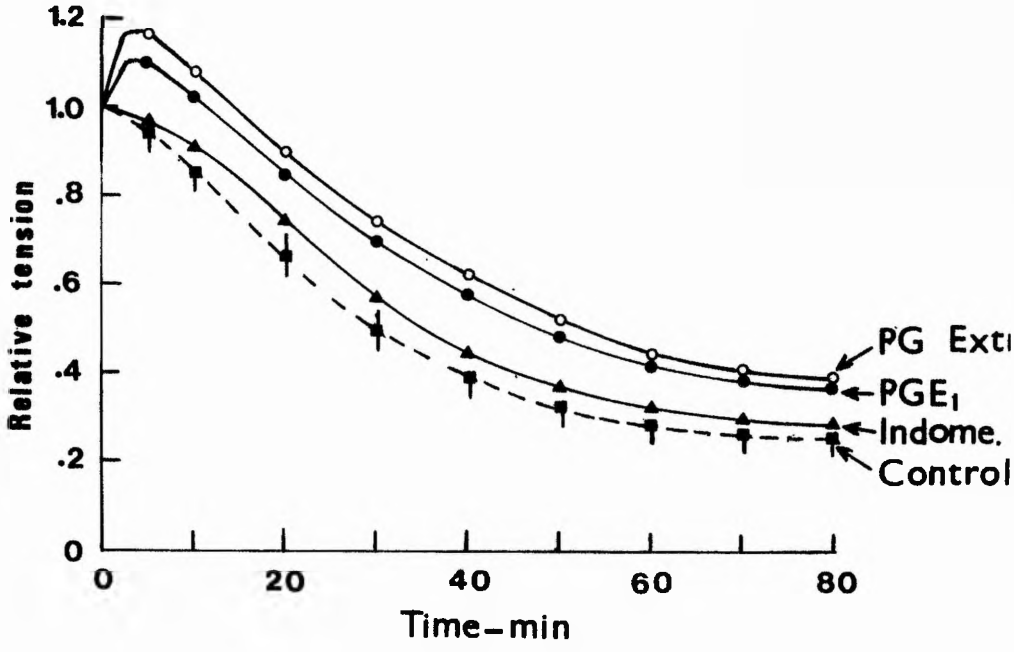
Effects of authentic PGE_1 and PLS extracted from perfusates on the development of the hypodynamic state: Two kinds of experiments are described. In the first, 6 preparations were superfused with frog Ringer solution (50 ml) while the contractile force fell to a steady level (around 80 minutes). The perfusates were then pooled and treated with ethyl acetate to extract lipid soluble substances, including prostaglandins. The quantity of PLS in the perfusate was then bioassayed using the rat stomach strip (see appendix IID for detail). The effect of the extract on the development of the hypodynamic state was then assessed on a fresh half ventricle, using the other half as a control. In a second series of experiments, 6 ventricles were similarly superfused for period in excess of 80 minutes, but this time in frog Ringer solution containing 10^{-5}M indomethacin.

Figure 3.5.

Effects of 25 ng.ml^{-1} (estimated by bioassay) PG ethyl acetate extract (open circles), 25 ng.ml^{-1} authentic PGE_1, (solid circles) and 3 ng.ml^{-1} (estimated by bioassay) 'indomethacin treated' PG ethyl acetate extract (solid triangles) on the time course of the decline in isometric force. The control response (means $\pm$ S.E's for 3 preparations) is depicted by the broken line (solid squares). PG ethyl acetate was obtained from circulating perfusates of 6 preparations that were made hypodynamic. The 'indomethacin treated' PG ethyl acetate extract was obtained from perfusates of 6 preparations made hypodynamic following treatment with 10^{-5}M indomethacin (see appendix II D for PG extraction and bioassay procedures). Abscissa; Time (min). Ordinate; Relative tension (expressed as multiple of the initial level).

The data for each curve were taken from one experiment following stimulation with either authentic PGE_1, or the unknowns.

Stimulation parameters: Pulse width, 5msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate, 100 ml.min^{-1}. Temp, 18.5^{o}C.


1.2
1.0
.8
.6
.4
.2
0
Relative tension
0
20
40
60
80
Time–min
PG Ext
PGE1
Indome.
Control

The pooled perfusates were extracted with ethyl acetate, bioassayed for PLS using the rat stomach strip, and the effect of the extract on the development of the hypodynamic state tested in a fresh preparation. For comparison, the effects of perfusing ventricles with authentic PGE_1 (25 ng ml^{-1}) were tested.

Fig. 3.5 shows the results of these experiments. The pooled extracts from the perfusate of normally - superfused preparations were made up to a prostaglandin concentration (calculated from the bioassay) of 25ng ml^{-1}. The effect of this on a fresh ventricle is shown by the open circles. The PG extract significantly delayed the development of the hypodynamic condition and, after 80 minutes, elevated the final steady-state tension attained as compared to the contral response (solid squares). The same concentration of authentic PGE_1 had a similar effect. In contrast, the indomethacin - treated ventricles yielded an ethyl acetate extract with a greatly reduced PG content, estimated after comparable dilution to be only 3 ng ml^{-1}. Its effects on the rate of development of the hypodynamic condition and the final steady-state level reached were accordingly much less (solid triangles).

<u>Relationship between the amount of PLS released and the extent of the hypodynamic depression:</u> In 8 experiments the ventricles were permitted to become partially or fully

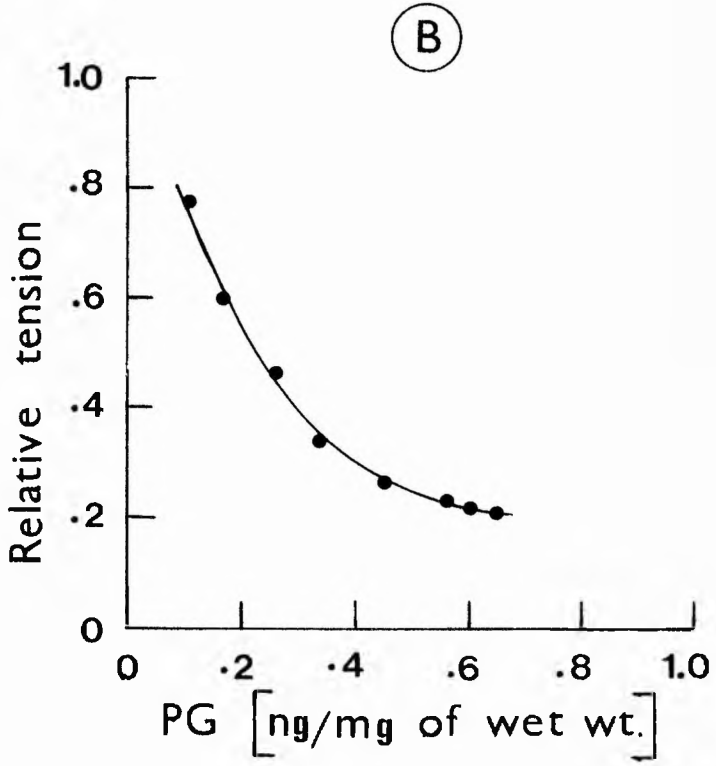
Figure 3.6.

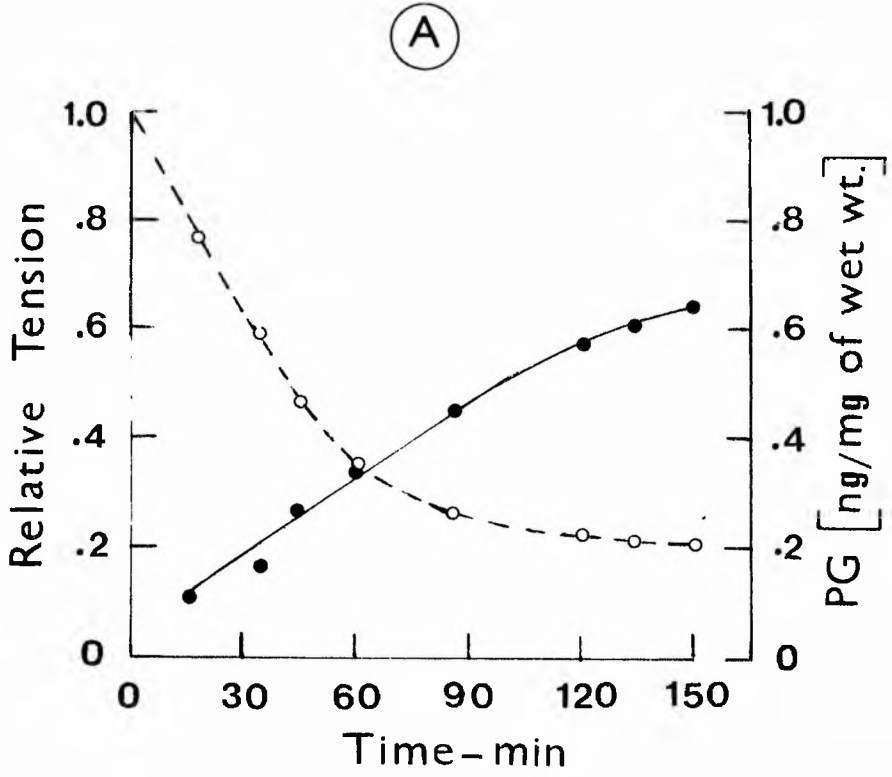
(A) The time course of the decline in contractile force (broken line, open circles) and the amounts of PG's and/or PLS (solid circles) released into the circulating perfusates for 8 preparations at various times during the development of the hypodynamic state. PG's and/or PLS in the perfusates were extracted with ethyl acetate and estimated by assaying on rat fundus strip using authentic PGE_1, as the bioassay control (see appendix II D for details). Abscissa; Time (min). Ordinate; Relative tension (expressed as a multiple of the initial control level); PG output (expressed as $ng.mg^{-1}$ wet ventricle).

(B) Relationship between the quantity of PG's and/or PLS released and the extent of the hypodynamic depression. PG's and/or PLS increase as the preparation becomes correspondingly more hypodynamic. Abscissa; PG release ($ng.ml^{-1}$ wet weight of ventricle). Ordinate; Relative tension.

Each point was taken from one preparation.

Stimulation parameters: Pulse width, 5 msec; amplitude, 10V; frequency, 30 min^{-1}. Flow rate, 100 $ml.min^{-1}$. Temp, 18.5^{o}C.


B
1.0
.8
.6
.4
.2
0
Relative tension
0
.2
.4
.6
.8
1.0
PG [ng/mg of wet wt.]


A
1.0
.8
.6
.4
.2
0
Relative Tension
PG [ng/mg of wet wt.]
0
30
60
90
120
150
Time-min

hypodynamic by superfusing with 200 ml of normal frog Ringer for varying lengths of time. The quantity of PLS obtained from the perfusates by ethyl acetate extraction (expressed as ng mg^{-1} wet ventricle) was subsequently bioassayed and the extent of the hypodynamic depression measured from the force records.

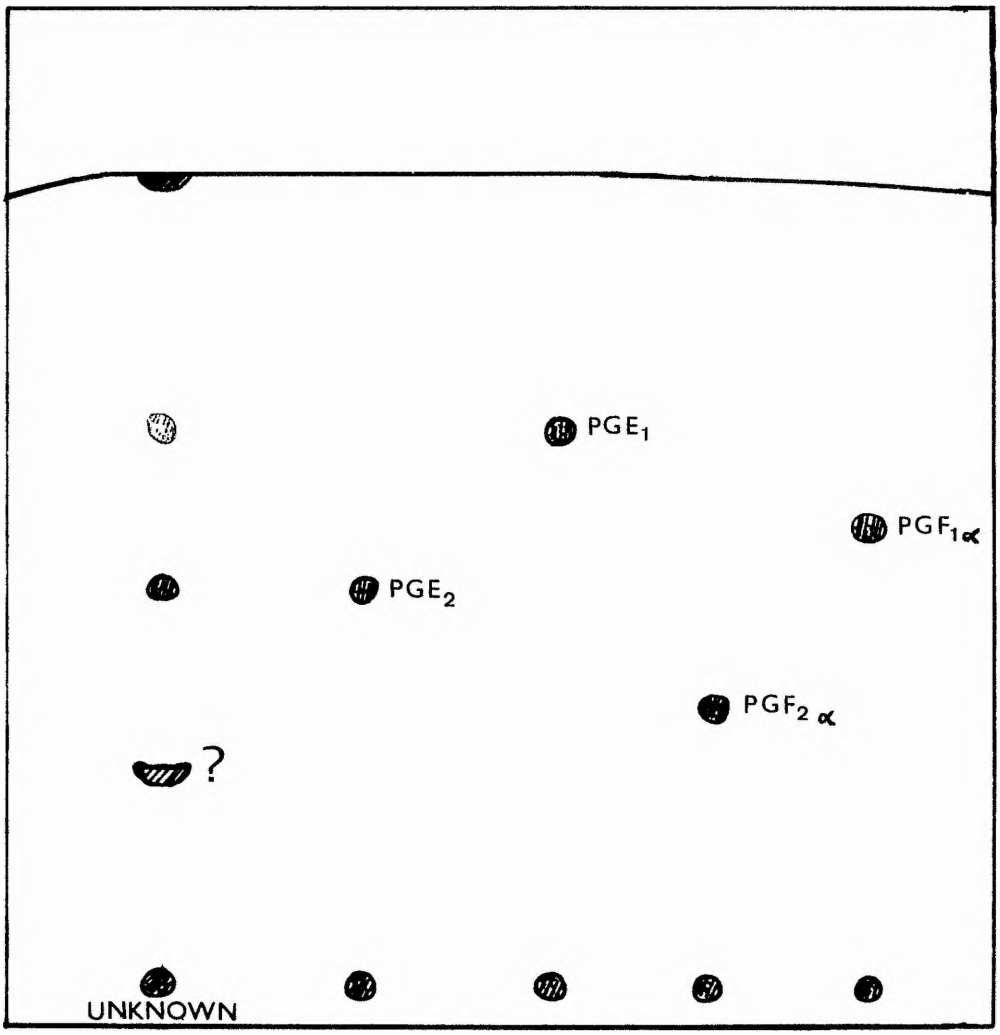
Fig. 3.6A depicts the quantity of PLS extracted (solid circles) and the hypodynamic depression (open circles) as a function of time; the levels of PLS in the extracts are seen to increase with increasing superfusing time, as the ventricle becomes correspondingly more hypodynamic. Fig. 3.6B shows the relationship between these two parameters. Contractility is plotted on the ordinate, force, given as a fraction of the initial (fresh) value, and the levels of PLS (ng mg^{-1} wet ventricle) on the abscissa.

Identification of PG by thin-layer chromatography (TLC):

The results of the preceding experiments provide indirect evidence for the loss of a PG or PLS from the ventricle during the development of the hypodynamic state. An attempt was made to identify which, if any, of the prostaglandins is present in the perfusate using TLC. Authentic samples of prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, A_1 and A_2 were run concurrently with samples of the extracted perfusates. The AI solvent system of Green and Samuelsson (1964) was used in one series of TLC's. In this system PG in the

Figure 3.7.

Chromatogram of authentic prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ and the unknown sample using the AII solvent system (Green & Samuelsson, 1964). Glass plates (0.40 x 20 x 20 cm) were coated with silica gel G(Merk) containing) 10% silver nitrate (see appendix II D for detail). Authentic prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ and the extracted prostaglandin-like material were applied on plates as single spots using micropipettes. Prostaglandins in the unknown sample have R_f values of 0.52 and 0.72 which correspond to PGE_2 (bright spot) and PGE_1 (faint spot), respectively. Also present on the chromatogram is a conspicuous spot (?) with R_f value 0.28 which does not correspond with authentic prostaglandins E, F or A series.


PGE1
PGF1α
PGE2
PGF2α
?
UNKNOWN

extract has an R_f value which corresponds closely with that of the E series of the authentic prostaglandins. In Green and Samuelsson (1964) AII solvent system, it was found to correspond with PGE_1 and PGE_2 (Fig. 3.7). In the latter, there was also present a conspicuous spot with an R_f value of around 0.28, which did not correspond with any of the known samples. It is possible that this unidentified substance is a PG precursor or metabolite. Further work is required to identify this component of the extract.

DISCUSSION

These experiments have demonstrated clearly that both the rate of development of the hypodynamic condition and the degree to which the steady-state contractile response is depressed are markedly influenced by experimental conditions; increasing the flow rate, the volume of circulating perfusate and stimulation frequency were all found to accelerate the rate of decline in contractility and, in addition, to result in progressively smaller steady-state responses. The results were found to be highly repeatable and this has made it possible to standardise the superfusion procedure in such a way as to provide a stable preparation which serves as a control for assessing the effects, described later, of treating test preparations with the various inotropic agents studied. This is an important point, particularly with the experiments which are concerned with the effects of exogenous ATP and isoprenaline on intracellular cyclic nucleotide levels. In the majority of experiments, the following conditions were used: stimulation frequency, 30 min^{-1}; flow rate, 100 ml min^{-1}; volume of circulatory perfusate, 1 litre.

The results are consistent with Clark's (1913) contention that an essential factor is lost from the fibres during superfusion and this is in some way responsible for the

observed deterioration in the condition of the heart. In this context, the results obtained by varying the rates of perfusion deserve special mention, since it is found that this has a marked effect on the final steady-state contractile force, as well as on the rate of development of the hypodynamic condition. This was an unexpected finding. If it is assumed that a fixed pool of Clark's factor exists within the fibres, then varying perfusion rate will only influence the rate at which the preparation becomes hypodynamic, and not the final condition of the ventricle. It therefore seems probable that this is an incorrect assumption, and that the essential factor is being continually resynthesised. Thus, a balance is struck between its loss from the fibres and the rate at which it is replenished, so that low flow rates favour its retention in the fibres, whereas higher flow rates result in a greater loss.

The experiments with indomethacin and arachidonic acid provide circumstantial evidence that the agent which is being lost, which Clark concluded was a lipid, is in reality a prostaglandin (PG) and/or a prostaglandin - like substance (PLS). Indomethacin, a potent inhibitor of prostaglandin synthetase activity (Vane, 1971; Flower & Vane, 1974), potentiates the hypodynamic depression, whereas arachidonic acid, the chief fatty acid precursor in prostaglandin biosynthesis (Samuelsson, 1972), delayed its onset and

elevated the final steady-state tension attained. Indomethacin has been shown to depress myocardial prostaglandin levels and to reduce the amounts released from the heart by several authors (Chanh, Junstad & Wennmalm, 1974; Block et al, 1975) and Gudbjarnason and Hallgrimsson (1974) have reported a beneficial effect of arachidonic acid in the response of the heart to overstimulation. These latter authors also reported that modification of the fatty acid composition of the cardiac membrane phospholipids greatly influences the development of myocardial necrosis resulting from overstimulation with isoprenaline.

More direct evidence for the involvement of PG or PLS was obtained by bioassaying the perfusates using the rat stomach strip. These experiments reveal a clear relation between PG or PLS concentrations in the perfusates and the degree of hypodynamic depression; long perfusion times produce higher levels of PG or PLS in the perfusates and the contractility of the ventricle becomes more depressed as compared with shorter times. Block et al (1975) also demonstrated that the perfused rabbit heart releases PLS into the perfusates and that the levels increase as the perparation deteriorates. Moreover, Piper and Vane (1971) have suggested that disturbance of the cell membrane is the major factor which induces PG release. Traumatic stimuli, such as mechanical stimulation and the formation of gaseous emboli have also been shown to stimulate PG

release from rabbit heart (Block, Poole & Vane, 1974).

Thin-layer chromatography of extracts of the perfusates provides evidence that the development of the hypodynamic state is accompanied by the release of significant amounts of PGE_1, and PGE_2 together with a PLS of unknown identity (Fig. 3.7). The findings that PGE_1 and PGE_2 are released are particularly interesting, since the E series of prostaglandins is known to exert positive inotropic effects on the heart. In this study, superfusion of fresh ventricles with frog Ringer solution containing PGE_1 (and PGE_2 but data not presented) delayed the development of the hypodynamic condition. Moreover, in other experiments, also not presented here, both PGE_1 and PGE_2 were found to cause temporary reversal of the hypodynamic depression.

In summary, the results of the experiments described in this chapter implicate the loss of PGE_1 and PGE_2 and a related PLS from the heart as possible factors contributing to the observed deterioration in its contractility. Establishing a causal relation is of course difficult; however, the work described in the next chapter shows clearly that the development of the hypodynamic depression is also accompanied by marked changes in the levels of intracellular cyclic 3', 5' - nucleotides, and it is well known that the E series of prostaglandins (Sobel & Robison, 1969; Klein & Levey, 1971b) is involved in the biosynthesis of cyclic AMP in the myocardium.

C H A P T E R IV

INTRACELLULAR CYCLIC 3',5'- NUCLEOTIDE LEVELS AND VENTRICULAR CONTRACTILITY DURING THE DEVELOPMENT OF THE HYPODYNAMIC CONDITION.

INTRODUCTION

Evidence was presented in the preceeding chapter which implicated leakage of PGE_1 and PGE_2 and/or a PLS as a possible factor contributing to the time - dependent depression of contractility seen during superfusion of the isolated half ventricle. The experiments described in this chapter have revealed that changes in levels of intracellular cyclic 3', 5' - nucleotides also occur during the development of the hypodynamic state; the decline in isometric twitch tension is accompanied by a progessive decrease in the levels of cyclic AMP and a concommitant increase in the levels of cyclic GMP. Assays of cyclic 3', 5' - nucleotide levels in the circulating perfusate have shown that some of the changes can be accounted for by leakage of small amounts of cyclic AMP and cyclic GMP from the ventricle, but that the major changes must occur as a result of alterations in the metabolism of cyclic AMP and cyclic GMP. Evidence for a causal relationship between the altered levels of cyclic AMP and cyclic GMP and the observed depression of the contractile response was obtained from experiments in which the time course of the development of the hypodynamic state was studied in the presence of exogenous derivatives of cyclic AMP and cyclic GMP.

The most striking finding is that there is a highly significant correlation between the extent of the hypodynamic depression and changes in the ratio of cyclic AMP/cyclic GMP; thus, for example, a 50% reduction in the force of contraction is invariably associated with a 50% reduction in the cyclic nucleotide ratio. This is a recurrent finding, as will be seen in subsequent chapters, concerned with the effects of two inotropic agents on the contractile performance of the heart (exogenous ATP and isoprenaline), and it is the main theme of this thesis.

Measurements of intracellular cyclic 3', 5' - nucleotide levels during the development of the hypodynamic state.

In these experiments the levels of intracellular cyclic AMP and cyclic GMP were measured at different times during the development of the hypodynamic state. Isolated ventricles were divided into two halves, one of which was 'crush - frozen' immediately (see Methods) and then assayed for cyclic AMP and cyclic GMP and for total protein. The values obtained from these freshly frozen half ventricles provided control levels. The second half of the ventricle was rendered partially or wholly hypodynamic by superfusion with frog Ringer using the standard conditions (stimulation frequency 30 min^{-1}; perfusion rate 100 ml min^{-1}; volume of circulating perfusate 1 litre), after which

it was 'crush - frozen' and cyclic nucleotide levels and total protein estimated. The observed changes in cyclic 3', 5' - nucleotide levels in the test half ventricle was expressed as a multiple of the level obtained in the control half ventricle.

In the second series of experiments, the superfusates were assayed for cyclic AMP and cyclic GMP in order to investigate the possibility of leakage of either or both cyclic nucleotides from the preparation.

Fig. 4.1 shows the decline in contractile force (solid triangles) and the measured levels of cyclic AMP (solid circles) and cyclic GMP (open circles) for 10 half ventricles frozen at different times during the superfusion procedure. Each point represents data from one ventricle, and all values are plotted as multiples of those observed in the freshly frozen (control) half ventricle.

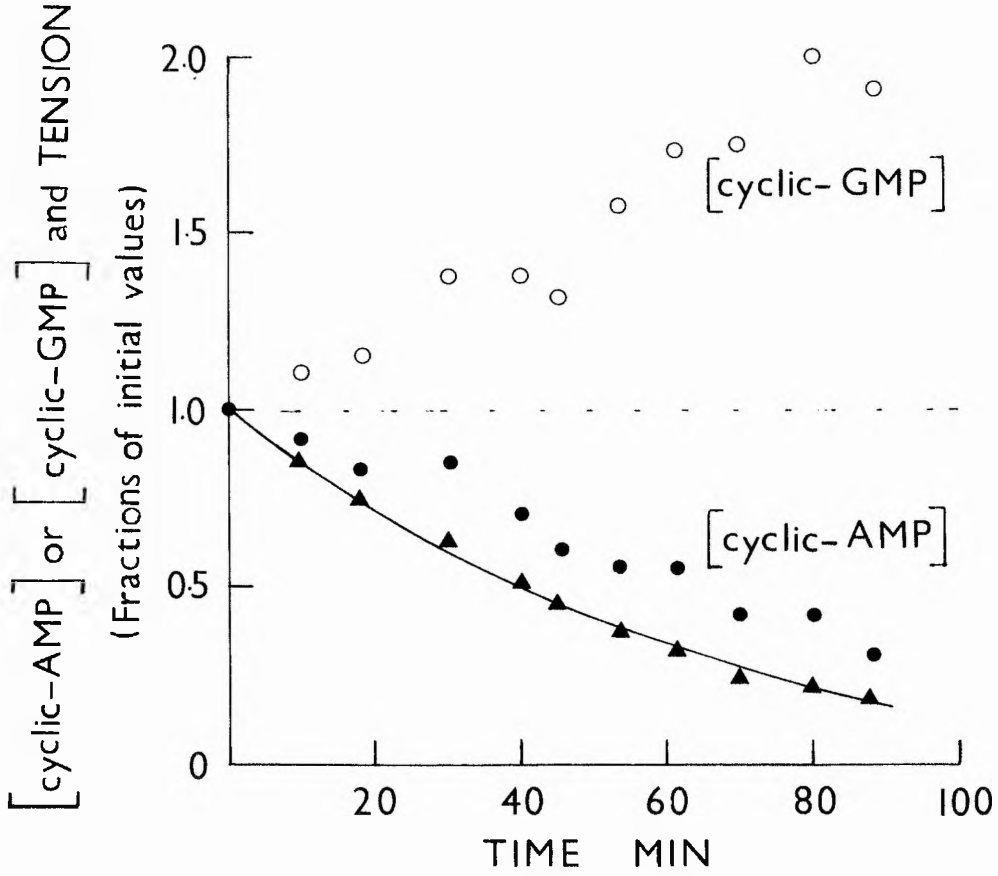
The data show a progressive decline in the level of cyclic AMP and an increase in the level of cyclic GMP accompanying the time dependent depression of isometric twitch tension. After 90 minutes of superfusion the level of cyclic AMP has fallen to around 0.3 times the value obtained in control ventricles, and that of cyclic GMP has increased to around 1.9 times the control level. Mean values for the levels of cyclic AMP and cyclic GMP in freshly frozen ventricles

Figure 4.1.

Time course of the decline in isometric twitch tension (solid triangles) together with observed changes in intracellular cyclic AMP (solid circles) and cyclic GMP (open circles) levels during the development of the hypodynamic state. The control (initial) tension is denoted by the horizontal broken line. Each point represents data from one ventricle. Tension and cyclic nucleotide levels are expressed as multiples of the initial (control) values. The decline in twitch tension is accompanied by a progressive reduction in cyclic AMP and a rise in cyclic GMP. Abscissa; Time (min). Ordinate, Relative tension and cyclic AMP or cyclic GMP levels (fraction of initial values).

Mean values ($\pm$ S.E's) for levels of cyclic AMP and cyclic GMP in freshly frozen ventricles (10 preparations) were found to be 15.80 $\pm$ 0.31 pmol mg^{-1} protein and 0.55 $\pm$ 0.04 pmol mg^{-1} protein respectively. Recovery of known amounts of cyclic AMP and cyclic GMP were 90.80% and 89.72% respectively.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate, 100 $ml.min^{-1}$. Temp. 18.5°C.


[cyclic-AMP] or [cyclic-GMP] and TENSION
(Fractions of initial values)
2·0
1·5
1·0
0·5
0
20
40
60
80
100
TIME MIN
[cyclic-GMP]
[cyclic-AMP]

(10 preparations) were found to be 15.80 $\pm$ 0.31 pmoles mg^{-1} protein and 0.55 $\pm$ 0.04 pmole mg^{-1} protein, respectively. In real terms then, these figures represent substantial changes, ca. 11 pmoles cyclic AMP mg^{-1} protein and 0.5 pmole cyclic GMP mg^{-1} protein.

Cyclic nucleotide levels in the superfusates: Levels of cyclic AMP and cyclic GMP in the superfusate were sampled at different times during the development of the hypodynamic state. Small but significant levels of both were detected. A total of 3.0 pmoles of cyclic AMP mg^{-1} heart protein appeared after 70 minutes of perfusion, and 0.10 pmole of cyclic GMP mg^{-1} heart protein. Control levels of cyclic AMP and cyclic GMP were 20.55 and 0.12 pmoles mg^{-1} protein respectively, and intracellular levels at the end of the 70 minute superfusion period were 7.99 and 0.25 pmoles mg^{-1} protein, respectively. Thus, not more than 15% of the observed reduction in intracellular cyclic AMP can be accounted for by leakage from the fibres. It is probable that this leakage occurs from fibres which are damaged during the dissection procedure.

Relationship between changes in cyclic 3', 5' - nucleotides and contractile force: Fig. 4.2 shows the relationship between the reduction in contractile force and the decrease in cyclic AMP on one hand (A) and the increase in cyclic GMP (B) on the other. Both sets of data yield

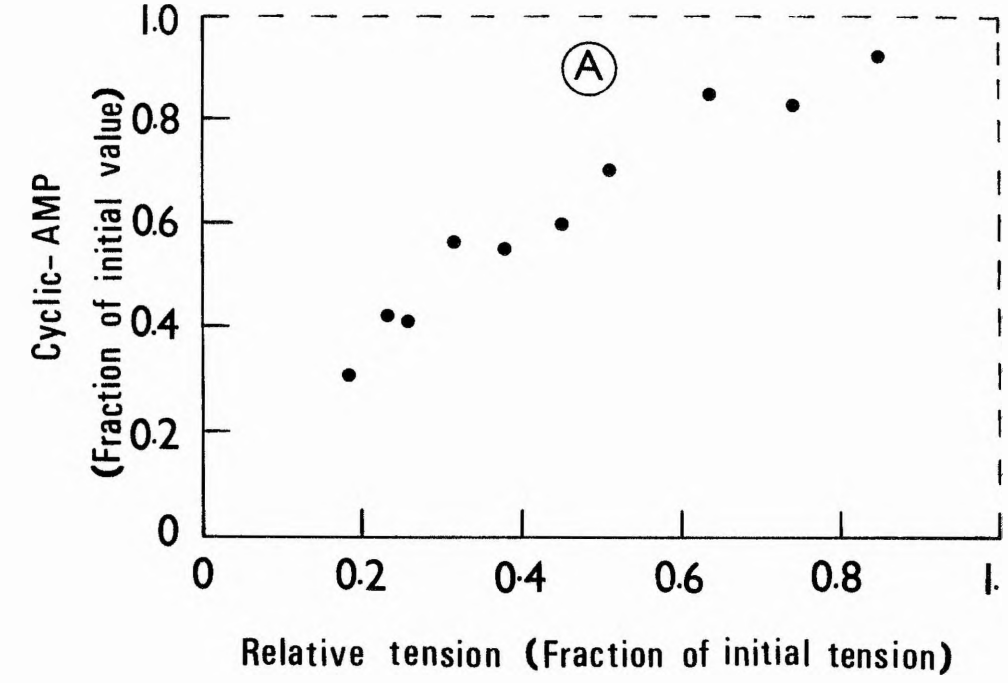
Figure 4.2.

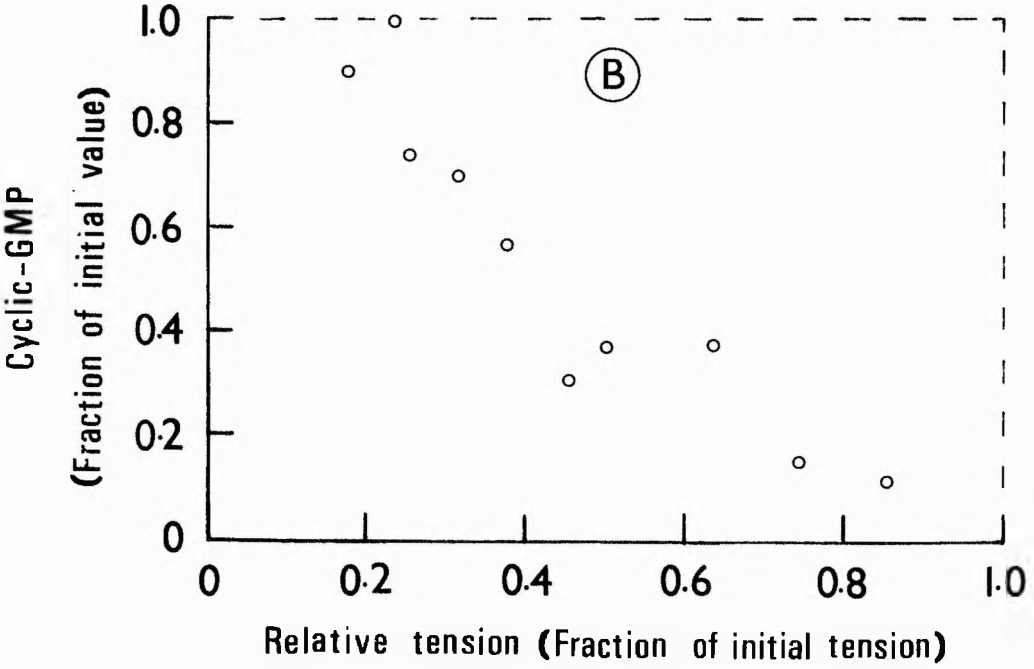
Data taken from Fig.4.1.

(A) Relationship between the decline in isometric force and the reduction in intracellular cyclic AMP levels. Correlation coefficient $\pm$ S.E. of estimate: 0.93 ± 0.14, $n = 10$, $P < 0.001$. Abscissa; Relative tension (fraction of initial tension). Ordinate; Cyclic AMP (fraction of initial value).

(B) Relation between the decrease in contractile force and elevated intracellular cyclic GMP levels. Correlation coefficient $\pm$ S.E. of estimate: -0.84 ± 0.11, $n = 10$, $P < 0.001$. Abscissa; Relative tension (fraction of initial tension). Ordinate; Cyclic GMP (fraction of initial value).

Note that the correlation coefficient is positive in (A) and negative in (B).


A
Cyclic-AMP
(Fraction of initial value)
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.
Relative tension (Fraction of initial tension)


B
Cyclic-GMP
(Fraction of initial value)
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
Relative tension (Fraction of initial tension)

statistically significant correlation. There is a positive correlation between contractile force and intracellular cyclic AMP (correlation coefficient $\pm$ S.E. of estimate : 0.933 ± 0.138, $n = 10$, $P < 0.001$) and a negative correlation between contractility and intracellular cyclic GMP levels (correlation coefficient $\pm$ S.E. of estimate : -0.859 ± 0.11, $n = 10$, $P < 0.001$).

These results raise the interesting possibility that the contractile capacity is regulated by a balance between the two nucleotides : cyclic AMP exerting a force - promoting action, and cyclic GMP opposing it. If this is the case, a graphical plot of the relationship between contractile force and the ratio of cyclic AMP/cyclic GMP might be expected to yield a more precise correlation. Fig. 4.3 shows such a plot. Here the ratio of cyclic AMP/cyclic GMP for the wholly or partially hypodynamic ventricles is expressed as a fraction of cyclic AMP/cyclic GMP of corresponding control half ventricle, thus:

$$\frac{\text{cyclic AMP/cyclic GMP (hypodynamic)}}{\text{cyclic AMP/cyclic GMP (control)}}$$

and plotted against contractile force, again expressed as a fraction of the twitch tension generated by the control preparation:

Figure 4.3.

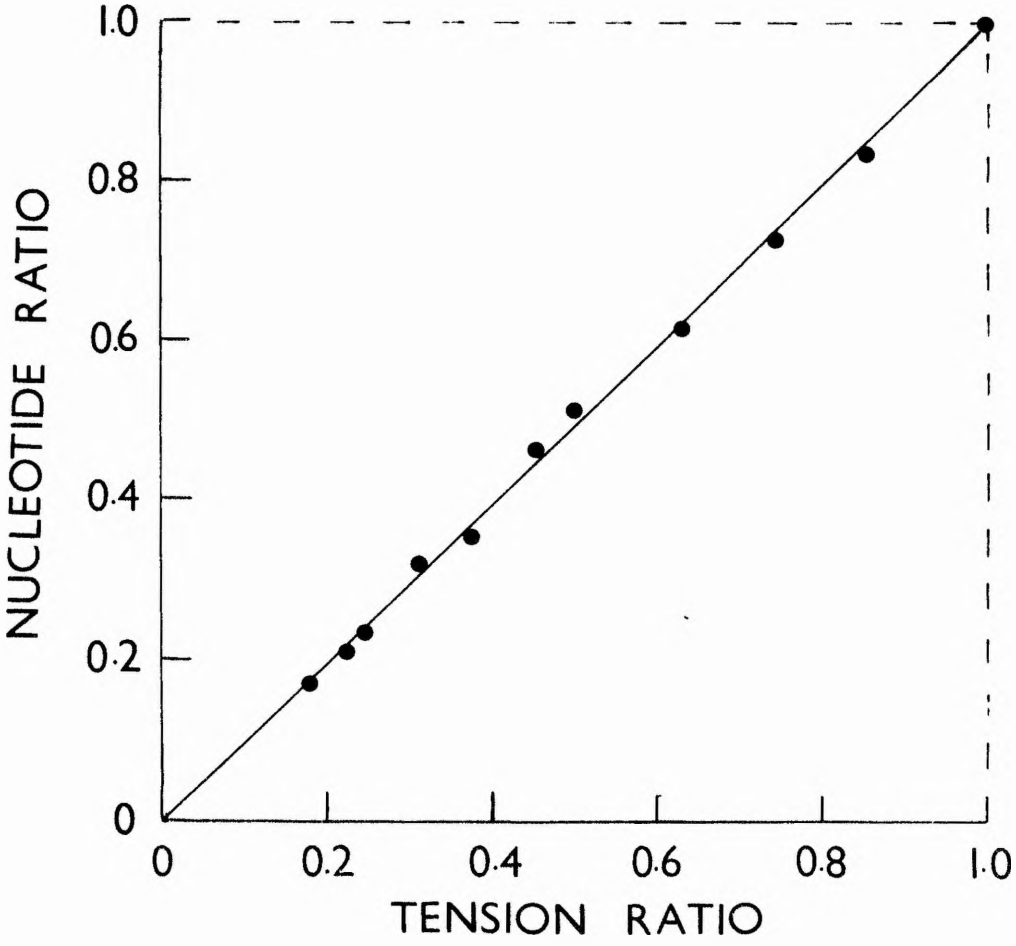
Data from Fig.4.1.

Relation between isometric force and intracellular cyclic 3', 5' - nucleotide ratio. Both parameters are expressed as multiples of the control values. Correlation coefficient $\pm$ S.E. of estimate: 0.98 $\pm$ 0.10, n = 10, $P < 0.001$. The solid line is drawn by eye to emphasise a direct 1:1 proportionality between contractile force and the cyclic nucleotide ratio. The resulting decrease in the ratio [cyclic AMP] / [cyclic GMP] parallels closely the decrease in the contractility. Abscissa; Tension ratio,

$$\left[\frac{P\ (hypo)}{P\ (control)}\right]$$

Ordinate: Nucleotide ratio.

$$\left[\frac{\text{cyclic AMP/cyclic GMP (hypo)}}{\text{cyclic AMP/cyclic GMP (control)}}\right]$$


1.0
0.8
0.6
0.4
0.2
0
NUCLEOTIDE RATIO
0
0.2
0.4
0.6
0.8
1.0
TENSION RATIO

$$\frac{P \text{ (hypodynamic)}}{P \text{ (control)}}$$

This shows clearly that the decrease in ventricular contractility almost exactly parallels the decrease in the cyclic nucleotide ratio. Statistically, the data are highly significant (correlation coefficient $\pm$ S.E. of estimate : 0.983 ± 0.102, $n = 10$, $P < 0.001$).

Is there a causal relation between intracellular cyclic 3', 5' - nucleotide levels and contractile force? The results of the preceding section strongly suggest that a cause and effect relationship exists between the instantaneous levels of the two cyclic nucleotides and the capacity of the ventricle to produce force, and that the observed changes in the ratio of cyclic AMP/cyclic GMP are not simply a byproduct resulting from the diminished contractility.

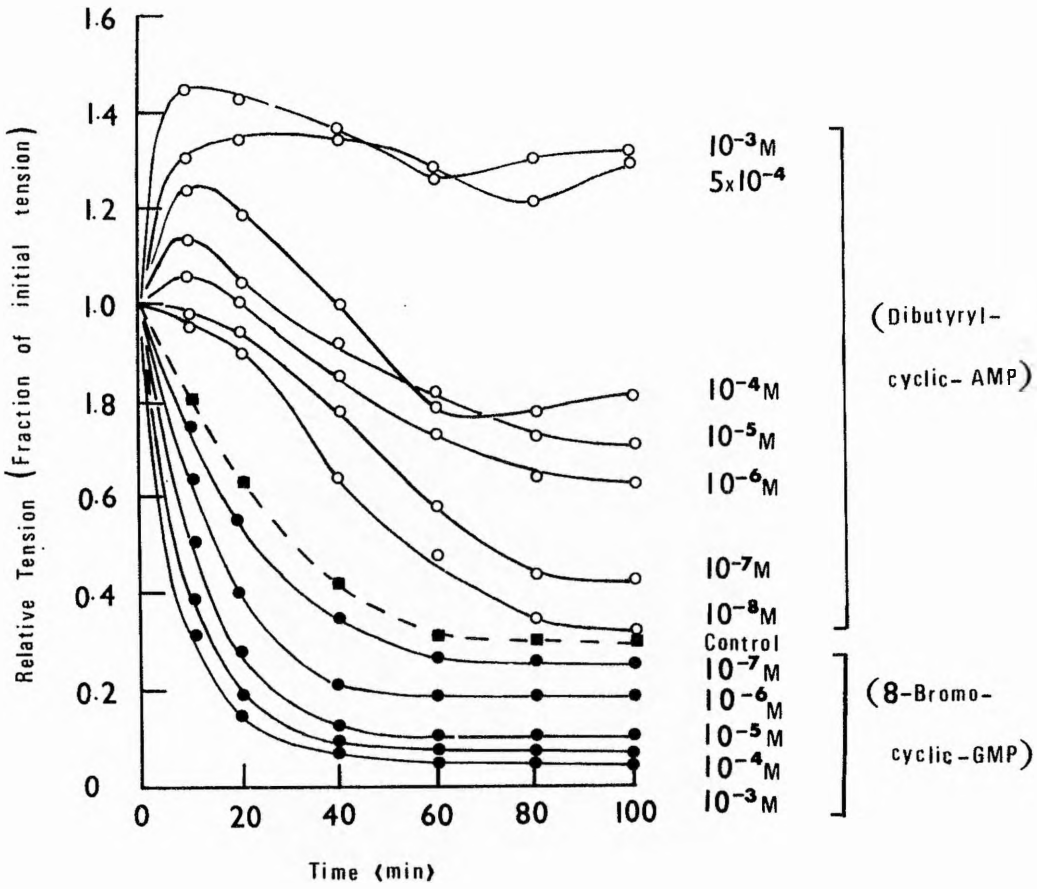
In order to investigate this question further, experiments were made in which isolated ventricles were superfused with Ringer solution containing either exogenous dibutyryl cyclic AMP (DB cyclic AMP) or exogenous 8 - Bromo cyclic GMP (8-Br cyclic GMP) and the effect on the time course of the development of the hypodynamic condition and the extent of the hypodynamic depression were assessed. The aim of making these experiments was to artificially manipu-

Figure 4.4.

Time course responses of the decline in isometric twitch tension during exposure to exogenous (10^{-3} - 10^{-8}M) dibutyryl cyclic AMP (open circles) and (10^{-3} - 10^{-7}M) 8 - Bromo cyclic GMP (solid circles). The control tension response (solid squares) is depicted by the broken line for comparison. Cyclic AMP delays the development of the hypodynamic state, whereas 8 - Bromo cyclic GMP accelerates it. Dibutyryl cyclic AMP ($> 10^{-3}$M) prevents the decline in twitch tension (up to 8 hours). Abscissa; Time (min). Ordinate; Relative tension (expressed as a multiple of the initial (control) value).

The data for each curve, following exposure to either dibutyryl cyclic AMP or 8 - Bromo cyclic GMP, were obtained from 1 preparation.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate: 100 $ml.min^{-1}$. Temp, 18.5^{o}C.


Relative Tension (Fraction of initial tension)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Time (min)
10⁻³M
5x10⁻⁴
10⁻⁴M
10⁻⁵M
10⁻⁶M
10⁻⁷M
10⁻⁸M
Control
10⁻⁷M
10⁻⁶M
10⁻⁵M
10⁻⁴M
10⁻³M
(Dibutyryl-cyclic-AMP)
(8-Bromo-cyclic-GMP)

late the levels of cyclic AMP and cyclic GMP within the fibres in order to alter the cyclic nucleotide ratio.

The results are summarized in Fig. 4.4. The dashed curve shows the time course of the decline in twitch tension for control superfused ventricles. The upper and lower families of curves show the effects of superfusing with DB cyclic AMP and 8-Br cyclic GMP, respectively. It can be seen that superfusion with exogenous DB cyclic AMP produces a dose - dependent reduction in the rate of decline in twitch tension and also leads to elevated steady-state (after 100 minutes) levels, whereas superfusion with 8-Br cyclic GMP has converse effects, causing acceleration of the rate at which the twitch declines and a further reduction in the steady-state level ultimately attained. Dose - response curves for the effects of DB cyclic AMP and 8-Br cyclic GMP on the steady-state depression and on the time course of the responses are shown in Fig. 4.5A & B respectively.

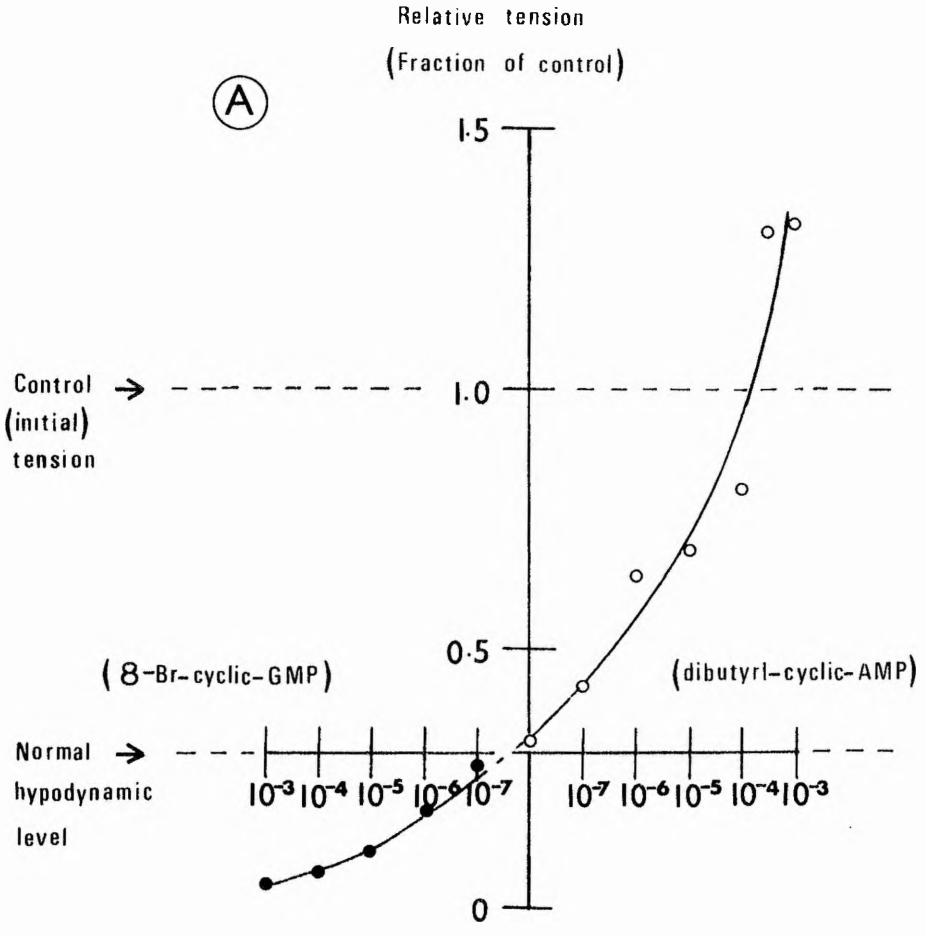
The effects of treating <u>fully hypodynamic</u> preparations with DB cyclic AMP or 8-Br cyclic GMP are presented in a subsequent chapter (VI). Briefly, these show that DB cyclic AMP produces a long - lasting potentiation of the twitch, whereas 8-Br cyclic GMP has the reverse effect, producing an even greater depression.

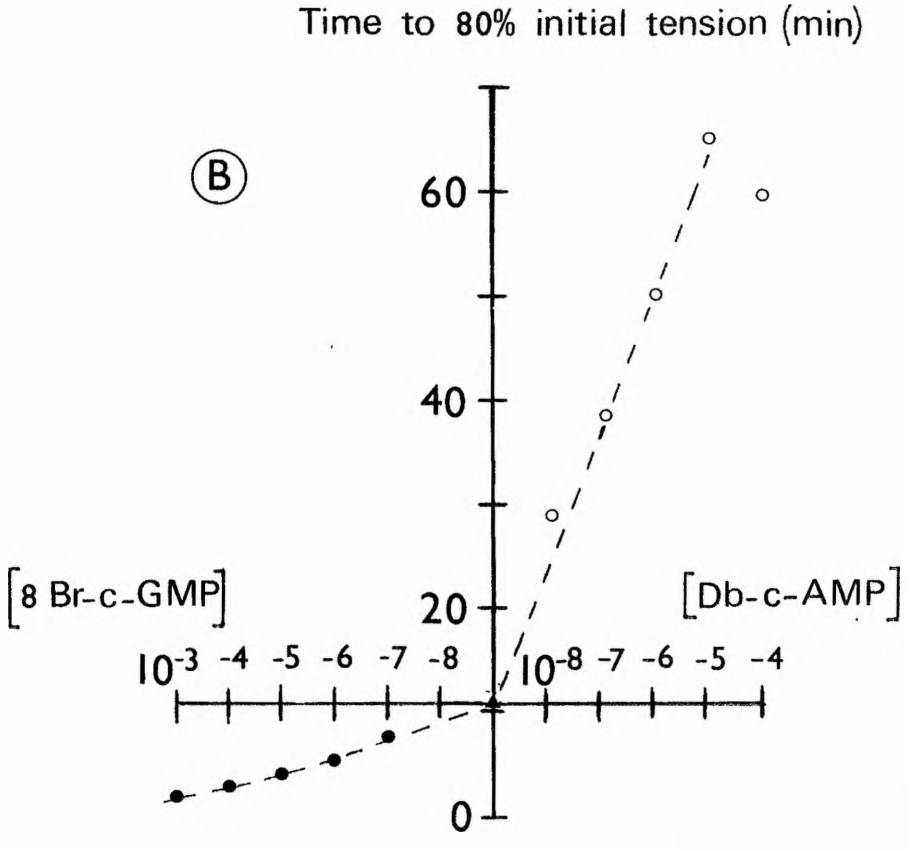
Figure 4.5.

Data taken from Fig.4.4.

(A) Effects of exogenous (10^{-3} - 10^{-8}M) dibutyryl cyclic AMP (solid circles) and (10^{-3} - 10^{-7}M) 8 - Bromo cyclic GMP (solid circles) on the final steady-state tension reached after 100 minutes. The upper broken horizontal line shows the control (initial) tension. The lower horizontal solid line depicts the normal (hypodynamic) tension attained after 100 minutes perfusion with normal frog Ringer solution. Dibutyryl cyclic AMP elevates the final steady-state level, whereas 8 - Bromo cyclic GMP depresses it in a dose-dependent fashion. Abscissa; Concentration (M) dibutyryl cyclic AMP and 8 - Bromo cyclic GMP. Ordinate; Relative tension (fraction of control value).

(B). Influence of exogenous (10^{-4} - 10^{-8}M) dibutyryl cyclic AMP (open circles) and (10^{-3} - 10^{-7}M) 8 - Bromo cyclic GMP (solid circles) on the rate of development of the hypodynamic state. The control is depicted by the (solid triangle) where the two axes intersect. Abscissa; Concentration (M) dibutyryl cyclic GMP and 8 - Bromo cyclic GMP. Ordinate; Time taken for tension to decay to 80% of the control (initial) level (min).


Relative tension
(Fraction of control)
A
1·5
1·0
0·5
0
Control
(initial)
tension
(8-Br-cyclic-GMP)
(dibutyrl-cyclic-AMP)
Normal
hypodynamic
level
10-3 10-4 10-5 10-6 10-7
10-7 10-6 10-5 10-4 10-3


Time to 80% initial tension (min)
B
60
40
20
0
[8 Br-c-GMP]
[Db-c-AMP]
10-3 -4 -5 -6 -7 -8
10-8 -7 -6 -5 -4

These results taken together provide support for the view that a causal relationship exists between the levels of cyclic AMP and cyclic GMP and ventricular contractility, a conclusion which is strengthened by results presented later, concerning the effects of exogenous ATP and isoprenaline on the ventricle.

DISCUSSION

Several factors have been identified as possible causal agents in the mechanism of the hypodynamic depression, including loss of a lipid substance (Clark, 1913; Szent-Gyorgyi, 1953), lowered intracellular ATP levels (Greiner, 1952), reduced calcium influx resulting from the loss of a hypothetical calcium binding site (Chapman & Niedergerke, 1970a, b) and an energetic deficiency which can be compensated by provision of pyruvate (Vassort & Ventura-Clapier, 1977). The present work identifies yet another change in the metabolic status of the ventricle which may well prove to be of significance; namely a reduction in intracellular cyclic AMP levels and the accompanying abnormally-elevated levels of intracellular cyclic GMP.

The close correlation between changes in the contractile state of the superfused preparation and alterations in the ratio of cyclic AMP/cyclic GMP is thought to be especially significant. It suggests that the two agents exert opposing influences on the mechanism of force production, a conclusion which finds considerable support in the literature. Nawrath (1976; 1977) found dose - dependent negative inotropic effects of exogenous 8 - Br cyclic GMP on rat atria and on cat papillary muscle, and increases in the levels of endogenous cyclic GMP are known to accompanying the negative

inotropic effects of acetylcholine (see also George et al, 1970, 1973, 1975; Watanabe & Besch, 1975; England, 1976). Conversely, treatment with exogenous DB cyclic AMP has been shown to produce positive inotropic effects (Kukovetz & Pöch, 1970; Skeleton et al, 1970; Ahren et al, 1971; Drummond & Hemming, 1972; Meinertz et al, 1973; Nawrath, 1976; Tsien, 1977) and many authors (see review by Tsien, 1977 and Introduction) have reported elevated cyclic AMP levels following stimulation of the heart with β- agonists such as adrenaline and isoprenaline.

The probability that the observed relation between altered cyclic nucleotide levels and contractility is a causal one is increased by the results reported in subsequent chapters, which show that this correspondence holds in other, physiologically - different situations. This makes it seem less likely that the observed changes in the levels of intracellular cyclic AMP and cyclic GMP during the development of the hypodynamic state are merely a consequence of impaired contractile performance. Time course experiments, showing potentiation of the hypodynamic condition following superfusion with 8 - Br cyclic GMP and, in contrast, improved contractility following superfusion with DB cyclic AMP, reinforce this view.

It is less clear what <u>initiates</u> the change in cyclic AMP and cyclic GMP levels. Evidence was presented earlier to

show that PGE_1 and PGE_2, together with a PLS, are released into the superfusates, and since PGE_1 and PGE_2 are known to stimulate adenylate cyclase activity (Klein & Levey, 1971b; Levey & Klein, 1973), their loss might result in a lower conversion of ATP to cyclic AMP within the fibres. Certainly, it is clear that relatively little cyclic AMP is actually lost into the perfusates and so the depressed levels must be due either to low adenylate cyclase activity or alternatively to increase cyclic AMP phosphodiesterase activity.

A literature search has failed to yield any information relating to the actions of prostaglandins on phosphodiesterase activity (see Hittelman & Butcher, 1973), nor it seems is there any information concerning the actions of prostaglandins on guanylate cyclase activity. It is difficult, therefore, to see why the levels of cyclic GMP should increase unless the level of cyclic AMP acts in some way to regulate the levels of cyclic GMP. It was pointed out earlier (main Introduction) that several situations have been documented in which the primary effect of a cardioactive substance (such as, for example, acetylcholine and isoprenaline) appears to be on <u>one</u> of the two cyclic 3', 5' - nucleotides, but in which changes in the levels of the <u>other</u> are detectable. Examples of 'phase-shifted' changes in cyclic AMP and cyclic GMP following treatment of hypodynamic ventricles with exogenous ATP and isoprenaline will be referred to later.

In conclusion, the results presented in Chapters III and IV suggest that the loss of PGE_1, and PGE_2 and/or a PLS from the ventricle may be responsible for initiating changes in levels of cyclic AMP and cyclic GMP, which in turn have an effect on force generating mechanism. Further discussion of the nature of this effect is deferred until later.

P A R T II

EFFECTS OF EXOGENOUS ATP AND RELATED COMPOUNDS ON VENTRICULAR CONTRACTILITY AND ON CYCLIC 3', 5'-NUCLEOTIDE METABOLISM.

A HISTORICAL REVIEW OF THE LITERATURE PERTAINING TO THE EFFECTS OF ATP AND RELATED COMPOUNDS ON THE HEART.

I N T R O D U C T I O N

In 1929 Drury and Szent-Gyorgyi found that crude extracts of bullock heart exhibited strong pharmacological effects on the heart and coronary vessels, producing marked changes in cardiac function. Since this time, the action on the heart of a wide range of purine and pyrimidine based nucleosides and their nucleotide derivatives have been investigated. These substances have profound chronotropic and inotropic effects on isolated atria, ventricles and whole hearts, as well as on the cardiovascular performance of intact animals, including Man.

The purpose of this Introduction is to review the literature concerning their effects, which although extensive, has yielded comparatively little information concerning the mode of action of these agents. This is due in part to the fact that different regions of the heart respond in different ways (Drury, 1936). In intact hearts the chronotropic effects exert dominant action, presumably by affecting pacemaker activity, and these to some extent obscure the equally important inotropic actions. The situation is even more confusing when one compares the reported effects of injection into whole animals. Moreover, it is clear that the hearts of different species respond in different

ways and that the nature of the response is dependent upon the physiological condition of the preparation.

Actions of ATP and related compounds on amphibian hearts: Lindner and Rigler (1931) demonstrated that the anoxic heart responds to ATP in low concentrations (10^{-7}M), showing an immediate but transient increase in the force of contraction, followed by a slower and longer lasting rise. Parnas and Ostern (1932) confirmed that ATP produces an immediate positive inotropic effect in perfused hypodynamic hearts, but reported that this was followed by heart block. Gillespie (1934) demonstrated only the blocking action of ATP. Later studies confirmed that ATP produces a characteristic triphasic inotropic effect on hearts depressed with quinine (Lichtneckert & Straub, 1949), potassium (Loewi, 1949) or calcium deficiency (Marshall & Andrus,1953) and on perfused hypodynamic hearts (Pettko & Straub, 1948; Kanda, Sekiya & Inoue, 1954). Kanda et all (1954) were able to demonstrate a triphasic inotropic effect of ATP and heart block. In higher concentrations, they reported that both ATP and adenosine 5'-diphosphate (ADP) produced an immediate increase in contractile force, followed by a short lasting negative inotropic effect and complete heart block; after which the force of contraction gradually increased and remained elevated for a further 100 min.

These authors also made the significant observation that the effects of ATP are not blocked by atropine, a result which has been confirmed by subsequent investigators (Schenberg, 1956; Versprille, 1966).

The actions of ADP are qualitatively similar to those of ATP, although there are quantitative differences: the inotropic effects are generally smaller (Pettko & Straub, 1949; Marshall & Andrus, 1953) and even in high concentrations, it fails to produce heart block (Schenberg, 1956). There is some disagreement concerning the effects of AMP. Loewi (1949) reported a positive inotropic effect, but this was not confirmed in subsequent studies of Kanda et al (1954). Adenosine is reported to have no effect on the frog heart (Kanda et al, 1954; Boyd & Forrester, 1968).

In a more recent series of papers Versprille (1963a, b; 1964) has documented the effects of other related nucleosides and their phosphates on the intact frog heart and on the isolated frog ventricle. An inotropic effect is obtained with cytidine -5′ - triphosphate (CTP), guanosine -5′ - triphosphate (GTP), inosine -5′ - triphosphate (ITP) and uridine -5′ - triphosphate (UTP), although the magnitude of the response and its shape differ with different nucleotides. Boyd & Forrester (1968) reported that low concentrations of ATP, ADP and UTP produced marked inotropic

responses, whereas ITP, CTP and GTP only acted in much high concentrations and produced qualitatively different responses. Finally, Versprille (1965) showed that ATP (10^{-3}M) has a negative chronotropic effect on the intact heart but a positive chronotropic action on the isolated ventricle. The negative chronotropic effect is not blocked by atropine (Versprille, 1966). These observations together account for some of the confusion regarding the mode of action of ATP, a point which is emphasised by observations on mammalian hearts (see below).

ATP has a marked effect on the cardiac action potential. It restores electrical excitability to frog ventricles previously paralysed with high potassium solution (Kotowski, Antoni & Fleckenstein, 1959) or in a calcium deficient medium (Antoni, Englstfeld & Fleckenstein, 1960). It has a dose-dependent positive inotropic effect on frog atria and this is accompanied by an increase in the overshoot and duration of the action potential (Goto, Yatani & Tsuda, 1976, 1977; Yatani, Goto & Tsuda, 1978). Moreover, at higher concentrations, the transient negative inotropic effect of ATP is associated with a decrease in the overshoot and a shortening of the duration of the action potential. Voltage-clamp experiments by these authors have demonstrated an increase in the slow inward calcium current in response of ATP.

Effects of ATP and related compounds on mammalian myocardium: Drury, (1936) emphasised that the principal sites of action on the heart of the various adenyl compounds may differ in different species. His conclusion has been reinforced by more recent work. In general, the chronotropic and inotropic effects on the atria are negative, but predominantly positive on the ventricle.

Mammalian atria: Drury and Szent-Gyorgyi (1929) showed that adenosine and adenylic acid exert a negative inotropic effect on dog atria. This was confirmed later by Drury (1932) and later by Wedd and Fenn (1933) who used isolated atria from dogs, guinea-pigs and rabbits. Drury and Szent-Gyorgyi (1929) also demonstrated that these actions on the atria were not blocked by atropine. More recent studies have confirmed that ATP, ADP, AMP and adenosine depress the contractility of dog (Emmelin & Feldberg, 1948), cat (Green & Stoner, 1950; Acierno, Burno, Burstein & DiPalma, 1952; Bertelli, Bianchi & Beani, 1972), rabbit (Bielschowsky, Green & Stoner, 1944; Emmelin & Feldberg, 1948; Marchetti, Merlo, Noseda & Ferrini, 1968; Bertelli et al, 1972) and rat (Hollander & Webb, 1957; Bertelli et al, 1972; Meinertz, Nawrath & Scholz, 1973) atria. Treatment of rabbit atria with atropine (Bielschowsky et al, 1944) does not prevent the action of ATP. A marked

but transient negative chronotropic effect of adenosine and adenine nucleotides on canine sinus node, which is unaffected by atropine, was reported by James (1965).

The depressant effects of ATP, ADP, AMP and adenosine on isolated rat, rabbit and guinea-pig atria are accompanied by a marked shortening in the duration of the action potential, with little or no effect on the size of the overshoot (Hollander & Webb, 1957; Bertelli et al, 1972; Meinertz et al, 1973).

Mammalian ventricular preparations: Early investigators using dog ventricles failed to obtain a response to either adenosine or adenylic acid (Drury & Szent - Gyorgyi, 1929; Wedd, 1931; Drury, 1932). However, Lindner and Rigler (1931) observed that adenosine increased coronary blood flow and contractile force and Drury (1932) reported a long-lasting, positive inotropic effect resulting from treatment with adenylic and guanylic acids. Gillespie (1934) found that ATP caused temporary heart block, followed by a positive inotropic effect. Green and Stoner's (1950) work revealed that ATP has a triphasic inotropic effect on isolated rabbit ventricle, but adenosine has no effect and AMP has only a small effect. Green and Stoner drew attention to the fact that ATP has effects which are

unlike those of the other members of the adenyl series.

Whole animals: Adenosine and adenine nucleotides depress contractility and slow the rate of beating in cats (Bielschowsky et al, 1944; Emmelin & Feldberg, 1948; Green & Stoner, 1950), dogs (Emmelin & Feldberg, 1948; Angelakos & Glassman, 1961; Marchetti et al, 1968), rabbits (Sydow & Ahlquist, 1954; Buckley, Tsuboi & Zeig, 1961), guinea-pigs (Rand, Stafford & Thorp, 1955) and rats (Versprille & Duyn, 1966; Versprille, 1966) hearts. Generally, atropine has no effect on these responses, but in the cat it has been found that atropinization and/or sectioning of the vagi prevents these actions (Bielschowsky et al, 1944; Emmelin & Feldberg, 1948).

Effects of the adenyl compounds in man have been investigated extensively. Honey, Ritchie and Thomson (1930) injected adenosine into normal human subjects and reported impaired A-V conduction, with bradycardia. Similar results were obtained by Jezer, Oppenheimer and Schwartz (1933). Rothmann (1930) using adenylic acid from crude muscle extracts, observed depression of sinus rhythm, but no A-V impairment, and Van De Velden (1932) also reported only bradycardia. Hartmann (1932) found an initial increase in heart rate followed by slight bradycardia. Richards' (1934) study of adenosine and adenylic acid

confirmed that these agents produce an initial tachycardia and that in 25% of the subjects tested, there is a disturbance in A-V conduction. He concluded that adenylic acid acts primarily on the conducting system of the heart. This conclusion is supported by later studies of Wayne, Goodwin and Stoner (1949) who found that ATP has a profound effect on conduction, causing changes in cardiac rhythm; small doses produce sinus slowing, prolongation of the P-R interval and heart block, whereas larger doses produce standstill of either the ventricles or of the whole heart. Intravenous injection of atropine does not influence sinus slowing induced by ATP, but it reduces the duration of any heart block.

Effects of exogenous ATP on cardiac metabolism: Endogenous ATP holds a key position as an energy donor in many cellular processes, and a deficiency (due for example to hypoxia) produces an irreversible weakening of the heart and a gradual cessation of activity. Marshall and Andrus (1953) reported that the administration of exogenous ATP could improve the performance of the hypoxic heart. Moreover, Harary and Slater (1965) found that exogenous ATP could overcome the inhibitory effects of oligomysin and iodoacetate (inhibitors of oxidative and anaerobic energy production, respectively) on the contractile per-

formance of isolated cells derived from rat heart. Loss of phosphocreatine and glycogen from hypothermic dog hearts is also mitigated by the injection of ATP into the coronary system (Fedeleśova, Ziegelhoffer, Valachovic & Hubka, 1966; Fedeleśova, Ziegelhoffer, Krause & Wollenberger, 1969). The latter authors showed that intracellular adenosine nucleotides and phosphocreatine levels were greater in ventricles treated with exogenous ATP than in control hearts. These observations have been confirmed for the anoxic perfused dog heart (Fedeleśova, Ziegelhoffer & Styk, 1968; Ziegelhoffer, Fedeleśova & Siska, 1971/72). Fedeleśova et al (1968) conclude that ATP exerts its action by direct entry through the cell membrane although several studies have produced contradictory evidence on this point (see below).

Does ATP cross the cell membrane? It is a commonly held belief that ATP is unable to pass through the cell membrane (Boyle & Conway, 1941; Glynn, 1968), although there is some evidence to the contrary. Forrester and Hamilton (1975) claimed that ATP is released from cat soleus muscle and evidence for its entry into this muscle is provided by the work of Chaudry and Gould (1970). Forrester (1972) demonstrated that ATP appears in the circulation of the occluded, exercising human forearms and he proposed that

it is derived from muscle cells. Paddle and Burnstock (1974) demonstrated elevated ATP levels in the coronary circulation of perfused guinea-pig hearts under anoxic condition, although it is not clear whether this was derived from nervous tissues, vascular smooth muscle or from the heart cells. More recently, Forrester and William (1977) identified ATP in the fluid medium surrounding heart cells isolated from rat ventricles, especially under hypoxic conditions. They suggested that this is derived from the myocardial cells.

Thus there is considerable evidence that ATP can leak out of the cell; evidence regarding its ability to enter cells is less convincing. On balance, it appears more likely that ATP is first dephosphorylated, with liberation of adenosine and inorganic phosphate, and that adenosine then crosses the cell membrane and is rephosphorylated on the inside. Hoffmann and Okita (1965) working with perfused guinea-pig hearts, and Hatori, Miyazaki and Nakamura (1969), working with rat hearts, used a radioisotopic method to investigate this question. Mixtures of adenosine 8 - ^{14}C labelled triphosphate and adenosine triphosphate γ - ^{32}P of known isotopic ratio were employed. Their results were consistent with the idea of indirect entry. Similar results have since been obtained by

others (Krause & Wollenberger, 1968; Fedeleśova et al, 1969; Ziegelhoffer et al, 1971/72).

The site of dephosphorylation of ATP is not known. There is evidence that dog coronary artery homogenates are capable of releasing inorganic phosphate from ATP (Carr, Bell Bradyhouse & Kantz, 1953) and that coronary tissues contain 5-nucleotidase (Reis, 1951). Antoni et al (1960) concluded that in the frog heart, dephosphorylation occured on the outer surface membrane.

It is important to note that some at least of the effects of ATP are not mediated by a dephosphorylation - penetration - rephosphorylation mechanism. Recent work by Yatani et al (1978) using AMP - PNP, a non hydrolyzable analogue of ATP (Yount, Babcock, Ballantyne & Ojala, 1971), has shown that the actions of this substance on frog atria are essentially the same as those of ATP. The question of direct entry of ATP into myocardial cells or of indirect entry is as yet unresolved.

Present Study: The work reported in this section was undertaken to investigate the effects of ATP and related compounds on ventricular contractility and on intracellular cyclic 3, 5- nucleotide metabolism. Chapter V is concerned with the effects of exogenous ATP on the mechanical

and electrical properties of the ventricle. This work led to the formulation of a working hypothesis on which the remainder of the work in this thesis is based. In Chapter VI, indirect evidence for the involvement of cyclic 3′, 5′- nucleotides in mediating the ATP-induced response is presented. Chapter VII is concerned with changes in the levels of intracellular cyclic AMP and cyclic GMP elicited by ATP. Chapter VIII describes the effects of ATP and some related compounds on ventricular contractility.

Preliminary accounts of some aspects of this work were presented to the Physiological Society (Flitney, Lamb & Singh, 1977, 1978).

C H A P T E R V

EFFECT OF ATP ON MECHANICAL AND ELECTRICAL PROPERTIES OF THE VENTRICLE.

I N T R O D U C T I O N

In this chapter, the characteristic form of the response of the superfused ventricle to treatment with exogenous ATP is described in some detail. Evidence is presented to show, first, that it is not mediated by the release of neurotransmitters from nerve ending within the preparation; and secondly,that changes in the duration of the action potential, consistent with an effect of ATP on the slow inward calcium current, occur throughout the response. A working hypothesis is also described (in the Discussion) which actually formed the basis for a more detailed investigation of the mechanism of action of ATP, presented in subsequent chapters.

Characteristic form of the inotropic response to ATP.

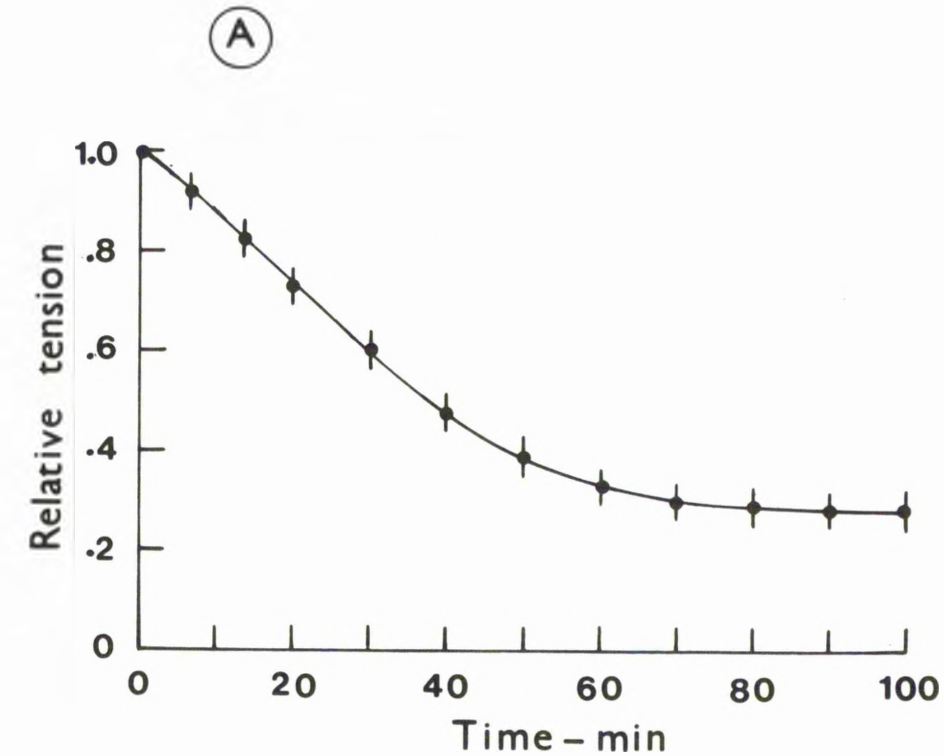
Fig.5.1A shows the time course of the development of the hypodynamic condition prior to exposure of ventricles to ATP. The perfusion rate was 100 ml.min^{-1}, stimulation frequency 30 min^{-1} and the total volume of circulating fluid was 1 litre. Contractile force fell to around 25 - 35% of the initial value over a period of 70 to 80 minutes.

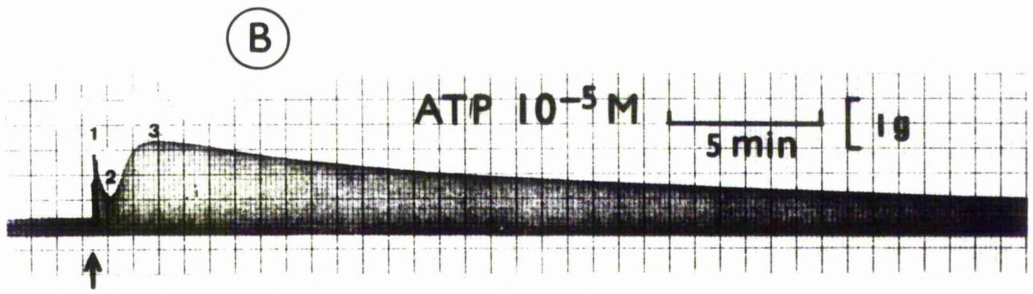
Figure 5.1.

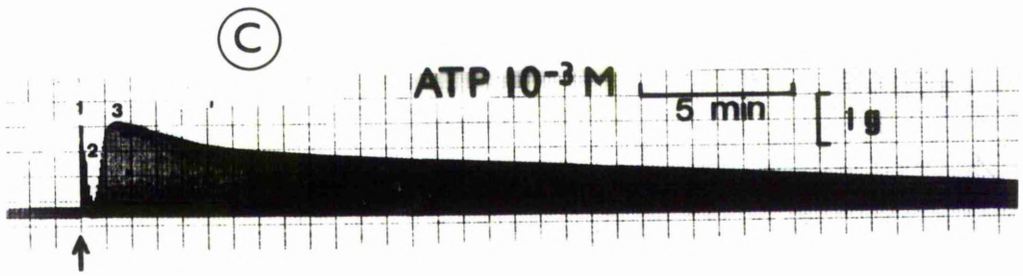
(A) Time course of the development of the hypodynamic condition prior to treatment with ATP. Each point represents the mean $\pm$ S.E. taken from 10 preparations. Contractile force declines to about 30% of the initial value after about 80 minutes. Abscissa; Time (min). Ordinate; Relative tension (fraction of initial (control) value).

(B & C) Original chart recordings showing inotropic responses to two concentrations of ATP: 10^{-5}M (B) and 10^{-3}M (C) on the hypodynamic ventricle. Note that ATP exerts a characteristic triphasic inotropic response comprising (1) an immediate positive inotropic effect which is maximal within 2-4 beats (2) a second inhibitory effect, reaching a maximum within 15-18 beats and (3) a long-lasting secondary potentiation (about 80-100 min) which is maximal after 1.5 - 2 min. The arrows indicate the times of application of ATP.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate: 100 $ml.min^{-1}$. Temp, 18.5^{o}C.


A
1.0
.8
.6
.4
.2
0
Relative tension
0
20
40
60
80
100
Time – min


B
ATP 10⁻⁵ M
5 min
1 g
1
2
3


C
ATP 10⁻³ M
5 min
1 g
1
2
3

Original chart recordings of the response to two concentrations of ATP, 10^{-5} and 10^{-3}M, are shown in Fig.5.1B. Although these differ in detail (see pages 104 - 106) they illustrate the general form of the ATP-induced response. Generally, it comprises an initial, rapidly-developing positive inotropic effect, which is maximal after 2-4 beats (hereafter referred to as the 1st phase), followed by a period in which the twitch amplitude is somewhat reduced, sometimes far below the control level (2nd phase) and superseded by a slowly-developing, secondary increase in contractile force (3rd phase) which is maximal after 1.5 - 2 minutes and which may persist for up to 100 minutes.

Log-dose response curves to varying concentrations of ATP:

The effect of varying [ATP]$_o$ (range 10^{-10} - 10^{-3}M) on the form of the response was studied. Fig.5.2A illustrates in diagramatic form the three parameters which have been measured. The results are presented as a family of curves in Fig. 5.2B. Contractile force in the presence of ATP is expressed as a multiple of the control (hypodynamic) level prior to treatment.

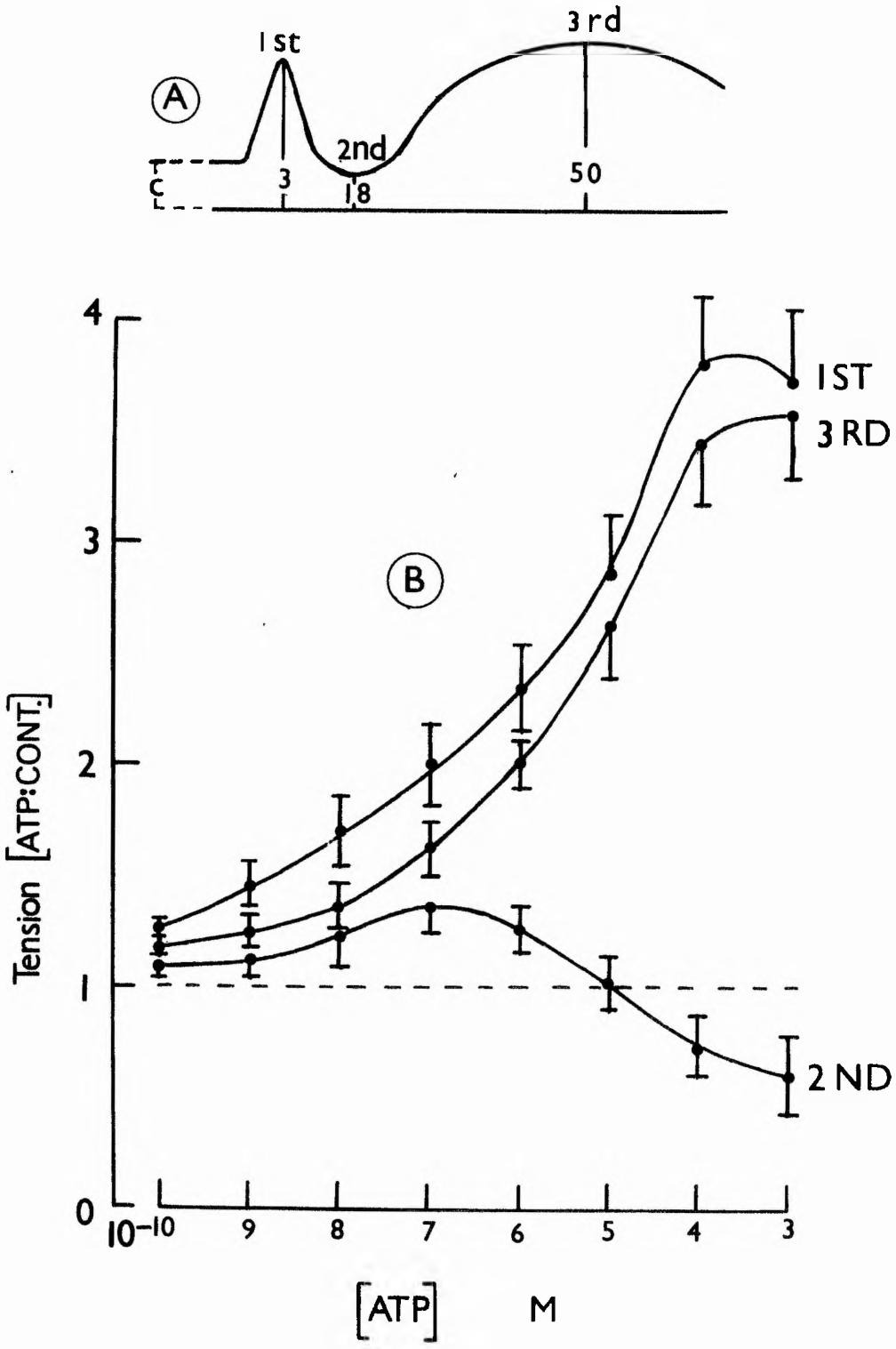
These curves show several features which are of interest. The effect of varying concentrations of ATP on the third component of the response (50th twitch; Fig.5.2A) produces

Figure 5.2.

(A) Characteristic triphasic form of ATP-induced inotropic response. The four parameters measured were: (i) control (c) hypodynamic level (ii) 1st phase (3rd twitch) (iii) 2nd phase (18th twitch) and (iv) 3rd phase (50th twitch). Results are presented as family of curves in (B).

(B) Log-dose response curves for 1st, 2nd and 3rd components of the response (range from 10^{-10} to 10^{-3}M). Maximum tension reached during 1st, 2nd and 3rd phases is expressed as a multiple of the control (hypodynamic) level. Each point represents mean $\pm$ S.E. taken from 10 preparations.
Abscissa; Concentration of ATP (M).
Ordinate; Tension Ratio (ATP/control).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, 18.5°C.


A
1st
2nd
3rd
3
18
50
B
1ST
3RD
2ND
Tension [ATP:CONT.]
0
1
2
3
4
10⁻¹⁰
9
8
7
6
5
4
3
[ATP] M

a sigmoidal log-dose response curve, rising to a maximum at 10^{-3}M of around 3.60x, the initial value. The amplitude of the first component (3rd twitch; Fig.5.2A) shows a similar increase with increasing $[ATP]_o$, but with a slight downward trend at higher concentrations (10^{-3}M). The amplitude of the 2nd component (18th twitch; Fig.5.2A) varies in a different manner, it increases for concentrations $< 10^{-7}$M but thereafter it becomes progressively reduced, falling below the control level at around 10^{-5}M ATP. In some preparations, 10^{-3}M ATP was seen to halt the ventricle temporarily.

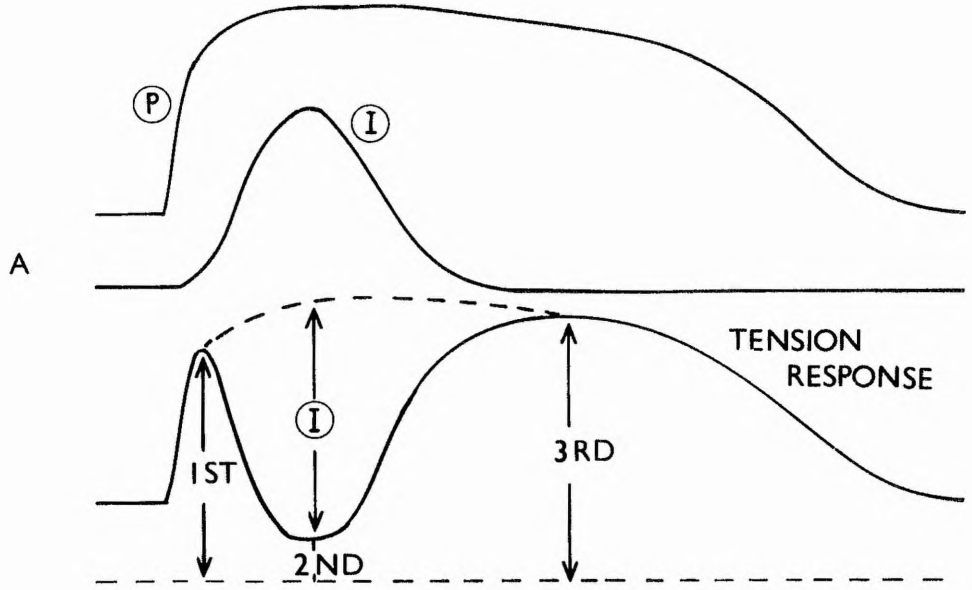
The form of the curve for the 2nd component of the ATP induced-response is particularly significant. It shows clearly that the reduction in twitch amplitude is not simply due to the absence of a positive inotropic effect, since contractile force falls substantially below the pretreated control value, and for this reason, it is concluded that the 2nd phase is actually a manifestation of an underlying inhibitory effect of ATP. Indeed, it suggests that ATP exerts a dual effect on the ventricle: a rapidly-developing and long-lasting positive inotropic effect and, superimposed upon it, a somewhat delayed and transient negative inotropic effect; and that what is recorded represents the resultant of these two opposing influences (Fig.5.3A).

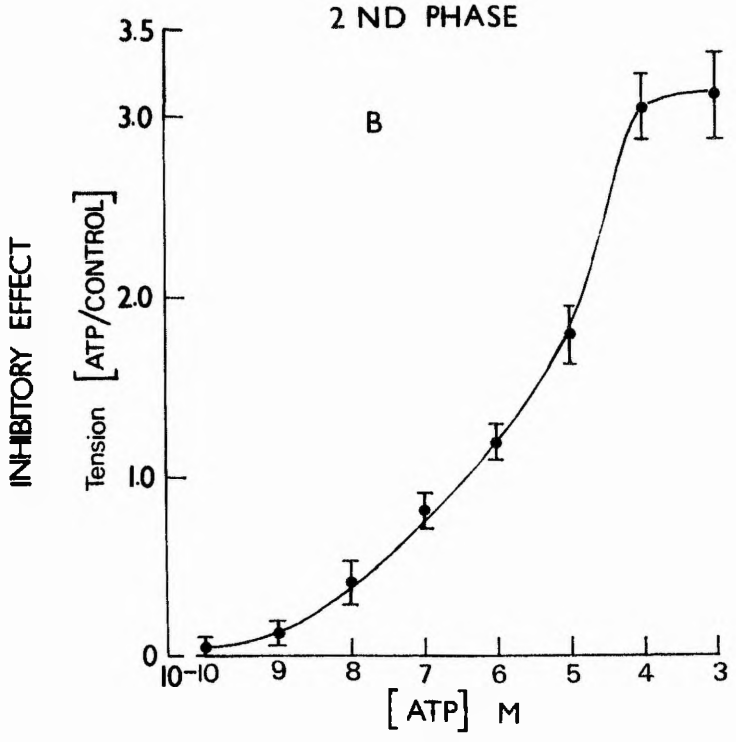
Figure 5.3.

(A) The upper trace (P) illustrates in a quantitative way the time course of a relatively long-lasting positive inotropic effect. The middle trace (I) depicts the time course of the postulated inhibitory effect. The lower trace shows the time course of the tension response resulting from these two opposing influences (P and I). The inhibitory effect (I) is obtained by subtracting the observed tension during the 2nd phase from the mean value of the 1st and 3rd phases. The results of 10 experiments for $[ATP]_o$ range from 10^{-10} to 10^{-3}M are shown in (B).

(B) Log-dose response curve for the inhibitory effect of the 2nd phase of the ATP-induced inotropic response. Each point is mean value $\pm$ S.E. Abscissa; Concentration of ATP (M). Ordinate; Inhibitory effect, Tension ratio (expressed as multiples of control values).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate: 100 $ml.min^{-1}$. Temp, 18.5^{o}C.


A
P
I
I
1ST
2ND
3RD
TENSION
RESPONSE


2 ND PHASE
B
INHIBITORY EFFECT
Tension [ATP/CONTROL]
3.5
3.0
2.0
1.0
0
10-10
9
8
7
6
5
4
3
[ATP] M

Effect of varying $[ATP]_o$ on the size of the inhibitory component: If the above supposition is correct, then the method of analysing the results for depicting the effects of ATP on the 2nd component of the response used earlier (Fig.5.3A & B) is inappropriate. The lower curve (Fig.5.3A) illustrates the correct procedure. This is to subtract the observed contractile force from the force which would have been recorded in the absence of any inhibitory influence. In practice, of course, it is impossible to do this accurately, since there is no way of telling how much force the ventricle would have produced had ATP exerted a positive inotropic response only. However, a reasonably objective assessment can be made by subtracting the amplitude of the 2nd phase (twitch 18th) from the mean value obtained for the first and third components (3rd and 50th twitch respectively) of the response.

The result of doing this is depicted in Fig.5.3B for varying $[ATP]_o$. The resulting curve shows a progressive increase in the estimated size of the inhibitory component with increasing $[ATP]_o$.

Does ATP exert its effect by releasing endogenous neurotransmitters?

The possibility that ATP exerts its effect by stimulating

Figure 5.4.

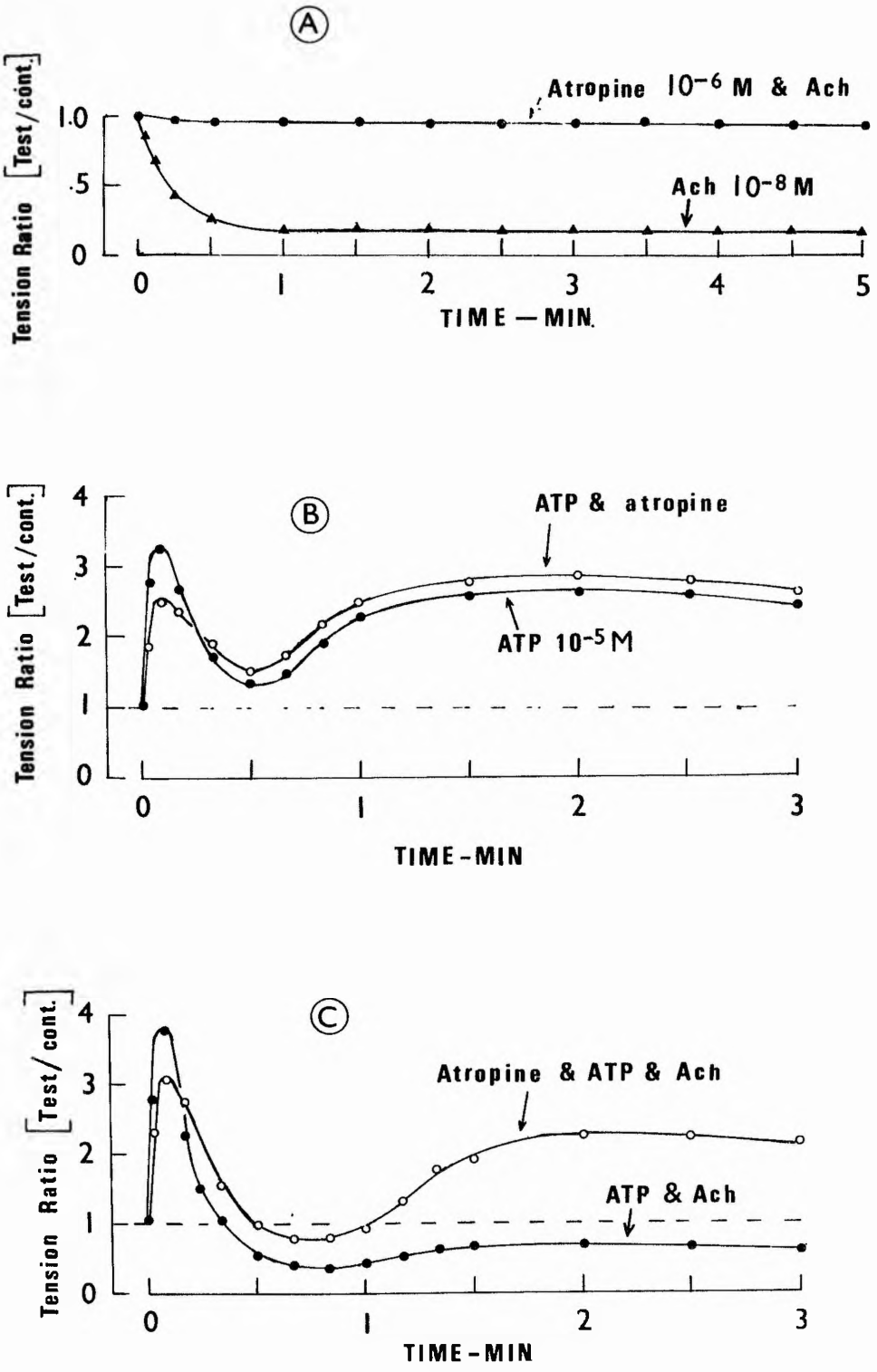
(A) Time course of the response to 10^{-3}M acetylcholine (solid triangles) and the same concentration of acetylcholine in the presence of 10^{-6}M atropine. Note that atropine almost completely abolishes the action of acetylcholine.

(B) Effects of 10^{-5}M ATP alone (solid circles) on the hypodynamic ventricle following pre-treatment with, and in the continuing presence of, 10^{-6}M atropine (open circles). The point to note here is that atropine reduces the initial component but has little effect on the 2nd and 3rd phases.

(C) Time course of the changes in contractile force during exposure of hypodynamic ventricle to Ringer solution containing a mixture of either 10^{-5}M ATP and 10^{-8}M acetylcholine (solid circles) or atropine (10^{-6}M), acetylcholine (10^{-8}M) and 10^{-5}M ATP. Acetylcholine greatly reduces twitch tension for the 2nd and 3rd components of ATP-induced response whereas atropine abolishes these effects.

The control tension response is shown by the broken horizontal line. Abscissa; Time (min). Ordinate; Tension ratio (expressed as a multiple of the control level prior to the addition of ATP, Ach or both)

Stimulation parameters: Pulse width 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate, 100 $ml.min^{-1}$. Temp, $18.5^{o}C$


A
Atropine 10⁻⁶ M & Ach
Ach 10⁻⁸ M
Tension Ratio [Test/cont.]
1.0
.5
0
0
1
2
3
4
5
TIME — MIN.
B
ATP & atropine
ATP 10⁻⁵ M
Tension Ratio [Test/cont.]
4
3
2
1
0
0
1
2
3
TIME-MIN
C
Atropine & ATP & Ach
ATP & Ach
Tension Ratio [Test/cont.]
4
3
2
1
0
0
1
2
3
TIME-MIN

the release of endogenous neurotransmitters has been tested by examining the effects of atropine and propranolol on the form of the response. Neither of these blocking agents has any marked effect on the ATP-induced response and it is therefore concluded that ATP influences the contractility of the ventricle in a way which does not involve either cholinergic or β - adrenergic receptors.

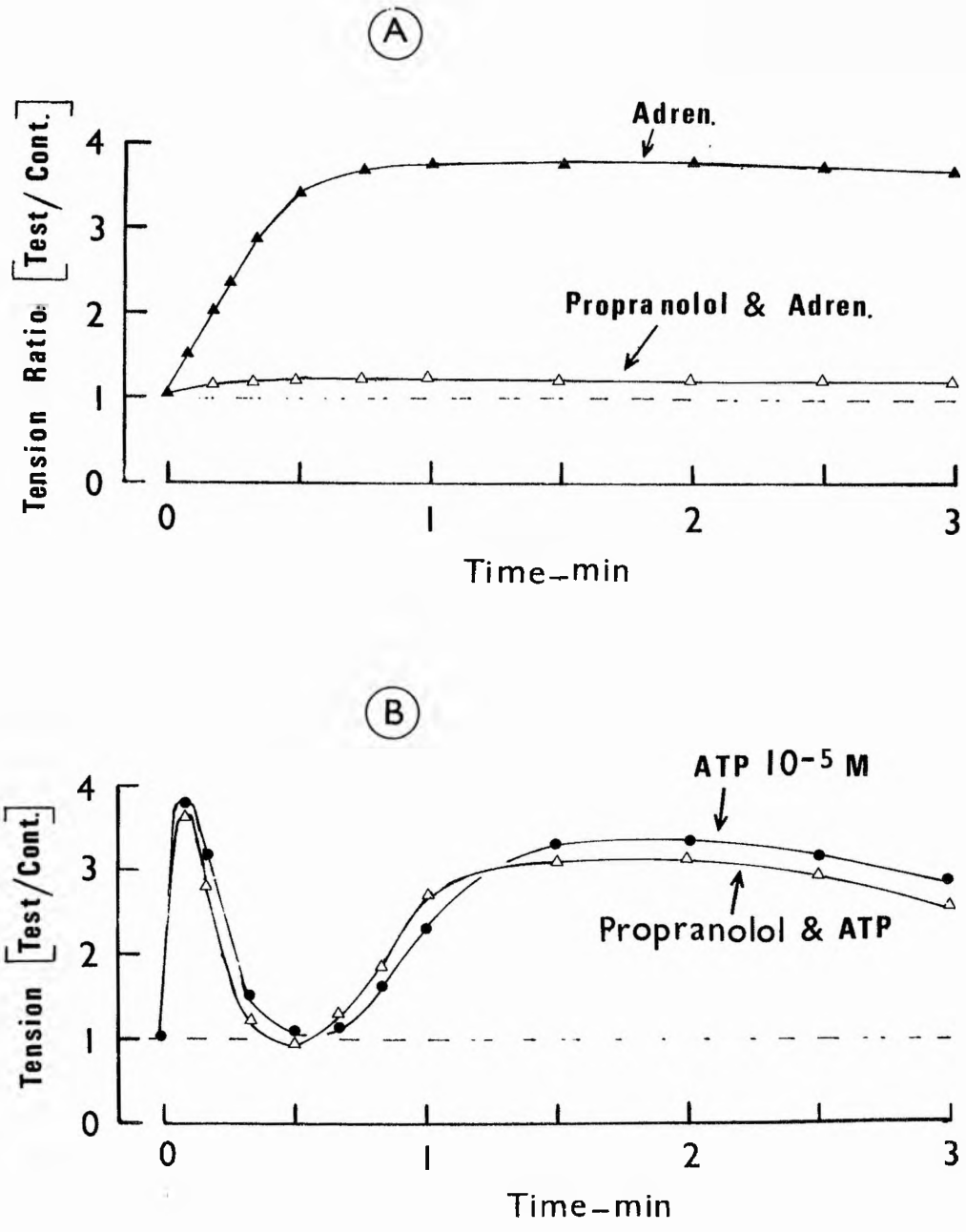
Effects of atropine on the ATP response: The response of the hypodynamic ventricle to acetylcholine above (10^{-8}M) is seen in Fig.5.4A (lower curve) and acetylcholine in the presence of 10^{-6}M atropine (upper curve). As expected, atropine almost completely blocks the acetylcholine-induced negative inotropic effect. In contrast to this, pretreatment of the hypodynamic ventricle with 10^{-6}M atropine has only a small effect on the ATP response. It reduces the first component somewhat (by 21%) but increases slightly the 2nd and 3rd components (Fig.5.4B). The effect of acetylcholine on the ATP-induced response is shown in Fig.5.4C (solid circles). The first phase is largely unaffected, whereas during the 2nd and 3rd phases of the response, the force of contraction is greatly reduced. Once again, this action is almost entirely prevented by pretreatment with atropine (open circles; Fig.5.4C) leaving a near-normal ATP response.

Figure 5.5.

(A) Effect of 10^{-6}M adrenaline (solid triangles) on the hypodynamic ventricle and following pre-treatment (for 5 min) with, and in the continuing presence of, 10^{-7}M propranolol (open triangles). Control tension is shown by the broken horizontal line. Note the powerful positive inotropic action of adrenaline which is almost entirely abolished by propranolol. Abscissa; Time (min). Ordinate; Tension ratio (expressed as a multiple of the control level prior to stimulation with adrenaline).

(B) Time course of changes in isometric force during exposure of hypodynamic ventricle to 10^{-5}M ATP alone (solid circles) and following pretreatment (for 5 min) with, and in the continuing presence of, 10^{-7}M propranolol. Control level is illustrated by the horizontal broken line. Propranolol has little effect on the response induced by ATP. Abscissa; Time (min). Ordinate; Tension ratio (expressed as a multiple of the control value prior to treatment with ATP).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate: 100 $ml.min^{-1}$. Temp, 18.5^{o}C.


A
Adren.
Propranolol & Adren.
Tension Ratio [Test/Cont.]
4
3
2
1
0
0
1
2
3
Time–min
B
ATP 10⁻⁵ M
Propranolol & ATP
Tension [Test/Cont.]
4
3
2
1
0
0
1
2
3
Time–min

Effect of propranolol on the ATP response: The effect of adrenaline (10^{-6}M) on the hypodynamic ventricle is shown in Fig.5.5A (upper curve). Adrenaline produces a powerful positive inotropic response, reaching a maximum after approximately fifteen twitches. This effect is almost entirely blocked by pretreating for 5 mins with 10^{-7}M propranolol, a β - antagonist. In contrast to this, propranolol has no marked effect on the ATP-induced response (Fig.5.5B).

Effects of ATP on the action potential: Intracellular microelectrode recordings have shown that ATP has a profound effect on the shape of the action potential. In this series of experiments, narrow strips of ventricle were used and the perfusion rate was reduced to only 20ml min^{-1} to avoid dislodging the microelectrode.

Initially, action potentials were recorded during the relatively long-lasting third phase of the response resulting from treatment with 10^{-3}M ATP, and these are described first. In subsequent experiments, action potentials were recorded at a specified time (between the 45th and 55th twitch) during responses elicited by varying $[ATP]_o$ and also continuously throughout the time course of a single ATP response. The effects of verapamil (α - Irosproyl -α

[(N-methyl-N-homoveratryl) - ϒ - aminopropyl] -3, 4 - di-methoxylacetonitrile hydrogen chloride), an agent which reduces transmembrane calcium movements (Kohlhardt, Bauer, Krause & Fleckenstein, 1972), on the ATP-induced action potential have also been investigated.

The results of these experiments suggest that the primary effect of ATP on the ventricle is to increase the magnitude of the slow inward calcium current.

Table 5.1: Membrane potentials from frog ventricular strips during the 3rd component of the response to 10^{-3}M ATP

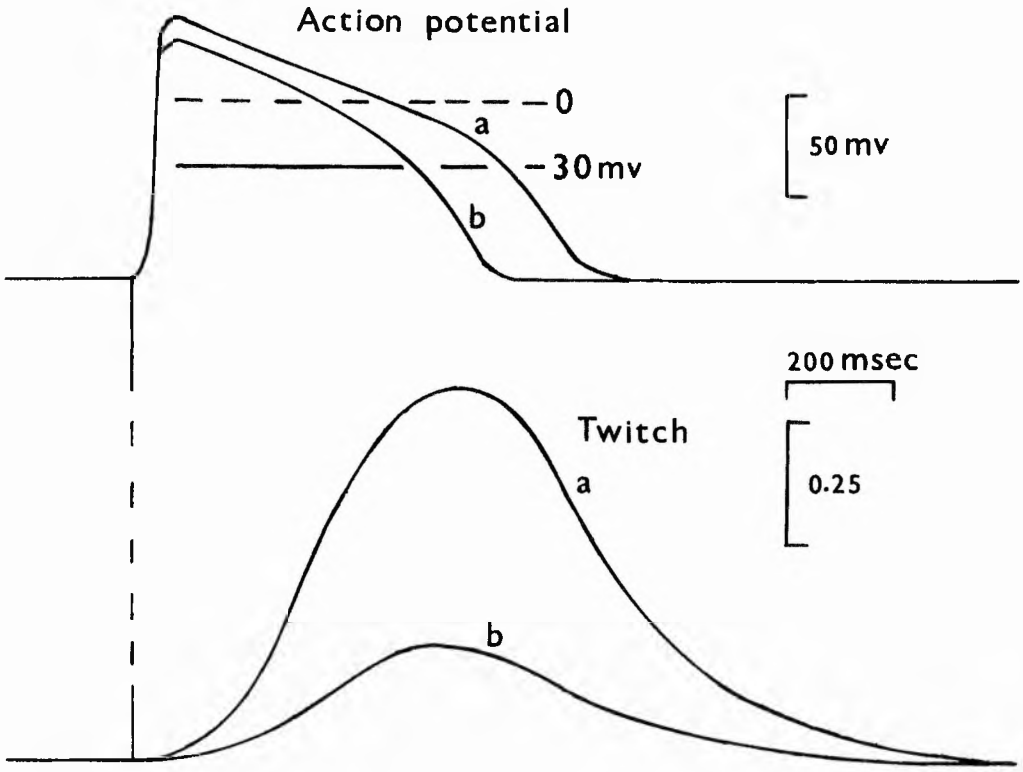
		Control $[Ca]_o$ 1mM	10^{-3}M ATP; $[Ca]_o$ 1mM
1.	Resting potential	78.36 ± 3.34 (14)	79.18 ± 3.86 (14)
2.	Action potential height	104.76 ± 4.10 (14)	116.78 ± 5.57 (14)
3.	Mean overshoot(2-1)	26.40	37.60
4.	Action potential duration at -30mV level (msec)	520 ± 12.9 (14)	705 ± 19.0
5.	Percent duration increase	-	35.5.
6.	Action potential duration at resting level	695 ± 14.0	951 ± 21.0 (14)

The figures in brackets denote the number of cells impaled from

Figure 5.6.

Effect of ATP (10^{-3}M) on action potential (a, above) and twitch amplitude (a, below) during the 3rd component (50th twitch) of the response. The control (prior to ATP treatment) action potential (b, above) and twitch tension (b, below) are shown for comparison. It can be seen that ATP produces an increase in the overshoot and duration of action potential and a marked augmentation of twitch contraction.

Stimulation parameters: Pulse width, 5 msec. frequency, 30 min^{-1}; amplitude, 25V. Flow rate: 20 $ml.min^{-1}$. Temp, 18-19^{o}C.


Action potential
0
a
−30 mv
b
50 mv
200 msec
Twitch
a
0.25
b

11 preparations.

Effect of ATP (10^{-3}M) on electrical properties of the ventricle recorded during the third component of the response: Data presented in Table 5.1 summarises the results of these experiments. Eleven preparations were used. The principal finding was an increase in the overshoot and duration of the action potential. ATP had no effect on the membrane potential between contractions. The overshoot increased by around 11mV, from 26.4 to 37.6 and the duration of the action potential, measured at -30mV level, which corresponds with the potential at which the calcium current becomes activated (Reuter & Beeler, 1969; Beeler & Reuter, 1970; Noble, 1975) increased from 520 $\pm$ 12.9 msec to 705 $\pm$ 19.0 msec.

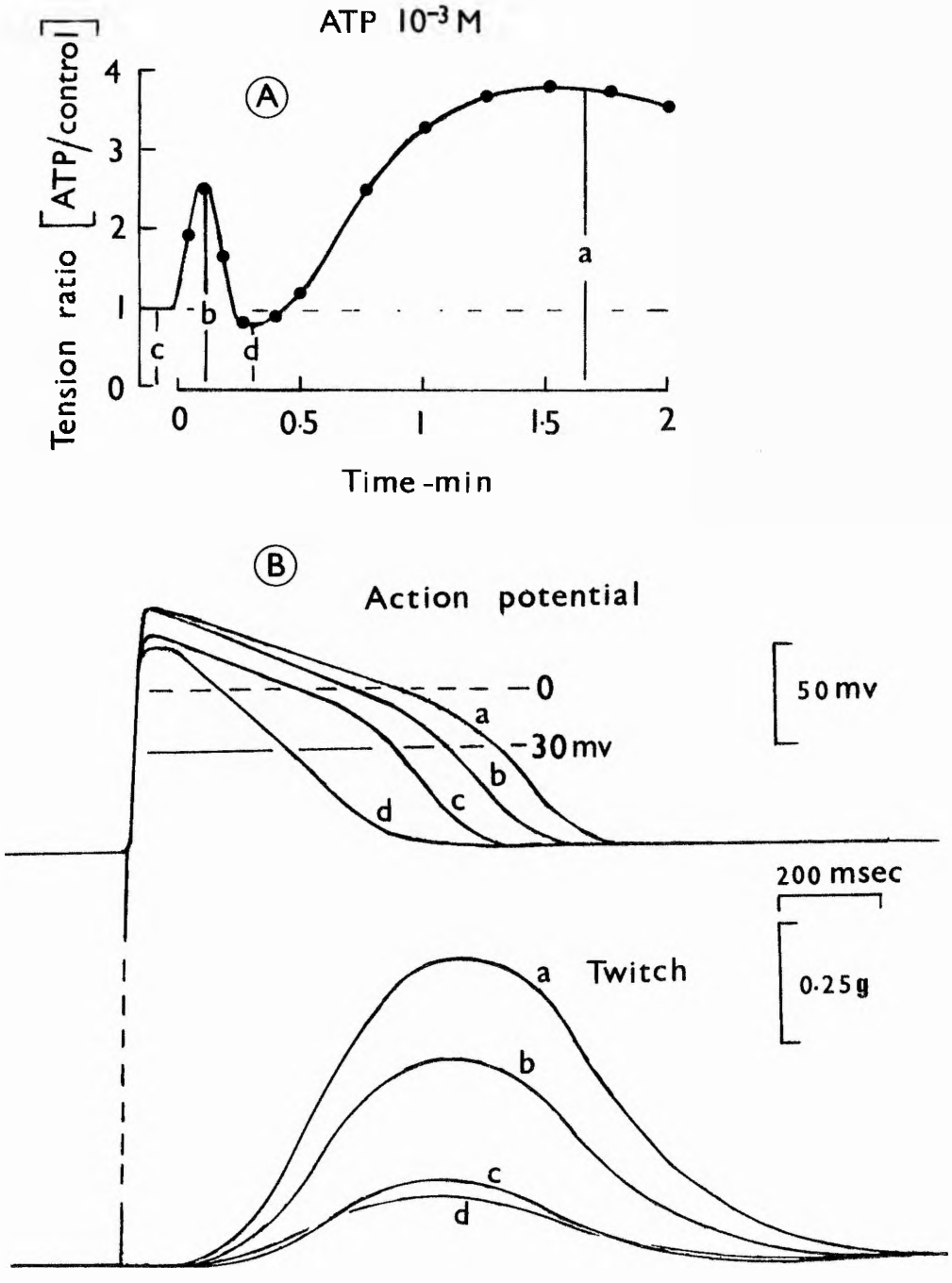
These changes in the shape of the action potential were accompanied by an increase in contractile force. Fig.5.6 shows oscilloscope recordings of action potentials and twitch tension before (b) and after (a) treatment with 10^{-3}M ATP. The action potential overshoot is increased from 27.5 mV to 38.0 mV and its duration at -30 mV level increased from 520 msec to 688 msec. Peak tension generated increase by 3.25 X control value but the time to peak tension remained unchanged. Thus the rate of rise in tension was increased by 3.57 times in the presence of ATP.

Figure 5.7.

(A) Time course tension response of hypodynamic ventricular strips to 10^{-3}M ATP. Membrane potentials were measured simultaneously at (i) control (c) (ii) peak of 1st phase (b) (iii) fully developed 2nd phase (d) and (iv) immediately after the peak of the 3rd phase (a) (see B, below). Abscissa; Time (min). Ordinate; Tension ratio (ATP/control).

(B) Effect of ATP (10^{-3}M) on action potential (above a, b, & d) and contraction (below a, b & d) for the 1st (3rd twitch), 2nd (18th twitch) and 3rd (50th twitch) components. The control action potential (above c) and twitch amplitude (below c) are shown for comparison). Note that during the 1st and 3rd phases ATP increases the overshoot and duration of the action potential, and enhances the twitch contraction as compared to the control, whereas during the 2nd phase the amplitude is reduced and action potential duration markedly abbreviated.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 25V.
Flow rate: 20 $ml.min^{-1}$. Temp, 18-19^{o}C.


ATP 10^{-3} M
A
Tension ratio [ATP/control]
4
3
2
1
0
a
b
c
d
0
0·5
1
1·5
2
Time-min
B
Action potential
0
−30 mv
50 mv
a
b
c
d
200 msec
Twitch
0·25 g
a
b
c
d

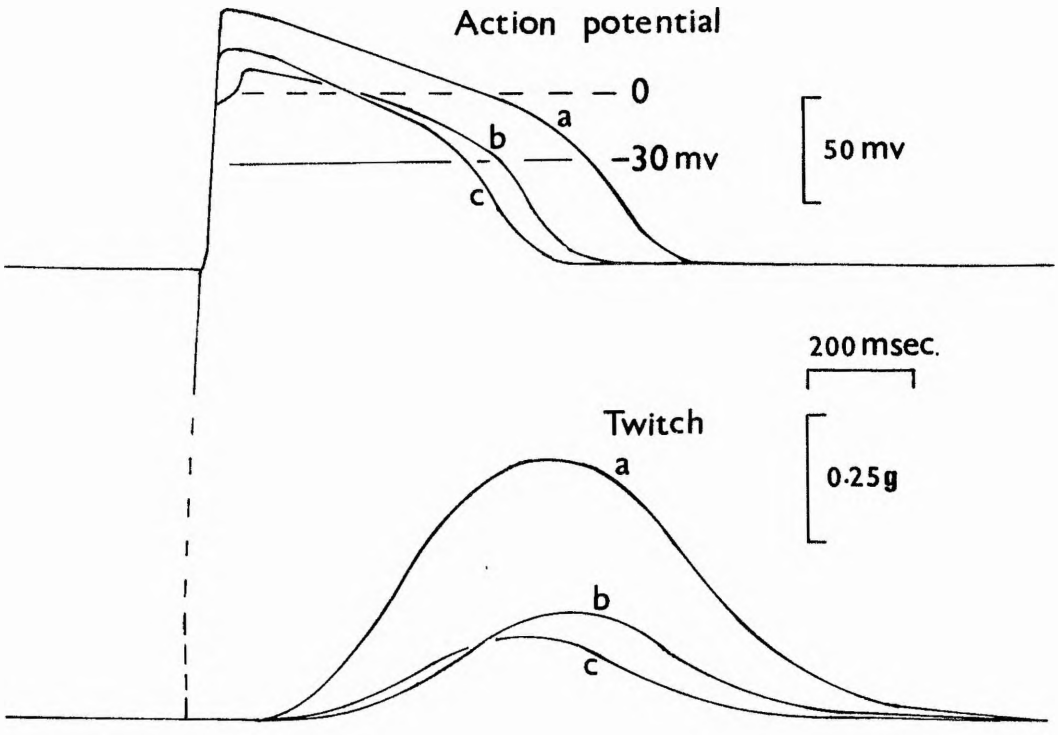
A comparison of the form of the action potential recorded during the 1st, 2nd and 3rd phases of the response and the shape of the corresponding twitches: A series of superimposed oscilloscope recordings of action potentials and twitches are shown in Fig.5.7B taken at the times indicated in Fig.5.7A viz: prior to the addition of ATP (c) at the peak of the first phase (b), during the fully developed second phase (d), and immediately after the peak of the 3rd phase (a). Changes in both amplitude and duration of the action potential correlate with the observed change in contractile force. During the 1st and 3rd phases the overshoot and duration were increased as compared to the controls, whereas during the 2nd component the action potential is reduced in amplitude and substantially abbreviated. It is notable that membrane repolarization commences just prior to or at the peak of the contraction during the 1st and 3rd phases of the ATP response, but that repolarization is almost complete before relaxation commences for both the control responses and during the second component of the ATP response.

Effects of verapamil on the contractile response and on the shape of the action potential: The marked effects of ATP on the form of the action potential, particularly on its duration, suggests that it acts by altering the magnitude

Figure 5.8.

Effect of 10^{-3} ATP alone on the action potential (a, above) and contraction (a, below) during the 3rd component (50th twitch) of the response, and in the continuing presence of 10^{-5} M verapamil (recordings above and below labelled b). The control responses for action potential (c, above) and twitch (c, below) are shown for comparison. The main point to note here, is that verapamil reduces both the overshoot and duration of the action potential, and the twitch tension normally elicited by ATP.

Stimulation parameters: Pulse width, 5 msec. frequency, 30 min^{-1}, amplitude, 25V.
Flow rate: 20 $ml.min^{-1}$. Temp, 18-19^{o}C.



a. ATP 10^{-3}M

b. Verapamil 10^{-5}M and
ATP 10^{-3}M

c. Control

of the slow inward calcium current, increasing it during the 1st and 3rd phases but reducing it during the 2nd. The effects of the calcium blocking agent verapamil on the response to ATP are illustrated in Fig.5.8. The control responses are shown in the two recordings labelled 'c' and the effects of ATP above (10^{-3}M) by records labelled 'a'. Verapamil (10^{-5}M) almost entirely abolished these effects of ATP (records b). ATP alone increases the duration of the action potential by around 29%, but in the presence of verapamil an increase of only 7% is seen. Verapamil also reduces the size of the overshoot.

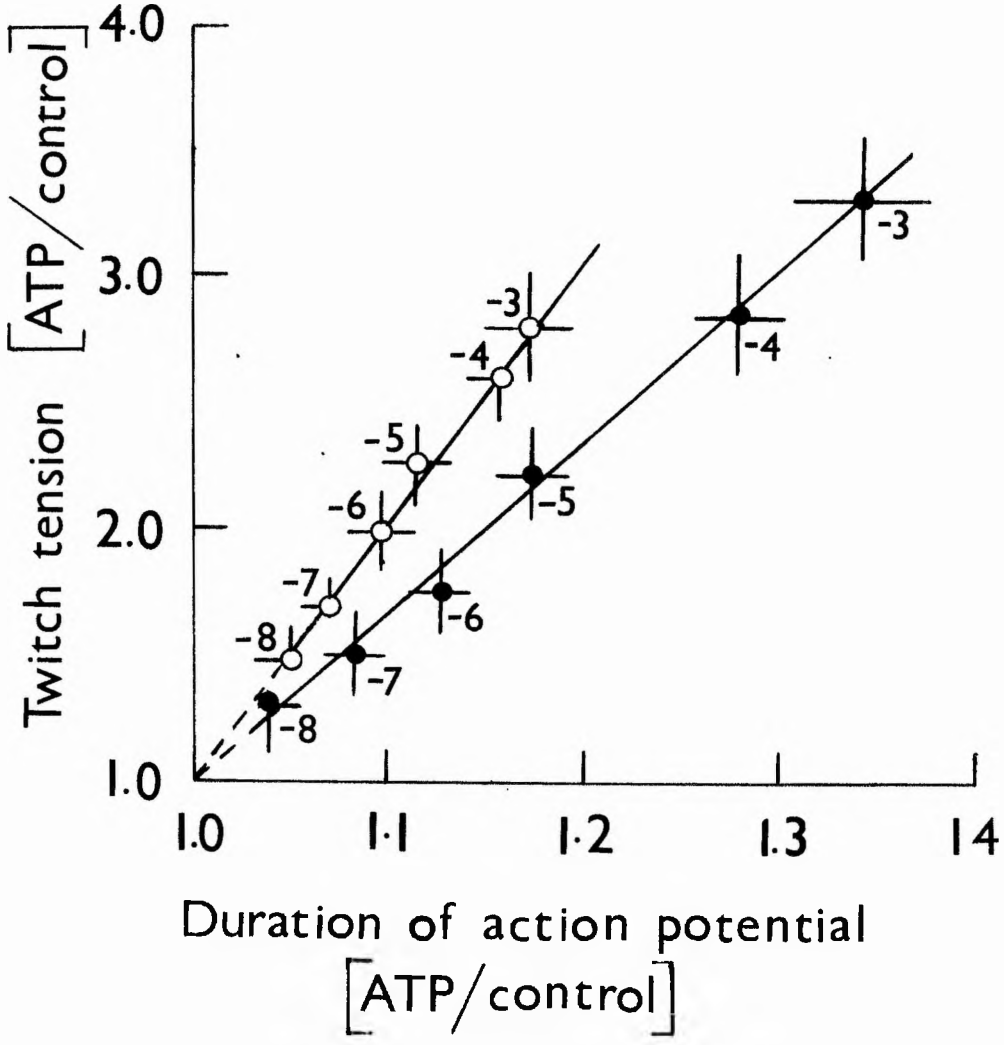
Relation between action potential duration and contractile force: Fig.5.9 shows the relationship between the action potential duration and contractile force for ATP ranging from 10^{-8} to 10^{-3}M. All values are expressed as multiples of the control values. The two curves show the relation for the 1st (open circles) and 3rd (solid circles) components of the ATP response.

These data reveal a clear correlation between the change in the contractile force and the action potential duration. Even more significant, however, is the fact that the slopes of the two curves are strikingly different. This implies that the relationship is a complex one. If it is assumed

Figure 5.9.

The relationship between action potential duration (measured at -30 mV level) and twitch potentiation for the first (open circles) and third (solid circles) components for ATP of 10^{-3} to 10^{-8}M. Both parameters are expressed as multiples of the control values. The values were obtained for the 3rd twitch and 50th twitch, respectively. Each point represents mean values $\pm$ S.E's during impalement of 10 cells taken from 6 preparations. The number beside each point denotes $[ATP]_o$ The correlation is highly significant for the first (correlation coefficient $\pm$ S.E. of estimate: 0.99 $\pm$ 0.03, n = 10, $P < 0.001$) and third (correlation coefficient $\pm$ S.E. of estimate: 0.99 $\pm$ 0.05, n = 10, $P < 0.001$) components. Note that the two slopes are statistically different ($P < 0.001$), the first component being steeper than the third. Abscissa; Duration of action potential (ATP/control). Ordinate; Twitch tension (ATP/control).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 25V.
Flow rate: 20 $ml.min^{-1}$. Temp, 18-19^{o}C.


Twitch tension [ATP/control]
4.0
3.0
2.0
1.0
-3
-4
-5
-6
-7
-8
-3
-4
-5
-6
-7
-8
1.0
1·1
1·2
1.3
1·4
Duration of action potential
[ATP/control]

that the duration of the plateau phase of the action potential by and large reflects the magnitude of the inwardly directed calcium current, then this result means that calcium entry must be having an indirect effect on the contractile mechanism. This point will be considered in more detail later.

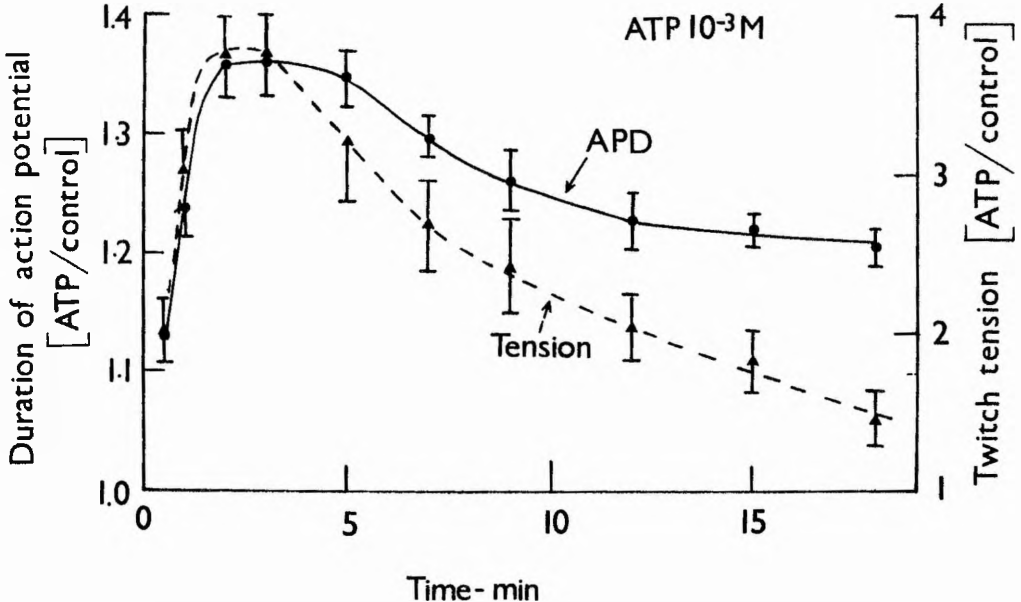
Changes in the shape of the action potential and twitch amplitude during the course of a single ATP (10^{-3}M) - induced response: It proved impossible to maintain a successful microelectrode impalement for the entire duration of the ATP response. However, the data in Fig.6.10 show results obtained from experiments lasting approximately 20 minutes in which the microelectrodes remained securely inside a single fibre. The data were collected during the entire rising phase of the 3rd component of the ATP response, up to and then beyond the peak. Both parameters increased in a parallel fashion during the rising phase, but thereafter, contractile force fell more rapidly than the decrease in the action potential duration.

This result again suggests that the relationship between the duration of the action potential and its effect on contractile force is not a straightforward one.

Figure 5.10.

Time course of the changes in action potential duration (solid circles) measured at -30 mV level and contractile force (solid triangles, broken line) during exposure to 10^{-3}M ATP for the slowly-developing and long-lasting third component of the response. Both parameters are expressed as multiples of the control values. Each point represents the mean $\pm$ S.E. taken from 7 preparations. Note that both action potential duration and tension increase in a parallel fashion during the rising phase, but thereafter isometric force declines more rapidly than action potential duration. Abscissa; Time (min); Ordinate; Duration of action potential and twitch tension (ATP/control).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 25V.
Flow rate: 20 $ml.min^{-1}$. Temp, 18-19^{o}C.


ATP 10-3 M
APD
Tension
Duration of action potential [ATP/control]
Twitch tension [ATP/control]
Time - min
1.4
1.3
1.2
1.1
1.0
4
3
2
1
0
5
10
15

DISCUSSION AND WORKING HYPOTHESIS

The characteristic triphasic form of the ATP - induced response is well documented - in the literature, for both mammalian (Green & Stoner, 1950) and amphibian preparations (Parnas & Ostern, 1932; Lindner & Rigler, 1931; Lichtneckert & Straub, 1949; Marshall & Andrus, 1953; Szent - Gyorgyi, 1953; Kanda et al, 1953; Versprille, 1963), but relatively little is known of the underlying mechanism. In this context, the manner in which the ventricle responds to differing $[ATP]_o$ has proved to be particularly informative. It is clear, for example, that the so-called second component of the response is not merely due to a temporary cessation of the positive inotropic effect of ATP, since during this phase contractile force was often observed to fall well below the initial control level, and in some instances contractility was temporarily abolished. For this reason, it is concluded that ATP exerts a delayed inhibitory effect on the ventricle.

At this point in the discussion we may reasonably exclude the possibility that the effects of ATP are mediated through the release of endogenous cholinergic or adrenergic neurotransmitters, since neither atropine nor propranolol has any significant effect on the responses. This is consistent with conclusions

arrived at by previous authors (Bielschowsky et al, 1944; Kanda et al, 1954; Schenberg, 1956; James, 1965; Versprille, 1966; Versprille & Duyn, 1966; Yatani et al, 1978). These same experiments also imply that ATP does not exert its effect by combining directly with either cholinergic or β-adrenergic receptors on the outer cell surface. Nevertheless, ATP arguably exerts effects on the ventricle which clearly resemble those that one might anticipate if it did interact with both kinds of receptors, and this raises the interesting possibility that the intracellular changes involved may have certain features which are also common to the mechanism of action of cholinergic and adrenergic transmitters.

This conclusion actually formed the basis of a hypothesis, summarised below, on which the remainder of the work described in this thesis is based.

WORKING HYPOTHESIS.

It is postulated that ATP exerts a dual effect on the ventricle, comprising a rapidly - developing, long-lasting positive inotropic effect, and superimposed upon it, a somewhat delayed and transient negative inotropic effect. It was pointed out earlier (Fig.5.3) that these assumptions could account for the overall shape of the response; in particular it provides an explanation for the downward trend of the log-dose response

curve for the first phase of the response, the argument being that the onset of a larger inhibitory component, induced at higher $[ATP]_o$ would tend to 'clip' the first phase earlier.

Since the involvement of endogenous neurotransmitters is excluded the question to be answered is: what is the nature of the positive and negative inotropic effects of ATP? It seemed possible that these two components of the ATP-induced response are mediated through changes in intracellular cyclic AMP and cyclic GMP, respectively. Evidence that the positive inotropic effects of β - agonists are accompanied by elevated level of intracellular cyclic AMP, and conversely, that the negative inotropic effects of acetylcholine are associated with elevated levels of cyclic GMP, was reviewed earlier (see main Introduction, pages 21-24, and 38). Thus, as a basis for further work, the component of the response labelled 'P' in Fig.5.3A was tentatively identified as representing the time course of the change in cyclic AMP level, and that labelled 'I' was taken to represent the accompanying changes in cyclic GMP levels.

It will be seen (Chapter VII) that these conclusions are broadly correct, although the precise time courses of the changes in cyclic AMP and cyclic GMP are not exactly as

predicted by Fig.5.3A. Indirect evidence pointing to the involvement of cyclic 3',5'-nucleotides in the ATP response is presented in Chapter VI, and the results of experiments involving direct measurements of intracellular cyclic AMP and cyclic GMP in Chapter VII. Further discussions on the mechanism of action of ATP, in particular, its marked effect on the action potential, is deferred until later.

CHAPTER VI

INDIRECT EVIDENCE FOR THE INVOLVEMENT OF CYCLIC 3, 5-NUCLEOTIDES IN MEDIATING THE ATP-INDUCED RESPONSE.

I N T R O D U C T I O N

This series of experiments was designed to establish whether or not it was worth pursuing the idea that intracellular cyclic 3', 5' - nucleotide matabolism is influenced by ATP. They were actually made before those described in Chapter IV, concerned with the changes in intracellular cyclic 3', 5' - nucleotide levels during the development of the hypodynamic condition.

The experiments fall into two broad groups.

Type 1 experiments: These experiments are concerned with the effects of ATP administered at various times during the development of the hypodynamic condition.

Type 2 experiments: These are concerned with the effects of ATP on preparations in which the levels of cyclic 3',5' nucleotides were artificially elevated by:

(i) Pretreatment with either DB cyclic AMP or 8-Br cyclic GMP alone.

(ii) Pretreated with a combination of theophylline (to inhibit cyclic 3', 5' - nucleotide breakdown by phosphodiesterases) and either DB cyclic AMP or 8-Br cyclic GMP.

(iii) Pretreatment with adrenaline to boost intracellular cyclic AMP levels.

These experiments will be considered in turn.

Type 1 experiments: effect of administering ATP at various times during the development of the hypodynamic state.

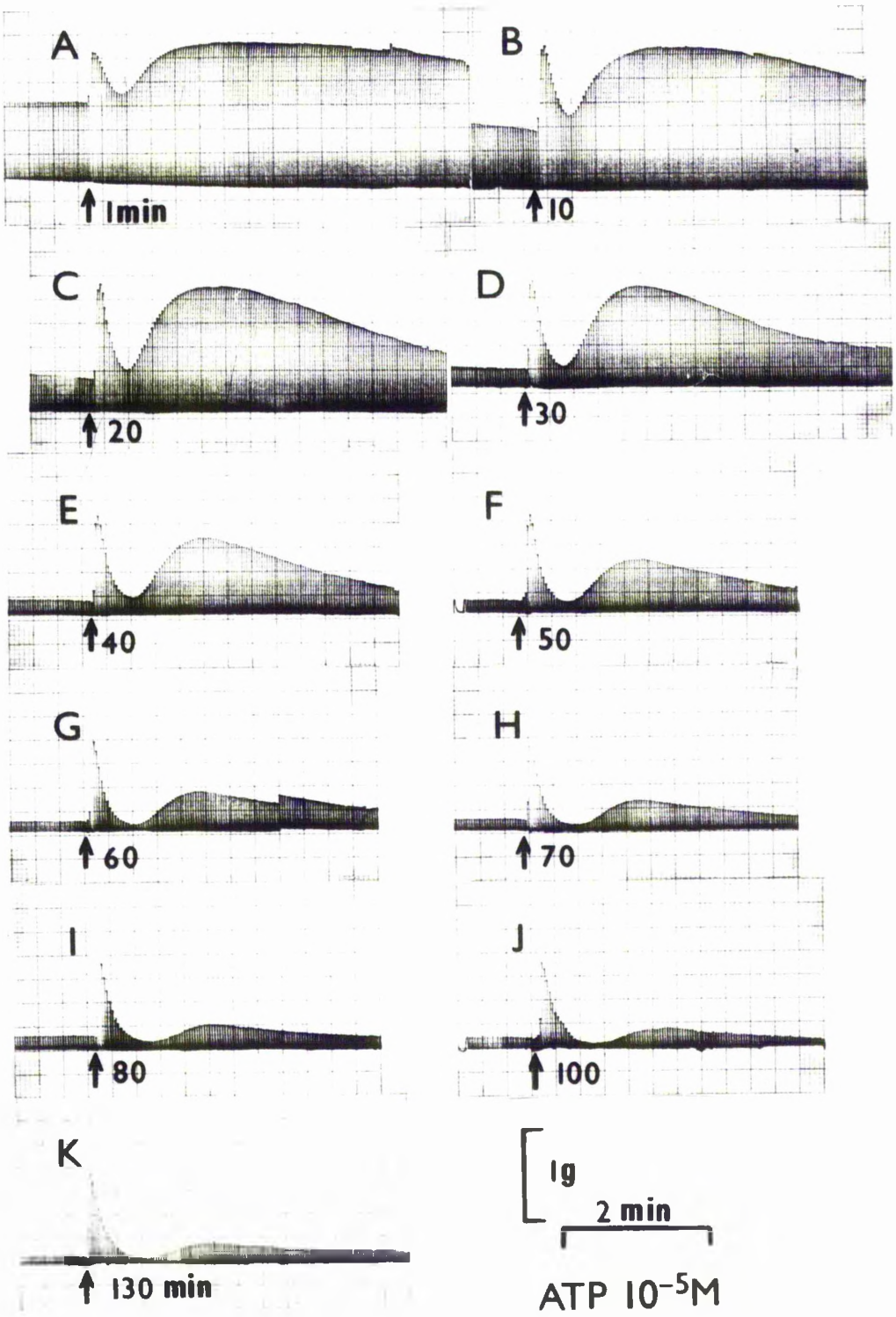
The gradual decline in contractility seen during the development of the hypodynamic condition was shown previously to be accompanied by a gradual reduction in intracellular cyclic AMP and an elevation of intracellular cyclic GMP levels. The relevant experiments, described earlier (Chapter IV), were in fact made prior to those described in this section. It was reasoned that if the hypodynamic depression is associated with, and the ATP-induced response mediated by, changes in intracellular cyclic 3', 5' - nucleotides, then the precise form of the response to ATP should vary throughout the early stages of perfusion, while the preparation is settling down to its final steady-state level.

The results of an experiment in which the ventricle was

Figure 6.1.

Original chart recordings (A-J) of isometric twitch tension following administration of 10^{-5}M ATP at various times during the development of the hypodynamic state. The arrows indicate the different times of application of ATP. Note that the character (size and form) of the response is altered as the hypodynamic depression is developed. The size of the 2nd phase increases as compared to the third component which decreases with increasing perfusion time. There is relatively little change in the response of the first component.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}, amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, 19°C.


A
1min
B
10
C
20
D
30
E
40
F
50
G
60
H
70
I
80
J
100
K
130 min
1g
2 min
ATP 10⁻⁵M

subjected to a standard dose of ATP (10^{-5}M) during the decline in contractility is shown in Fig.6.1. It can be seen that the character of the response is indeed affected by the extent to which the hypodynamic depression has developed. The most striking changes observed concern the size of the inhibitory (second component) and the size and form of the long-lasting third phase. The size of the inhibitory component clearly increases with increasing perfusion times; initially, up to 40 minutes of perfusion, the twitch tension remains above the control level during this phase, and only falls below it for periods in excess of around 50 minutes. The potentiation seen during the third phase decays more slowly in ventricles perfused for short periods as compared to those which have been superfused for a longer time. In contrast, the absolute size of the potentiation seen during the first component of the response is relatively constant.

It is not considered worthwhile attempting to explain the precise form of each response at this time. It is sufficient to note that the form of the ATP-induced response is affected by the degree to which the hypodynamic depression has been allowed to develop.

Type 2 experiment: effects of agents which are known to influence cyclic 3', 5' - nucleotide metabolism on the form of the ATP - induced response.

The three kinds of experiments described in this section were designed to artificially elevate the intracellular level of either cyclic AMP or cyclic GMP, by using their lipid soluble derivatives, or alternatively, by application of agents known to alter cyclic 3', 5' - nucleotide levels indirectly by a pharmacological action on the cells. The rationale is as follows: consider first the effect on the ATP response of artificially elevating intracellular cyclic AMP levels. If these can be raised sufficiently so that the capacity of the fibres to produce still more cyclic AMP is severely limited, then the result of subsequent treatment with ATP might be to elicit a negative inotropic response only, since presumably their capacity to produce additional cyclic GMP is unaffected by this procedure. The converse argument is applied in the case of artificially elevated cyclic GMP levels, where one might expect to obtain a monotonic, positive inotropic response only.

Effects of exogenous DB cyclic AMP and 8-Br cyclic GMP

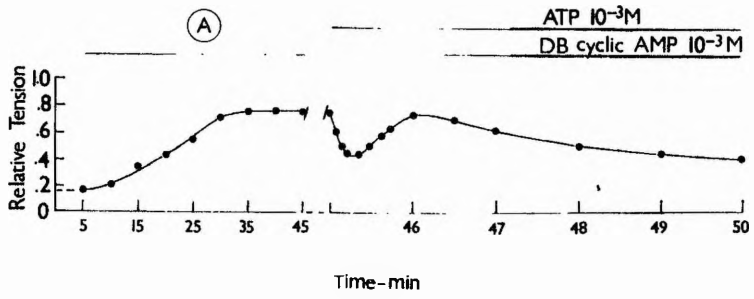
Figure 6.2.

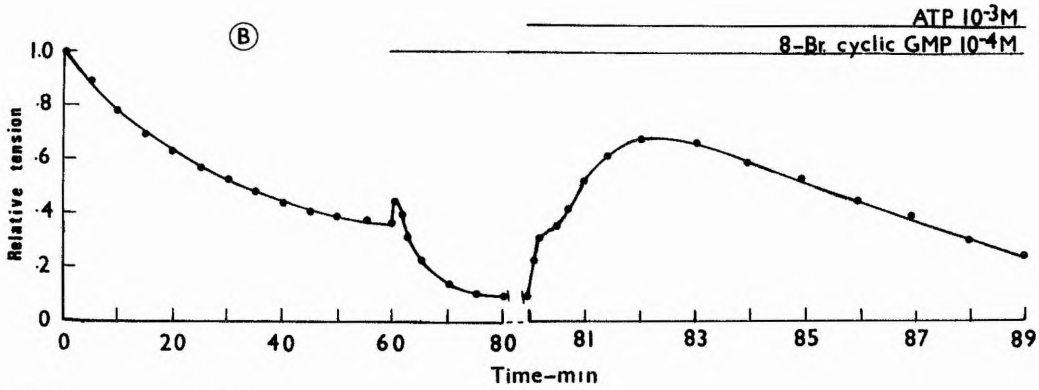
(A) Time course of the positive inotropic effect to 10^{-3}M dibutyryl cyclic AMP on the hypodynamic ventricle and the influence of 10^{-3}M ATP in the continuing presence of dibutyryl cyclic AMP. The point to note is that ATP exerts a marked inhibitory effect only. Abscissa; Time (min). Ordinate; Relative tension (fraction of initial (control) level.

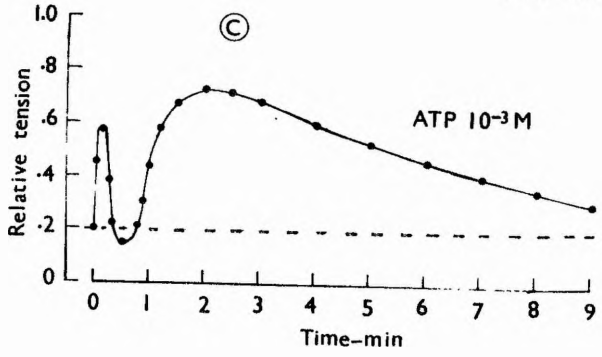
(B) Time course of the inotropic response to 10^{-4}M 8-Bromo cyclic GMP on the partially hypodynamic ventricle and the effect of subsequently administering 10^{-3}M ATP in its presence produces a monotonic positive inotropic effect. Abscissa; Time (min). Ordinate; Relative tension (fraction of initial control level).

(C) Influence of 10^{-3}M ATP (alone) on the hypodynamic ventricle. Note the normal triphasic nature of the inotropic response. Abscissa; Time (min). Ordinate; Relative tension (fraction of initial (control) value. The solid horizontal lines (in A & B) illustrate the duration of exposing the preparations to dibutyryl cyclic AMP and ATP.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate: 100 $ml.min^{-1}$. Temp, 19^{o}C.


A
ATP 10⁻³M
DB cyclic AMP 10⁻³M
Relative Tension
1.0
.8
.6
.4
.2
0
5
15
25
35
45
46
47
48
49
50
Time-min


B
ATP 10⁻³M
8-Br. cyclic GMP 10⁻⁴M
Relative tension
1.0
.8
.6
.4
.2
0
0
20
40
60
80
81
83
85
87
89
Time-min


C
ATP 10⁻³M
Relative tension
1.0
.8
.6
.4
.2
0
0
1
2
3
4
5
6
7
8
9
Time-min

on the ATP response: Fig.6.2 summarises the results of two experiments, one concerned with the effect of pretreatment with DB cyclic AMP (Fig.6.2A) and the other with the effects of pretreatment with 8-Br cyclic GMP (Fig.6.2B). Consider first the effect of pretreatment with DB cyclic AMP. The ventricle was superfused until it had become fully hypodynamic and DB cyclic AMP (10^{-3}M) was then administered for a period of 40 minutes. This produces a substantial positive inotropic effect, reaching a maximum after approximately 30 - 35 minutes. The effect of subsequently administering ATP (10^{-3}M) is to produce a large negative inotropic response. This is in fact followed by a return to the pre-ATP level and then a gradual deterioration approaching the original control twitch amplitude after approximately 10 minutes. Thus, the predominant effect of ATP under these conditions is to produce a lowering of isometric twitch tension.

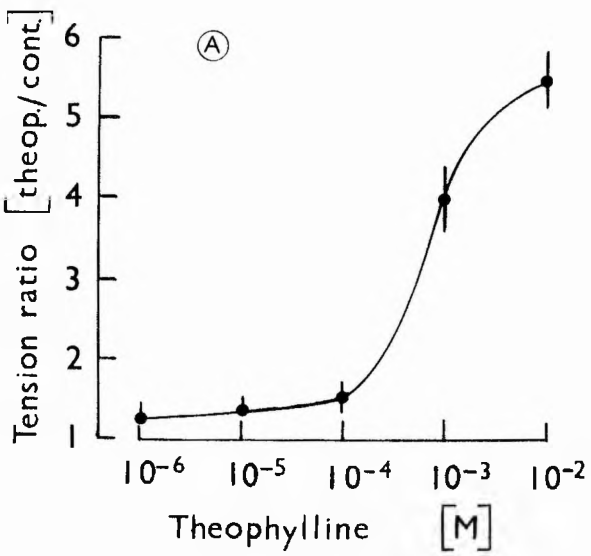
In contrast to this, Fig.6.2B shows the effect of pretreating the preparation with 8-Br cyclic GMP (10^{-4}M). The heart was allowed to become partially hypodynamic and then 8-Br cyclic GMP was administered for a period of about 20 minutes. This procedure depressed the contractile response down to about 10% of its initial value. ATP (10^{-3}M) was then administered. The resulting positive

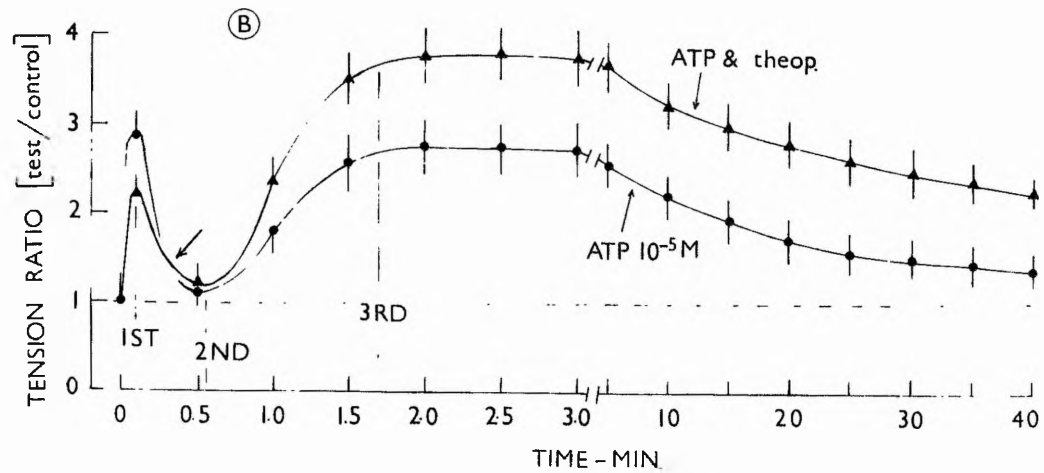
Figure 6.3.

(A) Dose-response curve for the positive inotropic effect of theophylline ranging from 10^{-6} to 10^{-2}M on the hypodynamic ventricle. Each point represents the mean $\pm$ S.E. for 10 preparations. Note that 10^{-4}M theophylline exerts only minimal direct effect on isometric force. Abscissa; Concentration (M) of theophylline. Ordinate; Tension response (theop./control).

(B) Time course of change in contractile force during exposure of 10 ventricles to 10^{-5}M ATP (solid circles) alone, and to the same concentration of ATP but in the presence of 10^{-4}M theophylline (solid triangles). Each point represents the mean $\pm$ S.E. Tension is expressed as a multiple of the control value prior to ATP stimulation. The control level is indicated by the horizontal broken line. Note that theophylline reduces the first phase but potentiates enormously the third component of the ATP-induced response.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, $19^{o}C$.


Ⓐ
Tension ratio [theop./cont.]
6
5
4
3
2
1
10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
Theophylline [M]


Ⓑ
TENSION RATIO [test/control]
4
3
2
1
0
ATP & theop.
ATP 10^{-5}M
1ST
2ND
3RD
0 0.5 1.0 1.5 2.0 2.5 3.0 10 20 30 40
TIME - MIN.

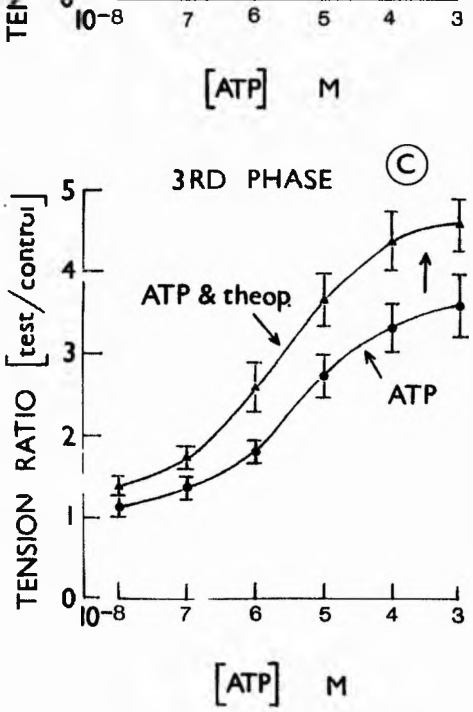
inotropic response shows only a small indication of a negative inotropic action of ATP; the large negative inotropic component seen on exposure of untreated hypodynamic ventricles to 10^{-3}M (Fig.6.2C) is almost absent.

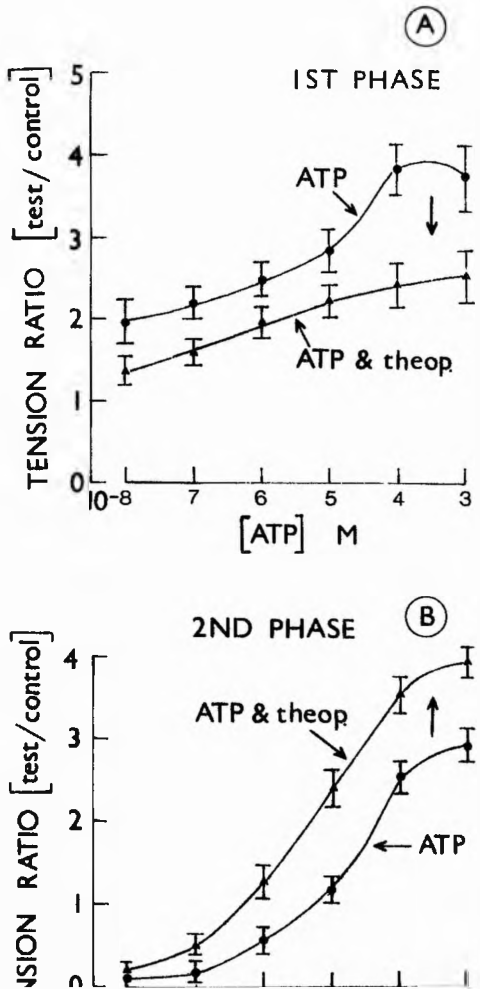
Effect of pretreatment with theophylline alone or with the theophylline and either DB cyclic AMP or 8-Br cyclic GMP on the ATP-induced response: Consider first the experiments concerned with the effects of pretreating the ventricle with theophylline on the form of the ATP response. Fig.6.3A shows a log-dose response curve for the effect of pretreating the hypodynamic ventricle with theophylline (10^{-6} - 10^{-2}M) alone. In the subsequent experiments concerned with the effect of theophylline on the ATP response, a concentration of 10^{-4}M was selected. It can be seen from Fig.6.3A that this concentration produces a relatively small potentiation of the hypodynamic twitch and would not therefore be expected to obscure to any great extent the effect of subsequent administration of ATP. Fig.6.3B shows the form of the response to ATP (10^{-5}M) alone and the response obtained after pretreating 10 ventricles with 10^{-4}M theophylline for a period of 10 minutes. There are two features of interest. First, the initial first component of the ATP response is reduced (from 2.90 to 2.20 x the control level), whereas the third phase of the

Figure 6.4.

Log-dose response curves to varying concentrations (10^{-3} - 10^{-8}M) ATP for the 1st (A), 2nd (B) and 3rd components of the inotropic response in the absence (solid circles) and in the presence of 10^{-4}M theophylline (solid triangles). Each point represents the mean $\pm$ S.E. from 10 preparations. Tension response is expressed as a multiple of the control value prior to ATP stimulation.
The important point to note is that theophylline potentiates the second (B) and third (C) phases but depresses the first (A) (as shown by arrows). The results suggest that under normal condition the 1st response is in reality clipped by the onset of the inhibitory effect.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, 19°C.


TEN
10⁻⁸ 7 6 5 4 3
[ATP] M
3RD PHASE
C
TENSION RATIO [test/control]
0 1 2 3 4 5
ATP & theop
ATP
10⁻⁸ 7 6 5 4 3
[ATP] M


A
1ST PHASE
TENSION RATIO [test/control]
5
4
3
2
1
0
ATP
ATP & theop
10-8
7
6
5
4
3
[ATP] M
B
2ND PHASE
ATP & theop
ATP

response is substantially increased, from around 2.70 x the control value to 3.70 x this level. This is considered to be a significant result within the context of the hypothesis outlined in the preceding chapter, where it was argued that the full size of the first response is prevented from appearing because it is distorted by the rapid onset of the second (inhibitory) component. Thus, theophylline appears to be increasing the magnitude of the negative inotropic component, and since the third phase is substantially increased, that of the secondary positive inotropic component is too. This is consistent with the non-specific nature of the inhibitory effect of theophylline on phosphodiesterase activity; it is known to inhibit both cyclic AMP and cyclic GMP phosphodiesterase activities.

This result is emphasised by the data in Fig.6.4, which shows log-dose response curves for the first (A), second (B) and third (C) components obtained with and without pretreatment with theophylline. The curves for the second component were obtained using the method of analysis outlined earlier (page 106). It can be seen from these data that the second and third components are increased by theophylline pretreatment at all ATP concentrations, whereas the first component is depressed.

Figure 6.5.

(A) Time course of the inotropic response to 10^{-3}M dibutyryl cyclic AMP and theophylline (10^{-4}M) on hypodynamic ventricle and the effect of 10^{-3}M ATP in the continuing presence of dibutyryl cyclic AMP and theophylline. It can be seen that ATP exerts only a large negative inotropic response. Abscissa; Time (min). Ordinate; Relative tension (fraction of initial (control) level).

(B) Inotropic influence of 8-Bromo cyclic GMP on the partially hypodynamic ventricle and the effects of subsequently administering theophylline (10^{-4}M) and ATP (10^{-3}M) in the presence of 8-Bromo cyclic GMP. It should be noted that ATP elicits a large monophasic positive inotropic effect; the negative inotropic component usually seen is almost completely suppressed. Abscissa; Time (min). Ordinate; Relative tension (fraction of initial (control) value.

(C) Effect of 10^{-3}M ATP alone on the hypodynamic ventricle. Note the presence of the three characteristic components of triphasic response.

The solid horizontal lines (in A & B) depict the times of exposing the preparation to the inotropic agents.

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V. Flow rate: 100 $ml.min^{-1}$. Temp, $19^{o}C$.

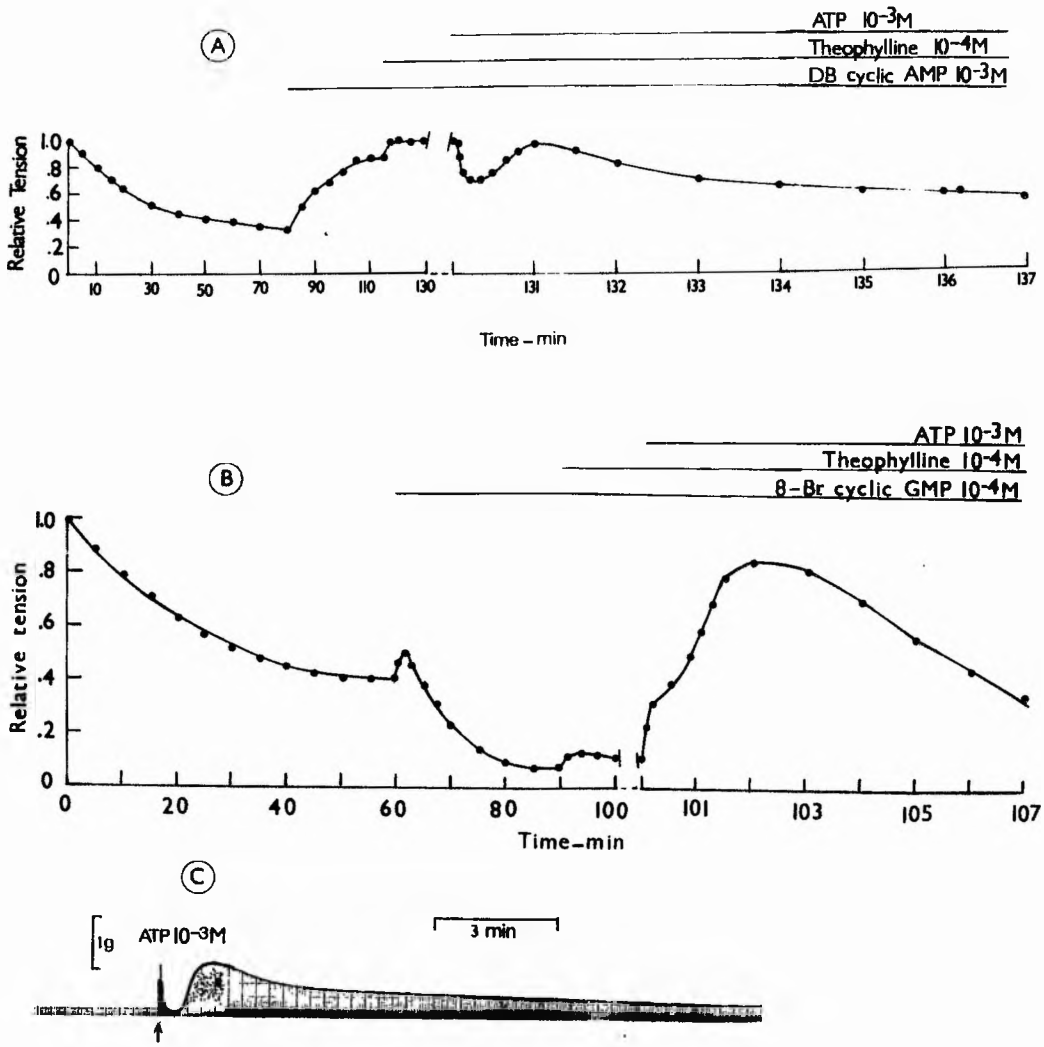

A
ATP 10-3M
Theophylline 10-4M
DB cyclic AMP 10-3M
Relative Tension
1.0
.8
.6
.4
.2
0
10
30
50
70
90
110
130
131
132
133
134
135
136
137
Time – min
B
ATP 10-3M
Theophylline 10-4M
8-Br cyclic GMP 10-4M
Relative tension
1.0
.8
.6
.4
.2
0
0
20
40
60
80
100
101
103
105
107
Time–min
C
1g
ATP 10-3M
3 min

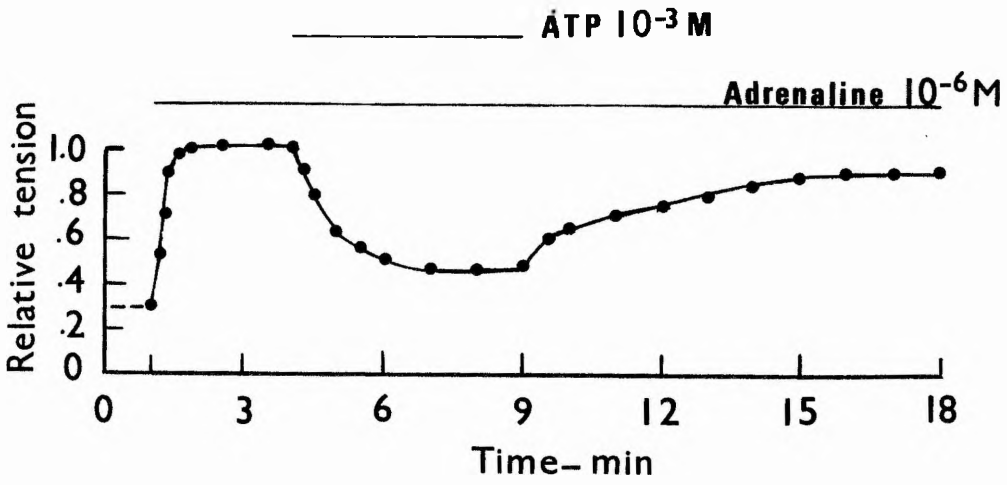
Fig.6.5A & B depict the results obtained in two experiments in which ventricles were pretreated with a combination of DB cyclic AMP (A) or 8-Br cyclic GMP (B) and theophylline. Qualitatively, these data give essentially the same results as were obtained with either DB cyclic AMP or 8-Br Cyclic GMP alone (Fig.6.2). The effect of ATP (10^{-3}M) alone is shown in Fig.6.5C for comparison.

Effects of pretreatment with adrenaline on the ATP response: The action of ATP following pretreatment with adrenaline is shown in Fig.6.6. The hypodynamic ventricle was stimulated with 10^{-6}M adrenaline and 3 minutes from the onset of the response it was treated with ATP (10^{-3}M). This produced a powerful inhibitory response which persisted in the continuing presence of adrenaline. The inhibitory effect was abolished by removing ATP from the superfusate.

Figure 6.6.

Positive inotropic influence of adrenaline (10^{-6}M) on the hypodynamic ventricle and the effect of subsequently administering 10^{-3}M ATP in the continuing presence of adrenaline. The solid horizontal lines indicate the duration of exposing the preparation to adrenaline and ATP. Note the powerful inhibitory effect only elicited by ATP and the augmentation of twitch tension following its removal from the circulating perfusate. Abscissa; Time (min). Ordinate; Relative tension (fraction of initial (control) value).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, 19°C.


ATP 10-3 M
Adrenaline 10-6 M
Relative tension
1.0
.8
.6
.4
.2
0
0
3
6
9
12
15
18
Time– min

D I S C U S S I O N

At the time, the results of these experiments provided sufficient encouragement to warrant embarking on a more detailed investigation of the possible effects of exogenous ATP on cyclic AMP and cyclic GMP levels within the ventricle. These experiments are presented in the next chapter. It will be seen that the results obtained are consistent with the working hypothesis, and for this reason, it is felt that a more detailed discussion of the present experiments would be superfluous. It is sufficient to note here that the hypothesis was able to predict, with some accuracy, the qualitative nature of the responses obtained as a result of manipulating the intracellular levels of each cyclic 3',5'-nucleotide separately, either directly, with suitable exogenous derivatives, or indirectly by using the appropriate pharmacological agents.

C H A P T E R VII

DIRECT EVIDENCE FOR AN EFFECT OF EXOGENOUS ATP ON INTRACELLULAR CYCLIC 3', 5' - NUCLEOTIDE LEVEL AND ITS RELATION TO THE CONTRACTILE RESPONSE.

INTRODUCTION

In these series of experiments ventricles were first rendered hypodynamic (using 'standard' conditions) and then subjected to treatment with 10^{-3}M ATP. At a pre-determined time during the response the ventricle was 'crush-frozen', extracted and then assayed for cyclic AMP, cyclic GMP and total protein using methods outlined in appendix II A-C. Untreated, hypodynamic half ventricles served as controls, and care was taken to ensure that the 'test' preparations were permitted to become hypodynamic to exactly the same degree as the control ones prior to the addition of ATP.

Time course of changes in cyclic AMP and cyclic GMP levels during the ATP-induced response: Fig.7.1A shows a representative sample of original chart recordings for an experiment of this type. The times at which different preparations were frozen and assayed are indicated in Fig.7.1B. Cyclic nucleotide levels in test and control ventricles were expressed originally in pmoles mg^{-1} of ventricle protein (see Table 7.1, appendix VII) and the ATP-induced levels expressed graphically as multiples of the control levels. (This was necessary because each data

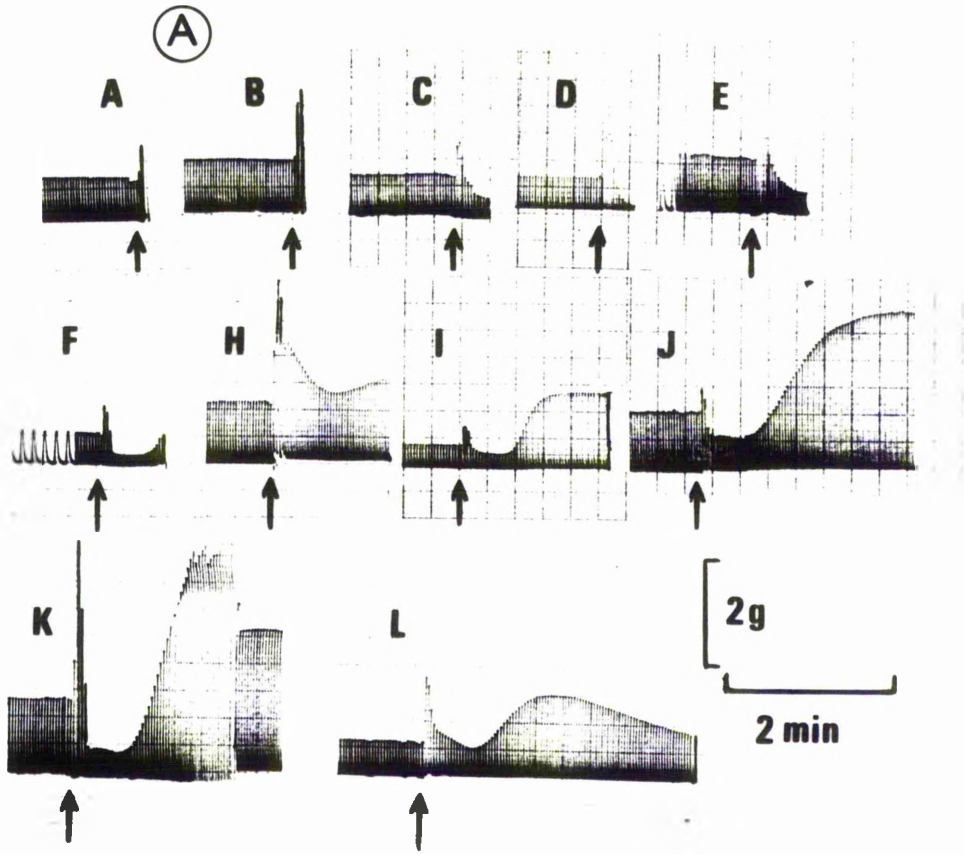
Figure 7.1.

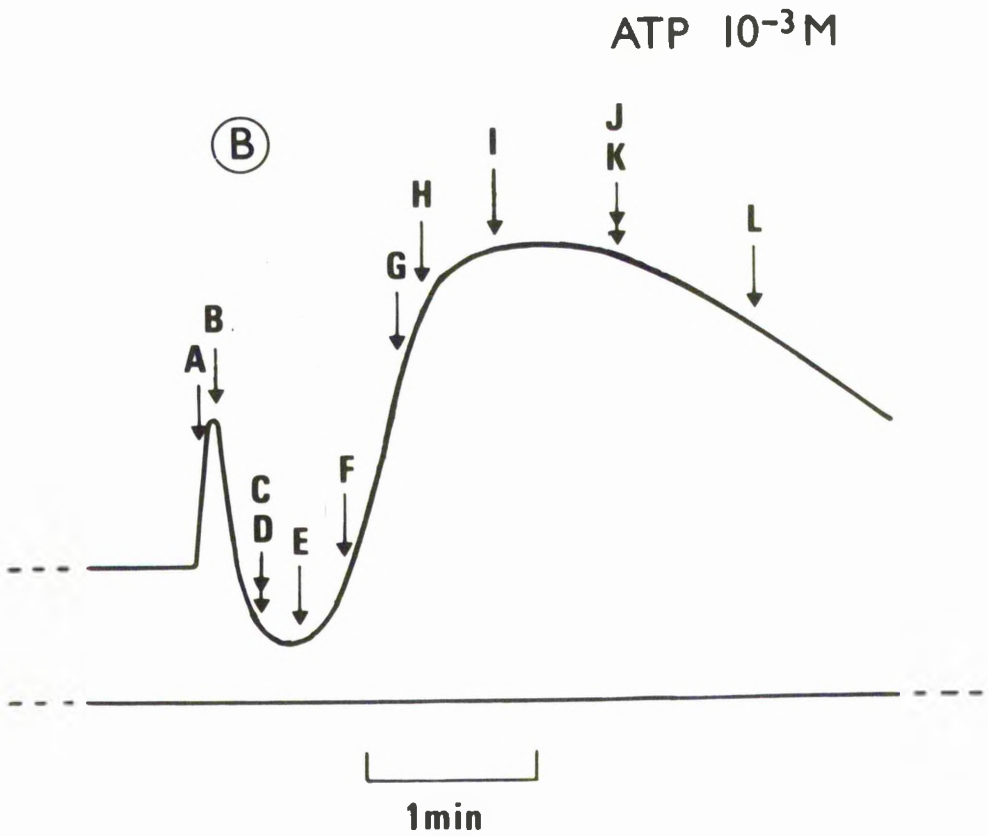
(A) A representative sample of original chart recordings (A - L) showing inotropic responses during exposure to 10^{-3}M ATP and the times of 'crush freezing'. The arrows indicate the times of application of ATP.

(B) A typical response to 10^{-3}M ATP. The letters A - L indicate the times at which the different preparations were frozen and assayed for cyclic AMP, cyclic GMP and total proteins.

Mean values ($\pm$ S.E's) for levels of cyclic AMP and cyclic GMP on the control (hypodynamic) ventricles (12 preparations) were 8.05 $\pm$ 0.43 pmol mg^{-1} protein and 0.91 $\pm$ 0.13 pmol mg^{-1} protein.

Stimulation parameters: Pulse width; 5 msec; frequency, 30 min^{-1}; amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, 18.5°C.


A
A
B
C
D
E
F
H
I
J
K
L
2g
2 min


ATP 10⁻³M
B
A
B
C
D
E
F
G
H
I
J
K
L
1min

point came from a different heart; the quantity of tissue available was too small to enable time course experiments to be made on a single heart).

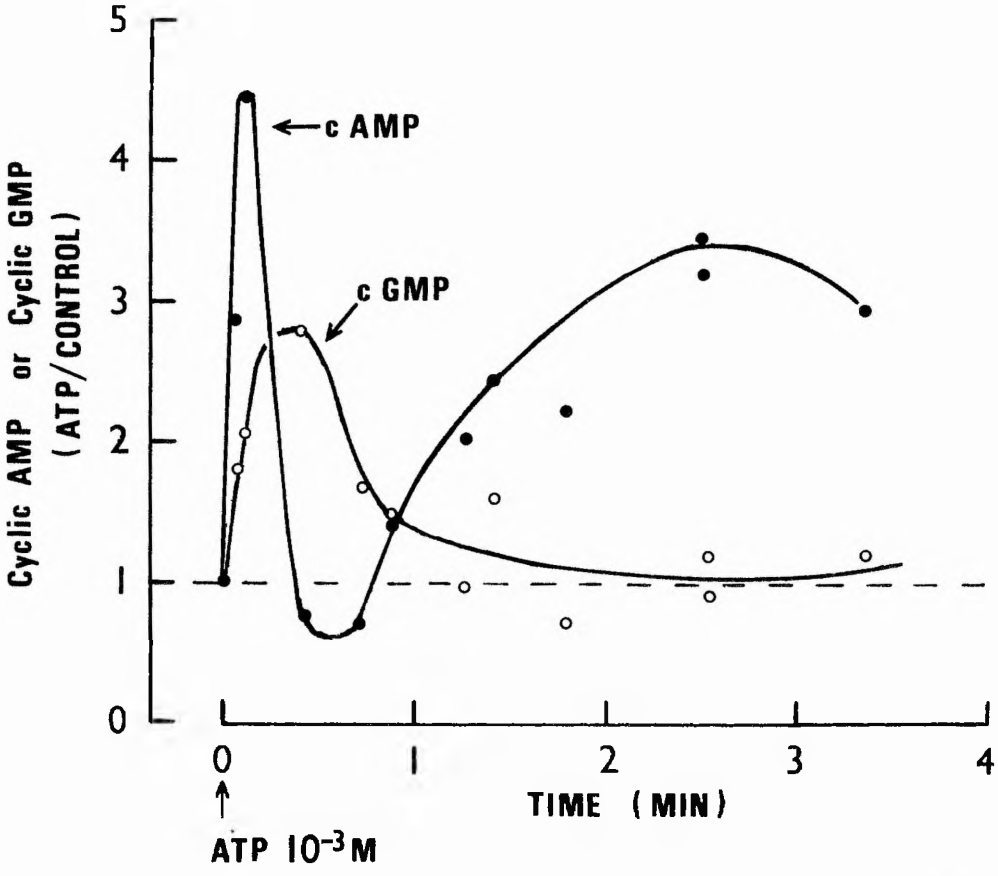
Fig.7.2 shows the time course of the changes in cyclic AMP (solid circles) and cyclic GMP (open circles) during the response to 10^{-3}M ATP. ATP induces a large and rapid increase in cyclic AMP, up to 4.5 x control level attained within approximately 5 - 8 seconds, followed by an equally abrupt fall, to around 0.6 x control level, reached some 30 - 40 seconds after the application of ATP. Cyclic AMP levels then rise again, more slowly, reaching a secondary peak of around 3.4 x the control value after 140 - 160 seconds. Thereafter, the level declines slowly.

The initial rapid rise and fall of cyclic AMP is accompanied by a slower rise and fall in cyclic GMP levels. Cyclic GMP reaches a peak of 2.8 x the control level within 20 - 25 seconds. It then returns slowly towards the control level, reaching it at around 80-100 seconds and remaining there throughout the rest of the response.

There are two important points to notice about these results. First, it is clear that ATP alters the levels of <u>both</u> cyclic AMP and cyclic GMP, and the relationship between their altered levels and isometric force is considered in the

Figure 7.2.

Time course of changes in levels of cyclic AMP (solid circles) and cyclic GMP (open circles), expressed as multiples of control hypodynamic values, measured at different times during the ATP (10^{-3}M) - induced inotropic responses (see Fig.7.1). The control level is indicated by the horizontal broken line. Note (a) the initial rapid rise and fall in cyclic AMP levels, followed by a slow secondary rise, (b) elevated levels of cyclic GMP correspond closely with the maximum inhibitory response and (c) cyclic GMP levels peak when cyclic AMP levels fall. Abscissa; Time (min). Ordinate; cyclic AMP and cyclic GMP levels (fraction of the control value).


5
4
3
2
1
0
c AMP
c GMP
Cyclic AMP or Cyclic GMP
(ATP/CONTROL)
0
1
2
3
4
TIME (MIN)
ATP 10^{-3}M

next section. Secondly, it is worth drawing attention to the fact that cyclic GMP levels peak at a time which coincides with the maximum rate of fall in cyclic AMP levels, and that cyclic AMP levels begin to rise again when cyclic GMP starts to fall. Taken at face value, this observation would suggest that cyclic GMP inhibits the formation of cyclic AMP and/or stimulates its conversion to 5' - AMP by phosphodiesterase activity.

Relationship between cyclic 3', 5' - nucleotide levels and contractile force: The interesting relationship found earlier between contractility and cyclic 3', 5' - nucleotide levels (specifically the ratio cyclic AMP/cyclic GMP) during the development of the hypodynamic condition is also seen to hold in the case of the ATP - induced response.

Fig.7.3A shows (A) the time course of the change in contractile force, indicated by the closed circles and by the solid line drawn in by eye to show the general shape of the responses; and (B) the corresponding changes in the ratio of cyclic AMP/cyclic GMP (open circles) in the presence of ATP, expressed as a multiple of the corresponding ratio for the control (hypodynamic) ventricle; viz:

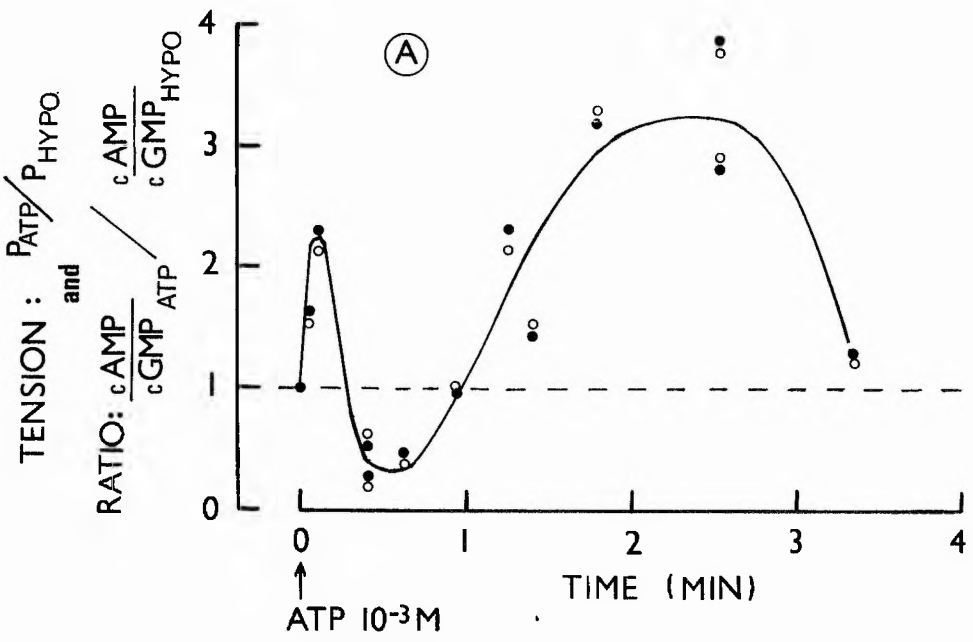
Figure 7.3.

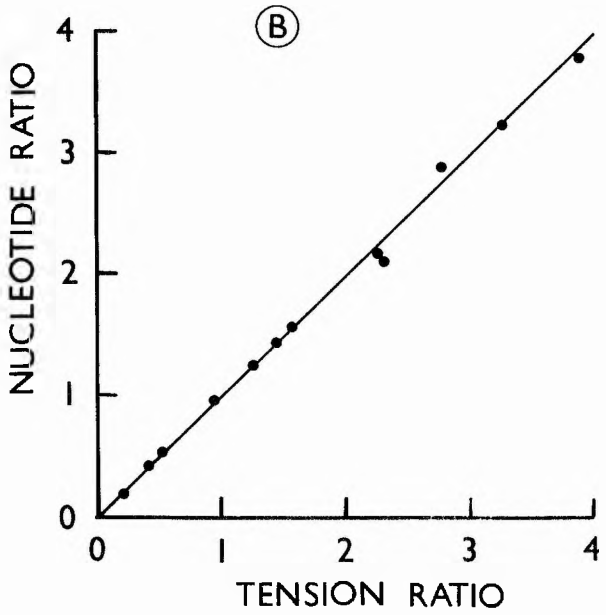
Data taken from Fig.7.1. and 7.2.

(A) Time course of changes in ratio of cyclic AMP/cyclic GMP (open circles) together with the accompanying contractile response (solid circles). Cyclic nucleotide ratio and isometric force are expressed as multiples of the control (hypodynamic) values. The solid line is drawn by eye to indicate the general shape of the response. The control level is indicated by the horizontal broken line. Abscissa; Time (min). Ordinate, Tension ratio $\left[P(ATP)/P(hypo)\right]$ and cyclic nucleotide ratio

$$\left[\frac{\text{cyclic AMP/cyclic GMP (ATP)}}{\text{cyclic AMP/cyclic GMP (hypo)}}\right]$$

(B) A graphical illustration of the relation between twitch tension and cyclic nucleotide ratio. The solid line is drawn with a slope of 1 to emphasise the fact that there is a direct 1:1 proportionality between these two parameters. Correlation coefficient $\pm$ S.E. of estimate: 0.98 ± 0.35, $n = 12$, $P < 0.001$.


A
TENSION : P_{ATP}/P_{HYPO}
and
RATIO: $\frac{cAMP}{cGMP}_{ATP}$ / $\frac{cAMP}{cGMP}_{HYPO}$
4
3
2
1
0
0
1
2
3
4
TIME (MIN)
ATP 10^{-3}M


B
NUCLEOTIDE RATIO
4
3
2
1
0
0
1
2
3
4
TENSION RATIO

$$\frac{\text{cyclic AMP/cyclic GMP (ATP)}}{\text{cyclic AMP/cyclic GMP (Control)}}$$

Once again, there is a striking correlation; the change in cyclic nucleotide ratio almost exactly parallels the change in contractile force. This relationship is emphasised further by plotting the cyclic nucleotide ratio against contractile force, as in Fig.7.3B. The data fall almost exactly on the solid straight line which was drawn in by eye to indicate the results to be expected on the basis of a linear 1:1 correlation. Statistically, the correlation is highly significant: correlation coefficient $\pm$ S.E. of estimate: 0.98 ± 0.35, $n = 12$, $P < 0.001$).

Compared to this, the correlation between the change in contractility and the change in either cyclic AMP or cyclic GMP levels is less convincing, although significant. These data are plotted separately in Fig.7.4A & B. The data for cyclic AMP yield correlation coefficient ($\pm$ S.E.) of 0.73 ± 0.46 ($n = 12$; $P < 0.01$) and those for cyclic GMP, -0.69 ± 0.36 ($n = 12$; $P < 0.01$). The raw data for

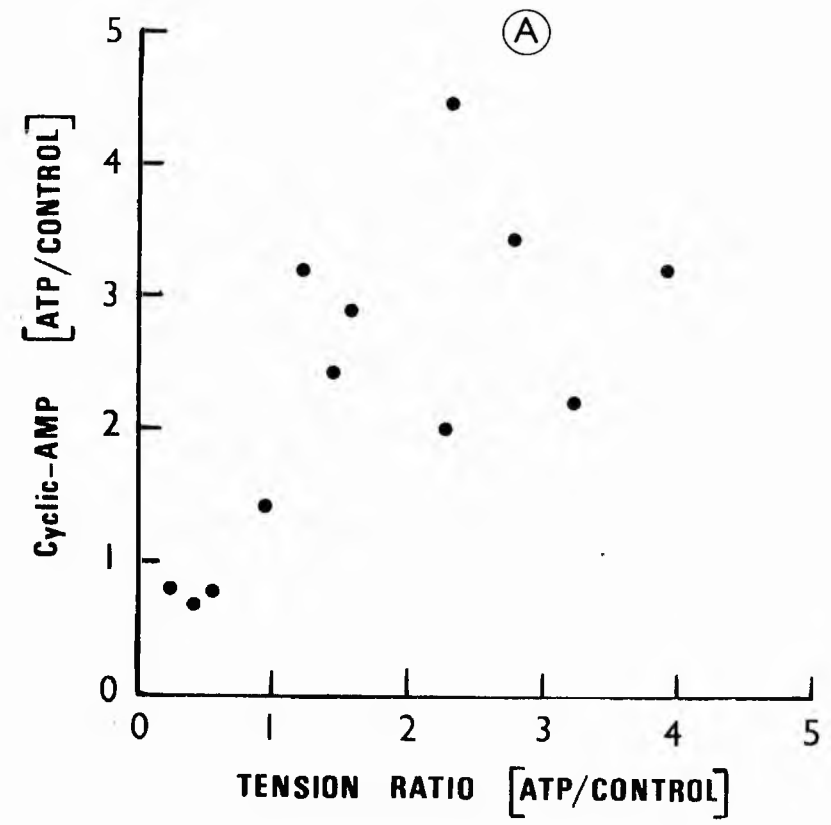
Figure 7.4.

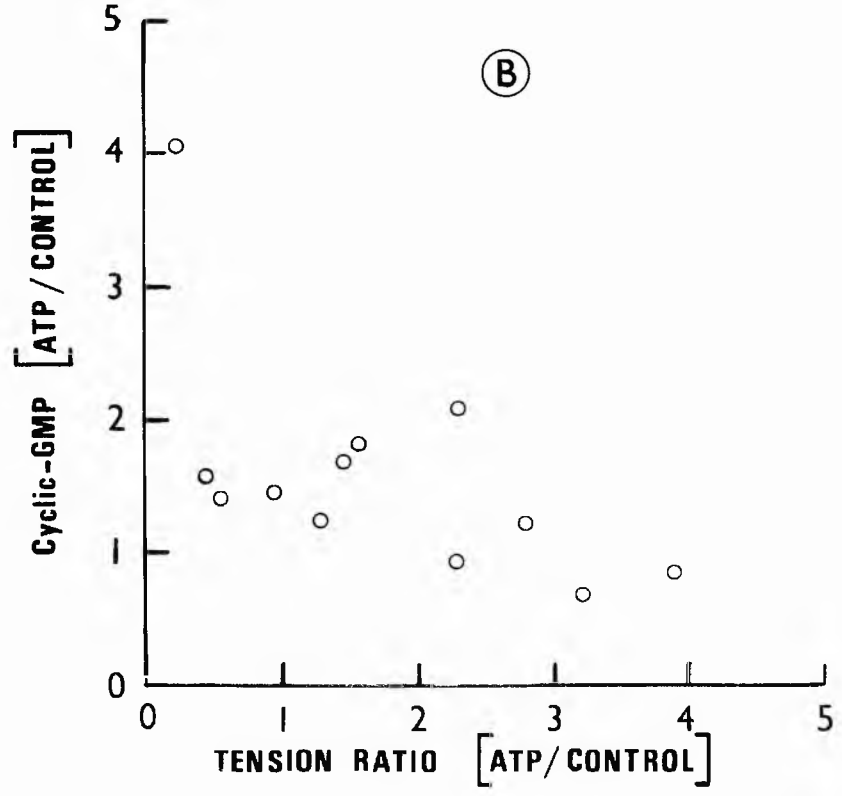
Data taken from Fig.7.1 and 7.2.

(A) Relationship between changes in contractile force and cyclic AMP levels (multiples of control (hypodynamic) values) during exposure to 10^{-3}M ATP. Correlation coefficient $\pm$ S.E. of estimate: 0.73 ± 0.46, $n = 12$, $P < 0.001$. Abscissa; Tension (ATP/control) Ordinate; cyclic AMP levels (ATP/control).

(B) Relation between changes in cyclic GMP levels and isometric force (multiples of control (hypodynamic) values) following stimulation with 10^{-3}M ATP. Correlation coefficient $\pm$ S.E. of estimate: -0.69 ± 0.36, $n = 12$, $P < 0.001$.

Note that the correlation between contractility and cyclic 3', 5'- nucleotide levels is positive in the case of cyclic AMP and negative in the case of cyclic GMP.


A
Cyclic-AMP [ATP/CONTROL]
TENSION RATIO [ATP/CONTROL]
0
1
2
3
4
5


B
Cyclic-GMP [ATP/CONTROL]
TENSION RATIO [ATP/CONTROL]
0
1
2
3
4
5

this experiment are collected in table 7.1, appendix VII.

D I S C U S S I O N

The experiments described in this chapter support the view that the triphasic response is in reality a manifestation of two opposing influences of ATP. They clearly indicate that ATP has a marked effect on intracellular levels of both cyclic AMP and cyclic GMP, in addition to its effect on ionic conductance at the surface membrane.

The close correlation between contractile force and cyclic nucleotide ratio, similar to that seen during the development of hypodynamic depression, is especially significant. The form of the ATP - induced response is unquestionably bizarre, yet the results yield a correlation between contractile force and cyclic nucleotide ratio which is just as convincing as that found during the development of the hypodynamic condition. Accordingly, it is felt that this result substantially reinforces the suggestion that both cyclic 3', 5' - nucleotides play an important role in regulating the contractile capacity of the ventricle, and that this is not exclusively a property of cyclic AMP alone.

C H A P T E R VIII

AN INVESTIGATION INTO THE MECHANISM OF ACTION OF ATP AND SOME RELATED PURINE AND PYRIMIDINE NUCLEOTIDES ON VENTRICULAR CONTRACTILITY.

INTRODUCTION

It is known from previous work that other nucleosides and their nucleotide derivatives have inotropic effects on the frog ventricle (see main Introduction, Part II) although the form of the response may differ from that seen with ATP. The experiments described in this Chapter are concerned with the effects of several ATP analogues on the form and size of the contractile response. This comparative approach is used in an attempt to relate molecular structure to differing pharmacological effects of the various agents used on the ventricle. The real point of issue is: what is the nature of the receptors (Burnstock, 1972)?

It was concluded from experiments presented earlier, concerning the classical receptor blocking agents atropine and propranolol, that ATP at least appears not to exert its actions either by releasing endogenous neurotransmitters, on the one hand, or by combining directly with cholinergic or β-adrenergic receptors on the other; neither atropine nor propranolol had any marked effects on the ATP-induced response. This result holds too for the other agents tested.

The various analogues of ATP which have been studied

produce differing responses. They can be broadly classified into groups on the basis of either (i) the form of the inotropic response; or (ii) whether they produce predominantly positive or predominantly negative inotropic effects; or (iii) on the basis of their relative potencies. For ease of description the various agents are here classified according to the form of the inotropic response, although emphasis is given, where appropriate, to their relative potencies.

Broadly speaking, the responses can be classified into one of three groups:

Type A response: This is the typical ATP-induced response, comprising three more or less distinct phases, already described in some detail and depicted diagrammatically in Fig.8.1A. This class includes ADP, AMP and CTP (concentration range from 10^{-7} to 10^{-3}M) as well as ATP.

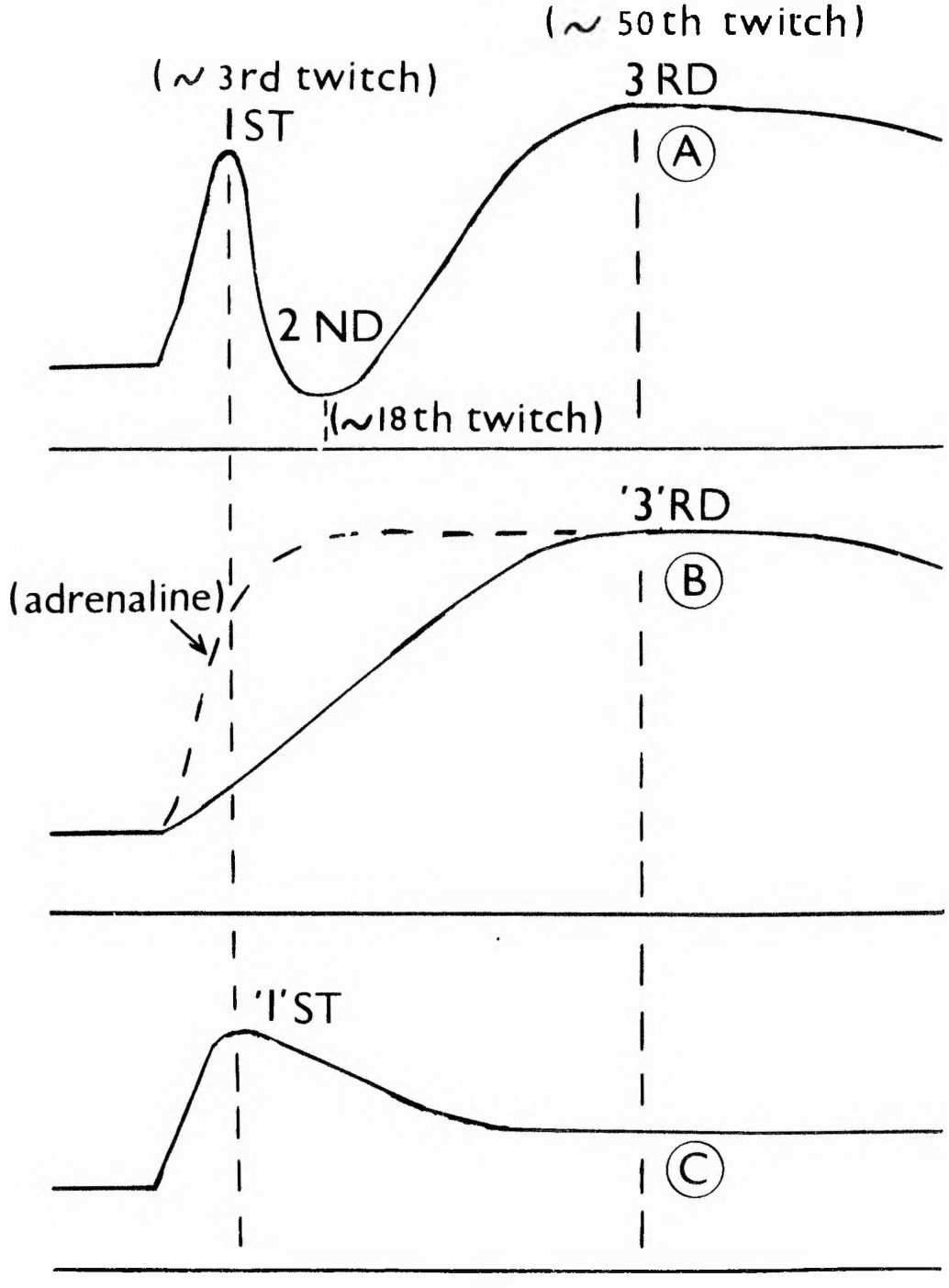
Type B response: This is a relatively-slowly developing positive inotropic response (Fig.8.1B). It reaches its maximum at a time corresponding to the peak of the third component of the type A response (approximately twitch No.50). ITP, GTP and UTP in concentrations ranging from 10^{-9} to 10^{-4}M produce type B responses. The inotropic effects of these agents develop more slowly than the

Figure 8.1.

(A) A graphical illustration of the characteristic triphasic inotropic response (Type A) induced by compounds such as ATP, ADP, AMP and CTP (range from 10^{-7} to 10^{-3}M). For analysing the magnitude of the 1st, 2nd and 3rd phases, the 3rd, 18th and 50th twitches were used respectively.

(B) The form of type B response (slowly-developing with peak tension (approximately 50th twitch) coinciding approximately with that for 3rd component of type A response induced by UTP, ITP and GTP (range from 10^{-9} to 10^{-3}M). The dashed curve illustrates the nature of the response elicited by adrenaline.

(C) A diagrammatic representation of type C (rapidly-developing, positive inotropic) response induced by ATP, ADP, AMP and CTP (concentrations 10^{-7}M). Note that this is a variant of type A response.


(~ 50th twitch)
(~ 3rd twitch)
3 RD
1 ST
A
2 ND
(~18th twitch)
'3'RD
(adrenaline)
B
'1'ST
C

response to adrenaline (dashed curve in Fig.8.1B).

Type C response: This is characterised by a rapidly - developing, positive inotropic response which reaches a maximum at a time coinciding with the first component of the type A response. Strictly speaking, it should perhaps be considered as a variant of the type A response, since it is shown by type A agents when they are used in very low concentrations ($< 10^{-7}$M). It is illustrated diagrammatically in Fig.8.1C.

Qualitative nature of type A and B responses and molecular structure.

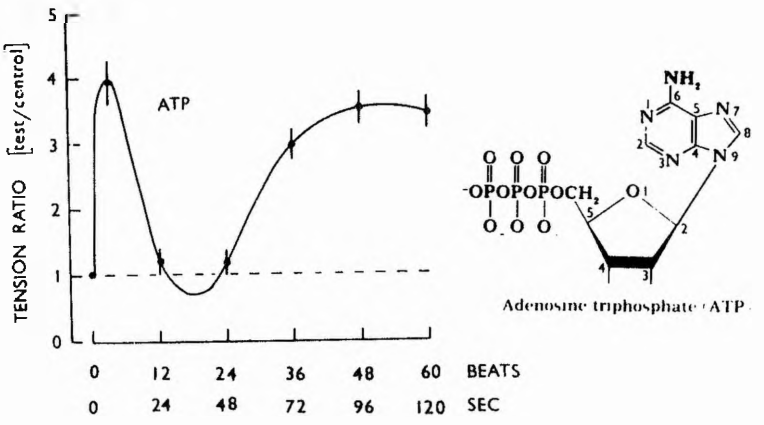
Fig.8.2 and 8.3 show the characteristic forms of the type A and type B responses respectively. Their molecular structures are also shown. The data presented are from 10 experiments at a concentration of 10^{-4}M and each point represents the mean value $\pm$ S.E.

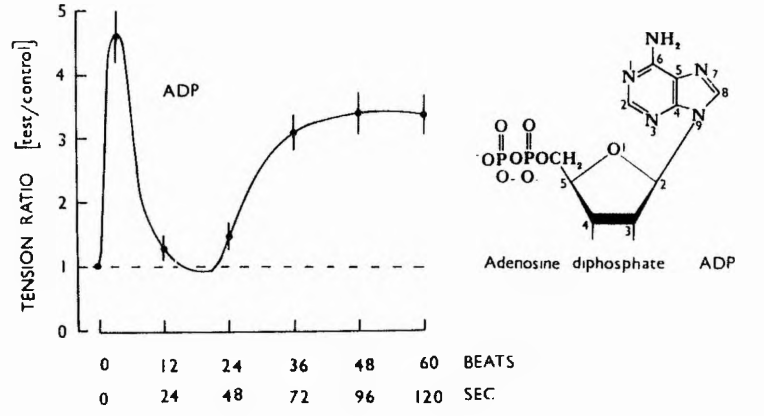
The type A agents studied included ATP, ADP and AMP (purine based nucleotides) and CTP (a pyrimidine based nucleotide). The type B agents studied included UTP (a pyrimidine based nucleotide) and ITP and GTP (both

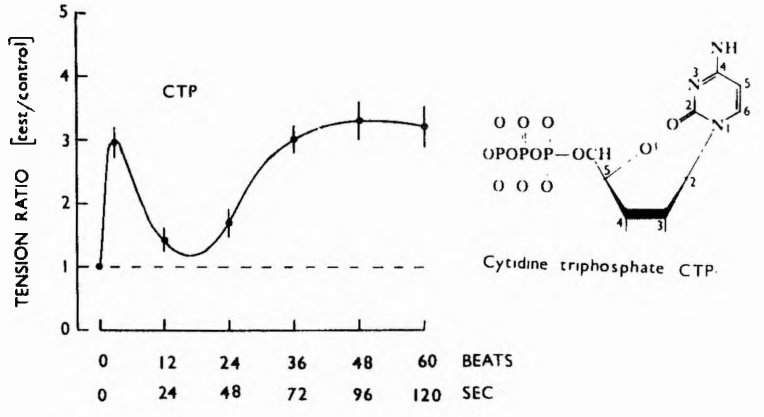
Figure 8.2.

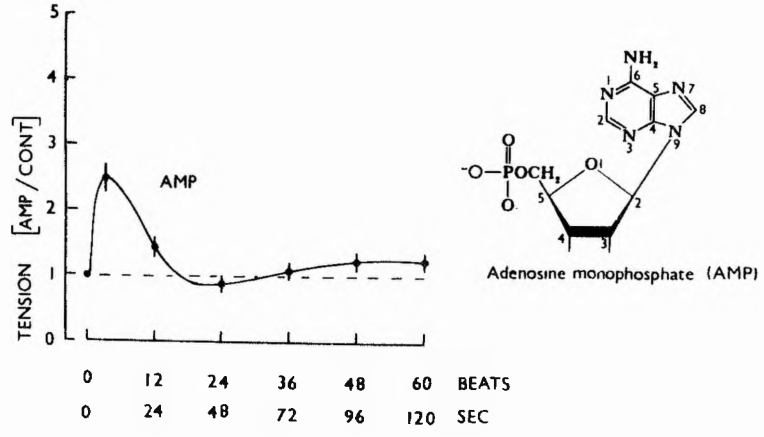
Time course of the changes in contractile force during exposure of the hypodynamic ventricle to 10^{-4}M type A agents (ATP, ADP, CTP and AMP). The molecular structure for each compound is shown for comparison. Tension is expressed as a multiple of the control value. Each point represents the mean $\pm$ S.E. taken from 10 preparations. Control tension level is indicated by the horizontal broken line. Note the triphasic form of the inotropic response induced by these compounds and their common structural features; namely, the presence of a phosphate group together with an $-NH_2$ moeity which is located at 6C (purine ring) or 4C (pyrimidine ring) position. Abscissa; Number of beats; Time (sec). Ordinate; Tension ratio (test/control).

Stimulation parameters: Pulse width, 5 msec; amplitude, 10V; frequency, 30 min^{-1}. Flow rate: 100 $ml.min^{-1}$. Temp, 19°C.


ATP
TENSION RATIO [test/control]
0 12 24 36 48 60 BEATS
0 24 48 72 96 120 SEC
Adenosine triphosphate (ATP)


ADP
TENSION RATIO [test/control]
0 12 24 36 48 60 BEATS
0 24 48 72 96 120 SEC
Adenosine diphosphate ADP


CTP
TENSION RATIO [test/control]
0 12 24 36 48 60 BEATS
0 24 48 72 96 120 SEC
Cytidine triphosphate CTP


AMP
TENSION [AMP/CONT]
0 12 24 36 48 60 BEATS
0 24 48 72 96 120 SEC
Adenosine monophosphate (AMP)

purine based nucleotides).

The principal conclusions to be drawn are as follows:

(i) The classification into type A and type B responses does not correspond with the classification of the agents into either purine or pyrimidine based nucleotides.

(ii) There is a common structural feature to those agents which produce a triphasic (type A) response; namely, the presence of a phosphate group together with an $-NH_2$ group which is located on the 6C (purine ring) or on the 4C (pyrimidine ring) position.

(iii) It appears from the results obtained with the adenine nucleotides that the amplitude of the third phase depends upon the number of terminal phosphate groups. Thus, the amplitude of the third phase is in the order: ATP $>$ ADP $>$ AMP. There is also an effect on the first phase, but this is less pronounced.

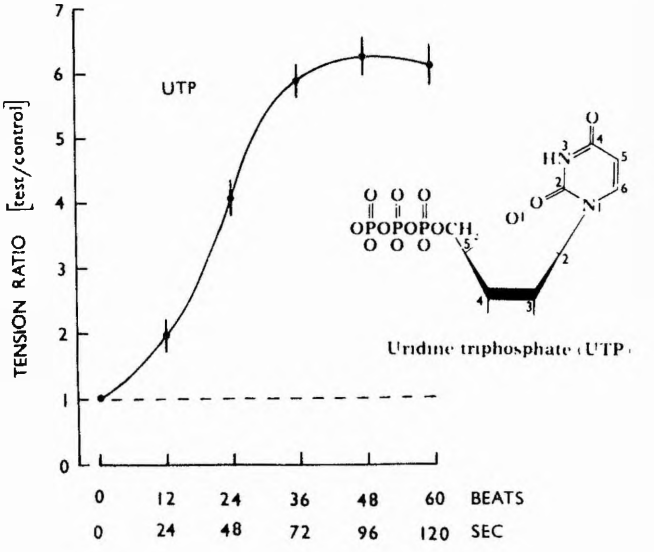
(iv) The molecular feature common to type B agents is the presence of a ketone group located at the same position as that occupied by the $-NH_2$ group in the case of the type A agents i.e. on the 6C (purine based nucleotides; ITP, GTP) or on the 4C (UTP; a pyrimidine based nucleotide) position.

Thus, the presence of the initial rapidly-developing inotropic

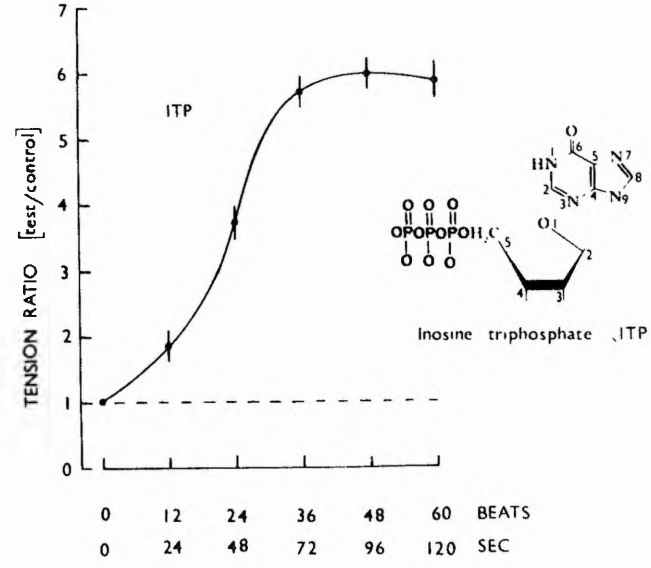
Figure 8.3.

Effect of 10^{-4}M type B agents (UTP, ITP and GTP) on the force of contraction of the hypodynamic ventricle. The molecular structure of each compound is shown for comparison. Tension is expressed as a multiple of the control value. Each point is the mean $\pm$ S.E. taken from 10 preparations. Control level is depicted by the horizontal broken line. Note that these compounds produce a relatively slowly-developing positive inotropic response and structurally, each possesses a terminal phosphate together with a ketone group at 6C (purine ring) or 4C (pyrimidine ring) position. It is interesting to note also that GTP has in addition, an $-NH_2$ group at 2C position of the purine ring and that the magnitude of the contractile response is far less than that observed with either UTP or ITP. Abscissa; Time (sec); Number of beats. Ordinate; Tension ratio (test/control).

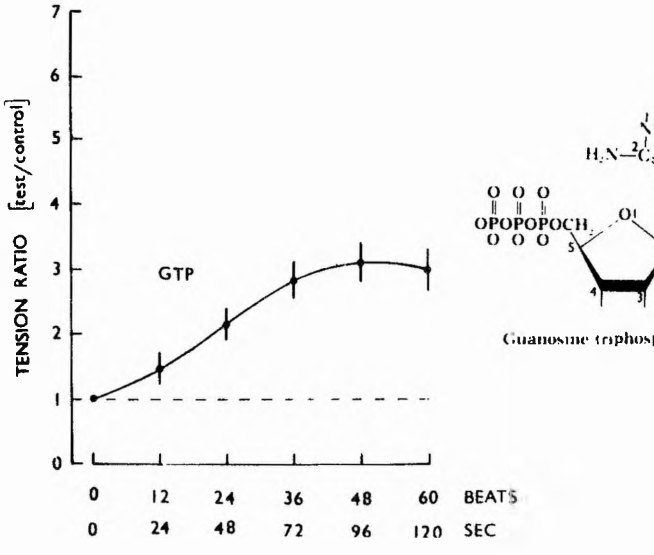
Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, $19^{o}C$.



Uridine triphosphate (UTP)



Inosine triphosphate (ITP)



Guanosine triphosphate (GTP)

response, seen in the case of type A agents, appears to require an $-NH_2$ group on the nucleotide base and a ribose phosphate.

(v) Of the type B agents studied, GTP gives a substantially smaller response than either ITP or UTP. The principal structural difference between ITP and UTP on the one hand, and GTP on the other, is the presence of an $-NH_2$ group in 2C position in the purine ring. The significance of this is emphasised by comparing GTP with ITP, both of which are purine based nucleotides.

It is worth mentioning briefly here that these formulae are planer projection of three dimensional structure. The apparent positional correspondence of the $-NH_2$ and $>C = O$ groups may not therefore reflect a true spacial correspondence.

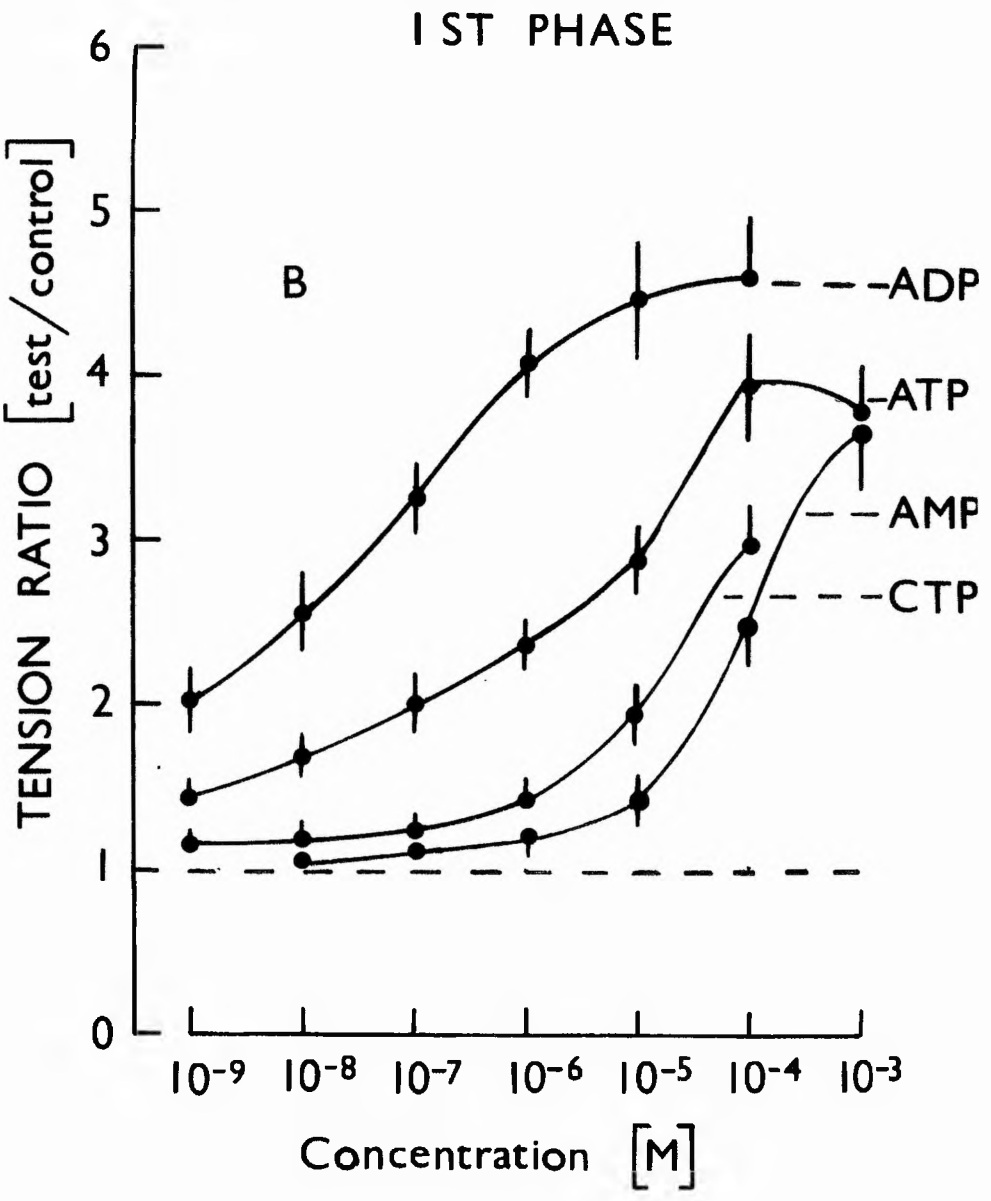
Quantitative observations on type A and type B responses and molecular structure.

In these experiments, the effects of differing concentrations of agents were studied, ranging from 10^{-9} to 10^{-3}M. The amplitude of the responses during the first, second and

Figure 8.4.

Concentration dependence of type A agents (ATP, ADP, AMP and CTP, range from 10^{-9} to 10^{-3}M) on contractile force during the first phase (3rd twitch) of the response. Tension is expressed as a multiple of the control value. Each point represents mean value $\pm$ S.E. taken from 10 preparations. The control tension response is depicted by the horizontal broken line.
Note the order of relative potencies of type A agents: ADP > ATP > AMP > CTP.
Abscissa; concentration (M). Ordinate; Tension ratio (test/control).

Stimulation parameters: Pulse width, 5 msec; amplitude, 10V; frequency, 30 min^{-1}.
Flow rate: 100 $ml.min^{-1}$. Temp, 19°C.


1 ST PHASE
B
TENSION RATIO [test/control]
6
5
4
3
2
1
0
ADP
ATP
AMP
CTP
10-9 10-8 10-7 10-6 10-5 10-4 10-3
Concentration [M]

third phases were measured at twitch numbers 3, 18 and 50 respectively (Fig.5.8; vertical dashed lines).

Log-dose response curves for the first component (type A agents only): Log-dose response curves for the initial, fast-developing positive inotropic effects (1st phase) for the type A agents is shown in Fig.8.4. The relative potencies are in the order: ADP $>$ ATP $>$ CTP $>$ AMP. These results can be expressed more quantitatively in terms of the dose required to produce a half maximal response (ED_{50}). These were as follows: ADP, 4×10^{-8}M; ATP, 1.5×10^{-6}M; CTP, 4×10^{-5}M; AMP, 4.5×10^{-5}M. Assigning the efficacy of AMP as unity, then CTP is 1.13x; ATP, 30x and ADP, 1,130x as effective. The maximum potentiations observed at 10^{-4}M (expressed as a multiple of the control value) were: ADP, 4.55 ± 0.30 x; ATP, 3.95 ± 0.30x; CTP, 2.98 ± 0.22x and AMP, 2.50 ± 0.17x. This particular concentration is the same as the one used for the experiments shown in Fig.8.2.

Log-dose response curves for the second component (type A) agents only: The method used to analyse the size of the second component was described previously (see Chapter V) and is shown diagrammatically in Fig.8.5A. Fig.8.5B shows log-dose response curves for the observed

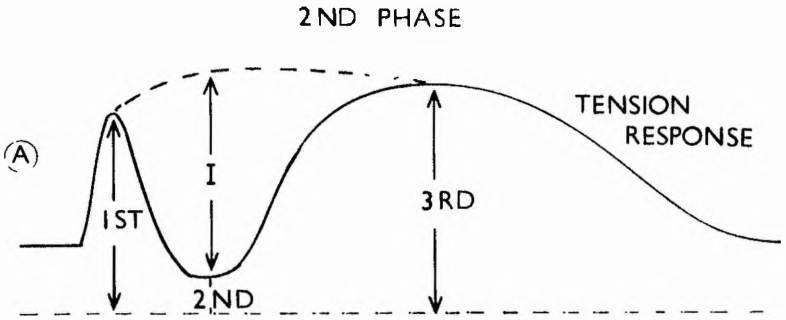
Figure 8.5.

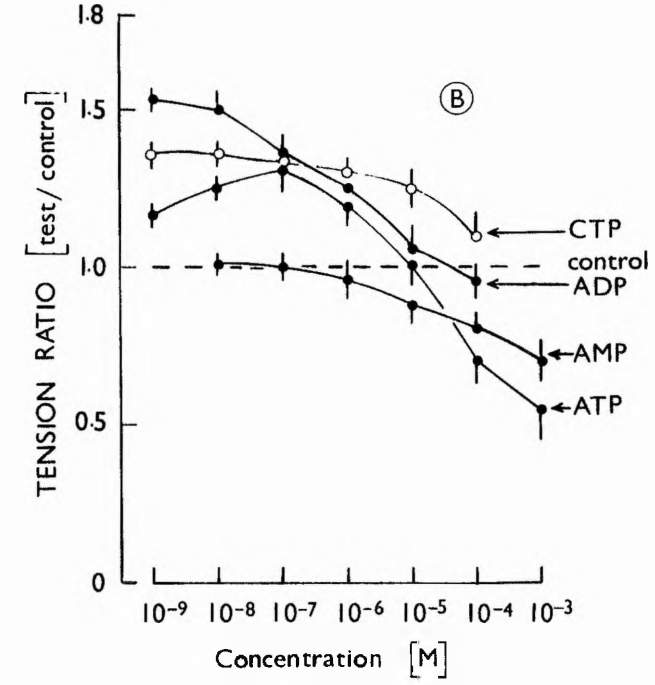
(A) A diagrammatic illustration of the inhibitory effect of the 2nd phase. The inhibitory effect (I) is obtained by subtracting the observed tension during the 2nd component from the mean value of the 1st and 3rd phases. The results of 10 experiments for type A agents (range from 10^{-9} to 10^{-3}M) are shown in (C).

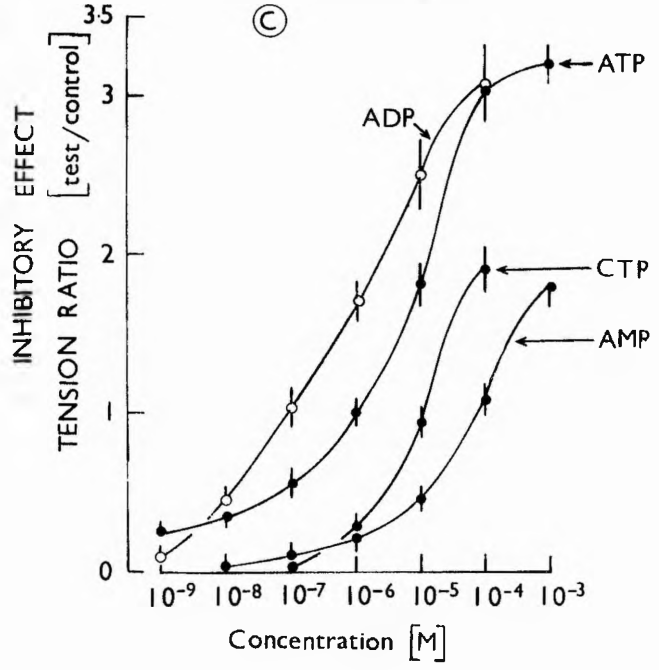
(B) Log-dose response curves for the observed tension responses elicited by type A agents (ATP, ADP, CTP and AMP) at concentrations ranging from 10^{-9} to 10^{-3}M. Note the decline in tension to below the control (hypodynamic) level (horizontal broken line) in the presence of high concentrations of ATP, ADP and AMP. Abscissa; Concentration (M). Ordinate; Tension ratio (expressed as multiples of control values).

(C) Log-dose response curves for the inhibitory effect of the 2nd phase of type A (ATP, ADP, CTP and AMP) agents-induced inotropic responses. Abscissa; Concentration (M). Ordinate; Inhibitory effect, tension ratio (expressed as multiples of control values).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min^{-1}; amplitude, 10V.
Flow rate: 100 $ml.min^{-1}$. Temp, $19^{o}C$.


2ND PHASE
TENSION RESPONSE
A
I
1ST
2ND
3RD


B
TENSION RATIO [test/control]
1·8
1·5
1·0
0·5
0
CTP
control
ADP
AMP
ATP
10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³
Concentration [M]


C
INHIBITORY EFFECT
TENSION RATIO [test/control]
3·5
3
2
1
0
ATP
ADP
CTP
AMP
10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³
Concentration [M]

tension response, and Fig.8.5C after analysis.

The relative potencies of the type A agents during the second phase are in the order: ADP > ATP > CTP > AMP. Corresponding ED_{50}'s are as follows:- ADP, 7.5×10^{-7}M; ATP, 7.5×10^{-6}M; CTP, 1×10^{-5}M; AMP, 2×10^{-5}M. Again taking the efficacy of AMP as unity, CTP is 2x; ATP, 2.66x and ADP, approximately 26x as effective.

The maximum twitch potentiations observed (as multiples of control values; 10^{-4}M) were ADP, 3.10 ± 0.26; ATP, 3.05 ± 0.17; CTP, 1.92 ± 0.12; AMP, 1.1 ± 0.08.

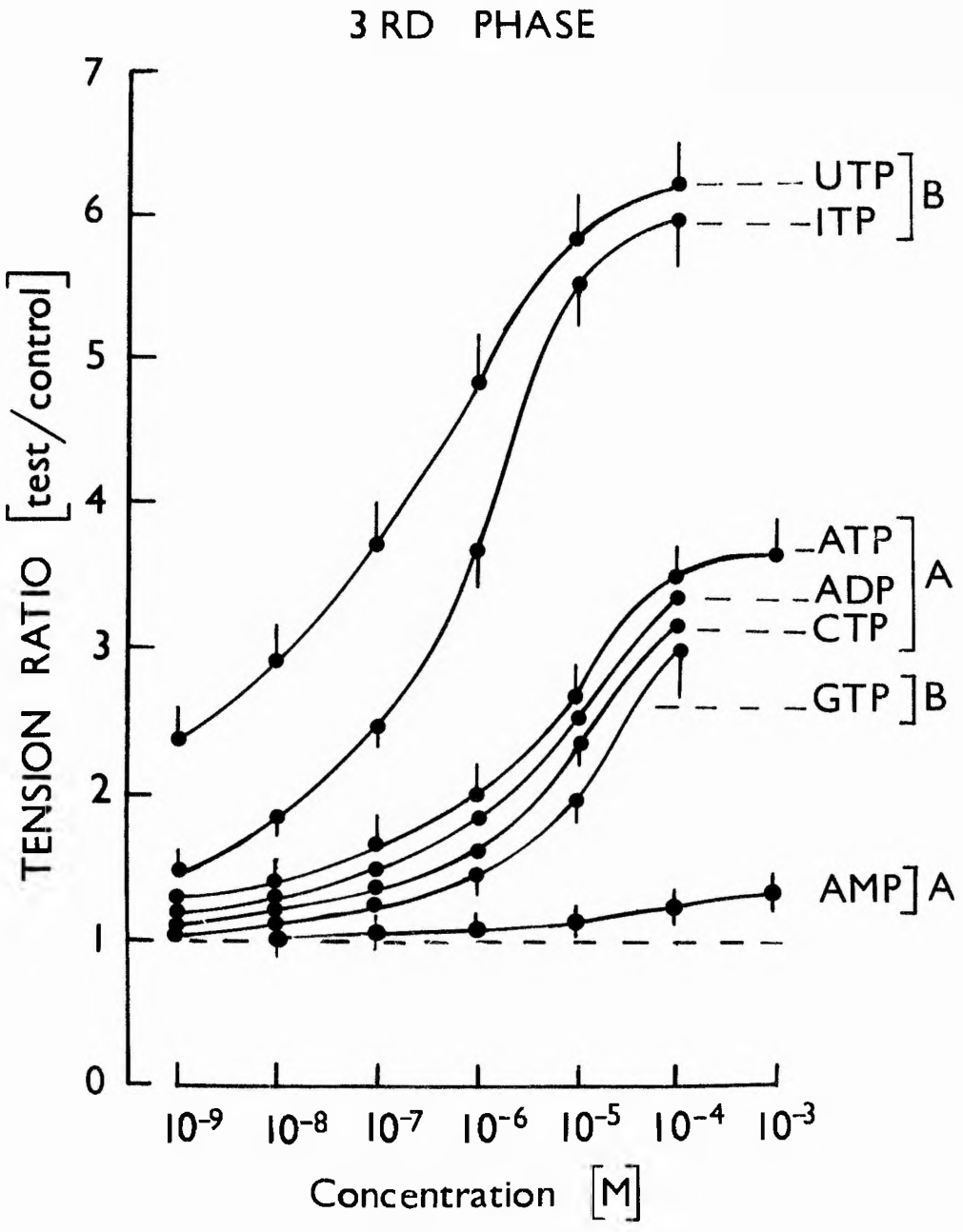
Log-dose response curves for '3rd' component (Types A and B responses): Both types A and B agents are considered here on the assumption (though not yet proven) that the type B response is essentially the same as the type A response without the first and/or second components.

Log-dose response curves for the third components are shown in Fig.8.6. The over all order of potency is: UTP > ITP > ATP > ADP > CTP > GTP > AMP. Considering the type A agents alone, the order is ATP > ADP > CTP > AMP. The ED_{50}'s for the type A agents were: ATP, 5.5×10^{-6}M; ADP, 6.5×10^{-6}M; CTP, 7.5×10^{-6}M; AMP,

Figure 8.6.

Log-dose response curves of types A (ATP, ADP, CTP and AMP) and B(UTP, ITP and GTP) agents (range from 10^{-9} to 10^{-3}M) for the '3rd' component of the contractile response. Tension is expressed as a multiple of the control value. Each point is the mean $\pm$ S.E. taken from 10 preparations. Control level is denoted by the horizontal broken line. The letters A and B beside the compounds denote the type. Note the order of relative potencies: UTP $>$ ITP $>$ ATP $>$ ADP $>$ CTP $>$ GTP $>$ AMP. Note also the variation in the sizes of the responses induced by these compounds. Abscissa; Concentration (M). Ordinate; Tension ration (ATP/Control).

Stimulation parameters: Pulse width, 5 msec; frequency 10mV; amplitude, 10V. Flow rate: 100 ml.min^{-1}. Temp. 19^{o}C.


3 RD PHASE
TENSION RATIO [test/control]
7
6
5
4
3
2
1
0
UTP
ITP
B
ATP
ADP
CTP
A
GTP
B
AMP
A
10-9
10-8
10-7
10-6
10-5
10-4
10-3
Concentration [M]

1×10^{-5}M. Taking the efficacy of AMP as unity, CTP is 1.3x, ADP, 1.5x and ATP, 1.8x as effective. Thus, there is relatively little difference in the potency of the various type A agents investigated.

Maximum twitch potentiations (expressed as multiples of control values) at 10^{-4}M were: ATP, 3.50 ± 0.16; ADP, 3.35 ± 0.25; CTP, 3.15 ± 0.20; AMP, 1.35 ± 0.02.

The relative potencies of the type B agents are: UTP $>$ ITP $>$ GTP. The ED_{50}'s were: UTP, 8.5×10^{-8}M; ITP, 8×10^{-7}M; GTP, 1×10^{-5}M. Expressing relative potency in terms of the effectiveness of GTP as unity gives: UTP, 118x and ITP, 12.5x as effective as GTP.

Maximum twitch amplitudes (expressed as multiples of the control values) at 10^{-4}M were as follows: UTP, 6.20 ± 0.23; ITP, 5.95 ± 0.28 and GTP, 3.00 ± 0.27.

Effect of a nucleoside (adenosine) on ventricular contractility.

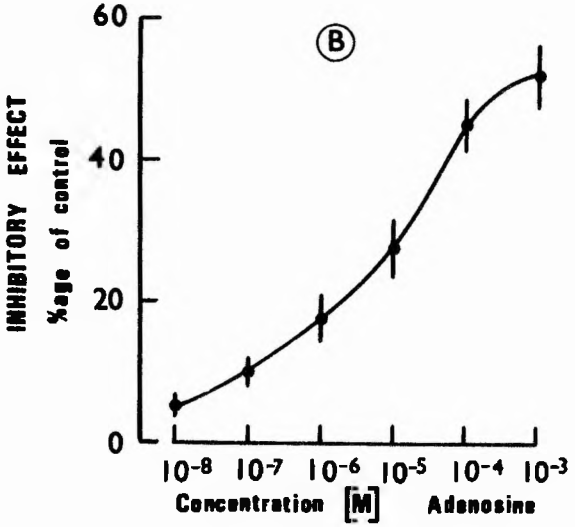
Adenosine differs from the type A and type B agents in that it exerts an exclusively negative inotropic effect. Fig.8.7A

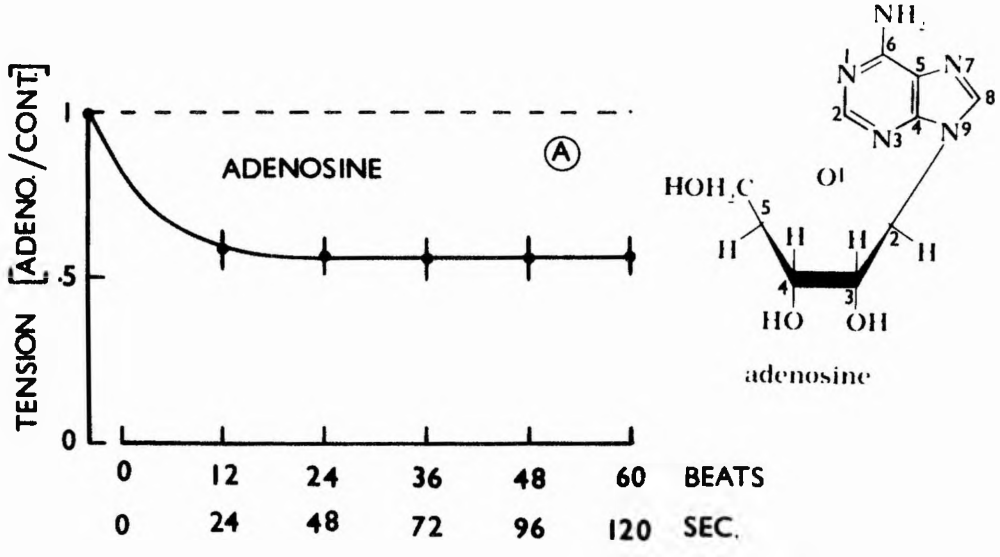
Figure 8.7.

(A) Time course of changes in contractile force following exposure of hypodynamic ventricle to 10^{-4}M adenosine. The molecular structure of the compound is shown for comparison. Tension is expressed as a multiple of the control value. Each point represents the mean $\pm$ S.E. taken from 10 preparations. The control level is shown by the horizontal broken line. Note the negative inotropic effect elicited by adenosine and the absence of a terminal phosphate in the molecule. Abscissa; Number of beats, Time (sec). Ordinate; Tension ratio (adenosine/control).

(B) Dose-response curve for negative inotropic action of adenosine (range from 10^{-8} to 10^{-3}M). Tension is expressed as a multiple of the control value. Each point represents the mean $\pm$ S.E. taken from 10 preparations. Abscissa; Concentration of adenosine (M). Ordinate; Inhibitory effect (% age of control).

Stimulation parameters: Pulse width, 5 msec; frequency, 30 min; amplitude, 10V.
Flow rate: 100 ml.min^{-1}. Temp, 19^{o}C.


B
INHIBITORY EFFECT
%age of control
60
40
20
0
10-8 10-7 10-6 10-5 10-4 10-3
Concentration [M] Adenosine


TENSION [ADENO./CONT]
1
.5
0
ADENOSINE
A
0
12
24
36
48
60
BEATS
0
24
48
72
96
120
SEC.
NH2
N
N7
8
2
N3
4
N9
HOH2C
5
OH
H
H
H
2
H
4
3
HO
OH

adenosine

shows the time course of the response to 10^{-4}M adenosine, together with the structure of the molecule. Immediately after the addition of adenosine the contractile force decreases, falling to a minimum by the 18th twitch (approximately 36 seconds). Fig.8.7B illustrates the dose-dependent negative inotropic response induced by varying concentrations from 10^{-8} to 10^{-3}M. Adenosine produced a maximum negative inotropic effect of 52 $\pm$ 4% of the control level at 10^{-3}M, with an ED_{50} of 8.5 x 10^{-6}M.

The effect of adenosine is of interest, since it differs from ATP, ADP and AMP by virtue of having no phosphate group attached to the ribose moeity. This difference in chemical structure must therefore be responsible for the difference between type A responses and the exclusively negative inotropic effect shown by adenosine.

D I S C U S S I O N

These experiments (and those described previously, pages 106-108) would appear to rule out either β-adrenergic or cholinergic receptors in mediating the response to ATP and other high energy nucleotides. It has also been shown that the broad classification of the responses into types A and B does not correspond with the classification of the various nucleotides tested into either purine or pyrimidine based nucleotides. However, a number of salient points emerged which may later prove to be a clue as to the nature of the receptor or receptors involved.

First, the type A responses are given by agents which contain one or more ribose-bound phosphate groups, together with a free $-NH_2$ group located on the 6C(purine) or 4C (pyrimidine) positions. When these are replaced by a ketone group (as in ITP, UTP and GTP), the initial rapidly-developing positive inotropic effect (1st phase) and the 2nd (inhibitory) phase produced by type A agents are both lacking and the response becomes a single, monotonic increase in contractility with a time course which resembles closely that for the 3rd phase of the type A response.

The data presented earlier (Chapter V) suggest that the initial effect of ATP may be on the slow inward calcium current, which in turn initiates changes in cyclic 3',5'-nucleotide levels. Since with the type A agents contractile force was seen to be closely correlated with changes in cyclic AMP and cyclic GMP, it is reasonable to suppose that the type B agents, which give a different kind of response, probably affect the metabolism of cyclic 3',5'- nucleotides in a different fashion. This remains to be established. It is possible, for example, that the initial increase in cyclic AMP levels seen with the type A agents is either absent altogether, or is effectively 'swamped' by a relatively large (but transient) increase in the levels of cyclic GMP. A more detailed comparative study of the effects of types A and B agents on cyclic 3',5'- nucleotide metabolism may yield useful information concerning the nature of the receptor involved in mediating these responses.

PART III

FURTHER EVIDENCE FOR A CORRELATION BETWEEN VENTRICULAR CONTRACTILITY AND INTRACELLULAR CYCLIC 3', 5'-NUCLEOTIDE LEVELS.

C H A P T E R IX

EFFECTS OF ISOPRENALINE ON CONTRACTILE FORCE AND INTRACELLULAR CYCLIC 3', 5'-NUCLEOTIDE LEVELS.

INTRODUCTION

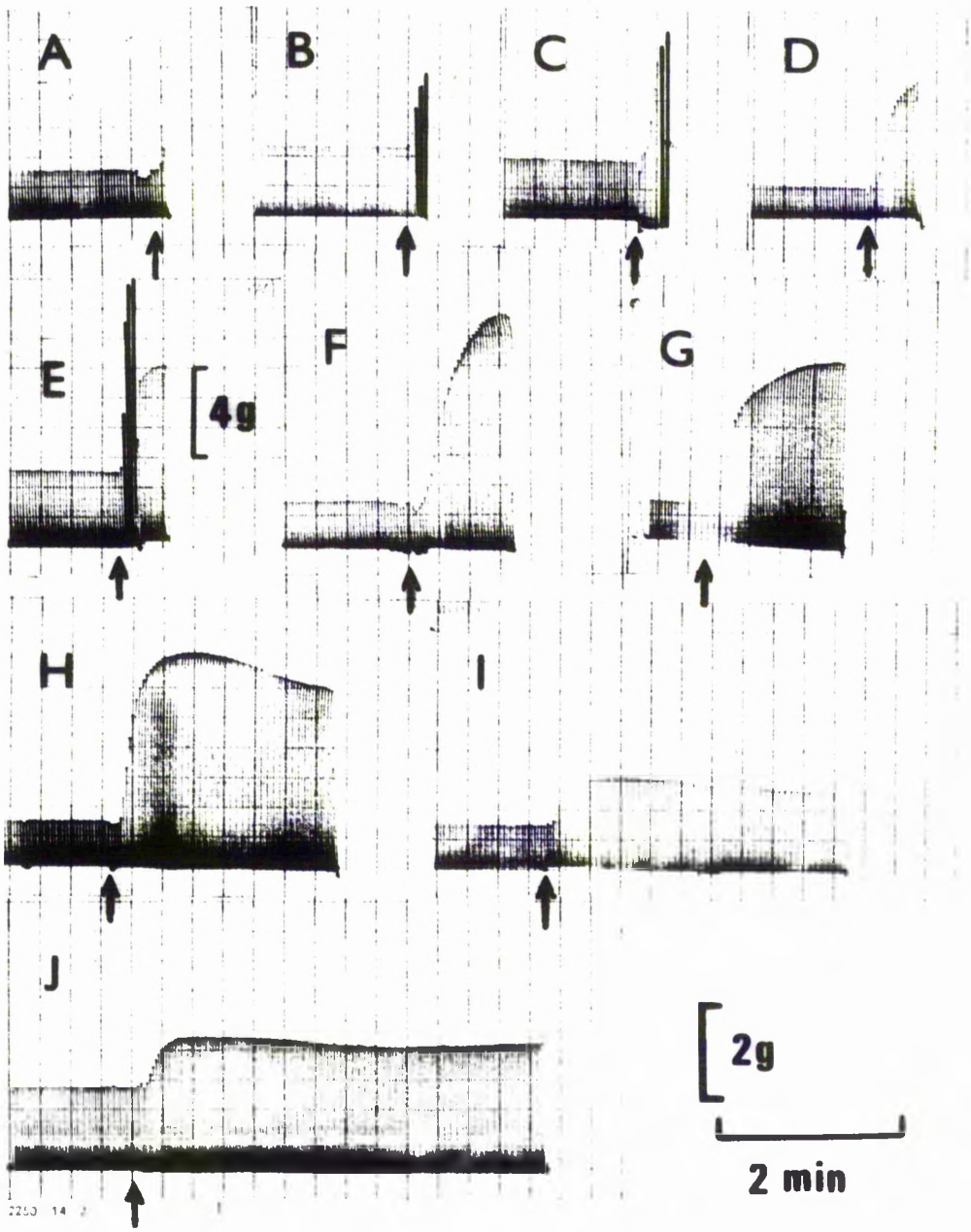
It was felt that the potential importance of the conclusion arrived at in the preceeding chapter - namely, that contractile force is regulated by an intracellular mechanism involving both cyclic 3', 5' -nucleotides - could be substantiated if the generality of the relationship between the cyclic nucleotide ratio and contractile force could be established in other physiologically - relevant situations. For instance, does it hold too during the response of the ventricle to the catecholamines, to acetylcholine, to ouabain or to the E series of prostaglandins, all of which are known to have marked inotropic effects on ventricular contractility. This chapter presents the results of experiments concerning the effects of a β-agonist, isoprenaline, on ventricular contractility and cyclic 3', 5'-nucleotide levels. Isoprenaline was selected because (a) it has a powerful positive inotropic effect on the heart and (b) it has been reported (England, 1976) to induce marked changes in intracellular cyclic AMP levels but to have little or no effect on the level of cyclic GMP.

Two kinds of experiments have been made. First, the time course of the changes in cyclic AMP and cyclic GMP were

Figure 9.1.

A representative sample of original chart recordings (A - J) showing the time course of the contractile responses following stimulation with 10^{-6}M isoprenaline, and also indicating the time of 'crush freezing' the preparation prior to analyses for cyclic AMP, cyclic GMP and total protein. The arrows indicate the times of application of isoprenaline. Note the change in the tension scale labelled E.

Stimulation parameters: Pulse width, 5 msec; amplitude, 10V; frequency, 30 min^{-1}.
Perfusion rate: 100 $ml.min^{-1}$. Temp, 19°C.
Note that in these time course experiments (10) ventricles from specimens of R. temporaria were used.



Isoprenaline 10^{-6}M

followed in the presence of isoprenaline; and secondly, the effects of varying the concentrations of isoprenaline on the relationship between cyclic 3', 5'- nucleotide levels and contractility were studied.

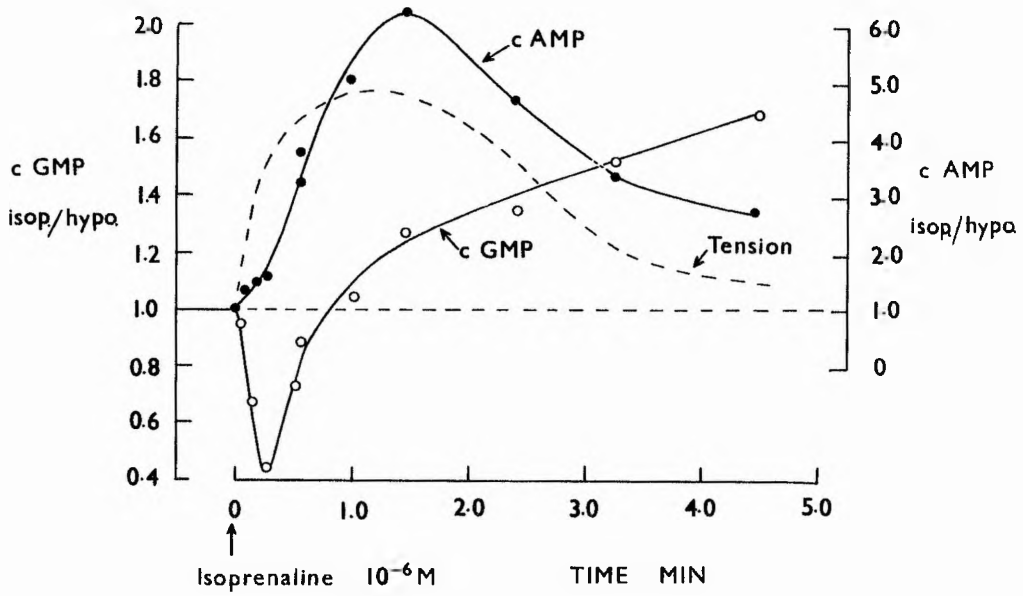
The results of these experiments have been published (Singh, Flitney & Lamb, 1978).

Time course of changes in cyclic AMP and cyclic GMP levels resulting from treatment with isoprenaline: The design of these experiments was identical to that used for investigating changes in cyclic nucleotide levels during the ATP-induced response. Isoprenaline (10^{-6}M) was applied to the hypodynamic ventricle and the resulting response terminated at varying times by 'crush - freezing' the preparation.

Fig.9.1 is a representative sample of chart recordings, showing contractile response and indicating the time of application of isoprenaline and the time of 'crush - freezing'. The time course of the change in force (dashed line), cyclic AMP levels (solid circles) and cyclic GMP levels (open circles) are plotted in Fig.9.2 (see table 9.1, appendix IX for raw data). At this concentration,

Figure 9.2.

Time course of changes in contractile force (dashed line) and intracellular cyclic AMP (solid circles) and cyclic GMP (open circles) during exposure of hypodynamic ventricles to 10^{-6}M isoprenaline. Both cyclic nucleotide levels and tension are expressed as multiples of the control (hypodynamic) values. Note the early increase in isometric tension response which corresponds more closely with the initial decrease in cyclic GMP level, to below the control (hypodynamic) value (horizontal broken line). The rise in cyclic AMP level is delayed and lags behind. Control (hypodynamic) values (mean $\pm$ S.E.) for cyclic AMP and cyclic GMP levels (pmol mg^{-1} total protein) were 9.39 $\pm$ 0.66 and 1.44 $\pm$ 0.15, respectively (n = 17). Abscissa; time (min). Ordinate; cyclic AMP and cyclic GMP levels (fraction of control (hypodynamic) value.


c GMP
isop/hypo
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
c AMP
c GMP
Tension
6·0
5·0
4·0
3·0
2·0
1·0
0
c AMP
isop/hypo
0
1·0
2·0
3·0
4·0
5·0
Isoprenaline 10^{-6} M
TIME MIN

isoprenaline induces a rapid and large positive inotropic response, the twitch amplitude rising to about 4.9 x the control level after approximately 60 seconds. Attention should be drawn to two points concerning the accompanying changes in intracellular cyclic 3', 5'- nucleotide levels.

First, the initial increase in contractile force corresponds most closely to a rapid fall (to around 40% of its initial value) in the levels of cyclic GMP, than to any change in cyclic AMP levels. Indeed, the data clearly show that the production of cyclic AMP lags behind the increase in contractility. This is a particularly significant observation, since if cyclic AMP alone was acting as a second messenger for isoprenaline, then any increase in its levels would necessarily preceed the change in contractile force.

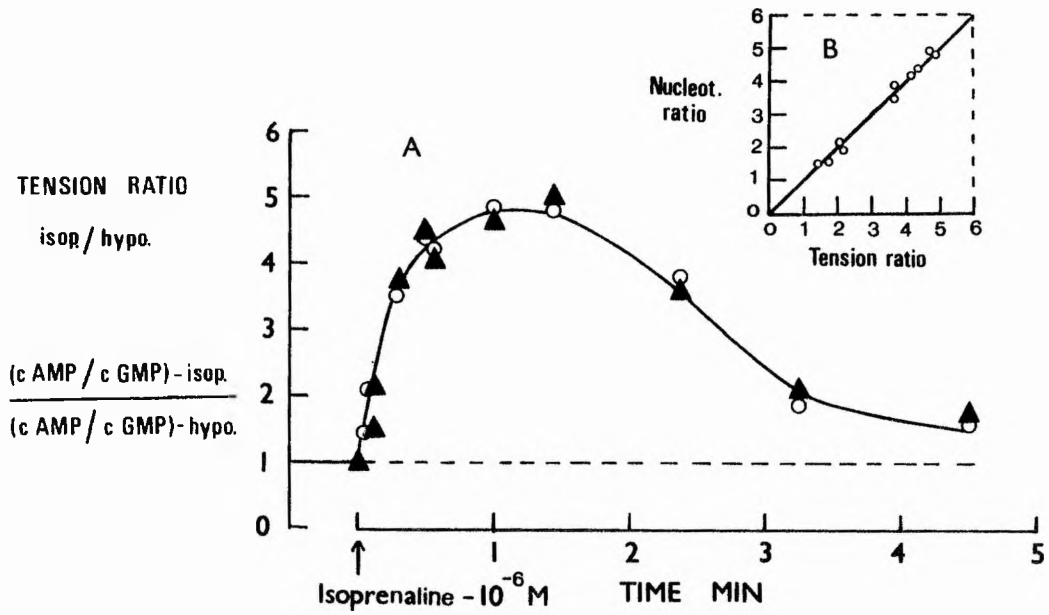
Secondly, the onset of the return of cyclic GMP towards its control level is marked initially by a slowing down in the rate of rise in cyclic AMP levels, and then shortly after cyclic GMP levels increase beyond the control level, cyclic AMP begins to fall. This observation is consistent with that made earlier concerning the ATP - induced response, where there was also an apparent inhibitory influence of cyclic GMP on cyclic AMP levels.

Figure 9.3.

Data taken from Fig.9.2.

(A) Time course of changes in isometric twitch tension (open circles) and cyclic nucleotide ratio (solid triangles) measured at various times during the response to 10^{-6}M isoprenaline. Both parameters are expressed as multiples of the control (hypodynamic) values (broken horizontal line). The solid line is drawn by eye to show the general shape of the responses. Abscissa; Time (min); Ordinate tension ratio and cyclic nucleotide ratio (fraction of the control (hypodynamic) level).

(B) Relationship between cyclic nucleotide ratio and contractile force (multiples of control values). The solid line is drawn with a slope of 1 to indicate a direct proportionality between the two parameters. Correlation coefficient $\pm$ S.E. of estimate: 0.99 ± 0.14, $n = 10$, $P < 0.001$. Abscissa; Tension ratio. Ordinate; Nucleotide ratio.


TENSION RATIO
isop / hypo.
(c AMP / c GMP) - isop.
(c AMP / c GMP) - hypo.
A
B
Nucleot. ratio
Tension ratio
Isoprenaline - 10⁻⁶M
TIME MIN

Unfortunately, it is again impossible to say whether this is due to an inhibition in cyclic AMP production or to its enhanced destruction.

The relationship between the cyclic nucleotide ratio and contractile force for the same data is presented in Fig.9.3A & B. Fig.9.3A shows the time course of the change in cyclic nucleotide ratio (solid triangles), together with the form of the contractile response (open circles), and Fig.9.3B depicts more clearly the relationship between these two parameters. As before, the solid line in Fig.9.3B is drawn in by eye to indicate a 1:1 relationship between a change in contractility and a change in the cyclic nucleotide ratio. Statistically, the correlation is highly significant: correlation coefficient $\pm$ S.E. of estimate: 0.99 ± 0.14, $n = 10$, $P < 0.001$.

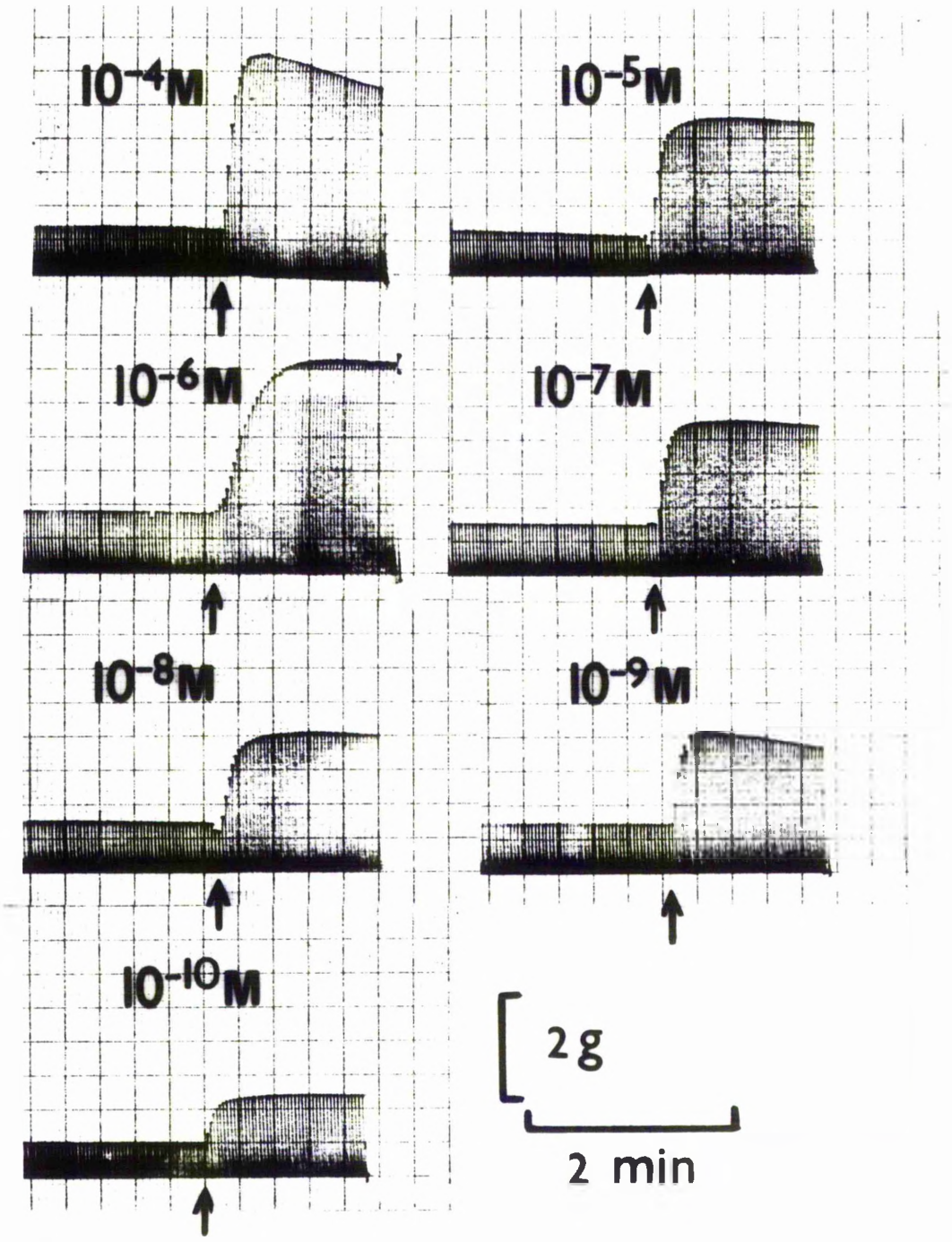
Log-dose response curve to isoprenaline: In these experiments hypodynamic ventricles were exposed to varying concentrations of isoprenaline (range from 10^{-10} to 10^{-4}M) Contractile force, cyclic AMP and cyclic GMP were this time measured close to the peaks of the resulting responses.

Fig.9.4 shows a representative sample of records. It should be noted that these experiments were made using

Figure 9.4.

A representative sample of original chart recordings of tension responses during exposure of hypodynamic ventricles to varying concentrations of isoprenaline (range from 10^{-10} to 10^{-4}M). The arrows indicate the times of application of isoprenaline. The preparations were superfused for approximately 100 seconds (peak of responses) prior to 'crush-freezing' and cyclic nucleotides measurements. Note that in these log-dose experiments (7) hearts from another species of frogs, R escuelenta were used.

Stimulation parameters: Pulse width, 5 msec; amplitude, 10V; frequency 30 min^{-1}; Perfusion rate: 100 $ml.min^{-1}$. Temp, 19°C.



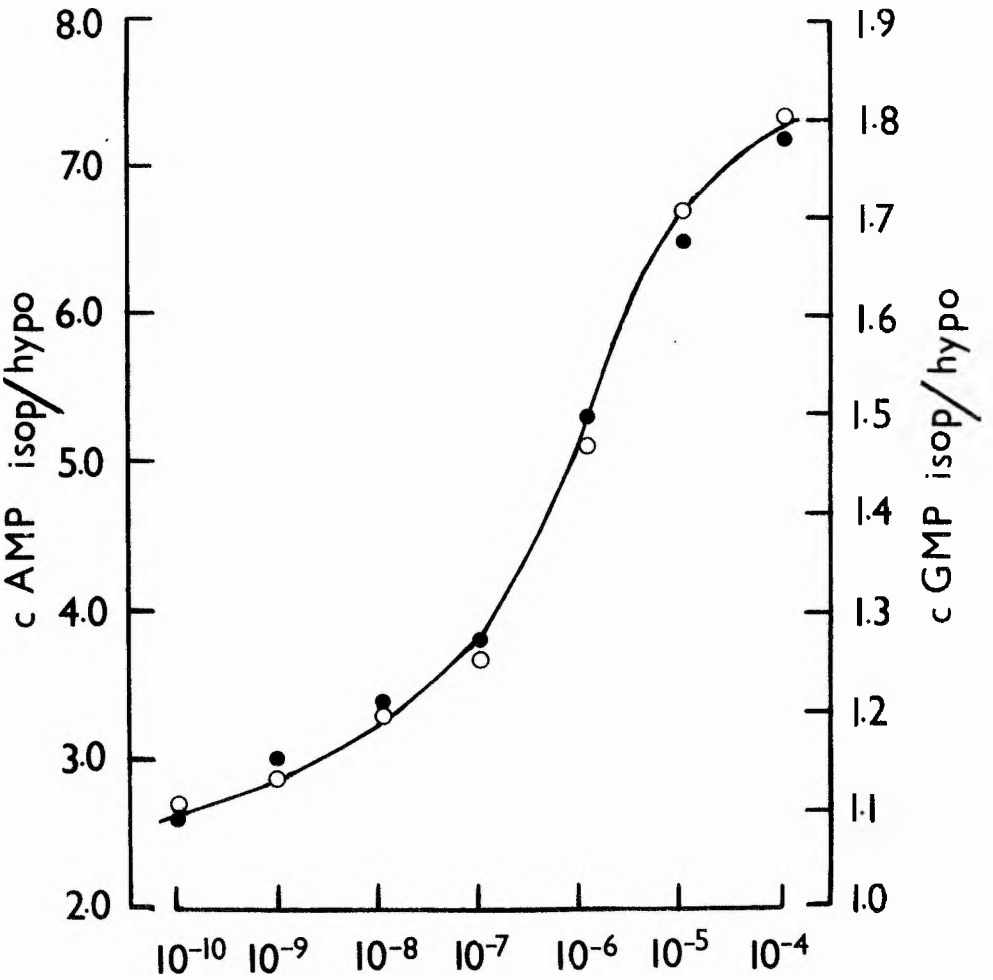
ISOPRENALINE

ventricles from speciments of R escuelenta and not R temporaria as were used in the time course experiments. The raw data of these experiments are presented in table 9.2. appendix IX.

Fig.9.5 shows log-dose response curves for cyclic AMP levels (solid circles) and cyclic GMP levels (open circles) in the range of 10^{-10} to 10^{-4}M isoprenaline. Both show similar sigmoidal curves with which AMP levels increasing approximately 3.5 x and cyclic GMP levels approximately 1.63 x over the range investigated. The relationship between the varying concentrations of isoprenaline and intracellular cyclic nucleotide ratio is depicted in Fig.9.6A (solid triangles) together with contractile force (open circles). Once again it is evident that the change in force is accompanied by a corresponding change in cyclic nucleotide ratio. The two parameters are plotted together in Fig.9.6B using the same method of representation as used before. The data again reveal a highly significant correlation (correlation coefficient $\pm$ S.E. of estimate: 0.99 ± 0.06, $n = 7$, $P < 0.001$).

Figure 9.5.

Log-dose response curves for intracellular cyclic AMP levels (solid circles) and cyclic GMP levels (open circles) following stimulation with varying concentrations of isoprenaline ranging from 10^{-10} to 10^{-4}M. Both parameters expressed as multiples of the control values. The two cyclic nucleotides show similar sigmoidal curves. Cyclic AMP and cyclic GMP increased approximately 3.5x and 1.63x respectively, over the range of $[\text{isoprenaline}]_o$ studied. Abscissa; concentration of isoprenaline (M). Ordinate; cyclic AMP and cyclic GMP levels (fraction of control value).


8.0
7.0
6.0
5.0
4.0
3.0
2.0
c AMP isop/hypo
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
c GMP isop/hypo
10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴
Concentration of isoprenaline (M)

D I S C U S S I O N

The experiments with isoprenaline provide further evidence for a close relationship between ventricular contractility and intracellular cyclic 3', 5' - nucleotide levels. These results extend the generality of the relationship and add further weight to the idea that cyclic AMP and cyclic GMP together participate in an intracellular control system which regulates the force of contraction. One particularly significant finding which deserves special comment concerns the changes which occur early on in the response to isoprenaline. In previous work, England (1976) reported an increase in cyclic AMP levels in rat hearts, but failed to observe any change in the levels of cyclic GMP. Inspection of his results (his Fig.5) shows clearly that cyclic GMP levels were not monitored early enough to detect the initial fall in cyclic GMP which, in the context of the present study, is the most significant effect of isoprenaline, since it is this which is chiefly responsible for altering the cyclic nucleotide ratio in the upward direction. To emphasise this point still further, it is quite clear from these experiments that cyclic AMP does not function as the sole second messenger for isoprenaline, for its production lags behind the increase in contractility.

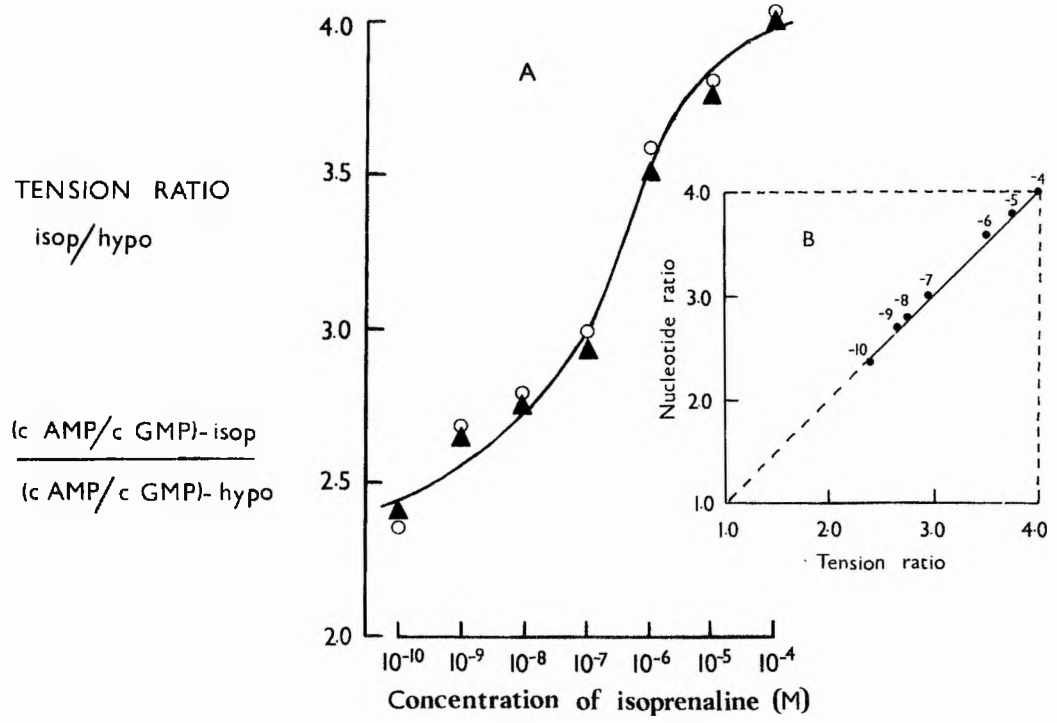
Figure 9.6.

Data taken from Fig.9.4. and 9.5.

(A) Effects of isoprenaline ranging from 10^{-10} to 10^{-4}M on contractile force (open circles) and intracellular cyclic nucleotide ratio (solid triangles). Abscissa; concentration of isoprenaline (M). Ordinate. Tension ratio $\left[\text{P(isop.)/P(hypo)}\right]$. Cyclic nucleotide ratio.

$$\left[\frac{\text{cyclic AMP/cyclic GMP (isop.)}}{\text{cyclic AMP/cyclic GMP (hypo.)}}\right]$$

(B) Relation between isometric force and cyclic nucleotide ratio. Both parameters are expressed as multiples of the control values. The number beside each point indicates the concentration (M) of isoprenaline. The solid line is drawn by eye to show the theoretical relationship between the two parameters, assuming a 1:1 proportionality. Correlation coefficient $\pm$ S.E. of estimate: 0.99 ± 0.57, $n = 7$, $P < 0.001$.


TENSION RATIO
isop/hypo
(c AMP/c GMP)- isop
(c AMP/c GMP)- hypo
4·0
3·5
3·0
2·5
2·0
A
B
Nucleotide ratio
Tension ratio
-4
-5
-6
-7
-8
-9
-10
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
Concentration of isoprenaline (M)

Special consideration too should be given to the results obtained by varying the concentrations of isoprenaline. These indicate first, that isoprenaline exerts dose - dependent effects on the levels of cyclic AMP and cyclic GMP, and secondly, that the relationship between ventricular contractility and altered 3', 5' - nucleotide ratio is maintained over the entire range of concentrations studied.

C H A P T E R X

GENERAL DISCUSSION

The work summarised in this thesis points clearly to the involvement of both cyclic AMP and cyclic GMP in an intracellular control system which in some as yet unknown fashion regulates the capacity of the ventricle to contract. This conclusion is based upon experiments concerned with three physiologically - distinct and apparently unrelated responses which (it transpires) all share a common feature; namely that changes in the ratio of cyclic AMP/cyclic GMP almost exactly parallel corresponding changes in contractile force.

This discussion is concerned primarily with the formulation of a hypothesis concerning the possible nature of the cyclic 3', 5' - nucleotide dependent control system. Its essential features are summarised in Fig 10.1. It focusses attention on two main questions of current interest:

First, what is the nature of the event/s that <u>initiate</u> changes in cyclic 3', 5' - nucleotide levels within the fibres?

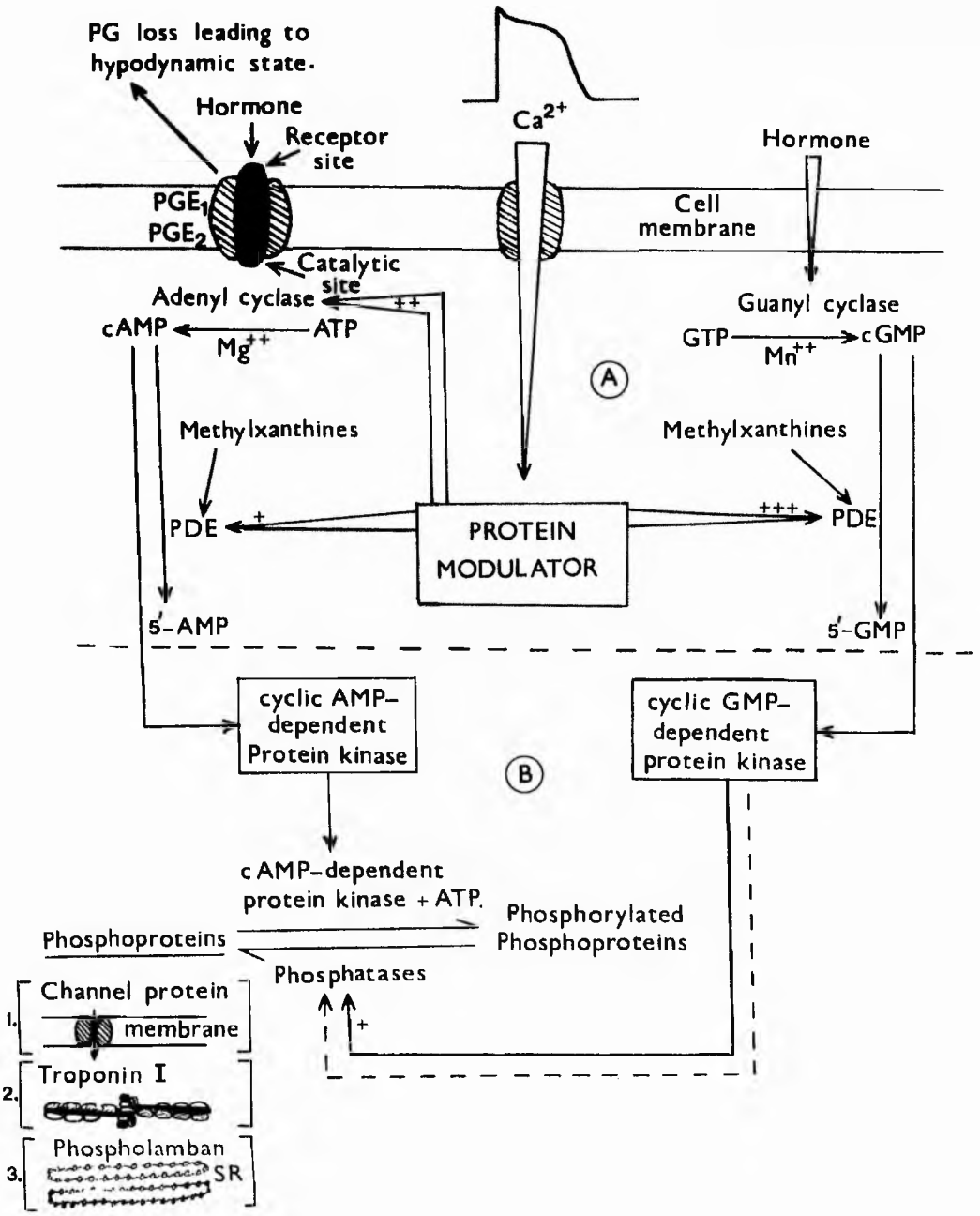
Secondly, how do cyclic AMP and cyclic GMP act in a co-ordinated manner to regualte the amplitude of the contractile response?

Figure 10.1

Possible mechanism for the involvement of intracellular cyclic 3', 5'- nucleotides in modulating cardiac contractility.

(A) The sites of actions of neurohumoral agents to activate adenylate cyclase and guanylate cyclase which catalyse cyclic AMP and cyclic GMP production, respectively. The influence of the protein modulator on cyclic 3', 5'- nucleotide production is also depicted.

(B) An illustration of the sites of actions of cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase.


PG loss leading to hypodynamic state.
Hormone
Receptor site
PGE1
PGE2
Catalytic site
Ca2+
Hormone
Cell membrane
Adenyl cyclase
++
cAMP
ATP
Mg++
Guanyl cyclase
GTP
cGMP
Mn++
A
Methylxanthines
Methylxanthines
PDE
+
PROTEIN MODULATOR
+++
PDE
5'-AMP
5'-GMP
cyclic AMP-dependent Protein kinase
cyclic GMP-dependent protein kinase
B
cAMP-dependent protein kinase + ATP.
Phosphoproteins
Phosphorylated Phosphoproteins
Phosphatases
+
1.
Channel protein
membrane
2.
Troponin I
3.
Phospholamban
SR

The principal aim of the discussion is to consider these two questions, and to attempt to relate the results of the present experiments to the well - established key position of calcium in regulating contraction.

What initiates changes in intracellular cyclic 3', 5' - nucleotide level?

(a) Direct stimulation of cyclases: Consider, first, the possibility of direct activation of enzymes which synthesise cyclic 3', 5' - nucleotides. There is abundant evidence that several important agents stimulate adenylate cyclase activity directly (Drummond & Severson, 1974; see main Introduction). Adenylate cyclase resides in the plasma membrane of the fibres (Øye & Sutherland, 1966; Drummond & Duncan, 1970; Wollenberger & Schulze, 1976) and is known to comprise separate receptor (or regulator) and catalytic subunits (Robison et al, 1971). A third component may also exist which couples events at the receptor with those taking place at the catalytic site (Lehotay, Lo & Levey, 1977). The receptor is thought to be located on the outside of the membrane and to be responsible for determining hormonal specificity, whereas the catalytic subunit is situated on the inner membrane surface and discharges its product intracellularly. The β - agonists are one group of agents which are thought to stimulate

cyclic AMP production directly by activating adenylate cyclase (Tsien, 1977).

Guanylate cyclase differs from adenylate cyclase in that it exists in both particulate and soluble forms (Kimura & Murad, 1974). Evidence for a direct effect of neurohumoral agents, such as acetylcholine, on guanylate cyclase activity is contradictory, although it is clear that several stimulate cyclic GMP production (George et al, 1970, 1973, 1975; Watanabe & Besch, 1975; England, 1976). White et al (1973) and Sulakhe et al (1975) obtained direct evidence for stimulation of guanylate cyclase by acetylcholine in broken cell preparations, but Kimura and Murad (1974) and Limbird & Lefkowitz (1975) were unable to confirm these results. These conflicting data suggest that acetylcholine may influence guanylate cyclase activity indirectly, through an intermediate step.

(b) Indirect stimulation of phosphodiesterases and adenylate cyclase: This thesis focusses attention on the significance of the ratio of cyclic AMP/cyclic GMP rather than on the role of cyclic AMP alone, and we must therefore look to a mechanism that could conceivably act in a co-ordinated way to alter the levels of both cyclic nucleotides. In this context, the properties of the newly discovered calcium - sensitive protein modulator (Teo

& Wang, 1973) are of paramount importance. These have been reviewed recently by Wang (1977). The protein modulator combines with calcium to produce an activated protein modulator–calcium complex (Teo & Wang, 1973; Brostrom & Wolff, 1974) which stimulates cyclic GMP and cyclic AMP phosphodiesterase activity, the former more so than the latter (Brostrom, Huang, Breckenridge & Wolff, 1975), and also adenylate cyclase activity (Cheung, Lin, Liu & Smoake, 1975; Gnegy, Costa & Uzunov, 1976). Thus, a change in the availability of calcium to the protein modulator, resulting either from altered Ca^{2+} entry during the action potential or from the effect this might have on the release of intracellular stores of calcium, would cause the protein modulator to act in a concerted fashion to raise or lower the cyclic 3', 5' - nucleotide ratio (Wang, 1977).

This, then, provides a mechanism whereby the nucleotide ratio might be varied in response to agents which affect the distribution of calcium. These include isoprenaline (Natham & Beeler, 1975), adrenaline (Reuter, 1975; Noble, 1975; Reuter & Scholz, 1977), dibutyryl cyclic AMP (Tsien et al, 1972; Tsien, 1973), PGE_1 (Mironneau & Grosset, 1976) and ATP (Goto et al, 1976, 1977 and present results), all of which enhance the slow inward current, and acetylcholine (Ikemoto & Goto, 1977; Giles & Tsien, 1975; Giles & Noble, 1976; Ten Eick, Nawrath, McDonald & Trautwein,

1976), 8-Bromo cyclic GMP (Nawrath, 1977) which reduce calcium entry. Isoprenaline, adrenaline, PGE_1 and dibutyryl cyclic AMP all exert positive inotropic effects, and we should therefore expect the cyclic 3', 5' - nucleotide ratio to increase; this has been shown to be the case for isoprenaline (Chapter IX). Conversely, acetylcholine and 8-Bromo cyclic GMP produce negative inotropic effects, and by the same line of reasoning, the protein modulator would respond to reduced Ca^{2+} entry by lowering the cyclic nucleotide ratio. This has yet to be confirmed. The actions of ATP are particularly informative in this context, for it produces both positive and negative inotropic responses. It has been seen that during the initial (lst phase) and during the third phase of the ATP - induced response, when contractile force is enhanced, the action potential duration is increased and the cyclic nucleotide ratio is elevated, whereas during the second (inhibitory) phase, contractile force is reduced, the action potential is abbreviated and the cyclic nucleotide ratio lowered.

The mechanism responsible for altering 3', 5' cyclic nucleotide levels during the development of the hypodynamic state is less clear, although there are two pertinent findings which would seem to have a bearing on this problem. Prostaglandins E_1 and E_2 are known to stimulate adenylate cyclase activity (Sobel & Robison, 1969; Klein & Levey,

1971b) and it was shown earlier (Chapter III) that both of these substances are lost from the ventricle during superfusion, together with a hitherto unidentified prostaglandin - like substance. It is conceivable, therefore, that this by itself would result in lowered cyclic AMP levels. Furthermore, hypodynamic depression is also known to be accompanied by diminished intracellular calcium levels (Boehm, 1914; Lieb & Loewi, 1918) and by reduced calcium influx (Chapman & Niedergerke, 1970a) and this would also lower the cyclic 3', 5' - nucleotide ratio via its effect on the protein modulator.

Finally, consideration must be given to the possibility that cyclic GMP levels per se act to regulate those of cyclic AMP, and vice versa. In all three responses studied in this thesis, it has been observed that whenever there is a significant change in the level of one cyclic nucleotide there is usually an opposing change in the other. Similar changes have been reported during the course of a single cardiac contraction. Wollenberger et al (1973) showed that both cyclic AMP and cyclic GMP undergo oscillatory changes which are synchronised with the cardiac cycle, in such a way that when cyclic AMP increases, during the rising phase of the twitch, cyclic GMP falls. It is impossible to say at this time whether this reflects a change in the activity of the calcium sensitive protein modulator, or whether it is due to a direct effect of one cyclic

nucleotide upon the synthesis and/or regulation of the other.

Whatever the mechanism, it is clear that the synthesis and/or degredation of both cyclic 3', 5' nucleotides is closely co-ordinated by a system which is capable of altering the nucleotide ratio very rapidly.

How do cyclic AMP and cyclic GMP together act to regulate force production?

Cyclic AMP is known to exert its regulatory effects by stimulating a class of protein - phosphorylating enzymes called cyclic AMP- dependent protein kinases (Greengard & Kuo, 1970). In recent years, several physiologically important phosphoprotein substrates for cyclic AMP-dependent protein kinases have been implicated in regulating myocardial contractility. Their functional state is known to be altered by an ATP - dependent phosphorylation of certain amino acid residues, catalysed by the activity of cyclic AMP - dependent protein kinases. These phosphoproteins include Troponin I (TN - I), a subunit of the regulatory protein complex (Reddy et al, 1973; Cole & Perry, 1975; Rubio et al, 1975; England, 1975, 1976; Solaro et al, 1976); phospholamban, a 22,000 dalton constituent of the sarcoplasmic reticulum (Kirchberger et al, 1975; Tada et al, 1975; Katz et al, 1975); and a sur-

face membrane-bound protein which is thought to be a structural component of the slow inward (calcium) channel (Krause et al, 1975; Wollenberger et al, 1975).

Phospholamban and the calcium channel protein are thought to affect the distribution of calcium, and hence its availability to the contractile proteins, by regulating transmembrane movements between the sarcoplasmic reticulum and myoplasm on the one hand (phospholamban) and between the extracellular fluid and fibre interior (channel portein) on the other. The role of TN - I is less clear. It is of course well established that it prevents interaction of actin and myosin in a resting muscle, and that this inhibitory effect is suppressed when TN - C interacts with calcium, but recent work, involving several different lines of research, indicates that its action may be more subtle and that it may also regulate the sensitivity of the contractile proteins to calcium. Thus, all three phosphoproteins are intimately associated with the physiological functions of calcium and their principal properties are therefore discussed in more detail below.

Phosphorylation of TN - I and calcium sensitivity of myofibrillar ATP ase: Bailey and Villar - Palasi (1971) first demonstrated that a purified protein kinase can catalyse phosphorylation of skeletal muscle TN - I in the presence of cyclic AMP. These workers postulated

that changes in the state of phosphorylation of TN - I might be involved in mediating the positive inotropic response to adrenaline. Work on cardiac myofibrillar phosphorylation followed the initial studies on skeletal muscle protein (Reddy et al, 1973; Reddy & Schwartz; 1974). Cole and Perry (1975) drew attention to three major differences between skeletal and cardiac TN - I which they considered to have functional significance.

(a) TN - I from cardiac muscle has more bound phosphate (approximately 2 moles phosphate $mole^{-1}$ TN - I) than that isolated from skeletal muscle.

(b) Phosphorylation of cardiac TN - I by cyclic AMP - dependent protein kinase is not blocked by TN - C, as is the case for skeletal muscle TN - I.

(c) Cardiac troponin differs from its skeletal muscle counterpart in its association with high amounts of endogenous cyclic AMP - dependent protein kinase (Reddy et al, 1973; Perry & Cole, 1974; Perry, 1975).

It is known that the increase in contractility elicited by β - adrenergic agents is accompanied by elevated levels of phosphorylation of TN - I; there is a time - dependent increase in ^{32}Pi incorporation into TN - I following

stimulation of rat (England, 1975, 1976) and rabbit (Solaro et al, 1976) hearts with varying concentrations of either isoprenaline or adrenaline. Significantly, the time course of the increase in phosphorylation closely parallels that of the increase in contractility. This is a particularly interesting observation in the light of the experimental results presented here, which show a similar parallelism between the increase in contractile force and in the ratio of cyclic AMP/cyclic GMP. It raises the possibility that the state of phosphorylation of TN - I is actually regulated by cyclic AMP and cyclic GMP acting together. We shall return to this point later.

The physiological influence of phosphorylation of cardiac TN - I is unclear. Rubio et al (1975) found that phosphorylation of TN - I in guinea - pig cardiac muscle increased the sensitivity of actomyosin ATP-ase to calcium. However, results obtained by Ray and England (1976) and Cole, Frearson, Moir, Perry and Solaro (1977) show that phosphorylation of TN - I causes a decrease in the calcium sensitivity of actomyosin ATPase from bovine and rat cardiac muscle. The discrepancy is difficult to explain, although it is possible that a species difference might be involved. Although the underlying mechanism must await further clarification, the experimental observations are quite clear; increased myocardial contractility is accompanied by increased phosphorylation of TN - I and by a corresponding increase

in the ratio of cyclic AMP/cyclic GMP.

Phosphorylation of phosphoproteins involved in regulating calcium distribution.

Phosphorylation of phospholamban: Several authors (Kirchberger et al, 1972; Tada et al, 1974; Katz et al, 1975; Kirchberger et al, 1975) have observed that phosphorylation of phospholamban from cardiac SR by cyclic AMP - dependent protein kinase increases the rate of uptake of calcium by isolated vesicles. In view of its known effects on cyclic AMP levels, Tada et al, (1974) have suggested that this might afford a basis for the well know "relaxant" effect of adrenaline. Conversely, Kirchberger and Raffo (1977) observed that dephosphorylation of phospholamban by phosphoprotein phosphatases is associated with a decrease in the rate of calcium pumping by cardiac SR fragments.

Phosphorylation of the calcium channel protein: It was mentioned earlier that several cardioactive substances appear to exert their inotropic actions by modulating the slow inward calcium current. There is now evidence that the degree of phosphorylation of the channel protein, catalysed by cyclic AMP - dependent protein kinase, is related to the magnitude of the inward Ca^{2+} current (Wollenberger, 1975; Krause et al, 1975; Hui et al, 1976; Sulakhe

et al, 1976; Will et al, 1976). On the basis of these observations it has been proposed that the surface membrane bound phosphoprotein is in reality an integral structural component of the calcium channel. It will be recalled that the positive inotropic effects of isoprenaline and ATP, both of which are accompanied by elevated cyclic nucleotide ratios, are also characterised by an increase in the slow inward current (Natham & Beeler, 1975; Goto et al, 1976; 1977).

A possible site of action for cyclic GMP.

The preceeding section summarises the main properties of three important phosphoproteins, two of which are involved in regulating the distribution of calcium and one of which may influence the sensitivity of myofibrillar ATPase to calcium. On evidence presently available there are grounds for arguing that the state of phosphorylation of one of these, namely TN - I, is determined by the relative amounts of cyclic AMP and cyclic GMP present in the fibres. This is based upon a comparison of the present results, showing a parallel increase in contractile force and cyclic nucleotide ratio, and those obtained by England (1975, 1976) and by Solaro et al (1976) working iwth mammalian hearts, who found a similar parallelism between contractility and ^{32}Pi incorporation into TN - I. If

this conclusion is correct (and it should be emphasised that the definitive experiments, measuring contractility, cyclic nucleotide ratios and ^{32}Pi incorporation into TN - I in a single species (or heart) has yet to be made) then it provides a strong clue concerning the mechanism of action of cyclic GMP. At present relatively little is known of its site of action in the heart, but by analogy with what is known about cyclic AMP, we should expect it to exert its effects by stimulating a specific class of protein kinases. A cyclic GMP - dependent protein kinase has in fact been found in the heart (Kuo, 1974) but direct evidence for the existence of a physiologically relevant phosphoprotein substrate is still lacking. The nature of the relationship between contractility and cyclic AMP/cyclic GMP implies that the two cyclic nucleotides play opposing roles in regulating contractility. Thus, it is reasonable to postulate that since cyclic AMP-dependent protein kinases phosphorylate phosphoprotein substrates, cyclic GMP - dependent protein kinase may be involved in a dephosphorylating mechanism. This line of reasoning leads to an important conclusion: namely; that the phosphoprotein phosphatases may serve as substrates for cyclic GMP - dependent protein kinases. The potential significance of this conclusion is that a similar mechanism may also determine the state of phosphorylation of a wide range of phosphoproteins in the heart, not only phospholamban and the channel protein, but perhaps also those phospho-

proteins which are known to be involved in energy metabolism.

Concluding remarks and scope for future study.

The present hypothesis forms a starting point for future work which will focus on the following lines of inquiry:

(1) The generality of the relationship between cyclic 3', 5' - nucleotide levels and contractile force: It is important to investigate in more detail the generality of this relationship. For example, is contractile force closely correlated with the cyclic 3', 5' - nucleotide ratio during the course of other responses, such as those to acetylcholine, the prostaglandins, ouabain, glucagon etc?.

(ii) The form of the isometric twitch: Future work will also be concerned with a detailed study of the form of the twitch under different conditions; first, when the ratio of cyclic AMP/cyclic GMP is high (for example, in response to isoprenaline); and secondly, when ratio of cyclic AMP/cyclic GMP is low (for example, during exposure to acetylcholine). A detailed comparison of the shape of the twitch (eg. rate of rise of tension, relaxation rates) under these two extreme conditions might pro-

vide important corroborative evidence relating to the sites of actions of the two cyclic nucleotides.

(iii) Relationship between cyclic 3', 5' - nucleotide ratio and the state of phosphorylation of troponin I: It is necessary to measure the levels of cyclic AMP and cyclic GMP together with the incorporation of ^{32}Pi into TN - I following treatment of the heart with various agents such as, acetycholine and isoprenaline, and to correlate observed changes with the contractile state of the heart.

(iv) Identity of phosphoprotein substrates for cyclic GMP - dependent protein kinases: It is important to obtain direct evidence for a stimulatory effect of cyclic GMP - dependent protein kinase on phosphatase activity.

(v) Relation between cyclic 3', 5' - nucleotide levels, contractility and the regulatory effects of calcium: Allen and Blinks (1978) recently reported experiments in which they looked at calcium transients (using the calcium - sensitive photoprotein aequorin) in frog hearts during single contractions. They examined the effects of acetyl-strophanthidin and isoprenaline, used in concentrations which were selected to give approximately the same degree of potentiation of the twitch, on the calcium transients. Isoprenaline was found to produce a larger calcium flux

than acetylstrophanthidin. Clearly, it would be of interest to look at the relationship between cyclic 3', 5' - nucleotide levels, phosphorylation of TN - I and contractility using these two agents.

A P P E N D I C E S.

A P P E N D I X II

(A-C) Protein estimation and cyclic 3', 5' - nucleotide assays.

(A) Protein estimation: The estimation of protein in the unknown samples was made according to the Biuret Test (Gornwall, Bardawill & David, 1949). The Biuret reagent was prepared as follows: 1.5g copper sulphate and 6.0g sodium potassium tartrate were dissolved in 500 ml distilled water. A volume of 300ml 10% sodium hydroxide was added to the content and the volume made up to 1 litre. The reagents were finally treated with 0.1% potassium iodide. Stock bovine albumen was made by dissolving 100mg of the crystalline substance in distilled water to give a final concentration of 10mg/ml.

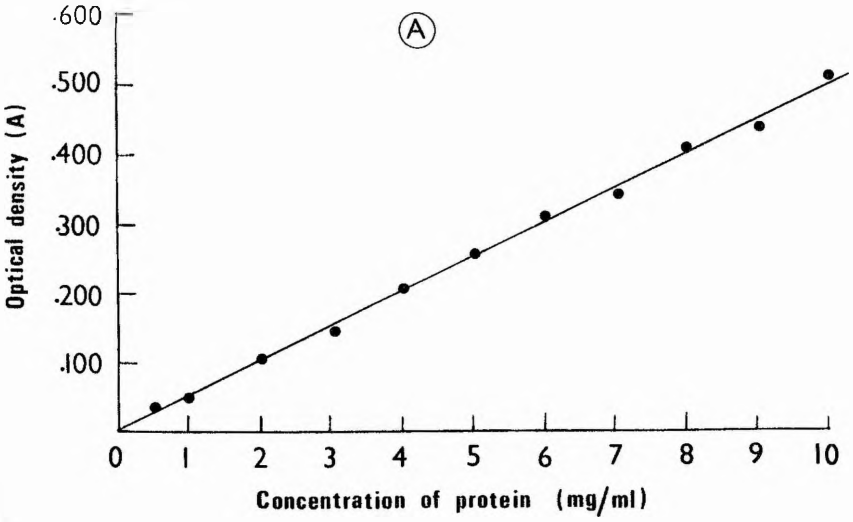
Varying dilutions, in duplicate, of bovine serum albumen were made ranging from 0.5mg ml^{-1} to mg ml^{-1}. To 200µl of the final volume of protein solution from each dilution 800µl of Biuret reagent was added. The contents were vortex mixed and allowed to stand for 30 minutes at room temperature. The blanks (200 µl distilled water) and unknown samples (200 µl), in duplicate, were treated similarly. After incubation period, the optical density of the solution in each pair of tubes, starting with the blanks, was determined at 500 nm using a Beckman (Model 24) spectrophotometer. The average reading from the blanks

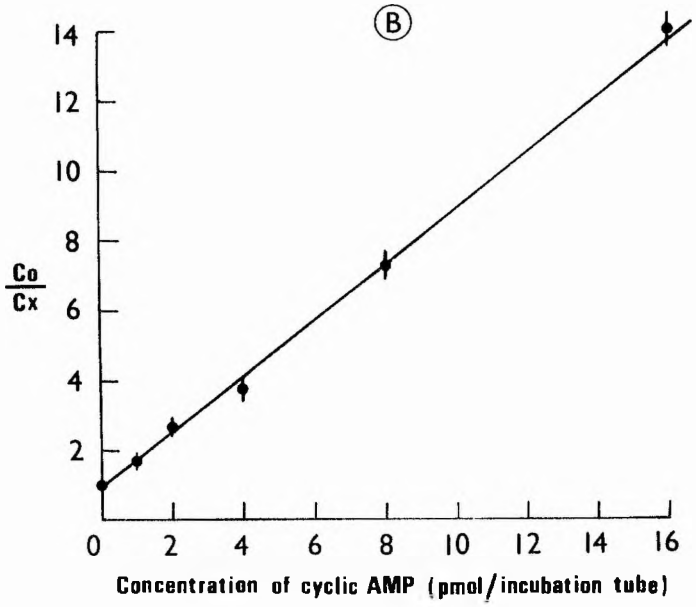
Figure 2.1. Appendix II

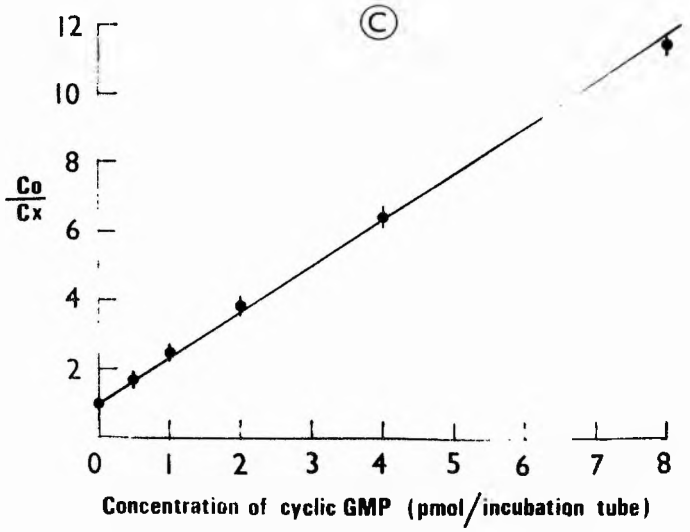
(A) A typical protein estimation standard curve. Abscissa; concentration of protein (mg/ml). Ordinate; Optical density (A).

(B) A typical cyclic AMP standard curve. Each point represents the mean $\pm$ S.E. taken from 10 experiments. Abscissa; concentration of cyclic AMP (pmol/incubation tube). Ordinate $\frac{Co}{Cx}$.

(C) At typical cyclic GMP standard curve. Each point represents the mean $\pm$ S.E. taken from 10 experiments. Abscissa; concentration of cyclic GMP (pmol/incubation tube) Ordinate; $\frac{Co}{Cx}$.


A
.600
.500
.400
.300
.200
.100
Optical density (A)
0 1 2 3 4 5 6 7 8 9 10
Concentration of protein (mg/ml)


B
14
12
10
8
6
4
2
Co/Cx
0 2 4 6 8 10 12 14 16
Concentration of cyclic AMP (pmol/incubation tube)


C
12
10
8
6
4
2
Co/Cx
0 1 2 3 4 5 6 7 8
Concentration of cyclic GMP (pmol/incubation tube)

was subtracted from that of each pair of known and unknown samples. A calibration curve (Fig.2.1A) was constructed using the data. The amount of protein in the unknown samples was determined from this curve.

(B) Cyclic AMP assay: The measurements of cyclic AMP were made according to the method described by the Radiochemical Centre Assay Kit (TRK 420).

Small test tubes suitable for centrifuging and in duplicate were placed into a rack which was kept in an ice-water bath. The tubes were labelled and various reagents added according to the protocol shown in table 2.1B. Each tube was capped and vortex mixed for 5 seconds. The ice bath containing the assay tubes was placed in the refrigerator at 2- 4^{o}C for 2 hours.

Tube No.	Reagent 1 (Buffer)	Standards (cAMP)	Unknowns	Reagent 3 (H^3) cAMP	Reagent 2 Binding Protein	
1,2	150	-	-	50	-	Charcoal blank
3,4	50	-	-	50	100	Zero dose
5,6	-	50	-	50	100	1pmol standard
7,8	-	50	-	50	100	2pmol standard
9,10	-	50	-	50	100	4pmol standard
11,12	-	50	-	50	100	8pmol standard
13,14	-	50	-	50	100	16pmol standard
15,etc.	-	-	50	50	100	Unknowns

All volumes are in microlitres.

Table 2.1 B: Cyclic AMP assay protocol.

After incubation, 100 µl of charcoal suspension (ice cold and continuously stirred) was added to each assay tube which was vortex mixed for 5 seconds. The tubes were centrifuged (10min, 3000 rpm, 0°C) approximately 5 minutes from the addition of the charcoal suspension. A volume of 200 ul of the clear supernatant was carefully removed and placed into scintillation vials, which contained 100ml Toluene/Triton X-100, for counting. Each vial was counted for 5 minutes using the Packard Tri Card Liquid Scintillation Spectrometer.

The count per assay tube was determined as count per minute (c.p.m.) after correction for instrument blank. The average c.p.m. of tubes 1 and 2 gave the blank. From the average c.p.m. of tubes 3 and 4 the blank was subtracted and this resulted in c.p.m. bound in the absence of unlabelled cyclic AMP (Co). The blank was also deducted from the average c.p.m. bound in the presence of the standards (tubes 5-14) and unknowns (tubes 15 etc) unlabelled cyclic AMP (Cx). The ratio $\frac{Co}{Cx}$ for each level of cyclic AMP was plotted against standard inactive cyclic AMP (pmol/incubation tube) to obtain a linear standard curve (Fig 2.1B) with an intercept of one on the ordinate. The concentration of cyclic AMP in each unknown is calculated from the calibration curve using the ratio $\frac{Co}{Cx}$ for each. The amount of cyclic AMP in the unknown is divided by the

total weight of protein in the ventricle giving the results in pmol mg^{-1} protein.

(C) Cyclic GMP assay: The analysis of cyclic GMP was done according to the method described by the Radiochemical Centre Cyclic GMP Radioimmunoassay Kit (TRK 500).

Small test tubes suitable for centrifuging and in duplicate were placed into racks which were kept throughout the assay in an ice-water bath. The tubes were labelled and the various reagents added according to the protocol in table 2.1C. The assay tubes were capped and vortex mixed for 5 seconds. The ice-bath containing the tubes was placed in the refrigerator at 2-4^{o}C for one and a half hours.

After incubation, 1ml ice cold $(NH_4)_2 SO_4$ solution (60% saturated) was added to each assay tube and vortex mixed for 5 seconds. The tubes were centrifuged (10 minutes; 3000 rpm; 0^{o}C) approximately 5 minutes after the addition of the ammonium sulphate solution. The supernatant was decanted and the precipitate dissolved in 1.1ml ice-cold distilled water. A volume of 1ml sample was removed from each tube and added to vials containing 10ml Toluene-Triton X-100 scintillant for counting. Each vial was counted for 5 minutes using the Packard (TRI CARD) Liquid Scintillation Spectrometer and the count per assay tube determined as count per minute (c.p.m.) after correction for the instrument blank.

Table 2.1C: Cyclic GMP assay protocol.

Tube No.	Reagent 1 (Buffer)	Standards (cGMP)	Reagent 5 (Blank)	Unknowns	Reagent $[H^3]$ cGMP	Reagent 2 (Antiserum	
1,2	100	-	-	-	50	50	Zero dose
3,4	-	100	-	-	50	50	0.5 pmol Std.
5,6	-	100	-	-	50	50	1.0 pmol Std.
7,8	-	100	-	-	50	50	2.0 pmol Std.
9,10	-	100	-	-	50	50	4.0 pmol Std.
11,12	-	100	-	-	50	50	8.0 pmol Std.
13,14	-	-	100	-	50	50	Blank
15,etc	-	-	-	100	50	50	Unknowns

All volumes are in microlitres.

The average c.p.m. of the blank (tubes 13 and 14) was subtracted from the average c.p.m. of tubes 1 and 2 to give Co (the c.p.m. bound in the presence of unlabelled cyclic GMP). The blank was also deducted from the average c.p.m. for each pair of assay tubes for the standards (tubes 3-12) and unknowns (tubes 15 etc.) to give Cx (the c.p.m.bound in the presence of the standard and unknown cyclic GMP). The ratio $\frac{Co}{Cx}$ was plotted against varying dilutions (pmol) cyclic GMP to give a linear standard curve (Fig 2.1C). The amount of cyclic GMP in the unknowns was calculated from the standard curve using $\frac{Co}{Cx}$ for each. The amount of cyclic GMP in each unknown is divided by the total weight of the ventricle giving the results in pmol mg^{-1} protein.

(D) Extraction, boiassay and identification of prostaglandins.

Extraction of prostaglandin - like substance: Samples of perfusates were acidified to pH3 with 1NHCl, and then extracted twice with equal volumes of ethyl acetate. The organic phase was washed neutral with distilled water and evaporated to dryness. Nitrogen was blown into the flask to remove traces of ethyl acetate and the residue was taken up in 30ml of 67% ethanol. The ethanolic solution was washed twice with 15ml of petroleum spirit (b.p. 40 - 60^{o}C), then evaporated to dryness. Prostaglandin - like substance in the final extract was either dissolved in 1ml Krebs solution and subjected to biossay or (b) 0.5ml

ethanol and subject to thin-layer chromatography.

Bioassay of prostaglandin - like substance: The final extract dissolved in Krebs solution was assayed for prostaglandin - like activity using the rat fundus strip. The stomach strip was prepared according to Vane (1957), mounted in an organ bath and immediately superfused at a rate of 5-10 ml min^{-1} with Krebs solution (composition (mM): NaCl, 118.4; KCl, 3.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 2.2; $NaHCO_3$, 24.9; $CaCl_2$, 2.6; glucose, 10; pH, 7.4) gassed with 5% CO_2 in O_2. The technique of superfusion was basically that of Gaddum (1953): the tissue was counterweighted with 0.5 - 2g and its movement was recorded with an isotonic lever writing frontally on a rotating drum.

To obtain specific responses of the assay organ to prostaglandin the following were conducted. A mixture of inhibitors containing atropine (2×10^{-6} M), mepyramine (3×10^{-6} M), methysergide (6×10^{-7} M), phentolamine (1×10^{-6} M) and propranolol (1×10^{-5} M) was added to the superfusing solution in order to render the tissue insensitive to acetylcholine, histamine, 5-hydroxytryptamine, noradrenaline and adrenaline while its sensitivity to prostaglandin was retained. In those experiments in which the heart was treated with indomethacin, the assay tissue was also superfused with the same drug at a concentration of 10 μM in order to inhibit the release of prostaglandin

from the rat fundus strip.

Authentic prostaglandin E_1 was used as bioassay standard. A sample measuring 0.5 ml either of known prostaglandin E_1 in Krebs or the unknown was assayed at 37°C. After superfusing the fundus strip for half an hour, varying concentrations of prostaglandin E_1 were used. The concentration of prostaglandin - like substance in the final extract was determined by bracket assay.

Thin-layer chromatography: The glass plates (0.4 x 20 x 20 cm) were coated by spreading a mixture of 50g silica gel G (Merk) and 110 ml of water using the Camag coater 21251 (thickness of coating approximately 0.3 mm). In some cases silver nitrate (5.g) was dissolved in the water before addition to the silica gel. The plates were activated by heating for about 30 minutes at 110 - 115°C before being used. Authentic prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, A_1 and A_2 and extracted prostaglandin - like material were applied on the plates as single spots using micropipettes.

The chromatoplates were placed in glass jars containing the AI (benzene - dioxane - acetic acid, 20: 20: 1) and AII (ethyl acetate - acetic acid - methanol, petroleum spirit - water, 110: 30: 35: 10: 100) solvent systems of Green and Samuelsson (1964) and covered with glass

plates. The plates were developed using the ascending technique, which was interrupted when the solvent front reached a point 2-4 cm from the top (75 - 100 minutes). After development, the plates were dried at 100°C and sprayed with 10% phosphomolybdic acid in ethanol and heated for 15 minutes at 120°C, which resulted in the appearance of dark blue spots on a yellow back-ground. The R_f values of the spots from the authentic and unknown samples were compared.

(E) DRUGS.

Drugs were freshly prepared as stock concentrated solutions in distilled water or in frog Ringer solution immediately before use. Final dilutions were made from stock.

The following drugs were used:-
O- acetylcholine chloride (BDH), adenosine (Sigma), adenosine 5'- triphosphate (Sigma), adenosine 5'- diphosphate (Sigma), adenosine 5'- monophosphoric acid (Sigma), adenosine 3', 5' - cyclic monophosphate (Sigma), adrenaline tartrate (BDH), arachidonic acid (Simga), atropine sulphate (BDH), 8-Bromo guanosine 3', 5' - cyclic monophosphate (ICN Pharmaceuticals), cytidine 5'- triphosphate (Sigma), N^6, 0^2- dibutyryl adenosine 3', 5' - cyclic monophosphoric acid (Sigma), guanosine 5' - triphosphate (Sigma), guanosine 3', 5' - cyclic monophosphoric acid (Sigma), indomethacin lactose (Merk, Sharpe and Dhome Ltd), inosine 5' - triphosphate (Sigma), DL -

isoprenaline hydrogen chloride (Sigma), mepyramine bimaleate (May and Baker Ltd), methysergide bimaleate (Sandos Ltd), phentolamine (Ciba), propranolol hydrogen chloride (Sigma), prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, A_1 and A_2 (Upjohn Company Ltd), uridine 5' - triphosphate (Sigma) and verapamil (Pfizer).

A P P E N D I X IV

Table 4.1. Intracellular cyclic 3',5'- nucleotides and contractility of the hypodynamic frog ventricle.

Time (sec)	Control (normodynamic) pmol mg^{-1} protein		Development of the hypodynamic state pmol mg^{-1} protein		Cyclic nucleotide ratio	Tension ratio
	c GMP	c AMP	c GMP	c AMP		
9	0.65	17.17	0.72	15.85	0.83	0.85
18	0.70	16.39	0.81	13.64	0.72	0.74
30	0.66	15.5	0.92	13.31	0.61	0.63
40	0.69	14.98	0.95	10.49	0.51	0.50
45	0.60	15.0	0.78	8.80	0.46	0.45
52	0.52	17.36	0.82	9.55	0.35	0.37
61	0.45	16.40	0.78	9.00	0.32	0.31
70	0.40	14.65	0.70	6.05	0.24	0.25
80	0.44	15.14	0.88	6.42	0.21	0.23
87	0.40	15.11	0.75	4.59	0.17	0.18

Mean values ($\pm$ S.E's) for the levels of cyclic AMP and cyclic GMP in freshly frozen ventricles (10 preparations were found to be 15.80 $\pm$ 0.31 pmol mg^{-1} protein and 0.55 $\pm$ 0.04 pmol mg^{-1} protein, respectively. Recovery of known amounts of cyclic AMP and cyclic GMP were 90.80% and 89.72%, respectively.

Table 4.2. Cyclic nucleotide levels in the superfusates during the development of the hypodynamic state

Time (min)	Cyclic AMP (pmol mg^{-1} protein)	Cyclic GMP (pmol mg^{-1} protein)	Tension (Fraction of initial value)
10	1.17	0.112	0.88
20	1.58	0.115	0.68
30	1.81	0.114	0.47
40	2.01	0.113	0.31
50	2.35	0.094	0.25
60	3.10	0.110	0.19
70	3.25	0.089	0.16

Intracellular cyclic AMP and cyclic GMP levels for the freshly frozen ventricle (initial level) and the control (hypodynamic)

ventricle were 20.55 and 0.122 pmol mg^{-1} protein and 7.99 and 0.245 pmol mg^{-1} protein, respectively.

A P P E N D I X VII

Table 7.1. Effects of ATP (10^{-3}M) on force of contraction and cyclic nucleotide levels in isolated frog ventricle.

Time - course experiments

Time (sec)	Control (hypodynamic) pmol mg^{-1} protein		ATP (10^{-3}M) pmol mg^{-1} protein		Cyclic nucleotide ratio	Tension ratio
	c GMP	c AMP	c GMP	c AMP		
4	1.25	9.5	2.26	27.48	1.59	1.57
6	0.85	6.28	1.76	28.14	2.10	2.30
24	0.34	7.78	1.39	5.92	0.19	0.21
24	0.35	6.35	0.48	4.68	0.54	0.53
38	0.27	8.61	0.43	5.76	0.42	0.42
50	1.04	9.55	1.5	13.33	0.96	0.94
75	1.08	8.72	1.00	17.40	2.13	2.28
84	1.26	9.38	2.10	22.55	1.44	1.44
106	1.11	9.05	0.73	19.20	3.25	3.22
152	1.45	9.47	1.23	30.29	3.77	3.86
152	0.50	6.29	0.60	21.76	2.86	2.76
200	1.39	5.64	1.66	16.36	1.25	1.25

Mean values ($\pm$ S.E's) for levels of cyclic AMP and cyclic GMP in the control (hypodynamic) ventricles (12 preparations) were 8.05 $\pm$ 0.43 pmol mg^{-1} protein and 0.91 $\pm$ 0.13 pmol mg^{-1} protein, respectively.

Interference from ATP (10^{-3}M). The cross reactivity of 10^{-3} ATP with cyclic AMP binding protein and cyclic GMP antiserum was investigated to establish if ATP interferes with the assay. The results are shown in table 7.2. and suggest that ATP does not cross-react in these assays at 10^{-3}M. These results are in agreement with the findings of the Radiochemical Centre Product Information (1977) in which it is reported that ATP does not interfere with (a) the cyclic AMP assay, at concentrations up to 5 mM which represents approximately one million fold-excess over the tritium-labelled cyclic AMP or (b) the cyclic GMP assay, at concentrations up to 1mM which represents a 5000,000-fold excess over tritiated cyclic GMP.

Table 7.2.

Conditions		Average c.p.m.	% age cross reactivity
Control	c AMP, 8 pmol	625.4	-
Test	c AMP, 8 pmol + 10^{-3}M ATP	623.0	0.38
Control	c GMP, 4 pmol	440.5	-
Test	c GMP, 4 pmol + 10^{-3}M ATP	438.4	0.47

A P P E N D I X IX

Table 9.1: Effects of isoprenaline on force of contraction and cyclic 3',5'- nucleotide content in isolated frog ventricular strips.

(a) Time-course experiments. Isoprenaline (10^{-6}M) R temporaria.

Time (sec)	Control (hypodynamic) pmol mg^{-1} protein		Isoprenaline (10^{-6}M) pmol mg^{-1} protein		Cyclic nucleotide ratio	Tension ratio
	c GMP	c AMP	c GMP	c AMP		
4	1.04	9.55	0.99	13.27	1.45	1.41
8	1.22	10.49	0.83	15.32	2.14	2.08
16	1.44	11.93	0.63	19.07	3.65	3.49
30	1.46	11.97	1.06	37.84	4.44	4.30
33	1.57	10.20	1.40	38.35	4.18	4.20
60	1.48	9.30	1.55	46.76	4.70	4.88
87	1.32	7.34	1.70	46.15	4.84	4.83
144	1.35	6.53	1.82	30.65	3.70	3.87
196	1.28	7.50	1.96	25.16	2.20	1.90
268	1.25	6.87	2.14	20.17	1.71	1.59

Mean values ($\pm$ S.E's) for levels of cyclic AMP and cyclic GMP in control (hypodynamic) ventricles (17 preparations) were 9.19 $\pm$ 0.38 pmol mg^{-1} protein and 1.48 $\pm$ 0.05 pmol mg^{-1} protein, respectively.

(b) Dose-dependent experiments. Isoprenaline (10^{-4} - 10^{-10}M).

R escuelenta

Conc. (M)	Time (Sec)	Control (hypodynamic) pmol mg^{-1} protein		Isoprenaline (10^{-4} - 10^{-10}M) pmol mg^{-1} protein		Cyclic nucleotide ratio	Tension ratio
		c GMP	c AMP	c GMP	c AMP		
10^{-4}	96	1.63	7.96	2.96	57.36	3.99	4.02
10^{-5}	96	1.66	8.55	2.85	55.70	3.80	3.75
10^{-6}	104	1.84	10.01	2.70	52.06	3.60	3.50
10^{-7}	92	1.81	10.70	2.29	40.57	3.00	2.93
10^{-8}	91	1.67	10.00	2.01	33.61	2.79	2.75
10^{-9}	91	1.62	10.07	1.85	30.84	2.69	2.65
10^{-10}	92	1.52	8.64	1.67	22.46	2.36	2.40

Interference from isoprenaline. In the present study cross reactivity of isoprenaline (10^{-6}M) with cyclic AMP binding protein and cyclic GMP antiserum was done to investigate whether the β-agonist interferes with the assay procedures. The results are summarised in table 9.2. and suggest that the extent of cross reaction is negligible.

Table 9.2.

Conditions		Average c.p.m.	% age cross reactivity
Control	c AMP, 8 pmol	625.4	-
Test	c AMP, 8 pmol + 10^{-6}M isop.	622.5	0.46
Control	c GMP, 4 pmol	440.5	-
Test	c GMP, 4 pmol + 10^{-6}M isop.	438.8	0.38

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Effects of ATP on the hypodynamic frog ventricle

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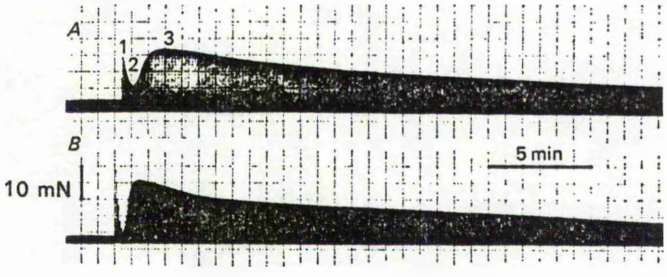
The effects of exogenous ATP on the electrical and mechanical properties of the hypodynamic frog ventricle have been investigated. Strips of ventricle were mounted horizontally and attached to a strain gauge. The inner surface was superfused with frog Ringer (100 ml. min^{-1}) containing ATP (10^{-9} to 10^{-3} M) while the preparation was stimulated electrically (0·2–1 Hz; 10 V square pulses; 5 msec duration). Fig. 1 shows the response



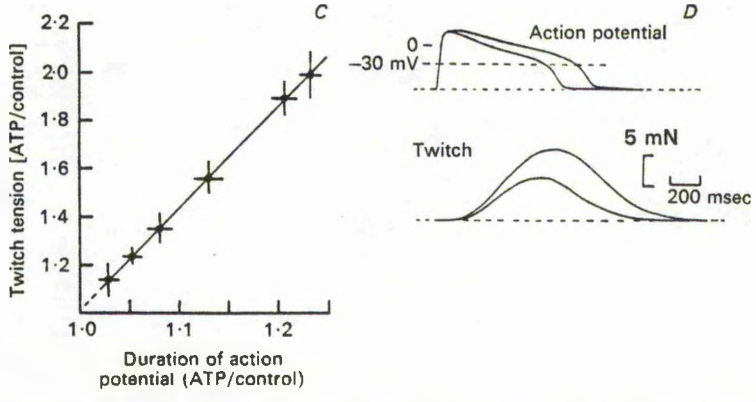


Fig. 1, *A*, *B*, chart-recordings of two responses to ATP, 10^{-5} M (*A*) and 10^{-3} M (*B*). *C*, the relation between twitch tension and action potential duration for concentrations of ATP ranging from 10^{-9} to 10^{-3} M. The values were obtained for the 50th twitch, recorded during the third phase of the response. Mean values ± 1 S.D. are shown (n = 10 for each point). *D*, isometric twitches (lower records) and action potentials (upper records) recorded in normal Ringer and in presence of 10^{-3} M ATP (50th twitch).

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to 10^{-5} M (*A*) and 10^{-3} M (*B*) ATP. There is an immediate, positive inotropic effect (1), followed by a period when the twitch is depressed, sometimes to below the control (hypodynamic) level (2), superseded by a longer-lasting ($\sim$ 80 min) potentiation (3), which is maximal after 1–2 min (cf. Versprille, 1963). Pretreatment with theophylline (10^{-4} M, 10 min) enhances the third component of the ATP response, but depresses the first. ADP produces a qualitatively similar response to ATP, as does AMP in high (10^{-3} M) doses, but adenosine has a small inhibitory effect. ATP affects the time course of the action potential (Fig. 1*D*). Fig. 1*C* shows the relationship between action potential duration (measured at -30 mV) and twitch potentiation, recorded during the third phase, for [ATP] of 10^{-8} to 10^{-3} M (slope of regression line $= 4{\cdot}173 \pm 0{\cdot}013$; $P < 0{\cdot}001$; $n = 6$).

The observations suggest that ATP facilitates entry of Ca^{2+} into the fibres and that its inotropic actions are mediated through changes in intracellular levels of cyclic-AMP and -GMP (Nawrath, 1976).

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Intracellular cyclic nucleotides and contractility of the hypodynamic frog ventricle

By F. W. Flitney, J. F. Lamb and J. Singh. *Department of Physiology and Pharmacology, University of St Andrews, St Andrews, Scotland*

Intracellular cyclic AMP and cyclic GMP have been measured in isolated frog ventricles at various times during the development of the hypodynamic state. Ventricular strips were superfused with frog Ringer (100 ml. min^{-1}; 1–8 hr) and stimulated at 0·5 Hz (square pulses, 10 V, 5 msec) through silver-wire electrodes. Preparations were frozen in liquid nitrogen and assayed for cyclic AMP, cyclic GMP and total protein.

The decline in twitch tension is depicted in Fig 1*A* (triangles), together with observed changes in intracellular cyclic AMP and cyclic GMP. Tension and cyclic nucleotide levels are expressed as multiples of the initial (control) values. There is a progressive reduction in cyclic AMP, accompanied by a rise in cyclic GMP; the resulting decrease in the ratio [cyclic AMP]/[cyclic GMP] parallels closely the decrease in isometric force (Fig. 1*B*).

Superfusion with Ringer solution containing exogenous cyclic nucleotides affects both the time course of the decline in twitch tension and the steady-state level attained in a dose-dependent fashion: 8-bromo-cyclic GMP accelerates the decay of tension and decreases the steady-state level, whereas dibutyryl cyclic AMP delays the development of the hypodynamic state and elevates the steady-state value. Dibutyryl-cyclic AMP ($> 10^{-3}$ M) prevents the decline of twitch tension (up to 8 hr) and produces steady-state levels up to 1·3 × the initial (control) value.

These results suggest that the ratio [cyclic AMP]/[cyclic GMP] is an important determinant of contractile force in the isolated frog ventricle.

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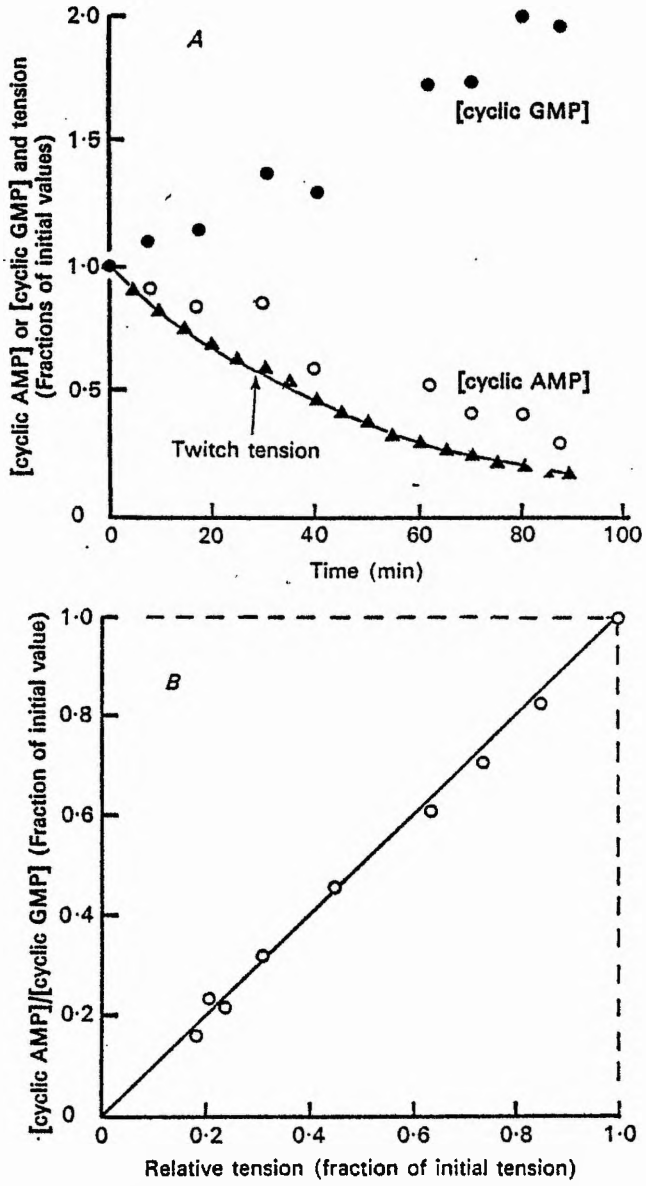

Fig. 1. *A*, the decline in twitch tension and changes in cyclic nucleotide levels. *B*, relation between relative tension and the ratio [cyclic AMP]/[cyclic GMP].

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Effects of exogenous ATP on contractile force and intracellular cyclic nucleotide levels in the hypodynamic frog ventricle

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We (Flitney, Lamb & Singh, 1977*a*) postulated that the inotropic effects of ATP on the hypodynamic frog ventricle are mediated through changes in cyclic nucleotide levels, and demonstrated (Flitney, Lamb & Singh, 1978) that the decline in isometric force during the development of the hypodynamic state is accompanied by a decrease in cyclic AMP and a rise in cyclic GMP. We now offer direct evidence for a close correlation between changes of twitch tension and [cyclic AMP]/[cyclic GMP] following treatment of hypodynamic ventricles with exogeneous ATP.

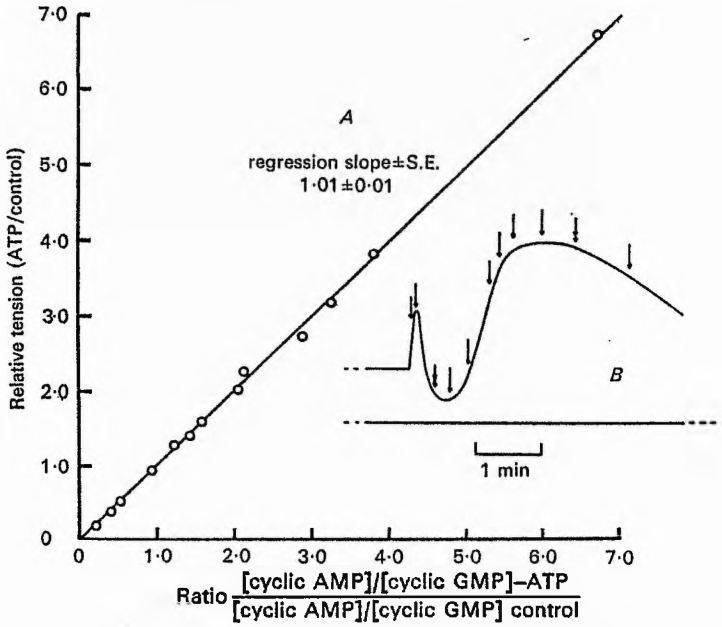

Fig. 1. Relation between relative force and cyclic nucleotide ratios (*A*) measured at various times (*B*, arrows) during the inotropic response to 10^{-3} M-ATP.

Hypodynamic ventricular strips were superfused with frog Ringer containing 10^{-3} M-ATP. Preparations were frozen at different times during the ATP response (Fig. 1*B*) and assayed for cyclic AMP, cyclic GMP and total protein. Fig. 1*A* shows the relation between twitch tension and [cyclic AMP]/[cyclic GMP]. Isometric force and cyclic nucleotide ratios are expressed as multiples of the control (hypodynamic) values. The observed linear relation (slope of regression line + S.E.: $1{\cdot}01 \pm 0{\cdot}01$; $n = 13$; $P < 0{\cdot}001$) suggests that ATP exerts its effect through changes in intracellular cyclic nucleotides and lends further support to the view (e.g. Nawrath, 1976) that these substances are important factors in determining myocardial contractility.

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EFFECTS OF ISOPRENALINE ON CONTRACTILE FORCE AND INTRACELLULAR CYCLIC 3′,5′-NUCLEOTIDE LEVELS IN THE HYPODYNAMIC FROG VENTRICLE

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1. Introduction

The increase in contractility which occurs in response to β-adrenergic stimulation of the heart is thought to be mediated through changes in the levels of intracellular adenosine 3′,5′-cyclic monophosphate (cyclic AMP) [1–5]. The possibility that guanosine 3′,5′-cyclic monophosphate (cyclic GMP) is also involved in regulating the contractile response of the heart has received comparatively little attention. This is somewhat surprising since it has been shown that the negative inotropic response to acetylcholine is associated with a rise in intracellular cyclic GMP [6–8], and that the levels of both cyclic AMP and cyclic GMP undergo oscillatory changes which are synchronised with the cardiac cycle [9]. In addition, we demonstrated that the development of the hypodynamic condition [10] and the inotropic response of the isolated frog ventricle to stimulation with exogenous ATP [11,12] are characterized by changes in the levels of both cyclic 3′,5′-nucleotides. Moreover, in each instance a striking correlation exists between changes in isometric twitch tension and the ratio of (cyclic AMP)/(cyclic GMP).

This paper presents results which show changes in the levels of cyclic AMP and cyclic GMP following stimulation of the hypodynamic frog ventricle with the β-agonist isoprenaline and here too there is a clear correlation between the magnitude of the contractile response and the change in the cyclic nucleotide ratio.

2. Methods

2.1. *Heart perfusion*

Hearts from frogs, *Rana temporaria* and *Rana esculenta*, were isolated and superfused at room temperature (18–20°C) by the method in [13]. The perfusion rate was kept constant at 100 ml. min^{-1} and the preparation was stimulated (Ag wire electrodes) at 30 min^{-1} (square, 5 ms pulses, 10 V). The ventricular strip was allowed to become hypodynamic and subsequently superfused with frog Ringer containing isoprenaline. At a predetermined time during the isoprenaline response it was freeze-clamped in liquid nitrogen. Another strip taken from the same ventricle was allowed to become hypodynamic to the same extent as the first and then freeze-clamped in liquid nitrogen. The latter served as the control strip.

2.2. *Extraction and assay for cyclic nucleotides*

Frozen ventricular strips were pulverized into a powdered extract. Cyclic AMP and cyclic GMP from the latter were extracted and assayed by the techniques in [14] and [15], respectively. The amount of protein present in the extract was estimated by the method depicted by [16]. Cyclic nucleotide results were expressed as pmol cyclic AMP or cyclic GMP/mg protein.

3. Results and discussion

Figure 1 shows the time course of the changes in intracellular cyclic AMP and cyclic GMP following superfusion of isolated frog (*R. temporaria*) ventricular strips with 10^{-6} M isoprenaline. The accompanying contractile response is also shown (dashed line) for comparison. There are two points to emphasise. First, the early increase in contractile force

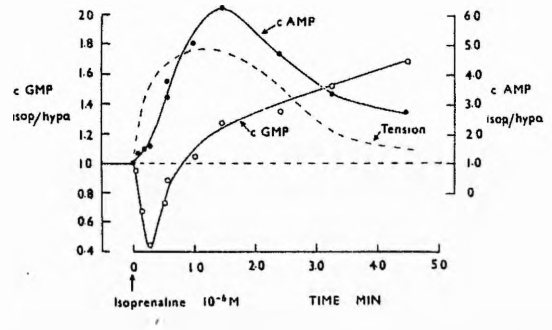

Fig.1. Time courses of changes in contractile force (dashed line) and intracellular levels of cyclic AMP (solid circles) and cyclic GMP (open circles) during exposure of the frog ventricle to 10^{-6} M isoprenaline. Control (hypodynamic) values (mean ± SE) for cyclic AMP and cyclic GMP levels (pmol mg^{-1}. total protein) were 9.39 ± 0.66 and 1.44 ± 0.15, respectively (n = 17).

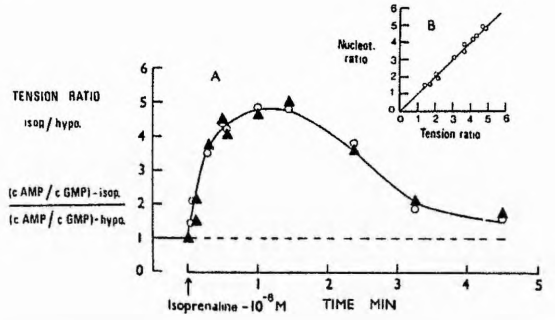

Fig.2. A. Time course of the change in isometric twitch tension (open circles) and cyclic nucleotide ratio (solid triangles) measured at various times during the response to 10^{-6} M isoprenaline. Both parameters expressed as multiples of the control (hypodynamic) values. B. Data taken from fig.1. Cyclic nucleotide ratio plotted against contractile force. Solid line drawn with slope of 1. Correlation coefficient ± SE of estimate: 0.995 ± 0.143, n = 10, $P < 0.001$.

corresponds more closely with an initial decrease in the level of cyclic GMP; the rise in the level of cyclic AMP is delayed and lags somewhat behind the contractile response. This result is of considerable interest since it was demonstrated in mammalian hearts [17–19] that a rise in cyclic AMP preceeds the onset of the inotropic response; indeed, this provides essential corroborative evidence in support of the 'second messenger' role for cyclic AMP [1]. Secondly, cyclic GMP levels reach to the control value (horizontal broken line) at about the time when the contractile response is maximal and further increase is accompanied by a fall in isometric twitch tension and a decrease in the level of cyclic AMP.

It was found earlier that a striking parallelism exists between the time course of the change in ventricular contractility and the ratio of the two cyclic nucleotides during both the development of the hypodynamic condition [10] and during the inotropic response to exogenous ATP [12]. The results of fig.1. yield a similar correlation, as shown in fig.2A. This depicts the change in the ratio of cyclic AMP/cyclic GMP, that is:

$$\left[\frac{\text{cyclic AMP/cyclic GMP (isop)}}{\text{cyclic AMP/cyclic GMP (hypo)}}\right]$$

with time (solid triangles), together with the accompanying tension response (open circles). Statistically, the correlation is highly significant (correlation coefficient ± SE of estimate: 0.995 ± 0.143, n = 10, $P < 0.001$) and reveals a clear correspondence between a change in the cyclic nucleotide ratio and the accompanying change in force (fig.2B, inset).

The effects of altering the concentration of isoprenaline on cyclic nucleotide levels and on contractile force for seven preparations is shown in fig.3,4. In

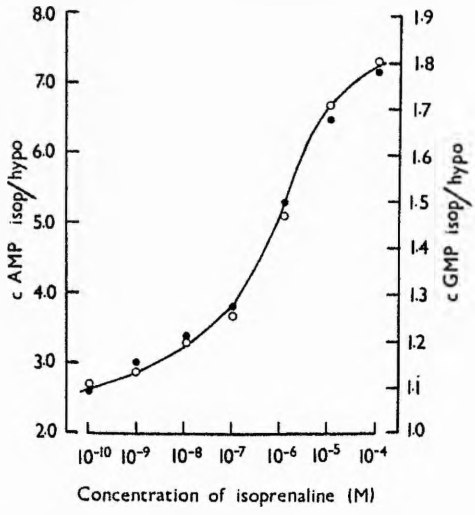

Fig.3. Log dose–response curves for concentrations of isoprenaline ranging from 10^{-10}–10^{-4} M. Solid circles, cyclic AMP; open circles, cyclic GMP. Preparations superfused for approximately 100 s prior to freeze-clamping. Both parameters expressed as multiples of control values.

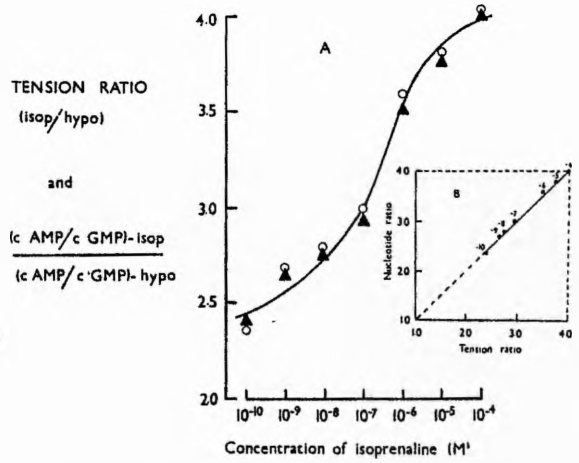

Fig.4. A. Effects of isoprenaline ranging from 10^{-10}–10^{-4} M on contractile force (open circles) and cyclic nucleotide ratio (solid triangles; data from fig.3). B. Relation between isometric force and cyclic nucleotide ratio (multiples of control values). Correlation coefficient ± SE of estimate: 0.996 ± 0.57, $n = 7, P < 0.001$.

these experiments hearts from another species of frogs, *R. esculenta*, were used. Figure 3 shows log dose–response curves for the increase in cyclic AMP (solid circles) and cyclic GMP (open circles) measured ~ 100 s after superfusing with the drug when the response was maximal. The change in the ratio of cyclic AMP/cyclic GMP with varying concentrations of isoprenaline and the corresponding change in isometric twitch tension, measured at the time of freeze-clamping each preparation, are shown in fig.4. The data again reveal a clear correlation (fig.4B. inset: correlation coefficient ± SE of estimate: 0.996 ± 0.057, $n = 7, P < 0.001$) between the cyclic nucleotide ratio and contractile force.

These results, and those referred to earlier [10,12], lead us to postulate that cyclic AMP and cyclic GMP are important components of a control mechanism which regulates the capacity of the ventricle to produce force. The observed correlation between the ratio of the two cyclic nucleotides and isometric force suggests that they play opposing roles in the regulatory process, a feature which is seen in several other biological systems [20]. Both cyclic AMP and cyclic GMP are thought to exert their regulatory effects on cellular metabolism by activating a number of protein kinases, and several substrates for cyclic AMP-dependent protein kinases have been implicated in regulating myocardial contractility, troponin I (TN-I), a subunit of the regulatory protein complex [21]; phospholamban, a 22 000 dalton protein constituent of the sarcoplasmic reticulum [22]; and a surface membrane-bound protein, thought to be a component of the slow inward (calcium) current channel [23]. It has been previously demonstrated that perfusion of the rat hearts with isoprenaline produces a time-dependent increase in the state of phosphorylation of TN-I, which almost exactly parallels the time course of the resulting contractile response [24]. The existence of a similar relationship between the contractile force and the ratio cyclic AMP/cyclic GMP raises the possibility that the state of phosphorylation of TN-I and perhaps of other phosphoproteins involved in regulating force production may be determined by the relative amounts of intracellular cyclic AMP and cyclic GMP. A cyclic GMP-dependent protein kinase has been isolated from the heart [25], although to date there have been no reports of a naturally-occuring substrate for this enzyme in the heart. The phosphatase enzymes responsible for dephosphorylating protein substrates would appear to be plausible candidates in the light of the present results.

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C. 13

Release of prostaglandins from the superfused frog ventricle during the development of the hypodynamic state

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In a previous communication (Flitney, Lamb & Singh, 1978) we reported changes in intracellular cyclic 3′,5′-nucleotide levels during superfusion of isolated frog ventricular preparations. We now present evidence that the loss of two prostaglandins, PGE_1 and PGE_2, and a prostaglandin-related substance (PRS) also accompany the development of the hypodynamic condition.

First, agents which affect PG biosynthesis influence both the rate of development and the degree of hypodynamic depression. Indomethacin, a PG synthetase inhibitor (Vane, 1971), *accelerates* the rate at which the hypodynamic state develops and *depresses* the final steady-state twitch tension, whereas arachidonic acid, the principal precursor of PG biosynthesis (Samuelsson, 1972), *delays* the rate at which the twitch tension declines and *elevates* the final steady-state level. Secondly, bioassay of ethyl acetate extracts of superfusates from ventricles allowed to become either partially or wholly hypodynamic provides evidence for a time-dependent decrease in intracellular PG levels which correlates with the observed decrease in contractile force (correlation coefficient $\pm$S.E. $0{\cdot}98 \pm 0{\cdot}100$, $n = 8$, $P < 0{\cdot}001$). Thirdly, thin-layer chromatography of pooled extracts, using Green and Samuelsson's (1964) A II solvent system, revealed the presence of PGE_1 and PGE_2, together with a hitherto unidentified PRS with an R_f value 0·28.

These results implicate the loss from the ventricle of PGE_1 and PGE_2, together with a PRS, as possible factors contributing to the observed deterioration in the contractile response. They broadly support Clark's (1913) contention that leakage of a lipid from the fibres may be responsible for the development of the hypodynamic state. Establishing a casual relationship is of course difficult, but in this context, it is worth noting that authentic PGE_1 and PGE_2 both delay the onset of the hypodynamic condition and also potentiate the contractile response of the 'fully' hypodynamic ventricle. Moreover, the identification of prostaglandins in the circulating superfusates adds significance to our earlier observations on changes in cyclic 3′,5′-nucleotide levels, since the E series of prostaglandins is known to stimulate adenylate cyclase activity (Klein & Levey, 1971). Thus, leakage of prostaglandins and/or the PRS may initiate changes in intracellular cyclic 3′,5′-nucleotide levels, which in turn affect the capacity of the ventricle to contract.

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