SOME EFFECTS OF TRIFLUOPERAZINE AND SODIUM NITROPRUSSIDE ON THE ISOLATED FROG VENTRICLE

Thesis in partial fulfilment of the requirements for the degree of Master of Science

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Declaration

I hereby declare that the thesis I have submitted in partial fulfilment of the requirements governing candidates for the degree of Master of Science, in the University of St. Andrews, entitled 'Some effects of trifluoperazine and sodium nitroprusside on the isolated frog ventricle', is the result of my own work. 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 Dr. F.W. Flitney.

Certificate

I hereby certify that Mehdi Mohsenin Moshiri has spent four terms engaged in research work under my 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 Master of Science.

Academic Record

I first matriculated at the University of St. Andrews in October 1976 and graduated with a B.Sc. (Honours) in Medical Biology in July 1980. I matriculated as a postgraduate research student of the Department of Physiology and Pharmacology, University of St. Andrews in October 1980. At present I am studying for the degree of MB BChir. at Darwin College, University of Cambridge School of Clinical Medicine.

Aristotle said: "All men, naturally want to know. The sign of this is the pleasure which they get from using their senses, among which I choose as the outstanding one that of vision, because this is the one most conducive to our knowledge of anything and makes plain many differences."

Even though it was not possible to <u>see</u> the second messengers, I had the good fortune to <u>feel</u> them. Believe me when they hit you, it is like being resurrected.

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ABBREVIATIONS

TFP	Trifluoperazine
SNP	Sodium nitroprusside
cAMP	Adenosine 3',5'-cyclic monophosphate
cGMP	Guanosine 3',5'-cyclic monophosphate
Ca^{++}	Ionised Calcium
Na^{+}	Ionised Sodium
K^{+}	Ionised Potassium
Mg^{++}	Ionised Magnesium
Mn^{++}	Ionised Manganese
H^{+}	Ionised Hydrogen (proton)
S.R	Sarcoplasmic Reticulum
$[\ ]_o$	concentration

SUMMARY

1. A study has been made of the response of the superfused (hypodynamic) frog ventricle to treatment with trifluoperazine (TFP), an anti-psychotic drug which is known to inhibit the calcium dependent regulator protein, calmodulin; and sodium nitroprusside (SNP), a potent stimulator of guanylate cyclase activity.
2. Both TFP and SNP depress the twitch, in a dose dependent manner with ED_{50} values of 1.2×10^{-6} M ($T_{\frac{1}{2}}$ = 7 mins) and 2×10^{-5} M ($T_{\frac{1}{2}}$ = 9 mins) respectively.
3. The responses to both TFP and SNP were unaltered by atropine, propranolol and phentolamine in the superfusate.
4. The response to TFP was irreversible and was not antagonised by Theophylline, while SNP had a reversible effect, and this was antagonised by the presence of Theophylline.
5. The shape of the action potential was unaffected by TFP and SNP during the initial stages, first 10 minutes with TFP and first 2 minutes with SNP of a response, during which time the isometric twitch tension decreased by 15% and 25% respectively. Action potential duration and peak twitch tension showed a parallel decline during the remainder of the response.
6. SNP stimulates the production of both 3',5'-cyclic GMP and 3',5'-cyclic AMP. The time course of the change in contractility is paralleled closely by a corresponding reduction in the ratio cyclic AMP : cyclic GMP.
7. These results support the view that the three secondary messengers (Ca^{++}, cAMP and cGMP) are important in the regulation of Cardiac contractility. SNP results indicate that the relative proportions of cAMP and cGMP are important in this respect. The two cyclic nucleotides appear to function antagonistically, cyclic AMP augmenting contraction and cyclic GMP depressing it.

CHAPTER I

INTRODUCTION

Harvey (1628) was the first to recognise that the heart is a mechanical pump, which serves to circulate blood around the body. He wrote 'The blood goes around on its continuous course and perpetual inflow and moves in a circle; namely, so that all parts depending on it, may be kept by their prime innate heat in life and their vital vegetative existence, and perform all their functions;' [65]. However, the physiology of the heart did not begin to be understood until the late 19th century. Ringer (1883) observed that the frog heart stops beating when the extracellular medium is deficient in calcium, even though it was later found that the electrical excitability of the myocardium was not altered [82]. Thus it became clear that Ca^{++} was an important factor involved in the control of cardiac contractility. Later, it was established that certain hormones and exogenous agents also have an effect on cardiac performance.

Although these agents act at superficial sites to control the heart, one must ultimately look at the molecular events involved in cardiac function in order to understand their mode of action. It is here that Sutherland and Rall's [116] idea of 'second messenger' takes on significance. Second messengers are substances whose levels are changed as the result of the action of primary messengers (hormones and exogenous agents) on their target cells. So far three have been recognised, namely calcium (Ca^{++}), adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP), which can co-operate in either of two ways: monodirectionally, where the effect of one messenger enhances the other, such as cAMP facilitating entry of Ca^{++} [35]; or bidirectionally, where second messenger has an antagonistic action on the first, such as opposing actions of cGMP and cAMP in regulating the heart [58; 46].

In the course of this introduction I shall discuss the possible involvement of Ca^{++} and the two cyclic nucleotides in cardiac contractility.

In addition the role of cyclic nucleotide phosphodiesterases and calcium dependent regulator protein (calmodulin) in their metabolism will be reviewed. Finally, a brief review will be made of two agents whose effects have been investigated during the course of this study, namely trifluoperazine (TFP) and sodium nitroprusside (SNP).

MYOCARDIAL CONTRACTILITY

Cardiac excitation-contraction coupling, action potential and calcium

Excitation-contraction coupling is the mechanism by which electrical events occurring at the cell surface triggers contraction. As in skeletal muscles, contraction is initiated by an action potenital. However, the myocardial action potential is different from that of skeletal muscle in a number of ways. The cardiac cell has a resting membrane potential of -90mV (interior negative to exterior), and stimulation causes a rapid depolarization of the sarcolemma, at a rate which is similar to that of other excitable tissues (1m sec). The ionic basis of this process is due to a large transient increase in permeability of the cell membrane to sodium ion, Na^+ (inward Na^+ current). However, repolarization of the sarcolemma is a three phase process. Initially there is a short rapid repolarization which is followed by a plateau phase due to a prolonged inward Ca^{++} current activated at -45mV [102] together with some residual Na^+ currents. The third phase is brought about by a delayed increase in potassium ion (k^+) permeability, during which time k^+ diffuses out of the cell and thus restores the membrane potential ot it's resting level.

The spread of the action potential in cardiac muscle is aided by the presence of low resistance gap junctions at the intercalated discs between

adjacent cells, and this allows a synchronised stimulation of the myocardium to take place. The individual cells have tubular invaginations which penetrate into the muscle fibre (t-tubules). The action potential is conducted from the sarcolemma down the t-tubules to the junction of the sarcoplasmic reticulum.

Calcium plays an essential role in initiating myocardial contraction, even though actin and myosin alone do not require Ca^{++} to function. Calcium exerts it's initiating effect through the regulatory proteins on the thin filament: tropomyosin, and troponin complex consisting of tropopin C, I and T (TnC, TnI and TnT respectively). According to the steric-inhibition hypothesis, in the non-activated state, the tropomyosin strand masks active sites on the actin filament and prevents the myosin head from binding. Calcium initiates contraction by binding to TnC, thus inducing a conformational change in the troponin-tropomyosin complex. This causes tropomyosin to move aside from the active site, allowing the myosin heads to interact with the actin filaments.

The source of the calcium is of great importance. In skeletal muscle, it is stored in and released from the sarcoplasmic reticulum (S.R.). However, in cardiac muscle, the internal Ca^{++} stores, such as those contained within the S.R and mitochondria, are thought to act as buffers in controlling the concentration of free Ca^{++}, so as to prevent unphysiological intracellular levels of Ca^{++} [36]. Because of the smaller dimensions of cardiac muscle fibres, the entry of Ca^{++} through the sarcolemma is of great importance. There is basically two proposed components of Ca^{++} entry into the cell. Firstly there is a Ca^{++} component to the cardiac action potential, which constitutes the slow inward Ca^{++} current. The Ca^{++} entry during this phase is important in initiating contraction [93; 2). Secondly in amphibian heart, and to a lesser extent in mammalian heart, there is a carrier mediated Na^{+}:Ca^{++} exchange

mechanism at the sarcolemma [36]. However, the amount of calcium entering through the sarcolemma is about 10% of what is required to sustain contraction [95].

Cyclic nucleotides and cardiac contractility

Cyclic AMP: Murad, Chi & Sutherland [89] were the first to propose the possible involvement of cAMP in cardiac function. It is now established that cAMP is one important regulator of cellular activity, and as such it can be considered as a 'second messenger' in mediating cellular responses to a variety of hormones, neurotransmitters and drugs. cAMP is formed from intracellular adenosine 5'-triphosphate (ATP) by the enzyme adenylate cyclase which is located mainly in the plasma membrane of the myocardium [94; 133]. The compound was first discovered by Sutherland and Rall [116] in hepatocyte homogenates in the presence of ATP, magnesium ion (Mg^{++}) and adrenaline#glucagon and was initially termed mononucleotide, adenosine 3',5'-phosphoric acid (3',5'-AMP).

Robinson, Butcher, Øye & Sutherland [104] applied adrenaline to isolated perfused rat heart and observed an increase in cAMP levels which preceeded the contractile response and this was followed by an increase in phosphorylase-a levels. Catecholamines, in addition to increasing contractile force, alter the pattern of ionic movements, particularly of Ca^{++} and K^{+}. The plateau phase is prolonged and repolarization is accelerated. These events are all linked with stimulation of β-adrenoceptors and with increased levels of cAMP [28]. Since the 1960's the rise in cAMP levels following catecholamine administration has been investigated in cardiac tissues of a number of different species [28; 3].

The physical coupling of adenylate cyclase and the β-adrenoceptor is now a widely accepted concept [28]. However, stimulation of

β-adrenoceptors is not the only way of increasing levels of intracellular cAMP. This can be achieved by a variety of other procedures such as a) - inhibition of phosphodiesterases (see later page 12); b) - activation of adenylate cyclase independent of the β-receptor, as occurs with glucagon, which produces a positive inotropic response and is not blocked by β-adrenergic antagonists such as propranolol [122]; c) - exposure to cAMP derivatives such as dibutyryl cAMP, which crosses the cell membrane and has a positive inotropic effect [122; 28; 47].

Not all the studies reported support the idea of cAMP mediating the positive inotropic responses. Benfey and Carolin [8] reported that phenylephrine did not alter cAMP levels in rabbit heart although it stimulated twitch tension to increase. However, Drummond & Hemmings [27] reported that the above compound applied to perfused heart increased cAMP levels and activated phosphorylase. Endoh, Broddle & Schümann [30] reported that inotropic action of phenylephrine, particularly at low concentrations, is mediated through α-receptors which do not involve cAMP. Similar experiments with papaverine (a phosphodiesterase inhibitor) did not alter the pattern of response to adrenaline [66]. However, alternative explanations for this action have been put forward: "Suppose, that response to papaverine plus adenaline is the resultant of three factors: a) - a depressant effect of extracellular papaverine due to block of the slow inward current; b) - a stimulatory effect of intracellular papaverine through phosphodiesterase inhibition; and c) - an effect due to adrenaline. If the first two factors cancelled each other, one might expect the overall response to be similar to that due to the third factor" [122].

The above discussion for and against the involvement of cAMP alone as the mediator of inotropic responses in the heart clearly shows that the need for researching into the involvement of other intracellular messengers and their possible interplay with cAMP (see later pages 11, 13, 16).

Phosphorylation reaction, Ca^{++} movements & cyclic AMP: Cyclic AMP acts via a series of enzymes called cAMP-dependent protein kinases (PK) [63; 3]. These enzymes consist of a regulatory (R) and a catalytic (C) subunit. Upon coupling with cAMP, the C subunit dissociates and becomes free to act [11].

$$2cAMP + R_2\text{-}C_2 \rightleftharpoons R_2\text{-}cAMP + 2C$$

An excellent correlation was found by Corbin & Keely [23] between cAMP levels and the protein kinase (PK) activity ratio (PK-cAMP : PK + cAMP) in response to noradrenaline and a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine). In addition, it was observed that a large fraction of the intracellular cAMP is bound to both particulate and soluble proteins [120], and that the particulate bound cAMP is probably associated with the regulatory subunit of PK [28].

Protein kinase is a non-specific enzyme which catalyses the phosphorylation of a wide variety of proteins which may be membrane bound or soluble [28]. There are three principal mechanisms involved in regulating cardiac contractiligy. First, Ca^{++} movements intracellularly; second Ca^{++} fluxes across the sarcolemma; and third alteration of the sensitivity of the regulatory phosphoproteins to calcium.

A protein of 22,000 dalton molecular weight called phospholamban, which is a component of cardiac S.R [118], is one cyclic AMP-dependent phosphorylation site. This phosphoprotein has been extensively studied by Katy and his colleagues [28]. They found that phosphorylation of S.R microsomes correlated closely with an enhanced rate of Ca^{++} uptake. Indeed, they proposed that microsomal phosphorylation is the mechanism whereby catecholamines accelerate cardiac relaxation by enhacning Ca^{++} transport into S.R.

Secondly, the fact that Ca^{++} movement across the sarcolemma is an

important factor in mediating contraction led many to look at the sarcolemma for a possible cAMP dependent phosphorylation site. This resulted in the identification of surface membrane bound proteins which are thought to be structural components of the slow inward calcium current channels [132]. In 1976 Hui, Drummond & Drummond [67] reported a cAMP dependent phosphorylation of guinea pig ventricular plasma membrane which was due to endogenous protein kinase. In addition they reported that the sarcolemma bound Ca^{++} in an ATP-dependent manner, whereby addition of oxalate (in the presence of ATP) resulted in Ca^{++} accumulation. Similarly, noradenaline enhanced phosphorylation and rate of Ca^{++} uptake [28].

Finally, possible phosphorylation site was reported to be present on the troponin complex TnI and TnT [106]. The TnI component is the main site of phosphorylation, with a maximal incorporation of 2 moles of phosphate per mole. Incubation of cardiac troponin-tropomyosin complex with cAMP and protein kinase caused a rapid rate of phosphorylation [115]. Mope, McClellan & Winegrad [88] observed that Ca^{++} sensitivity was inversely related to the relative amoung of ^{32}P incorporated into TnI and that cGMP stimulated phosphatase, while cAMP activate the phosphorylating protein kinase. Thus, they concluded that the Ca^{++} sensitivity of cardiac myofibrils is regulated by phosphorylation of TnI, which is stimulated by cAMP and inhibited by cGMP. The correlation between contraction and phosphorylation of TnI has been examined in isolated rat hearts [33; 34]. Upon application of catecholamines, the increase in TnI phosphorylation closely paralleled the increase in contractility.

Cyclic GMP: guanosine 3',5'-cyclic monophosphate (cGMP), which is structurally similar to cAMP, was first discovered by Ashman, Lipton, Mellicow & Price [6]. Since then it has been shown to be present in every

cell system. Cyclic GMP is formed from GTP by the catalytic action of the enzyme guanylate cyclase. In contrast to adenylate cyclase, which is membrane bound, guanylate cyclase is found in both particulate and soluble forms, each of which has it's own distinct properties [70]. The myocardial guanylate cyclase has an absolute requirement for Manganese (Mn^{++}) and possibly requires a correct Mn^{++}:Ca^{++} ratio [73].

George, Polson, O'Toole & Goldberg [51] were the first to observe an increase in cGMP levels and a decrease in twitch amplitude when rat hearts were perfused with acetylcholine (Ach). These results suggested a possible relationship between cGMP and the contractile process. Indeed, further experiments by George, Kordowitz & Wilkerson [53] showed not only an increase in cGMP accompanying a decline in contractile force, but also a significant decrease in cAMP levels. Since then a number of other authors have also demonstrated a correlation between the decline in cardiac contractility, induced by acetylcholine, and a corresponding increase in cGMP levels. Atropine has been shown to inhibit both the decline in contractility and the increase in cGMP levels [77; 57; 54; 50].

One method of testing whether cGMP is involved in regulating contraction is to treat heart with lipid soluble derivatives of cGMP. Dibutyryl cGMP was shown to mimick the chronotropic effect of carbachol on spontaneously beating cultured cells [72]. Later 8-bromo cGMP was shown to have a negative inotropic action on cat papillary muscle, and to cause a change in transmembrane calcium movements [92]. Wattanabe & Besch [127] provided evidence that Dibutyryl cGMP antagonised the positive inotropic action of isoprenaline, and this observation is compatible with the idea that cGMP mediates the effect of cholinergic stimulation.

The role of cGMP in regulating cardiac contractility is less well

understood than that of cAMP. In 1977 a number of papers were published, questioning the role of cGMP as a mediator of cardiac contractility. Mirro, Bailey & Watanabe [87] used paced guinea pig atria and observed no correlation between cGMP content and electrophysiological effect of acetylcholine. They concluded that either cGMP is not responsible for mediating the effect of acetylcholine, or it is manufactured in discrete effector pools, which are not separately identifiable. Brooker [10] showed that approximately 100 times more carbachol is needed to elevate intracellular cGMP levels than is required to produce 90% reduction in twitch amplitude. Finally, Diamond, Ten Eick & Trapani [26] reported that in mammalian hearts acetylcholine may induce negative inotropic responses without any appreciable change in endogenous cGMP levels. Moreover, they found that sodium nitroprusside SNP (a stimulator of guanylate cyclase) produced a 17 fold increase in cGMP, but no decrease in contractility.

Despite the results of these so called 'dissociation' experiments, there is still considerable evidence (see later page 11) to support the idea that both cAMP and cGMP are together involved in regulating cardiac contractility.

Relationship between cAMP:cGMP and contractility: There is increasing evidence to show that both cAMP and cGMP are involved in regulating myocardial contractility. Goldberg, Haddon & Nicol [58] described the actions of the two cyclic nucleotides as opposing each other. He adapted the oriental Ying-Yang concept: "YING YANG symbolizes a dualism between opposing natural forces but also takes into account that under certain circumstances the forces may enter into a mutual interaction that results in a synthesis" [58]. He then goes on: "In it's simplest form the hypothesis defines cyclic GMP and cyclic AMP as biologic effectors involved in regulating cellular functions that are controlled bidirectionally".

The control, he said, can be one of two types; type A) - where cyclic AMP facilitates an action, while cGMP suppresses it; and type B) - where cGMP promotes the action, but cAMP inhibits it.

Wollenberger, Babskii & Krause [131]; Nawrath [92] and more recently Endoh [31] provided evidence that cAMP and cGMP have opposing roles in influencing myocardial contactility in mammalian hearts. Since 1977, Flitney & Singh have provided an increasing body of evidence in support of the idea that both cAMP and cGMP are together involved in regulating cardiac performance. Ventricular strips from frogs were stimulated and allowed to become hypodynamic (a concept which will be explained later on page 24). During the development of the hypodynamic state, the decline in contractile force was accompanied by the loss from the ventricles of prostaglandins E_1 and E_2 [42]. This finding supported Clark's idea that loss of a lipid substance is responsible for initiating the changes which lead to the hypodynamic state [18]. At the same time, there was a progressive decrease in cAMP and a corresponding increase in cGMP levels. The magnitude of the decline in contractility was found to be accompanied by a parallel decline in the ratio of cAMP:cGMP [45].

Further experiments with isoprenaline [110], uridine 5'-triphosphate [43] and adenosine [111] also showed a very close correlation between increasing (or decreasing) twitch tension and corresponding changes in ratio cAMP:cGMP. Moreover, the response to adenosine triphosphate [46], which is a complex triphasic one was also accompanied by corresponding changes in the ratio cAMP:cGMP [46]. These observations led Flitney & Singh to suggest that both cyclic nucleotides are involved in regulating the inotropic status of the ventricle [44].

To investigate this further, experiments were made using two cyclic nucleotide analogs, 8-bromo cGMP and di-butyryl cAMP. Increasing concentrations of 8-bromo cGMP depressed the twitch and this was accompanied

by a decline in endogenous cAMP levels and an increase in cyclic GMP [47]. Dibutyryl cAMP potentiated the force, again in a dose dependent manner [49]. In both kinds of experiments, the ratio cAMP:cGMP changed in parallel with the effect of each analogue on the twitch.

Possible role of cGMP in regulating cAMP metabolism: Beavo, Hardman & Sutherland [7] first reported that cGMP in micromolar quantities can stimulate the hydrolysis of cAMP in various tissues. This observation was later confirmed in rat heart by Terasaki & Appleman [119], who also established that in the presence of cGMP the cAMP-phosphodiesterase loses it's co-operativity for cGMP without any appreciable change in V_{max} of the extrapolated K_m values. More recently, Flitney & Singh [46] reported that the maximum rate of fall in cAMP levels coincided with the peak of the intracellular cGMP when frog ventricles were exposed to ATP. Later observations showed that the effects of acetylcholine and 8-bromo cyclic GMP on intracellular cAMP levels were attenuated by pre-treatment with theophylline (a phosphodiesterase inhibitor). It was concluded that endogenous cGMP may function to accelerate the hydrolysis of cAMP, by stimulating a cGMP-sensitive cAMP-phosphodiesterase [49]. A possible explanation is offered on pages 14 & 15.

Cyclic nucleotide phosphodiesterases & CALcium binding MODULator proteIN (CALMODULIN) and their inhibitors

There is a dynamic balance between rates of production and degradation of cyclic nucleotides. Hormones and other agents could act either to alter their synthesis, or to effect the rate at which they are converted to their inactive forms.

Phosphodiesterases (PDE): Cyclic AMP and cyclic GMP are converted into inactive 5'-AMP and 5'-GMP, respectively, by enzymes known as

phosphodiesterases. Phosphodiesterase inhibitors are often used to prevent the degradation of cyclic AMP and cyclic GMP, thereby increasing their intracellular concentrations.

The methylxanthines, such as theophylline, are one such class of compounds. They have been shown to produce a positive inotropic response in cardiac tissues. The increase in contractility has been attributed to elevation of cAMP levels [117; 72]. More recently, Argel, Vittone & Grassi [4] observed no change in contractility of perfused rat heart upon application of 2×10^{-4}M theophylline, but they noted a significant increase in both cAMP and cGMP levels. Flitney & Singh [49] showed that 10^{-4} M theophylline antagonised both the decline in contractility and the reduction in cAMP levels produced by acetylcholine or 8-bromo-cyclic GMP. However, not all effects of theophylline have been attributed to changes in cyclic nucleotides. Martinez & McNeill [85] found little or no effect on cAMP concentrations upon application of theophylline and they attributed the positive inotropic effect to changes in calcium metabolism; they suggested that theophylline inhibits Ca^{++} uptake by the S.R and promotes its release.

Papaverine is another commonly used agent with a potency of 10-1000X greater than theophylline. It's inotropic effect is again the subject of controversy. There are reports of positive inotropic responses in a number of different species, accompanied by an increase in both cAMP and cGMP levels [1; 31; 4]. In contrast, Henry, Dobson & Sobel [66] found no change in contractility when isolated rat hearts were briefly perfused with papaverine, but there was a significant increase in cAMP levels.

Calmodulin: Calmodulin is a ubiquitos protein which belongs to a family of homologous calcium binding proteins, including troponin C and parvalbumin [20]. Its varying functions are achieved by altering the

activities of intracellular enzymes and proteins which are involved in cyclic nucleotide metabolism, smooth muscle contraction, microtubule formation, mitotic a-paratus assembly, calcium fluxes and secretory processes [29; 16]. It consists of a monomer of 148 amino acid residues, with a molecular weight of 16,700 dalton, and an isoelectric point of pH=4. It has four Ca^{++} binding sites [16; 71]. It has been demonstrated that calmodulin is identical to the -subunit of phosphorylase kinase extracted from rabbit skeletal muscle [21; 109]. It is thought that the structure of calmodulin has been highly conserved during evolution, an indication of its importance as a regulator of many fundamental biological functions.

Calmodulin is an important regulator of the metabolism of the three secondary messengers: Ca^{++}, cAMP & cGMP. In the presence of 10^{-6} M Ca^{++}, it undergoes a conformation change and this stimulates several intracellular enzymes, including adenylate cyclase, cyclic nucleotide phosphodiesterases [12; 16; 124] and phosphorylase kinase [20; 125]. In mammalian brain it has been shown that calmodulin regulates both the synthesis and degradation of cAMP. This regulation can allow a sequential stimulation of synthesis and subsequent degradation of cAMP, resulting in a transient increase in cAMP [17]. Calcium-calmodulin dependent phosphodiesterase preferentially hydrolyses cGMP rather than cAMP at substrate concentrations in the micromolar range [12; 17]. The differences in activities of cAMP and cGMP phosphodiestrases have been investigated in some detail. Filburn, Colpo & Sacktor [37] demonstrated that the rate of hydrolysis of cAMP and cGMP were increased in the presence of free Ca^{++}, and that this rate was dependent on the concentration of phosphodiesterase and the type of cyclic nucleotide present. It was observed that with increasing phosphodiesterase concentration, cGMP hydrolysis became more dependent on Ca^{++} (this being due to a decrease

in apparent km of phosphodiesterase), while the reverse applied to cAMP hydrolysis, where cAMP had no such effect on cGMP hydrolysis (particular at low concentrations). Chromatographic fractionation illustrated two distinct types of phosphodiesterases (D_I and D_{II}). Cyclic GMP phosphodiesterase was of both D_I and D_{II} type, while cyclic AMP phosphodiesterase was only of D_I type. Similarly it was established that the D_I type was calmodulin dependent and was inhibited in the absence of Ca^{++}, while D_{II} was only partially masked in the absence of Ca^{++}. This observation was later substantiated by Weiss, Levin & Greenberg [130] who demonstrated that in the presence of 0.4µg calmodulin, the cGMP-phosphodiesterase activity was 3X that of cAMP-phosphodiesterase.

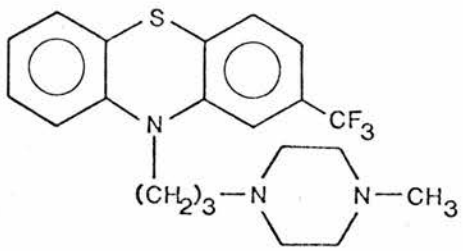
Thus it can be assumed that influx of calcium and the activation of calmodulin will lead to a transient increase in intracellular cAMP levels, whereas there will be a decrease in the levels of intracellular cGMP. This could explain a possible role for activated Ca^{++}-calmodulin complex in mediating reciprocal changes in cAMP and cGMP. Such a hypothesis could explain the existence of a correlation between the changes in twitch tension (ΔP) and changes in the ratio of cAMP:cGMP (ΔR). Both these changes could arise as the result of a common cause. Calcium activating the excitation-contraction coupling sequence for production of force, while simultaneously a calcium-calmodulin complex formation can bring about reciporcal changes in the intracellular levels of cAMP and cGMP.

Although intracellular activities of Ca^{++} have been under investigation for the past 30 years, the mechanism of it's action is only now beginning to be appreciated, largely due to recent researches on calmodulin and other calcium binding proteins. Many antipsychotic drugs have been shown to inhibit calmodulin activities [80]. One such drug is Trifluoperazine (TFP), and during the course of this study, some observations will be presented on the effect of this compound on the frog ventricule.

SCOPE OF THE PRESENT STUDY

The aim of the present study was to investigate further the hypothesis that all three of the secondary messengers (Ca^{++}; cAMP & cGMP) are involved in regulating cardiac contractility. In particular the possible role of cGMP in regulating contraction has been emphasised. The approach has been to superfuse the isolated frog ventricle with Ringer's solution containing trifluoperazine (TFP) or sodium nitroprusside (SNP).

Trifluoperazine (TFP): TFP is an anti-psychotic drug consisting of a phenothiazine nucleus with a piperazine chain attached at the C_{10} position.



It is related to chlorpromazine, which was first discovered as an anti-histamine in 1952. Clinically, TFP is used for treatment of psychosis, with a daily dose of 2-30mg. It is a white powder, soluble in water, but readily oxidizable and so must be kept air-tight and in a lightproof container when in solution [84; 60].

During the last 5 years, most research on TFP has been directed towards its inhibitory effect on calmodulin. Levin & Weiss [79] observed that TFP binds to calmodulin and inhibits its ability to activate phosphodiesterases. They also established that TFP inhibits the activation of adenylate cyclase by calmodulin. This was followed by reports that TFP inhibits the stimulation by calmodulin of phosphorylase kinase [109; 125].

Levin & Weiss [79] indicated that TFP was capable of binding at two difference sites: a high affinity, Ca^{++} dependent binding site and a low affinity Ca^{++} independent binding site. Of all the proteins they studied (catalase, cytochrome C, egg albumin, calmodulin) only calmodulin possessed the high affinity binding site. The calcium dependency of TFP binding to calmodulin is demonstrated by the fact that TFP-binding increases by 10X in the presence of Ca^{++} as compared to the absence of Ca^{++} [79; 96].

Further research into the effects of TFP on calmodulin has shown the following:

1) TFP binding is linear with increasing activator protein concentration.
2) TFP binding is concentration and Ca^{++} dependent at low TFP concentrations (2×10^{-9} - 10^{-5}M), whereas at higher concentrations (10^{-4}M) there is no Ca^{++} dependency.
3) TFP binding in the presence of Ca^{++} has a dissociation constant - (PD_{50}) of 10^{-6}M. At saturation level, 2 molecules of TFP become bound to one activator protein (high affinity site). The Ca^{++} independent low affinity site has a dissociation constant (PD_{50}) of 5×10^{-3}M, and at saturation level, 24 molecules of TFP are bound to one activator protein.
4) TFP binding is pH dependent at the high affinity site. Binding decreases as the pH is raised from 7.5 to 8.0. The low affinity site shows no pH dependency.
5) TFP binding is unaltered when Ca^{++} is substituted with Strontium (Sr), Nickel (Ni) or Zinc (Zn), but not with Magnesium (Mg).
6) TFP binding is reversible when Ca^{++} is withdrawn.
7) TFP has a low binding affinity for other proteins (egg albumin, cytochrome C, catalase).

8) A variety of other anti-psychotic agents such as chlorpromazine and pimozide can compete for the high affinity binding site [37; 79; 128; 129; 71].

Sodium nitroprusside (SNP): SNP is one of a family of nitroso-containing compounds with a ferro-cyanate nucleus

$$Na_2 [Fe (CN)_5 NO].2H_2O$$

Clinically SNP is used as a vasodilator for the treatment of hypertensive emergencies, congestive heart failure [19], acute myocardial infarction [15], and chronic ischaemic heart disease [86], with dose rates of 30-150 µg per kg body weight per minute. SNP is a ruby-red crystal which is soluble in water, but which readily decomposes in aqueous solution. As a result it must be prepared fresh, kept airtight and protected from the light [83].

SNP and related compounds have been shown to be important stimulators of guanylate cyclase activity, both in vivo and on the isolated enzyme [90]. They appear to act by generating nitric oxide, or a nitric oxide moiety which can be converted into nitric oxide [5]. Nitric oxide in turn oxidises the enzyme and alters its cationic requirement; the SNP-activated enzyme can utilize Mn^{++} or Mg^{++} equally well as a cationic co-factor [98]. In addition, SNP activation of guanylate cyclase occurs in the absence of Ca^{++} in the incubation medium, in contrast with other agents (notably the cholinergic agonists) all of which have an absolute requirement for Ca^{++} [90].

The results to be presented will show that both TFP and SNP depress ventricular contactility, in a dose-dependent manner. Theophylline antagonises the depressant effect of SNP, but has no antagonistic action on the TFP response. Both TFP and SNP stabilize the action potential duration, with TFP having a longer lasting effect. The negative inotropic effect of SNP is accompanied by quantitatively equivalent reductions in the ratio

cAMP:cGMP. The results provide further evidence in support of the idea that cAMP and cGMP are both involved in regulating the inotropic status of the ventricle. They also provide circumstantial evidence to suggest that cGMP may regulate cAMP metabolism by stimulating cAMP phosphodiesterase activity.

CHAPTER II

GENERAL METHODS & MATERIALS

Methods

All experiments were carried out on the isolated superfused frog ventricle. In general, adult males and females of the species Rana-temporaria, and a few specimens of R. pipiens were used. Animals were stored at 4°C for not less than 5 days before use. They were killed with a blow to the head and then pithed.

Dissection and mounting. Hearts were rapidly excised and the atria were removed. The ventricle was then cut into two cup-shaped pieces of tissue, one of which served as control for the other. The half-ventricles were then mounted horizontally, using entomological pins on a rubber bung inserted in the centre of the perfusion bath (Fig. 2.1.). The apex of the ventricle was attached to a force transducer (Devices Ltd. type 4151) by means of a fine stainless steel wire, and the transducer was mounted on a micromanipulator. At the start of each experiment, the length of both half-ventricles was increased stepwise (0.5 mm increments, 1.0 min intervals) until the optimal length giving the maximum contractile response was established (Fig. 3.0.1.). Twitches were recorded on a chart recorder or an oscilloscope.

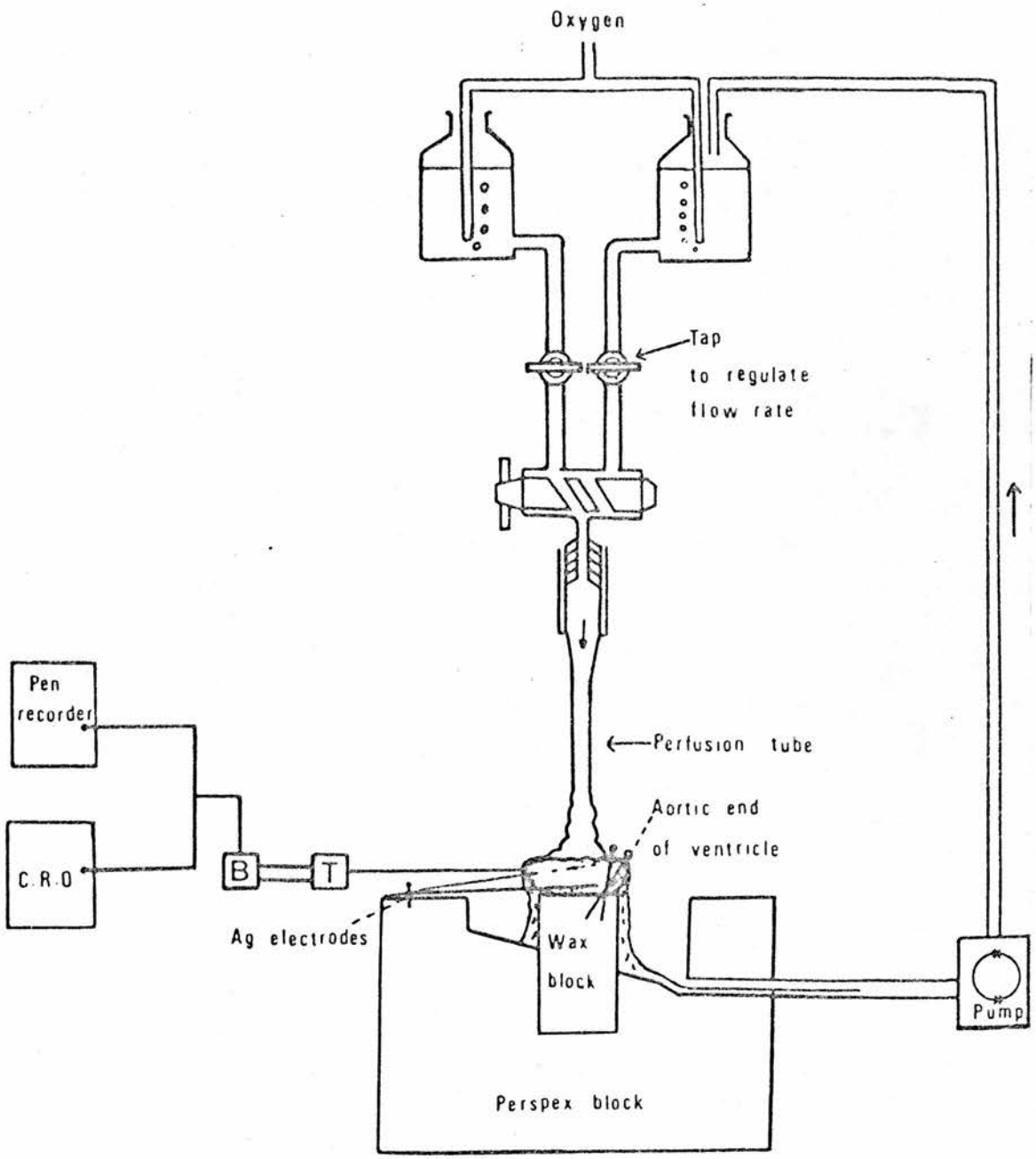
Electrical & Mechanical experiments

Perfusion procedure. With slight modification, the method of Lamb & MacGuigan [75] was used to superfuse the preparation in a closed circuit system with oxygenated Ringer solution (composition: (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1; Na_2HPO_4, 2.15; $NaHPO_4$, 0.85; glucose, 5.6; pH, 7.2). The vertical glass tube was placed 1.5 cm above the centre of the ventricle, and a flow rate of 100 ml min^{-1} was used. This was found to be sufficiently great to keep the ventricle 'inflated', thereby ensuring that the

Fig. 2.1

Vertical perfusion & recording apparatus

Flow of perfusion fluid is from one of two storage Mariotte bottles via a two-way tap and perfusion tube. 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.


Oxygen
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

tuberculae were in an 'opened' condition, providing rapid equilibration of solution with the fibres [75]. All experiments were carried out at room temperature (18-20°C). A change of solution was achieved by means of a two way tap. The solutions were stored in reservoirs approximately 60 cm above the chamber and the superfusate was recirculated by means of a Watson-Marlowe peristaltic pump.

Stimulation. All experiments were paced by electrical stimulation, via two Ag electrodes positioned on either side of the ventricle. Square pulses of 5 m sec duration and amplitude of 10 volts were employed at frequency of 30 min^{-1}.

Micro-electrode recording. The experimental conditions were modified for making membrane potential recordings as illustrated in Fig. 2.2. A narrow strip of tissue was used instead of a half-ventricle. The perfusion was carried out laterally and the rate was reduced to around 20 ml min^{-1} in order to avoid dislodging the microelectrode. Square pulses of 5 m sec duration, 10 volts amplitude were used at a rate of 30 beats per minute. Conventional 3 M KCl or 1 M K-Acetate filled floating microelectrodes (suspended by fine Tungsten wire) with tip resistance of 20-30 MΩ were employed. Electrical and mechanical responses were recorded on a dual-beam storage oscilloscope (Tektronix type 5103 N). Final analyses were made from enlargements of the photographic recordings of the traces (Fig. 3.II.7.).

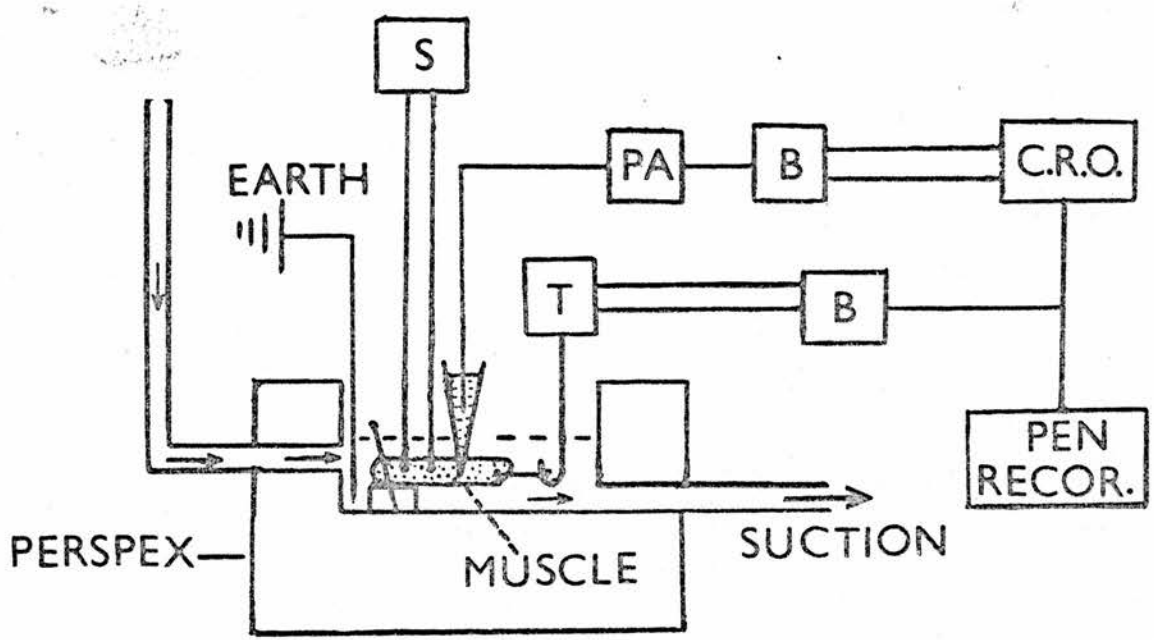
General Procedure. In all cases, the half-ventricle was superfused with frog Ringer solution until the twitch tension had reached 45 to 55% of its initial value. Fig. 2.3 is an original chart recording of two half-ventricles showing decline in twitch tension with time during a vertically perfused preparation. Once the twitch had declined to this level, a predetermined concentration of TFP or SNP was added to the test superfusate, while the

Fig. 2.2

Schematic diagram of experimental arrangement for measuring membrane potentials

The ventricle is perfused laterally with fluid flowing from one of two Mariotte bottles (Fig. 2.1.) to waste or to be recycled. One end of the ventricle is pinned onto the base of the chamber while the other is attached to an isometric (force) transducer (T). The output of the transducer is via a bridge (B) to the inputs of a pen recorder and a (C.R.O). Stimulation is as described before.

Membrane potentials are measured with 3 M Kcl filled microelectrodes and coupled via an Ag-Agcl wire to the high impedence inputs of a preamplifier (P.A.). The output of the preamplifier is fed via a bridge (B) to a storage oscilloscope (C.R.O.).


S
EARTH
PA
B
C.R.O.
T
B
PEN
RECOR.
PERSPEX
MUSCLE
SUCTION

control half continued to be superfused with Ringer solution. The test and control ventricles were perfused for the same period of time.

Intracellular cyclic nucleotide Extraction and Bioassay

Levels of endogenous 3',5'-cyclic AMP and cyclic GMP were measured at different times after exposure of the test half-ventricle to SNP. The experimental method is as described above.

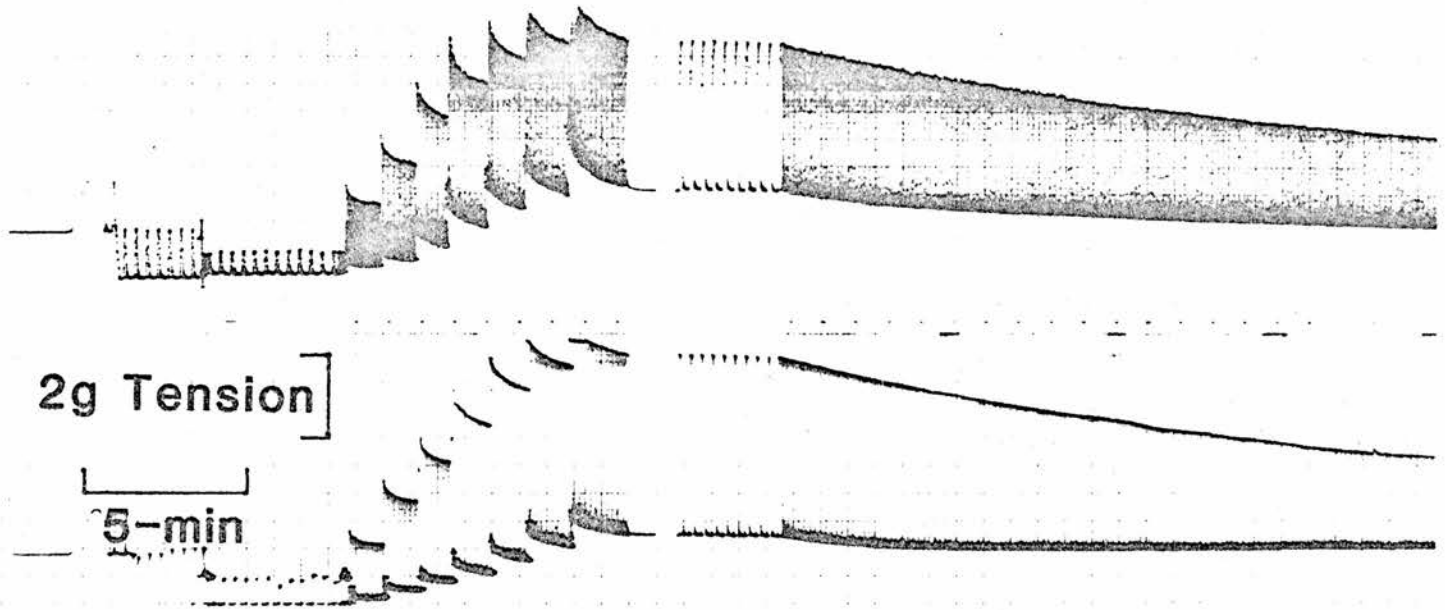
Freezing & Extraction procedure. At a predetermined time during the SNP response, the ventricles were 'freeze-clamped' in liquid nitrogen. The control half-ventricle was also 'freeze-clamped' after it had continued for the same period of time. Fig. 2.4. is an original chart recording of treated and untreated half ventricles at the time of freezing. The half ventricles were then individually pulverised with a precooled stainless steel mortar and pestle and then extracted with 2 ml of acidic ethanol (1 ml 1N HCl:100 ml ethanol). It was vortex mixed for 10 seconds and allowed to stand for a further 5 minutes in an ice-cold water bath. It was then centrifuged at 3000 rpm for a period of 5 minutes. The supernatant was removed and the pellet resuspended in 1 ml of ice-cold acidic ethanol and the centrifugation procedure repeated. The combined supernatants were evaporated to dryness using a stream of dry nitrogen. The residue from the extraction was dissolved in 1 ml of distilled water and the total protein content estimated using the Biuret method [59]. The dried extract was dissolved in 0.5 ml Tris-EDTA buffer (0.05 M Tris, pH 7.5, containing 4 mM EDTA). The resulting solution was used for cyclic nucleotide assays.

Bioassay procedure. Cyclic AMP and cyclic GMP were estimated using the Radiochemical Centre kits (TRK 432; TRK 500) [99; 100]. The cAMP assay

Fig. 2.3

Development of hypodynamic state in 'vertically-perfused' and electrically paced frog ventricles

This is an original chart recording showing the development of hypodynamic condition in two half ventricles. Preparations were superfused with frog Ringer at a rate of 100 ml per min at room temperature. The tissue was stimulated via two silver electrodes at a rate of 30 per min with square pulses, of 5 m sec duration 10V.

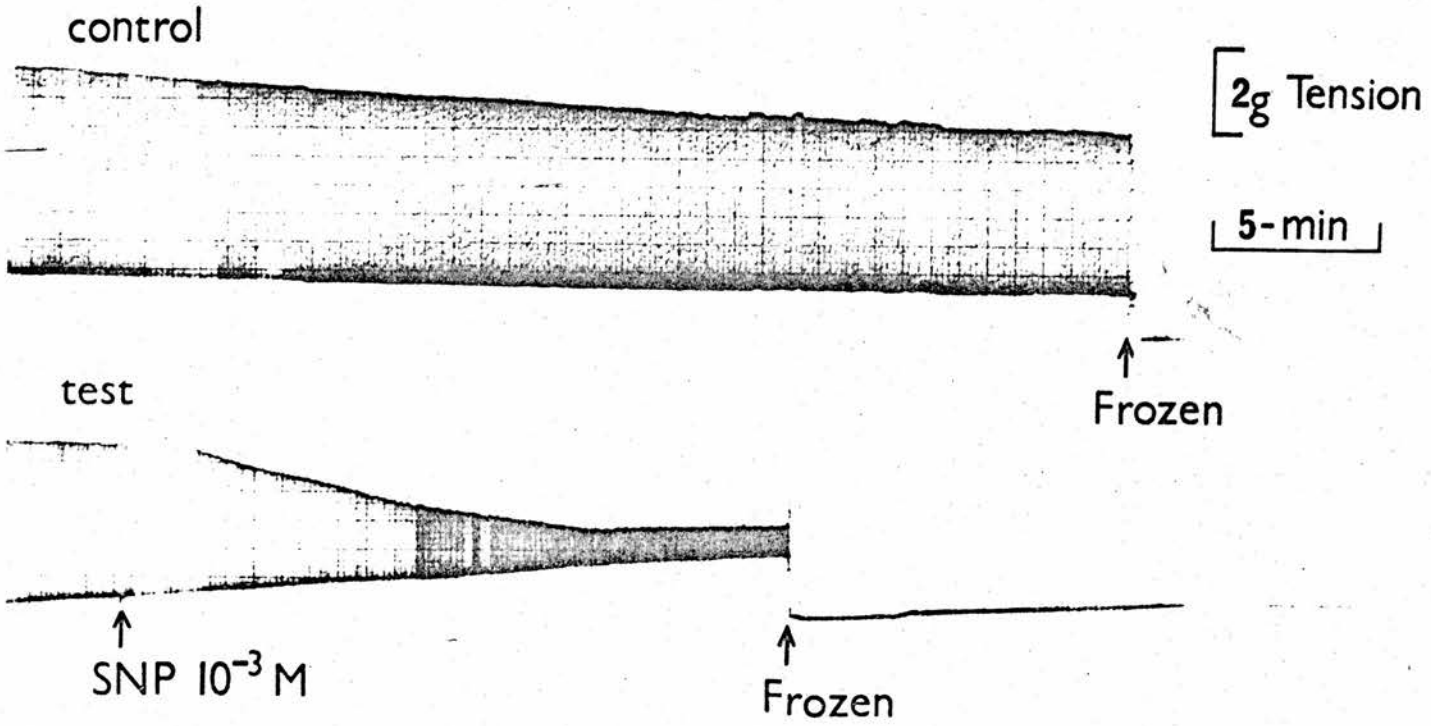

2g Tension
5-min

is based on a competition between the unlabelled (test) cAMP and a fixed quantity of the ^{3}H-labelled cAMP for binding to a protein which has a high affinity for the cAMP. The amount of labelled ^{3}H-protein is inversely related to the amount of unlabelled cAMP in the test sample. The separation of the cAMP bound to the protein from the unbound is achieved by addition of charcoal which takes up the free (unbound) cAMP. This is followed by centrifugation, after which an aliquot of the supernatant is removed for liquid scintillation counting. The cGMP assay is also based on the competition between unlabelled cGMP and a fixed quantity of the ^{3}H labelled cGMP for binding to an antiserum which has a high affinity and specificity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely related to the amount of cGMP present in the test sample. The measurement of radioactivity can be used to calculate the amount of cGMP present in the test sample. The separation of the cGMP bound to the antibody and the unbound is achieved by addition of ammonium sulphate, which precipitates out the antiserum. This is then removed by centrifugation. The precipitate containing the anti-body bound complex is dissolved in water and it's activity is determined by liquid β-scintillation counting. The lower limits of detection were around 0.05 p mole and linear calibration curves were obtained in the ranges 1-16 p mole (3',5'-cyclic AMP) and 0.5-8 p mole (3',5'-cyclic GMP).

Fig. 2.4

Freezing preparation

This is an original chart recording showing the effect of 10^{-3}M SNP on a test half-ventricle. The half-ventricles were frozen using precooled forceps at predetermined times. The control half-ventricle was allowed to continue for the same period of time as the test half-ventricle prior to freezing.


control
2g Tension
5-min
Frozen
test
SNP 10^{-3} M
Frozen

CHAPTER III

RESULTS

INTRODUCTION

The experiments to be described were all made using frog half-ventricles superfused with Ringer solution. The initial length of the half-ventricles were increased stepwise (0.5 mm increments, 1.0 min intervals) until the optimal length giving the maximum contractile response was established. Fig.3.0.2 and Table 3.0.1. summarise the results obtained in a series of 26 experiments in which the initial length, the final length and the stepwise increments in length between the two fixed points of the muscle (from the pins to the stainless steel hook) were measured accurate using a micrometer. During the course of these experiments, it was established that the optimal length which gives the maximum contractile response was reached when the length of the preparation was increased by between 1.9 to 2.1 X.

Preliminary results showed that the extent to which the twitch was depressed dependent upon the contractile state of the ventricle at the time of application of TFP or SNP. For this reason, all the experiments to be presented were performed on ventricles which were superfused with Ringer solution for periods ranging from 45 minutes in vertically perfused preparations to 140 minutes in horizontally perfused preparations (see later, page 26). This procedure results in a gradual decline in twitch amplitude, leading to a depressed but relatively stable condition termed the hypodynamic state [18].

The development of the hypodynamic state is accompanied by a variety of metabolic changes such as a fall in high energy metabolites, including endogenous ATP [64], creatine phosphate [123], and ionic changes such as a decline in calcium fluxes [13; 14]. More recent work from this laboratory [45] has shown that the process is also accompanied by the loss of prostaglandins (PG) from the tissue and by marked changes in cyclic nucleotide levels.

Table 3.0.1

Effect of stretch on the force of contraction

Results are expressed as mean ± S.E. Pmax is the maximum contractile force produced at optimal length of the tissue l_{max} (6.12 mm ± 0.28 mm). l_o is the initial length of the tissue prior to stretch (3.44 mm ± 0.1 mm), while l_x is the length after x increment increase in length.

lx/lo	relative to Pmax
1.0	0.00
1.10	0.24 ± 0.03
1.20	0.45 ± 0.09
1.30	0.56 ± 0.07
1.40	0.72 ± 0.07
1.50	0.80 ± 0.07
1.60	0.89 ± 0.05
1.70	0.94 ± 0.03
1.80	0.98 ± 0.02
1.90	0.99 ± 0.01
2.00	1.00
2.10	1.00

During the course of the development of the hypodynamic state, it was found that the decline in twitch was accompanied by a reduction in the duration of the action potential. In addition there was a difference in the rate at which the twitch declined, being significantly slower when superfused horizontally (at a rate of 20 ml min^{-1}) as compared to being perfused vertically at 100 ml min^{-1}. Accordingly, the results will be presented in three sections:

I) Changes in action potential and twitch during the development of the hypodynamic state.

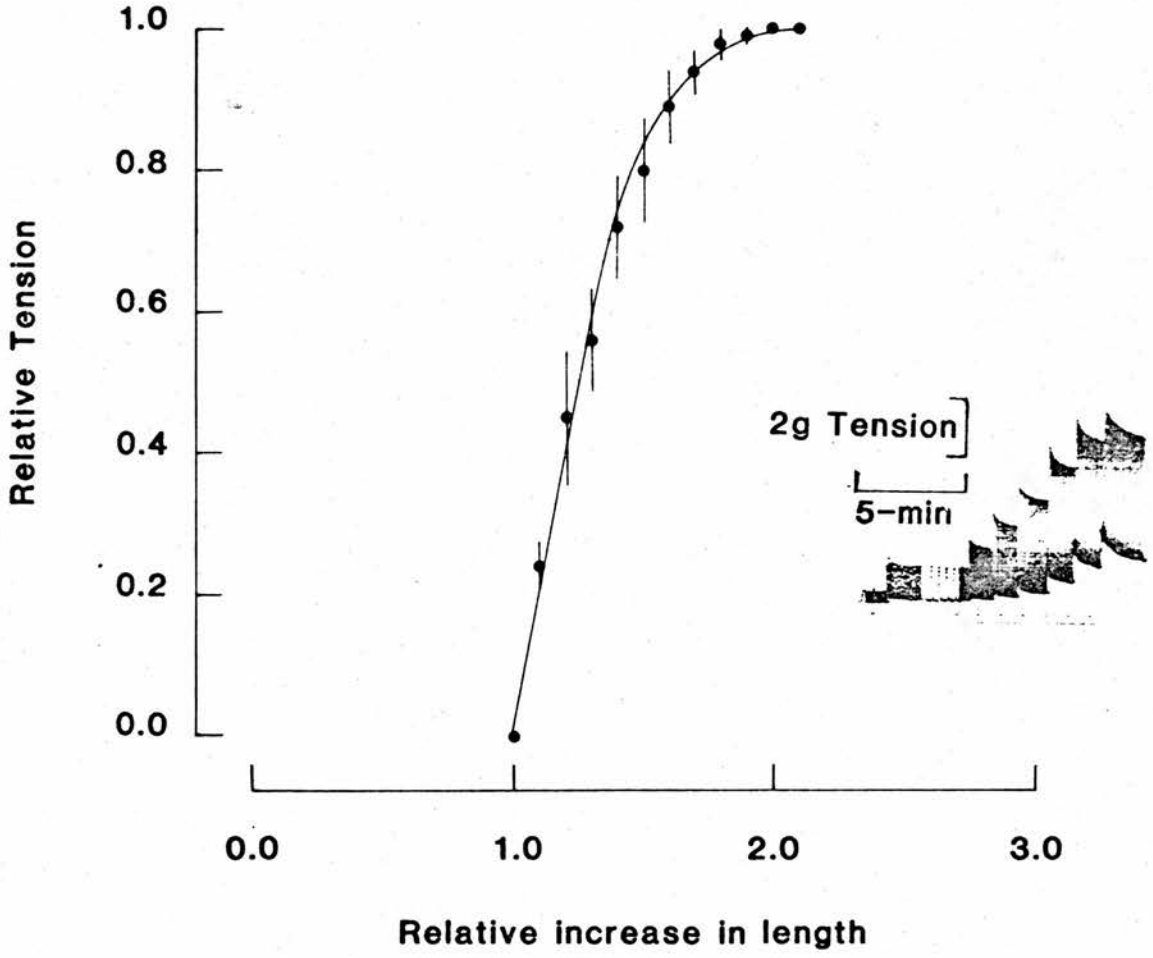
II) Effect of TFP on twitch tension and action potential.

III) Effect of SNP on twitch tension, action potential and cyclic nucleotide levels.

Fig. 3.0.2

Effect of stretch on isometric twitch tension in frog ventricle

The graph represents pooled data from a series of 26 experiments (mean ± S.E.). The length of the tissue was increased (0.5 mm increments, at 1 min interval) until the optimal length giving the maximum contractile response was established. (The inset is an original chart recording illustrating this point.)


1.0
0.8
0.6
0.4
0.2
0.0
Relative Tension
2g Tension
5-min
0.0
1.0
2.0
3.0
Relative increase in length

RESULTS PART I

CHANGES IN ACTION POTENTIAL AND TWITCH TENSION DURING THE DEVELOPMENT OF THE HYPODYNAMIC STATE

Rate of decline in tension under different perfusion condition. Fig. 3.I.1. is an original chart recording showing difference in the time course of the decrease in isometric twitch tension during superfusion of the ventricles in 'horizontally' and 'vertically' perfused preparations. This illustrates two points of interest.

1) The decrease in twitch tension in both 'horizontally' and 'vertically' perfused preparations is time dependent. Average rate of decline in tension was 0.36% per minute in horizontally perfused and 1.25% per minute in vertically perfused preparations.

2) The vertically perfused preparations show two distinct phases during the decline in the isometric twitch tension. There is an initial fast phase, where the rate of decline in twitch tension is 2% min^{-1}, whereas in the slow phase the rate of decline is 0.5% min^{-1}. This is less marked in the case of the horizontally perfused preparations (Fig. 3.I.2.).

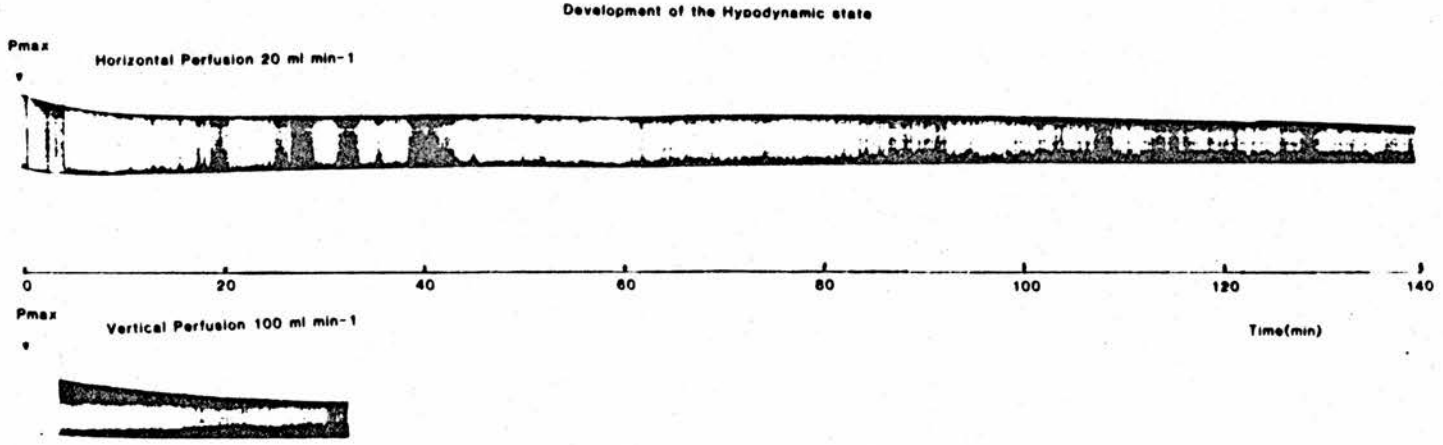
Changes in action potential duration during the development of the hypodynamic state. Fig. 3.I.3.A. summarises the results obtained in a series of 35 experiments in which strips of ventricle were superfused horizontally at a rate of 20 ml min^{-1}. During the course of the experiments intracellular microelectrode recordings were made of the shape of the action potential. The inset of Fig. 3.I.3.A. are original oscilloscope recordings of isometric twitch tension and action potentials (a, 10 minutes after the onset of the experiment & b, 70 minutes after the onset). These illustrate several features of interest.

(1) As the twitch decreases, there is a parallel reduction in the action potential duration, measured at the D-30mV level.

Fig. 3.I.1

Development of hypodynamic state in electrically paced frog ventricles

This is an original chart recording showing the development of the hypodynamic state. Horizontal perfusion was carried out at a rate of 20 ml min^{-1}, while vertical perfusion was carried out at a rate of 100 ml min^{-1}. Both tissues were stimulated via two silver electrodes at a rate of 30 per min with square pulses of 5 m sec duration 10V.


Development of the Hypodynamic state
Pmax
Horizontal Perfusion 20 ml min-1
0
20
40
60
80
100
120
140
Time(min)
Pmax
Vertical Perfusion 100 ml min-1

(2) The normal resting diastatic membrane potential is -86mV.

(3) In all experiments where action potentials and twitches were recorded at varying times over 140 minutes, the action potential duration decreased on average by 25%.

(4) The normal positive overshoot value was around +15mV, which is consistent with values obtained by Niedergerke & Orkand [93].

Relationship between changes in action potential duration and twitch tension. The relationship between changes in action potential duration and isometric twitch tension are shown in Fig. 3.I.3.B. The results are pooled data from a series of 35 experiments (mean $\pm$ S.E), using ventricles which had been superfused 'horizontally'. The graph shows a well-correlated decline in twitch amplitude and the corresponding action potential duration measured at D-30mV level (correlation coefficient = 0.95).

Fig. 3.I.2

Time course of the development of the hypodynamic state in vertically and horizontally perfused preparations

The graph represents pooled data from a series of 35 (horizontal perfusion) & 105 (vertical perfusion) experiments (mean ± S.E.). The preparations were set to give the maximum force of contraction initially which declined to 50% of that value before application of any agents.

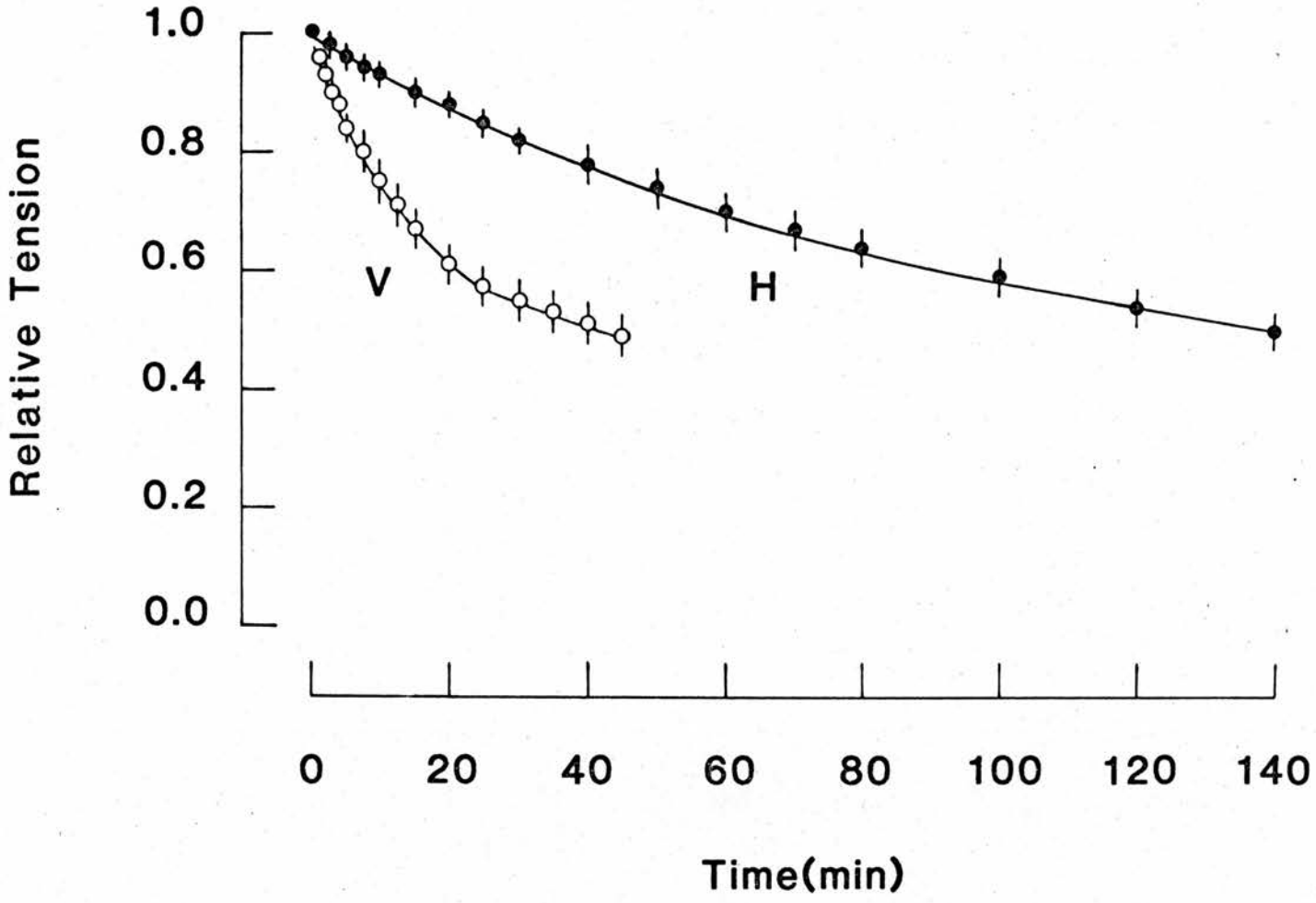

Relative Tension
1.0
0.8
0.6
0.4
0.2
0.0
V
H
0
20
40
60
80
100
120
140
Time(min)

Fig. 3.I.3

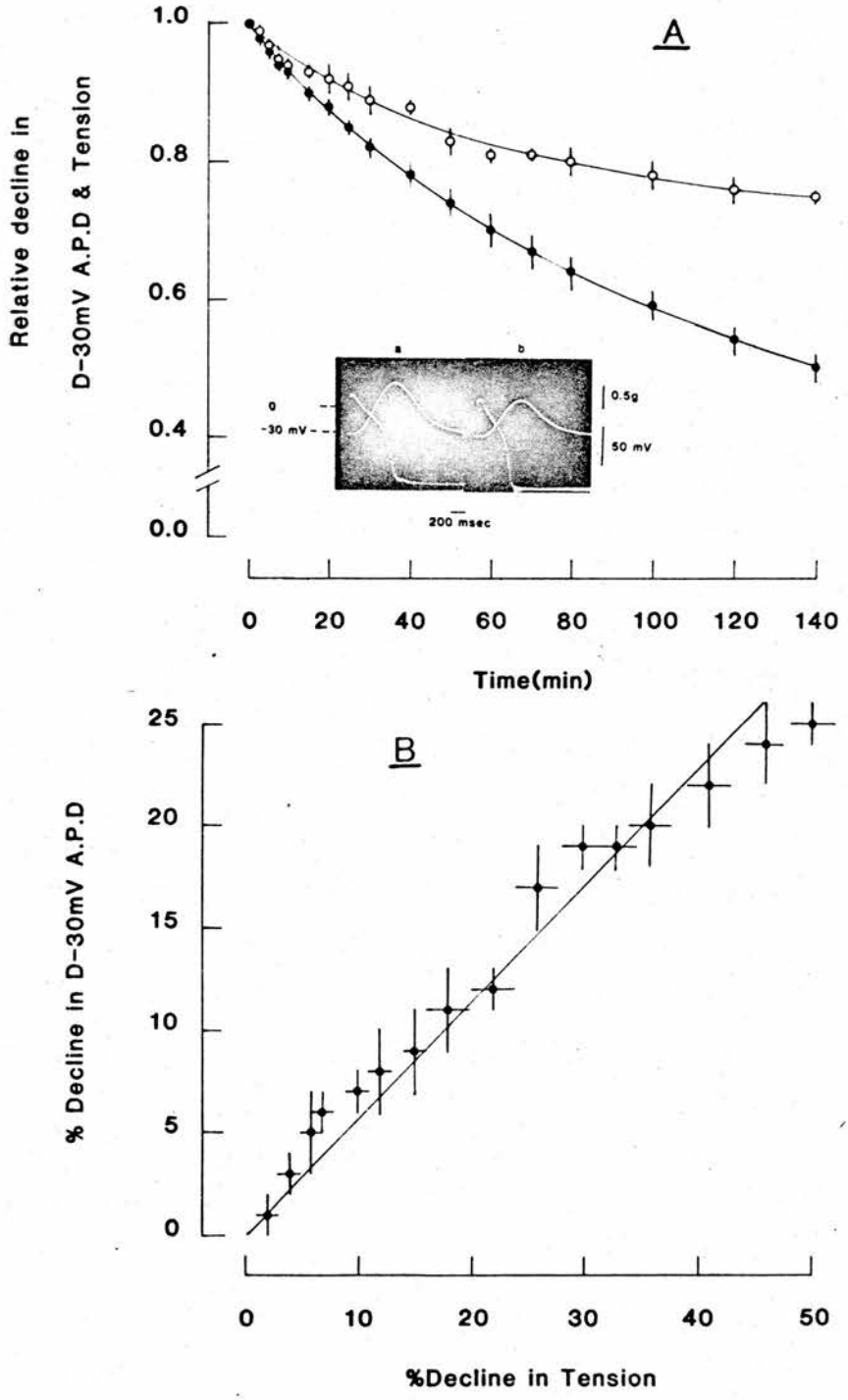
Changes in action potential duration & Isometric twitch tension during the development of hypodynamic state

A) Shows the decline in contractility (closed circle) and the corresponding reduction in action potential duration measured at D-30mV (open circle).

The insets are two original oscilloscope recordings illustrating the changes in twitch tension and shape of the action potential at times 10 min (a), and 70 min (b) after the onset of the experiment.

At the onset of the experiments on average the A.P.D. was 730 $\pm$ 40 msec.

B) The graph shows the correlation between the relative decrease in action potential duration and the twitch tension.


A
Relative decline in
D-30mV A.P.D & Tension
1.0
0.8
0.6
0.4
0.0
a
b
0
-30 mV
0.5g
50 mV
200 msec
0
20
40
60
80
100
120
140
Time(min)
B
% Decline in D-30mV A.P.D
25
20
15
10
5
0
0
10
20
30
40
50
%Decline in Tension

RESULTS PART II

EFFECT OF TFP ON TWITCH TENSION AND ACTION POTENTIAL

The experiments to be described show that TFP has a marked depressant effect on ventricular contractility. TFP effects on membrane potential are also investigated.

Characteristic inotropic effect of TFP

TFP effect on twitch amplitude. Fig. 3.II.1, shows the effects of two different concentrations of TFP on isometric twitch tension. The results are pooled data (mean ± S.E) from a series of experiments where the ventricles were superfused with 1×10^{-6}M or 5×10^{-5}M TFP. It illustrates two points of interest.

(1) TFP markedly depresses contractility, and this is true for concentrations as low as 10^{-9}M.

(2) The effect on the twitch is dose dependent. Higher concentrations increase the rate at which the contractile response declines (1×10^{-6}M $T_{\frac{1}{2}}$ = 7.5 min; 5×10^{-5}M $T_{\frac{1}{2}}$ = 0.33 min), and produce a more severe depression of the twitch.

Effect of using different concentrations of TFP. Fig. 3.II.2, summarises the results obtained in two sets of experiments; 'vertically' perfused preparations (V, open circles) and 'horizontally' perfused preparations (H, closed circles).

(1) Vertically perfused preparations were superfused with TFP at concentrations ranging from 10^{-8} - 10^{-4} M. The steady-state twitch tension attained after 30 minutes were recorded. The log-dose response curve is sigmoidal, with an ED_{50} value of 1.2×10^{-6} M (PD_2 = 6.08). It should be noted that concentrations of TFP used clinically are in the region of

Fig. 3.II.1

Effect of two different concentrations of TFP on twitch amplitude

A) Is an original chart recording showing the effect of 5×10^{-5}M TFP on twitch tension.

B) The graph is pooled data (mean ± S.E) from two series of experiments where the tissue was exposed to 1×10^{-6}M TFP (n = 20) & 5×10^{-5}M TFP (n = 4).

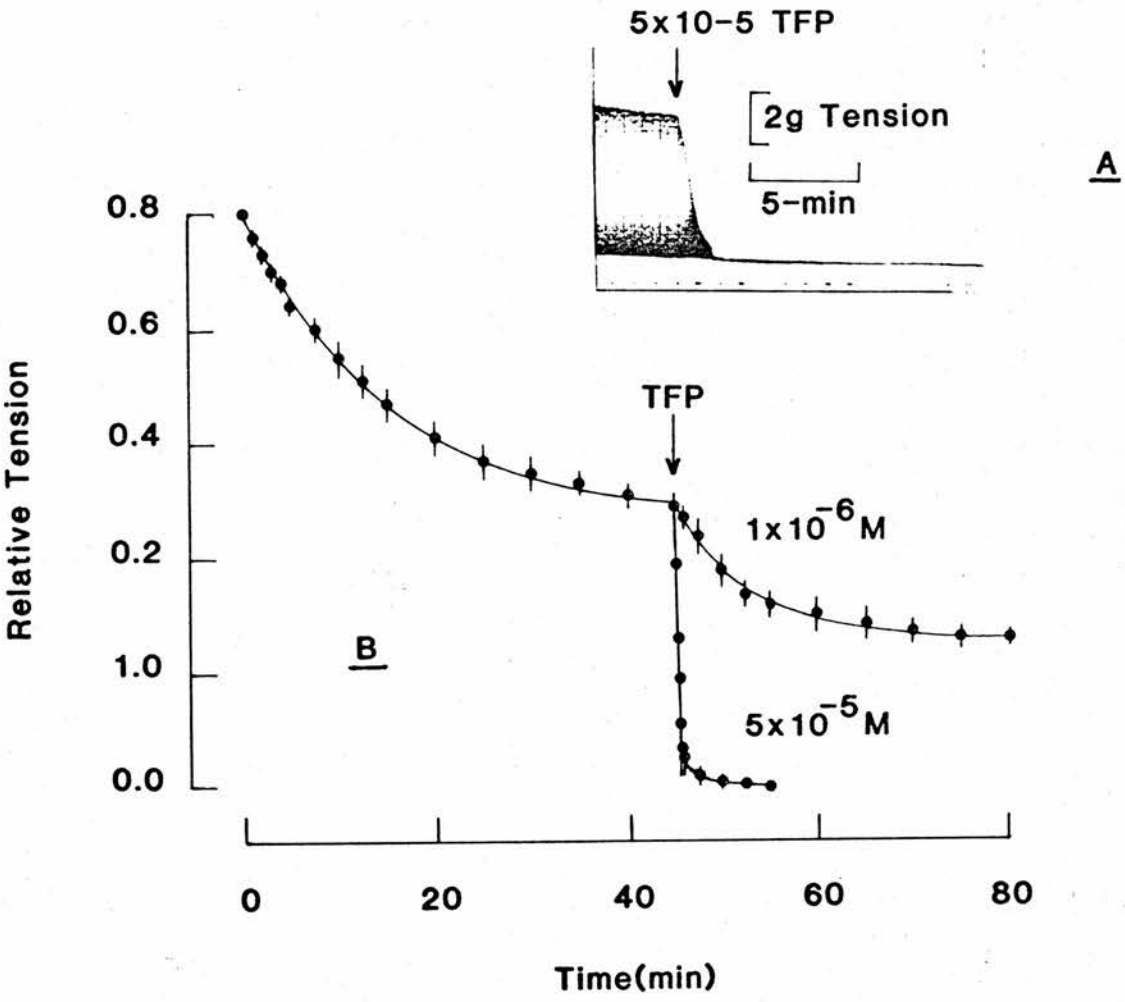
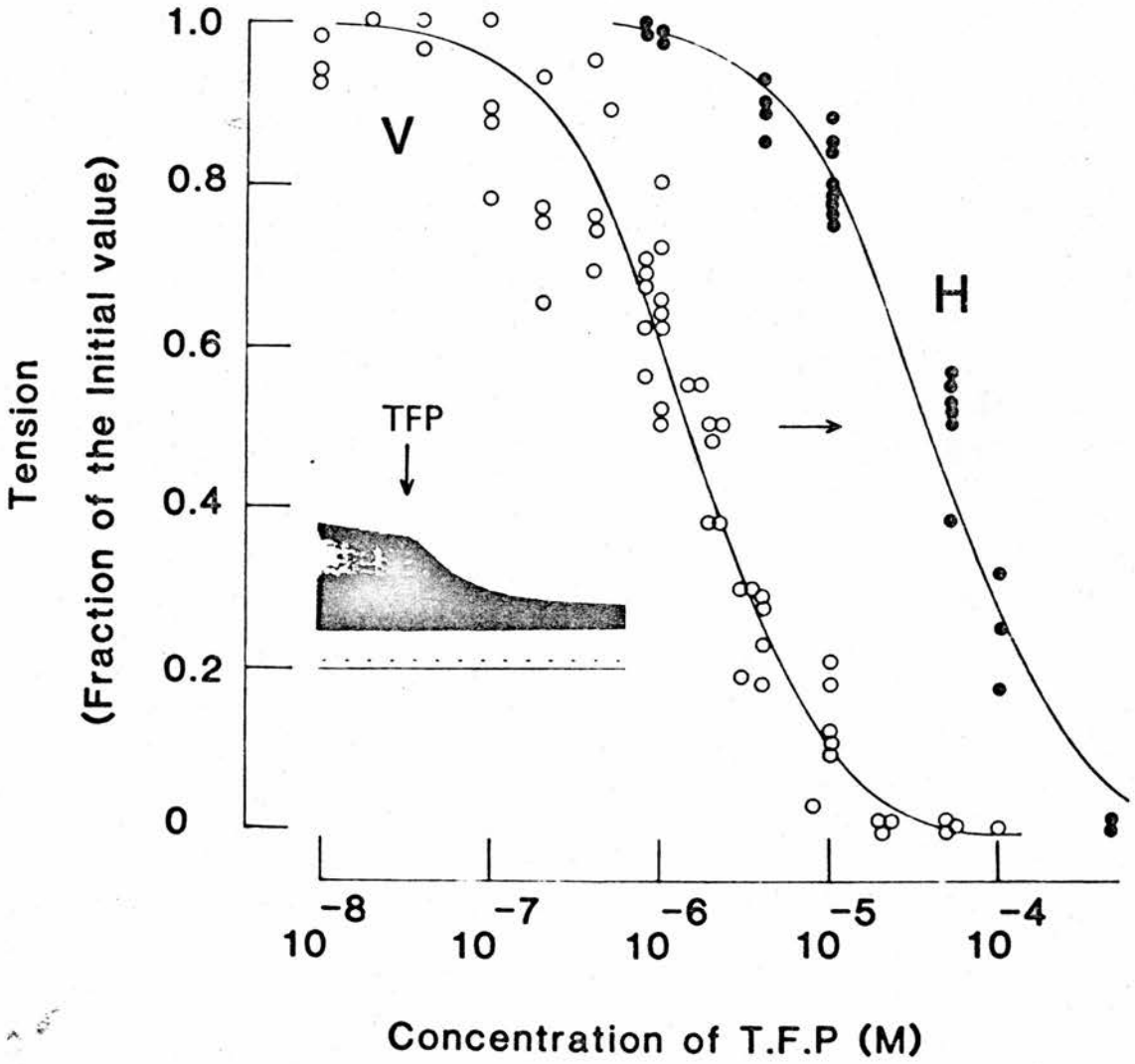

5x10-5 TFP
2g Tension
5-min
A
0.8
0.6
0.4
0.2
1.0
0.0
Relative Tension
TFP
1x10⁻⁶M
5x10⁻⁵M
B
0
20
40
60
80
Time(min)

Fig. 3.II.2

Dose-response curve of the action of TFP on vertically perfused (V) and horizontally perfused (H) frog ventricle

The graph shows the inhibitory effect of 10^{-8}- 5 x 10^{-4}M TFP. Both vertically perfused (V) open circles and horizontally perfused (H) closed circles preparations achieve 100% depression of the twitch at 2 x 10^{-5}M and 5 x 10^{-4}M respectively after 30 minute superfusion. Half maximum concentration ED_{50} for (V) = 1.2 x 10^{-6}M ≡ PD_2 of 6.08; ED_{50} for (H) = 3 x 10^{-5}M ≡ PD_2 of 5.49.

The inset is an original chart recording showing the effect of 5 x 10^{-6}M TFP on a hypodynamic frog ventricle.


1.0
0.8
0.6
0.4
0.2
0
Tension
(Fraction of the Initial value)
V
H
TFP
10^-8
10^-7
10^-6
10^-5
10^-4
Concentration of T.F.P (M)

5×10^{-6}M (calculated from summary in Martindale, the Extra-Pharmacopeia 27th edn) [84].

For each of the 10 concentrations of TFP considered, the half maximum response time $T_{\frac{1}{2}}$ was determined. Fig.3.II.3, shows the pooled data ($T_{\frac{1}{2}}$ min ± S.E) for each series of experiments. The shape of the curve is sigmoidal, with the smallest $T_{\frac{1}{2}}$ value (0.33 min) for 5×10^{-5}M TFP, and largest (11 min) for 10^{-8}M TFP. At the ED_{50} concentration (1.2×10^{-6}M) $T_{\frac{1}{2}}$ = 7 min.

(2) Horizontally perfused preparations were superfused with TFP at concentrations ranging from 8×10^{-7} - 5×10^{-4}M, and the steady state twitch tension attained after 30 minutes was recorded. The log-dose response curve is sigmoidal, with an ED_{50} value of 3×10^{-5}M (PD_2 = 5.49). This contrast between the degree of depression achieved by TFP in vertically and horizontally perfused preparations is interesting. The vertically perfused ventricles achieve the same amount of depression at concentrations of about 30 fold less than those perfused 'horizontally'. This difference in sensitivity of horizontally and vertically perfused preparations is not unique to TFP; it is also observed in SNP responses, where higher concentrations are needed to achieve the same degree of depression in horizontally perfused preparations as compared to vertically (see results part III).

Effects of cholinergic & adrenergic blockers. Fig.3.II.4, shows the results of an experiment in which half-ventricles were treated with 5×10^{-6}M TFP alone, or with TFP (5×10^{-6}M) and with a cholinergic antagonist (10^{-6}M atropine), or adrenergic antagonist, β-blocker (10^{-7} propranolol and an α-blocker (10^{-6}M phentolamine). The results show that none of the receptor blockers tested have any effect on the action of TFP.

Fig. 3.II.3

Half maximum response time for different concentrations of TFP superfused vertically

The graph shows pooled data from 10 experiments where the half maximum response time ($T_{\frac{1}{2}}$ min ± S.E) is measured for each concentration of TFP. 1.2×10^{-6}M TFP (ED_{50} value) has a $T_{\frac{1}{2}}$ of about 7 minutes.

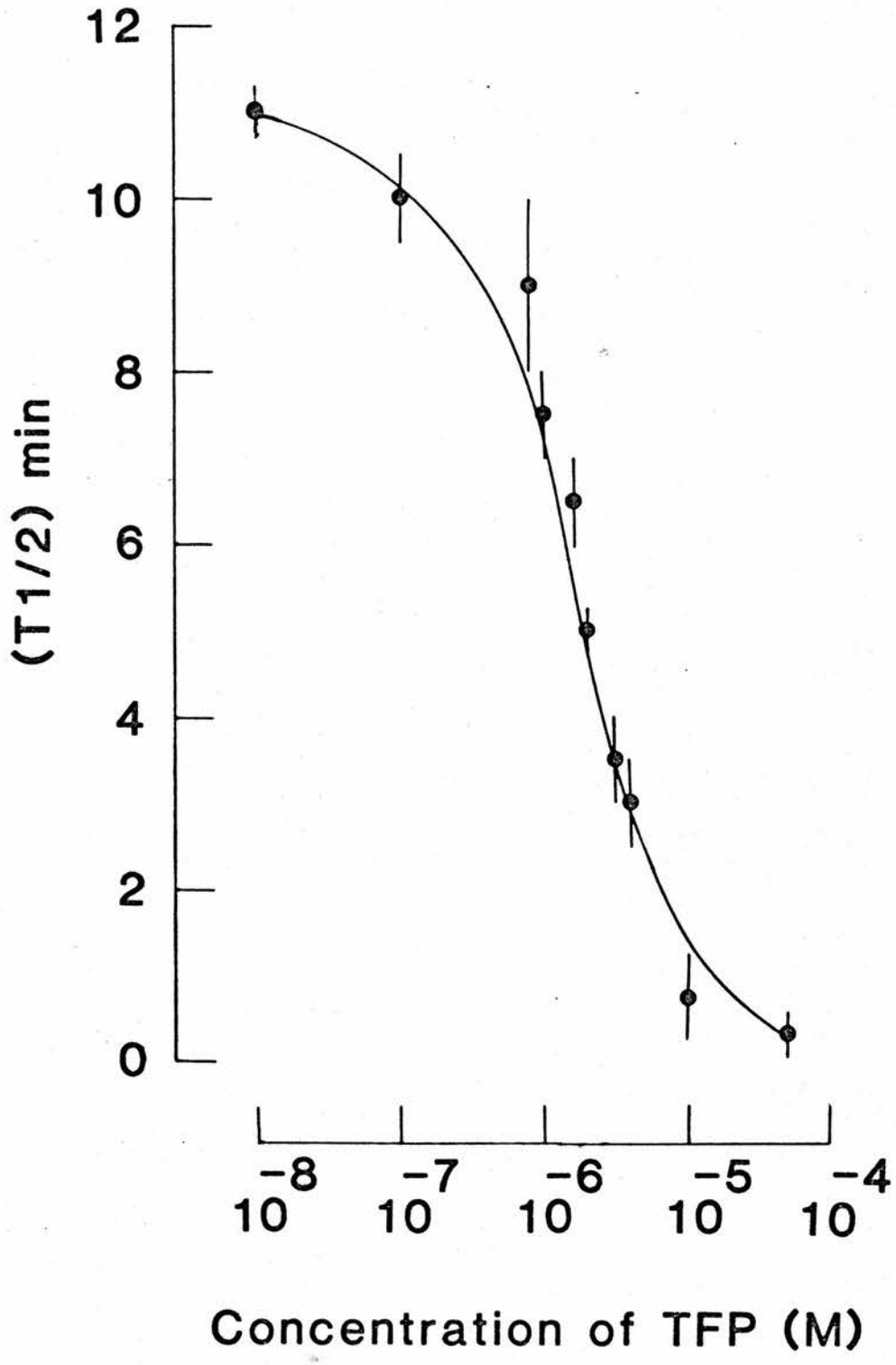
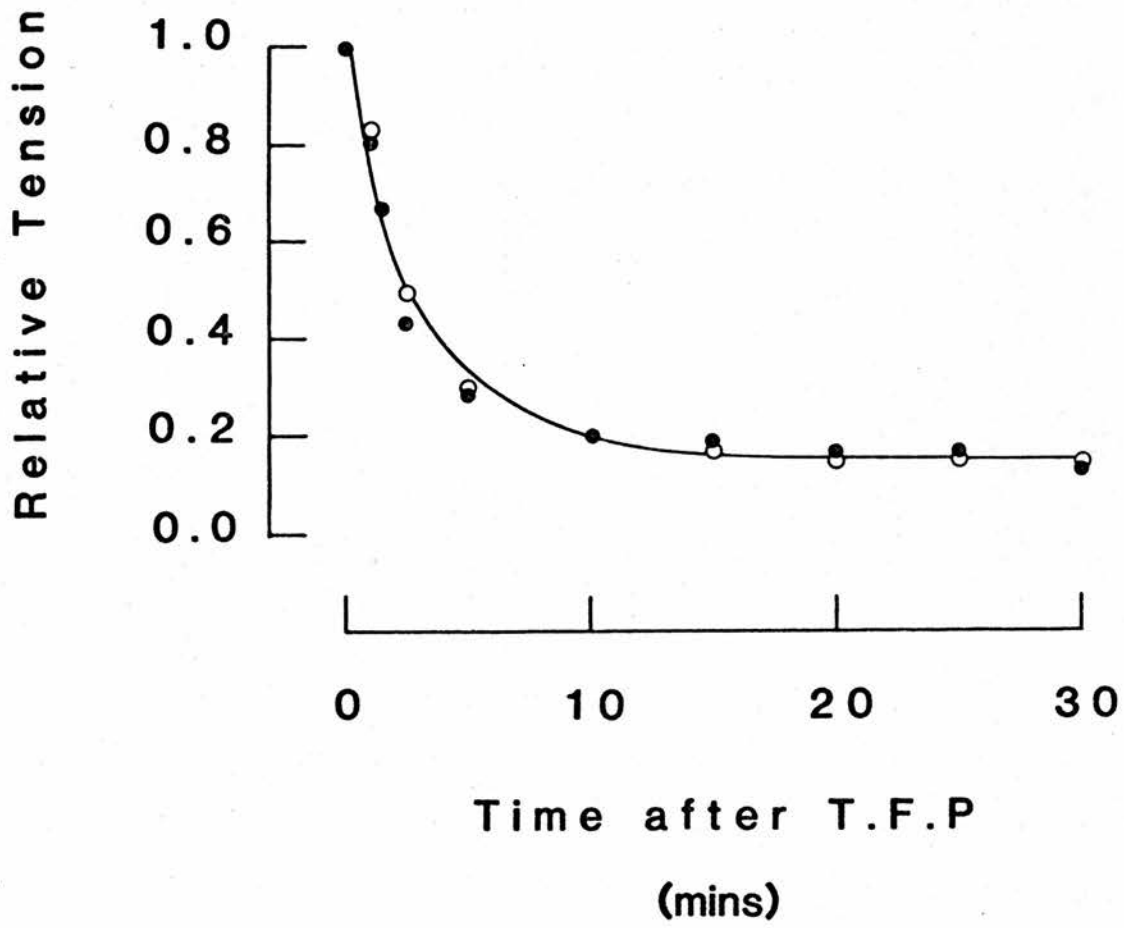

12
10
8
6
4
2
0
(T1/2) min
10^-8
10^-7
10^-6
10^-5
10^-4
Concentration of TFP (M)

Fig. 3.II.4

Effects of cholinergic & adrenergic blockers

The graph shows the result of one of a series of experiments in which half-ventricles (open circles) were exposed to 5 x 10^{-6}M TFP alone while their partners (closed circles) were in addition to 5 x 10^{-6}M TFP, treated with 10^{-6}M atropine, 10^{-6}M phentolamine and 10^{-7}M propanolol. There is no significant difference between TFP-responses obtained in the presence and absence of these blockers.


Relative Tension
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time after T.F.P
(mins)

Effect of the phosphodiesterase inhibitor, Theophylline. In general, phosphodiesterase inhibitors (e.g. methylxanthines such as caffine and theophylline) potentiate responses which are mediated by changes in 3',5'-cyclic nucleotide levels. However, recent experiments by Flitney & Singh [47] have shown that 3',5'-cyclic GMP suppresses 3',5'-cyclic AMP levels in the frog ventricle, by stimulating 3',5'-cyclic AMP phosphodiesterase activity. Their evidence in support of this hypothesis is i) the depressant effect of exogenous 8-bromo-cyclic GMP on the twitch accompanied by a marked reduction in endogenous 3',5'-cyclic AMP levels; and ii) both of these effects are antagonised (not potentiated) by theophylline.

It was therefore of interest to see whether theophylline augments the TFP response or blocks it. Experiments were conducted in which the ventricle was divided into two halves; one half (control) was superfused with 10^{-6}M TFP alone, and the other (test) was first pretreated with 10^{-4}M TFP, in the continuing presence of theophylline Fig. 3.II.5.

The results obtained in 5 experiments using 'paired' half ventricles are shown in Fig. 3.II.6. The open circles show the mean value $\pm$ S.E for the decrease in contractile force with time for TFP and theophylline. The data clearly demonstrates that prior treatment with theophylline does not have an antagonistic effect on the actions of 10^{-6}M TFP. These results differ from those obtained using theophylline & SNP, which will be discussed later (page 34).

Effects of TFP on electrical activity

Effect of TFP on the action potential. Fig. 3.II.7, shows superimposed oscilloscope recordings of twitch tension and action potential, before the addition of TFP, Fig. 3.II.7.Aa, 8 minutes after application of TFP,

Fig. 3.II.5

Effect of 10^{-4}M Theophylline on the ventricular response to 10^{-6}M TFP

This is an original chart recording showing the effect of 10^{-4}M theophylline on the depressant action of TFP.

A) Shows the test half ventricle being pretreated with 10^{-4}M theophylline. The rapid increase in twitch amplitude gradually returns to the control level within 30 minutes.

B) Shows the responses of both test & control half ventricles to 10^{-6}M TFP. The results shows that there is no significant change in the test twitch amplitude as compared with that of control. However, after the initial 10-15 minutes, the test half-ventricle begins to miss alternate beats, an event which is not observed in the control half-ventricle.

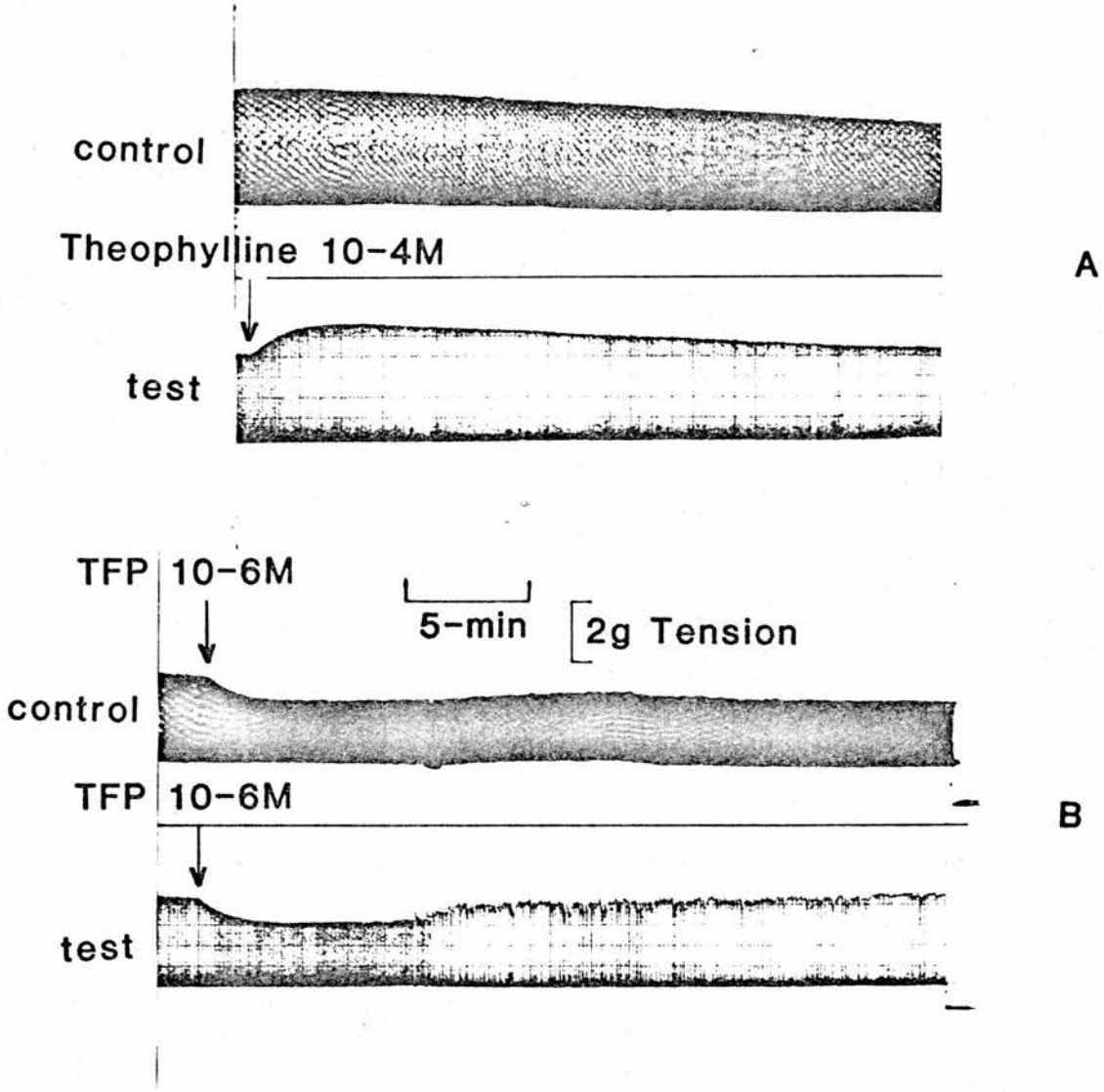

control
Theophylline 10-4M
test
A
TFP 10-6M
5-min
2g Tension
control
TFP 10-6M
test
B

Fig. 3.II.6

Effect of 10^{-4}M Theophylline on the ventricular response to 10^{-6}M TFP

The test half ventricles were treated with 10^{-4}M Theophylline for a period of 20-30 minutes prior to application of TFP. The graph shows that prior treatment with theophylline does not have an antagonistic effect on the actions of 10^{-6}M TFP.

The results are expressed as mean ± S.E, and (n) number of experiments = 5. The results show no significant difference between the test and control half ventricles response to TFP ($P<0.05$).

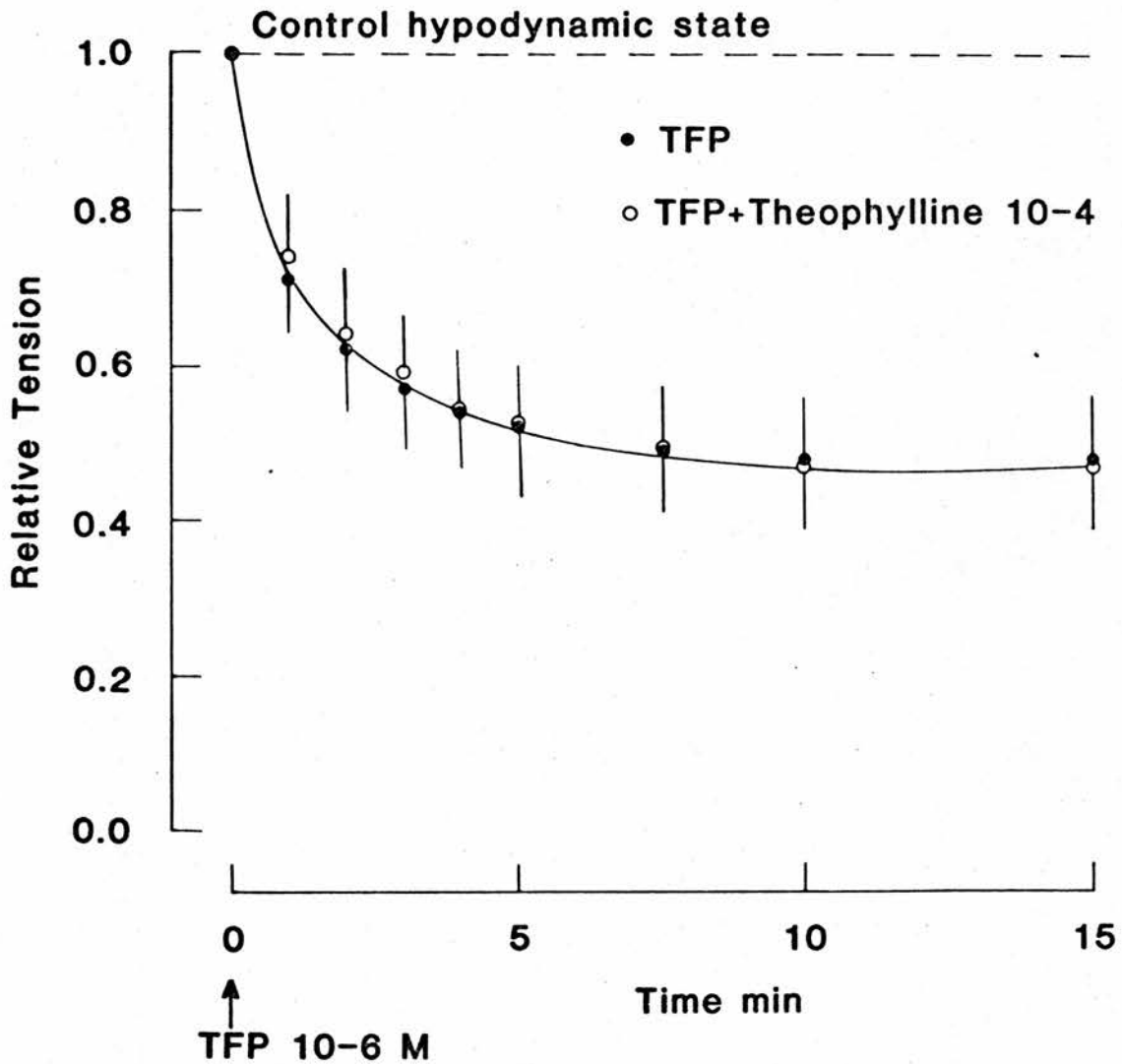

Control hypodynamic state
1.0
0.8
0.6
0.4
0.2
0.0
Relative Tension
• TFP
○ TFP+Theophylline 10-4
0
5
10
15
Time min
TFP 10-6 M

Fig. 3.II.7.Ab, and on reaching the steady state Fig. 3.II.7.Ac. There are several points of interest:

(1) There is an initial decline in twitch amplitude, without any decrease in time to peak tension.

(2) For up to 14% reduction in twitch tension (achieved over a 10 minute period) there is no change in the shape of the action potential. This stabilizing period was also observed at lower concentrations of TFP (10^{-5}M and 4 x 10^{-6}M).

(3) Action potential duration measured at -30mV level (D -30mV). These changes in action potential duration are paralleled by decreases in twitch amplitude.

The latter two points are illustrated more fully in Fig. 3.II.8.

Relationship between changes in the duration of the action potential and the decrease in contractile force. The relationship between changes in action potential duration (ΔD-30mV) and isometric twitch tension (ΔP) for 5 x 10^{-5}M TFP is shown in Fig. 3.II.9. The results were obtained from a series of 25 experiments in which the % decrease in twitch amplitude (mean ± S.E) and % decrease in D-30mV (mean ± S.E) were measured.

In general, there is no change in the action potential duration for a period up to 10 minutes (represented by points which lie on the dotted line, depicting zero change in the action potential duration). However, after this initial stabilizing period, there is a parallel decrease in D-30mV and in twitch amplitude (slope of regression line = 0.99, correlation for values other than those of x = 0 is 0.98).

These results clearly indicate that TFP has a stabilizing effect on the action potential.

Fig. 3.II.7

Effect of 5 x 10^{-5}M TFP on twitch tension and the shape of the action potential

A) Shows the effect of TFP on ventricular twitch tension and the size of the action potential; (a) control,(b) 8 minutes after addition of 5 x 10^{-5}M TFP. With 10% reduction in contractility, there is no change in action potential shape.

B) Shows the effect of TFP on ventricular twitch tension and the action potential; (a) control, (b) 30 minutes after addition of TFP. In this case however, there is 8% reduction in the duration of action potential (D-30mV), corresponding to 34% reduction in ventricular contractility.

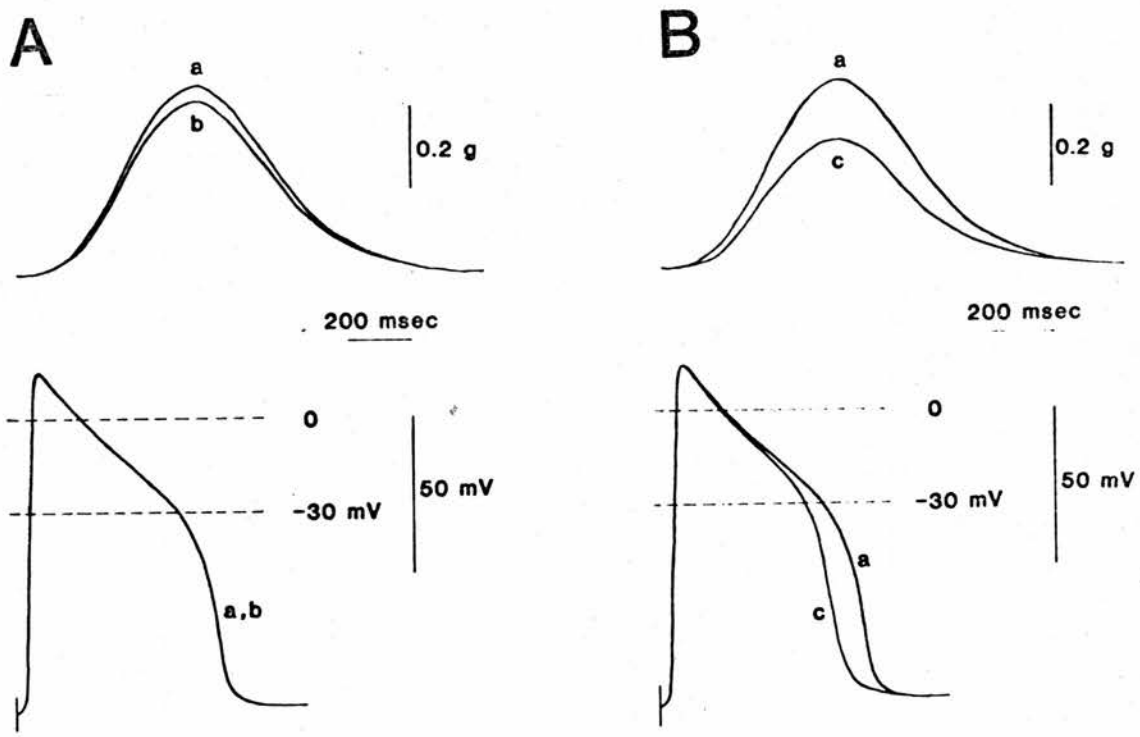

A
a
b
0.2 g
200 msec
0
-30 mV
50 mV
a,b
B
a
c
0.2 g
200 msec
0
-30 mV
50 mV
a
c

Fig. 3.II.8

Effect of 5×10^{-5}M TFP on ventricular contractility and the action potential duration (D-30mV)

The histogram shows the changes in contractility and the D-30mV action potential duration which follow application of 5×10^{-5}M TFP to a strip of ventricular tissue. The outer histogram (error bars up) represents the action potential duration (A.P.D.), while the inner histogram (error bars down) is that of twitch tension.

For the first '10 minutes' there is no change in APD, while the twitch tension declines by 15% of control value. However, after this initial stage, there is a parallel decline in both A.P.D. and twitch tension.

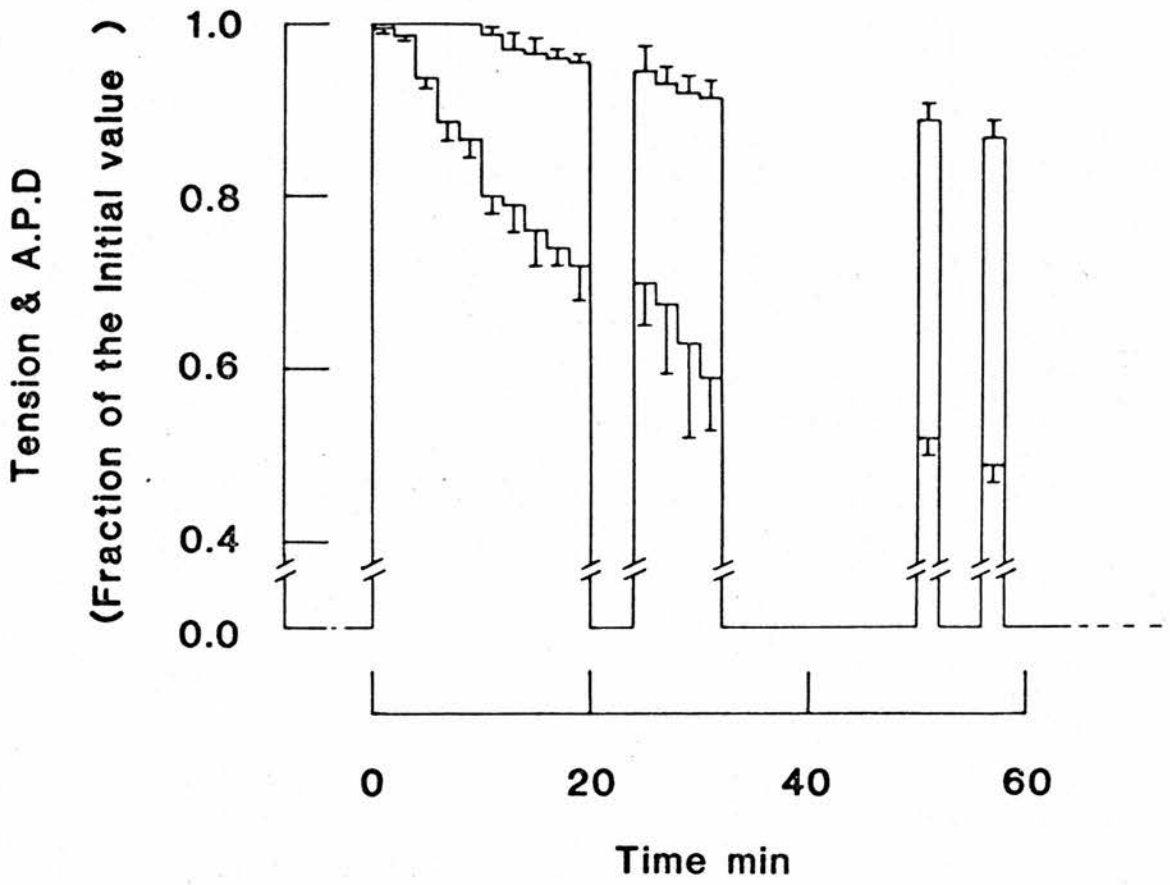
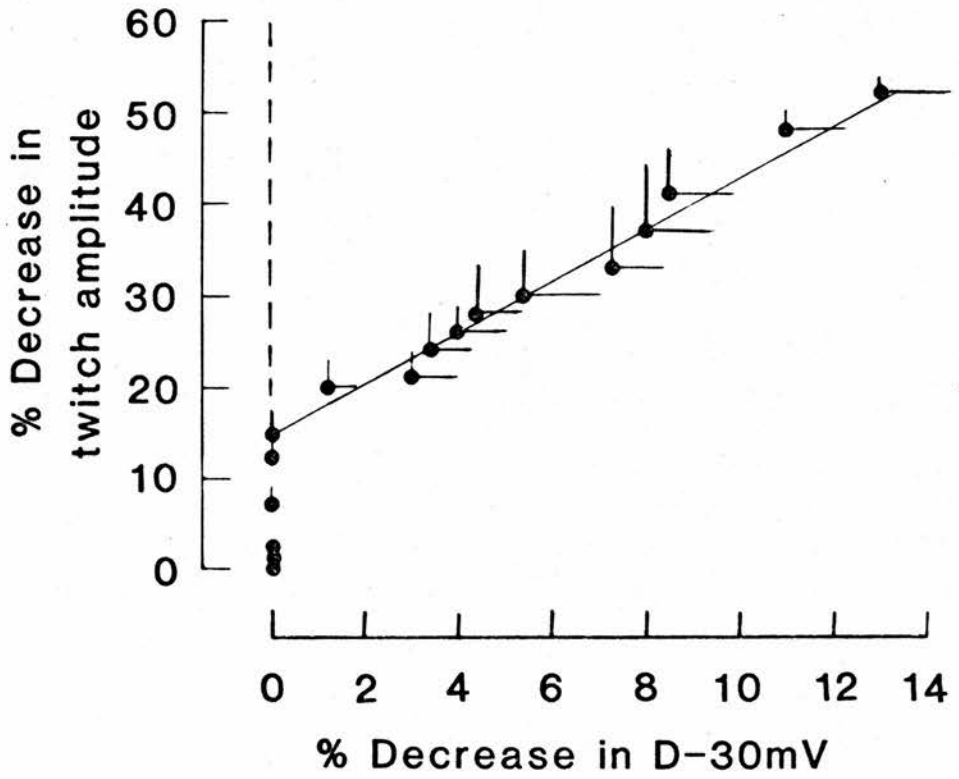

Tension & A.P.D
(Fraction of the Initial value)
1.0
0.8
0.6
0.4
0.0
0
20
40
60
Time min

Fig. 3.II.9

Relationship between twitch tension and the action potential duration (D-30mV)

The regression line represents the pooled data from a series of 25 experiments showing correlation between the % decrease in twitch amplitude (mean ± S.E) and % decrease in D-30mV (mean ± S.E).

The points which lie on the ordinate are excluded from the regression analysis. (Slope of regression line = 0.99; intercept on ordinate = 14.2; correlation coefficient = 0.98).


% Decrease in twitch amplitude
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
% Decrease in D-30mV

RESULTS PART III

EFFECT OF SNP ON TWITCH TENSION, ACTION POTENTIAL AND CYCLIC NUCLEOTIDE LEVELS

The experiments to be described show that SNP has a marked depressant effect on the twitch. In addition, the effects of SNP on membrane potential and endogenous 3',5'-cyclic nucleotide levels were investigated.

Characteristic Inotropic effects of SNP

SNP effect on twitch amplitude. Fig. 3.III.1, which is an original chart recording, shows the effects of varying concentrations of SNP on isometric twitch tension. This illustrates several features of interest.

1) SNP markedly depresses the twitch even when used at a concentration (10^{-4}M) which has been reported previously to have negligible effect (or even a small positive inotropic effect) on mammalian heart [26].

2) The depressant effect of SNP is fully reversible on superfusing the preparation with more Ringer solution (see also Fig. 3.III.2.).

3) A second (or subsequent) application of SNP at the same concentration depresses contractile force to a greater degree and more rapidly than the first application. This is shown clearly in Table 3.III.3. and Fig. 3.III.4. which summarise the results obtained from a series of experiments using 10^{-3}M SNP. The mean time taken for the twitch amplitude to fall to 50% of it's maximum response ($T_{\frac{1}{2}}$) was 2.5 minutes in the case of 'first' application, and this was reduced to 1.0 minutes for a 'second' application. The steady state twitch tension reached after 30 minutes was 31% for 'first', and 25% for 'second' applications.

4) The effect on the twitch is dose dependent (Fig. 3.III.1, and see below).

Fig. 3.III.1.

Effect of first and subsequent application of SNP

This is an original chart recording showing the effect of different concentrations of SNP on frog ventricle. SNP depresses ventricular contractility in a dose dependent and reversible manner. A 'second' application of SNP had a more rapid effect and reduced contractility to a greater extent.

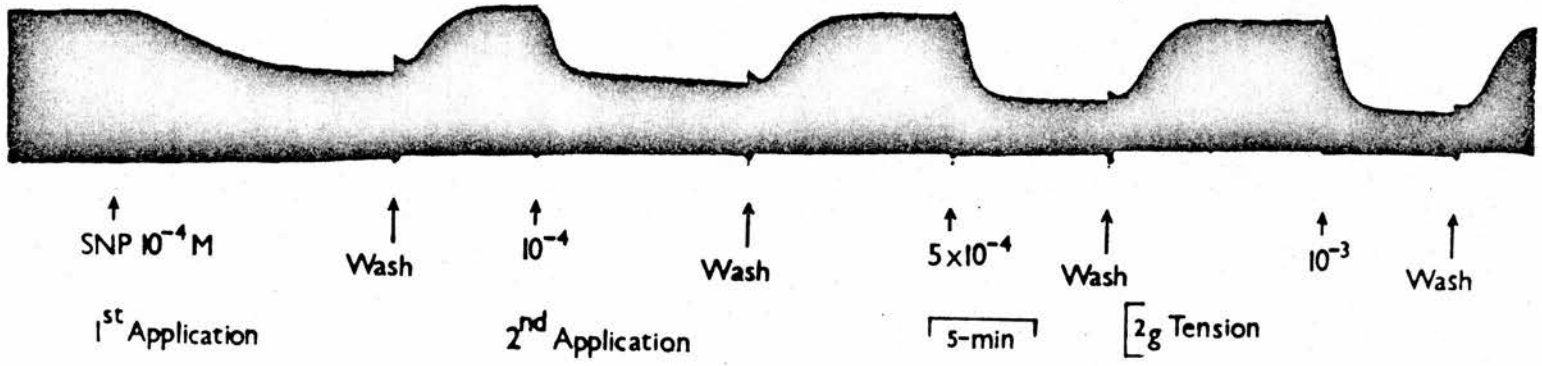

SNP 10^{-4} M
Wash
10^{-4}
Wash
5×10^{-4}
Wash
10^{-3}
Wash
1st Application
2nd Application
5-min
2g Tension

Fig. 3.III.2

Effect of 10^{-3}M SNP on isometric twitch tension and its recovery during washout

The graph represents pooled dats from a series of 10 experiments (mean ± S.E.). 10^{-3}M SNP was applied to the test half-ventricle once it had reached 50% of its initial tension. After 30 minutes, the ventricle was superfused with fresh Ringer solution.

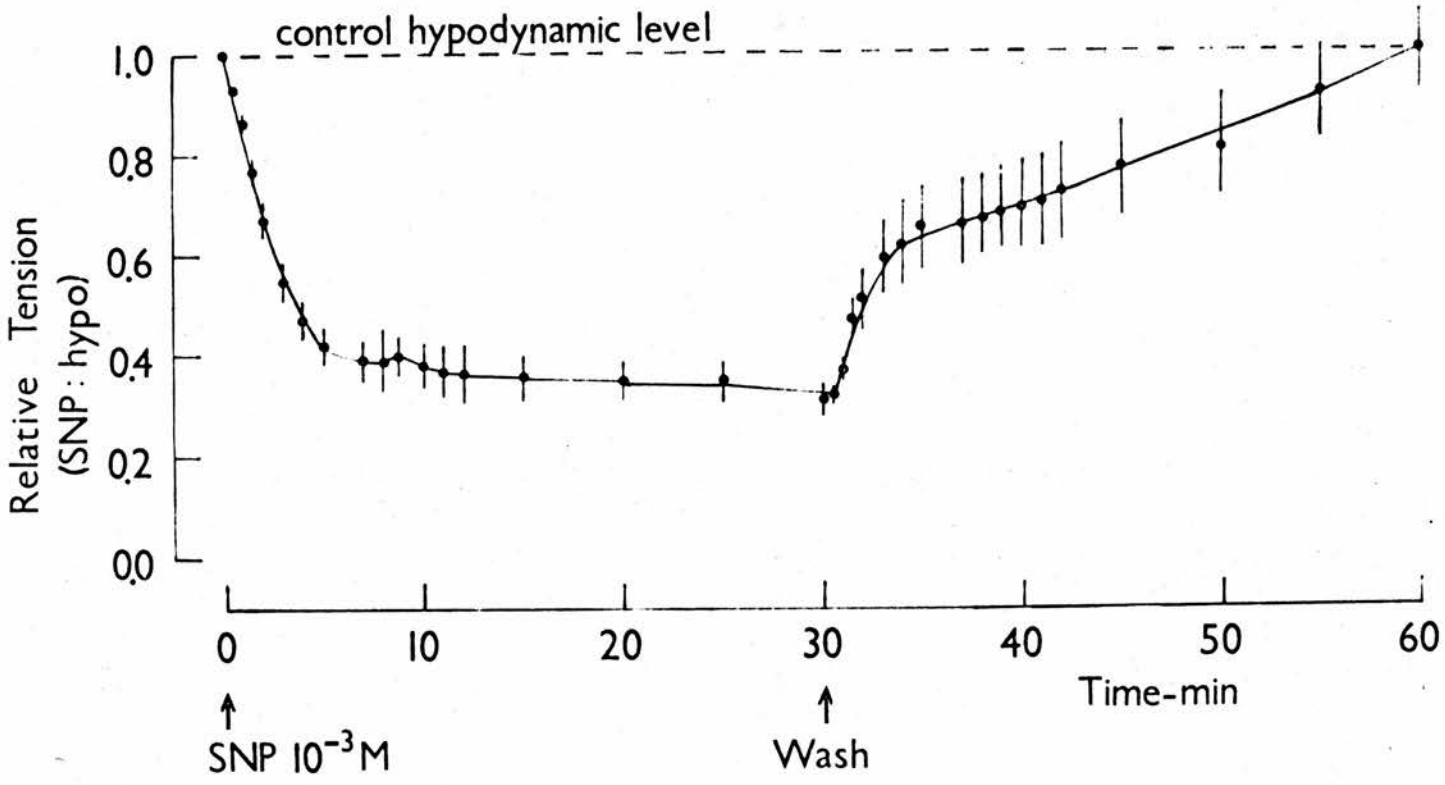

control hypodynamic level
1.0
0.8
0.6
0.4
0.2
0.0
Relative Tension
(SNP : hypo)
0
10
20
30
40
50
60
Time-min
SNP 10^{-3} M
Wash

Table 3.III.3

Comparison of 1st and 2nd application of 10^{-3}M SNP

The table shows pooled data (mean ± S.E) from 4-19 (10 ± 1) experiments where the ventricles were superfused with 10^{-3}M SNP on two consecutive occasions. The second exposure to SNP was carried out after the tissue had recovered its pre-SNP contractile amplitude.

* significantly different from the corresponding 1st application. Value ($P<0.05$).

** significantly different from the corresponding 1st application. Value ($P<0.01$).

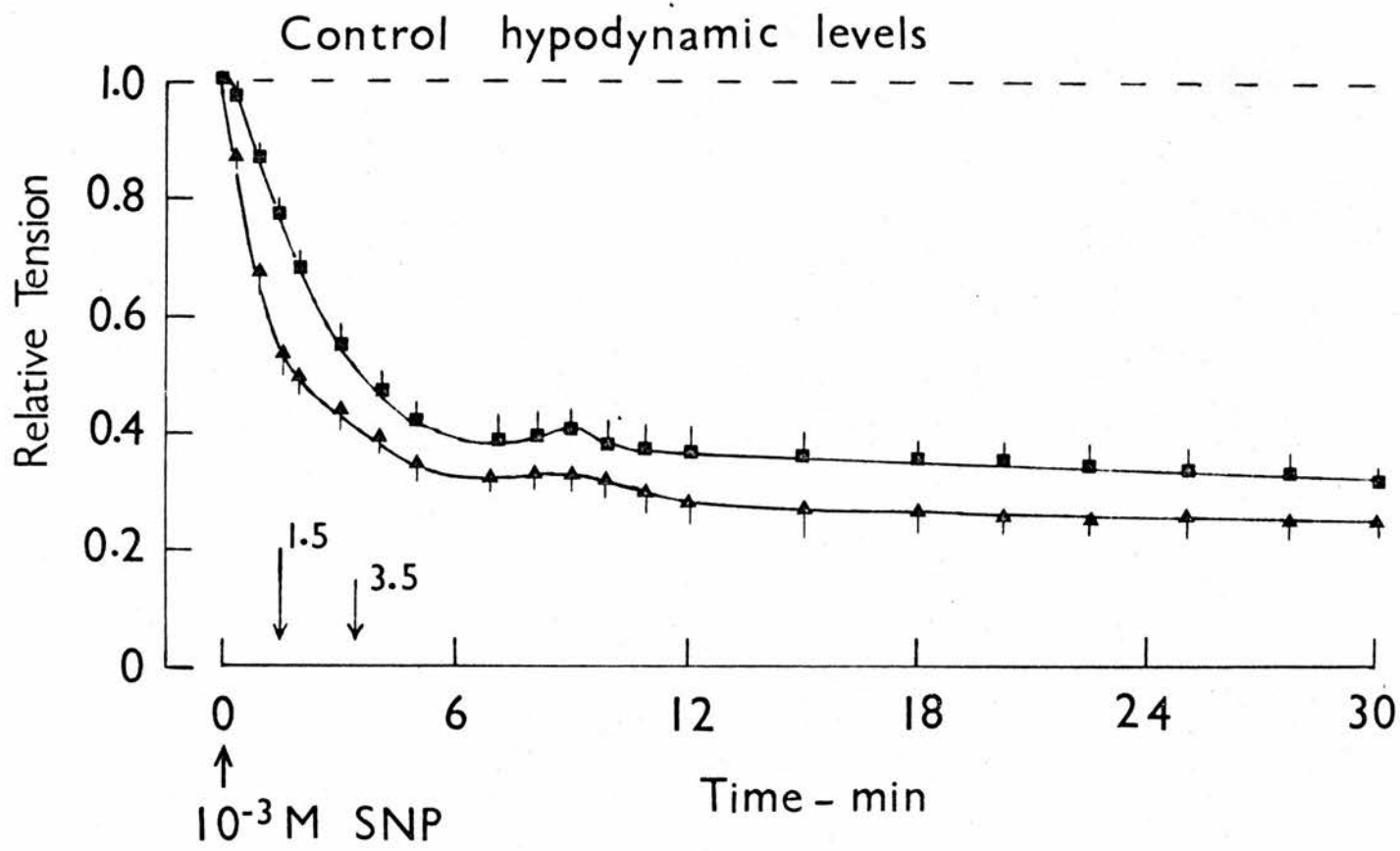
*** significantly different from the corresponding 1st application. Value $P<0.001$).

Time (min) after addition of SNP	Tension (% of Hypodynamic state) 1st applicn.		Recovery		2nd applicn.		
0.0	100	± 0	31	± 3	100	± 0	
0.5	93	± 1	31.8	± 0.7	86.7	± 1.8	**
1.0	86	± 1.4	37.3	± 2	67.3	± 4.1	***
1.5	77	± 2.7	47	± 4	53	± 5	***
2	67	± 3.5	51	± 6	49.6	± 4.3	**
3	55	± 4	59	± 9	44	± 4	***
4	47.5	± 3.7	62	±10	40	± 3.8	*
5	42	± 3.7	65	± 10	35	± 3.2	*
7	39.2	± 4	66	± 10	33	± 3.1	
8	39	± 6.1	67	± 10	33	± 3	
9	40	± 4	68	± 9	33	± 1.6	
10	38.5	± 4.6	69	± 10	31	± 3	
11	37	± 5	70	± 10	30	± 3.4	
12	36.5	± 5.7	72	± 10	28.5	± 4	
15	36	± 4.3	77	± 11	27	± 6	*
20	35	± 2.4	81	± 12	26.5	± 3	*
25	34.5	± 3.1	92	± 12	26	± 3	*
30	31	± 3	100	± 11	25	± 3	*

Fig. 3.III.4

Effect of 1st & 2nd application of 10^{-3}M SNP

The graph represents pooled data from a series of 10 experiments (mean ± S.E). 10^{-3}M SNP was applied once the test half ventricle had reached 50% of its initial tension. After 30 minutes, the ventricle was superfused with fresh Ringer solution until it fully recovered. Then it was re-exposed to 10^{-3}M SNP. 1st application of 10^{-3}M SNP (closed squares), gives a rapid decline in contractility $T_{\frac{1}{2}}$ of 2.5 minutes. 2nd application of 10^{-3}M SNP (closed triangles), gives a more rapid decline in contractility with $T_{\frac{1}{2}}$ of 1.0 min.


Control hypodynamic levels
Relative Tension
1.0
0.8
0.6
0.4
0.2
0
1.5
3.5
0
6
12
18
24
30
10-3 M SNP
Time - min

Effect of using different concentrations of SNP. Fig. 3.III.5. summarises the results obtained in a series of 7 experiments, in which ventricles were superfused with SNP at concentrations ranging from 10^{-7} to 10^{-2}M (n=8 ventricles for each concentration). All these data were obtained for 'first' application and the values represent the steady state twitch tension attained after 25 minutes.

The log dose response curve is sigmoidal, with an ED_{50} value of 2×10^{-5}M (PD_2 = 5.33), rising to a maximum degree of depression of 82% at 10^{-2}M SNP. It should be noted a) that in some experiments, concentrations as low as 10^{-9}M SNP produced a small depression of the twitch; b) the concentrations of SNP used clinically are in the region of 5×10^{-7}M (calculated from summary in Martindale, The Extra Pharmacopeia 27th edn.) [83].

For each of the 7 series of experiments considered, the half maximum response times ($T_{\frac{1}{2}}$) were measured. Fig. 3.III.6, shows the pooled data ($T_{\frac{1}{2}}$ min ± S.E) for each series of experiments. The shape of the curve is sigmoidal with the shortest time of 0.5 min for 10^{-2}M SNP, the longest time of 11 min for 10^{-7}M SNP and at ED_{50} concentration (2×10^{-5}M), the $T_{\frac{1}{2}}$ = 9 min.

Effects of cholinergic & adrenergic blockers. Fig. 3.III.7, shows the results of a number of experiments in which test and control half-ventricles were set up. The control half-ventricles were exposed to 10^{-4}M SNP, while the test halves were treated with 10^{-4}M SNP in the presence of a cholinergic antagonist (10^{-6}M atropine), β-adrenergic antagonist (10^{-7}M propranolol and α-blocker (10^{-6}M phentolamine). The results show clearly that these blockers have no effect on the inotropic actions of SNP, and suggest that SNP acts intracellularly, rather than via adrenergic or cholinergic pathways.

Fig. 3.III.5

Dose-response curve of the action of SNP on frog ventricle

The graph shows the inhibitory effects of 10^{-7}-10^{-2}M SNP. Maximum degree of depression obtained is about 82%, with a half maximum concentration ED_{50} of 2 x 10^{-9}M SNP ≡ PD_2 of 5.33.

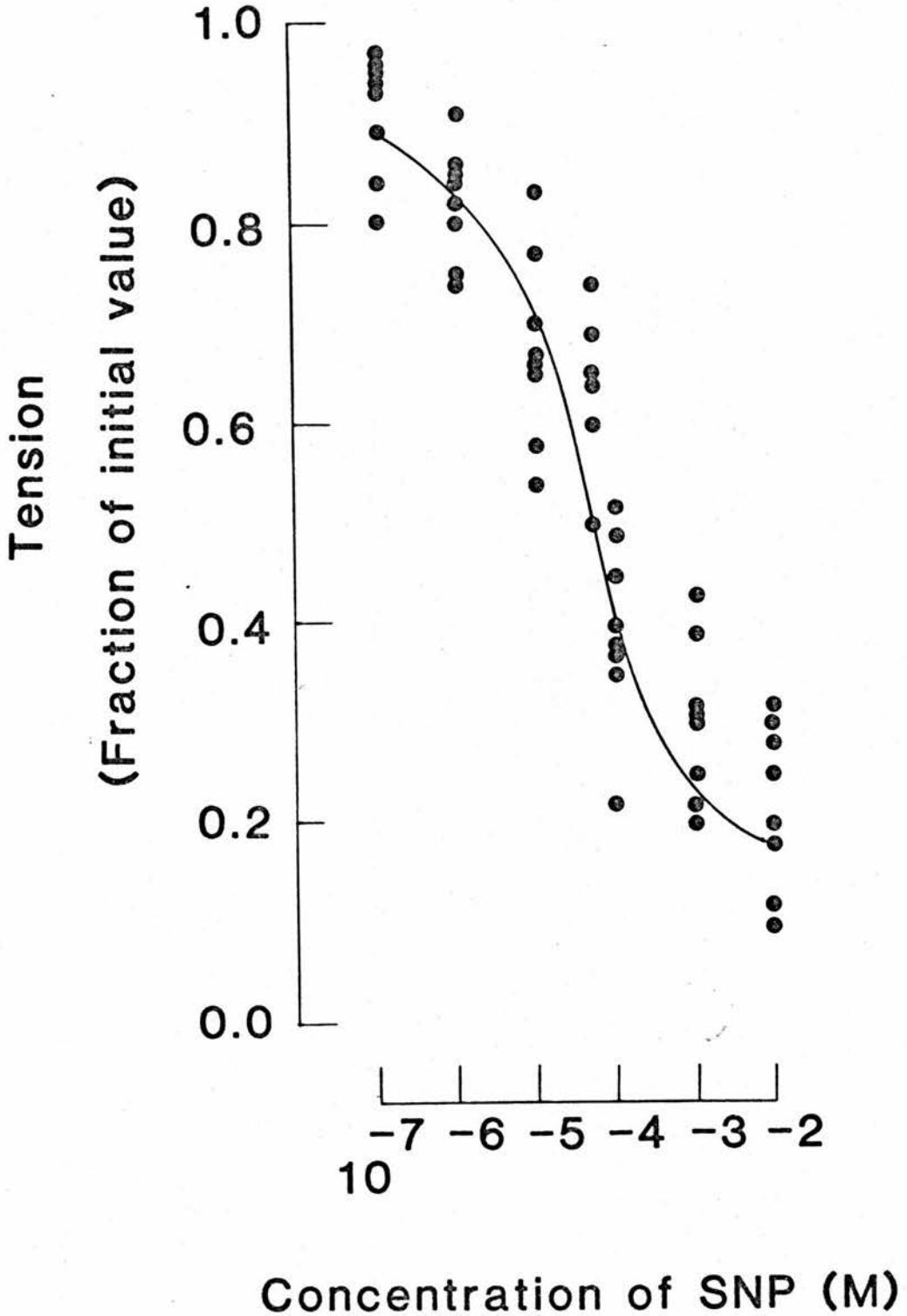

1.0
0.8
0.6
0.4
0.2
0.0
Tension
(Fraction of initial value)
−7 −6 −5 −4 −3 −2
10
Concentration of SNP (M)

Fig. 3.III.6

Half maximum response time for different concentration of SNP

The graph shows pooled data from series of experiments where the half maximum response time ($T_{\frac{1}{2}}$ min ± S.E) is measured for each concentrations of SNP. 2×10^{-5}M SNP (ED_{50} value) has a $T_{\frac{1}{2}}$ of about 9 min.

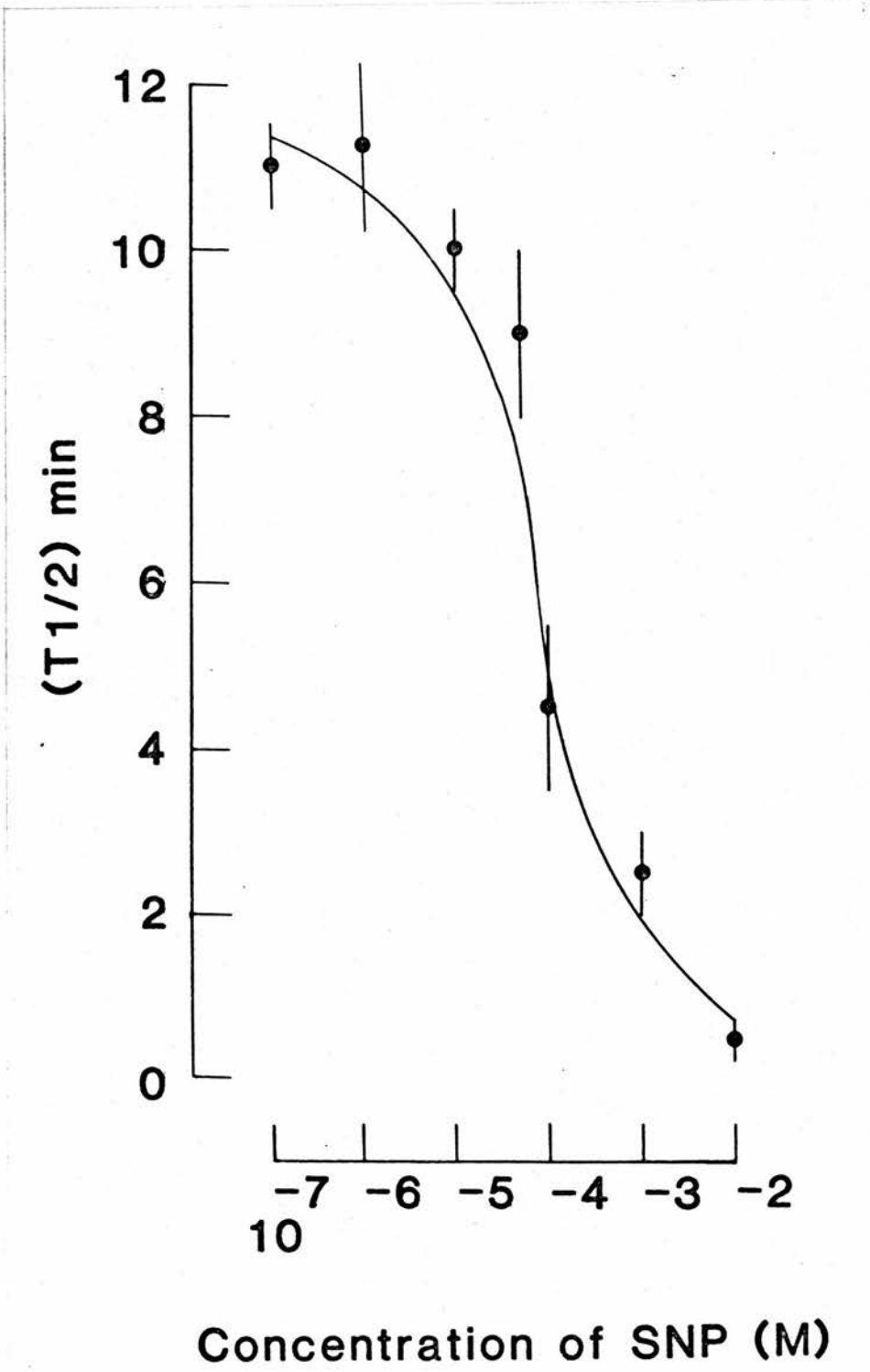
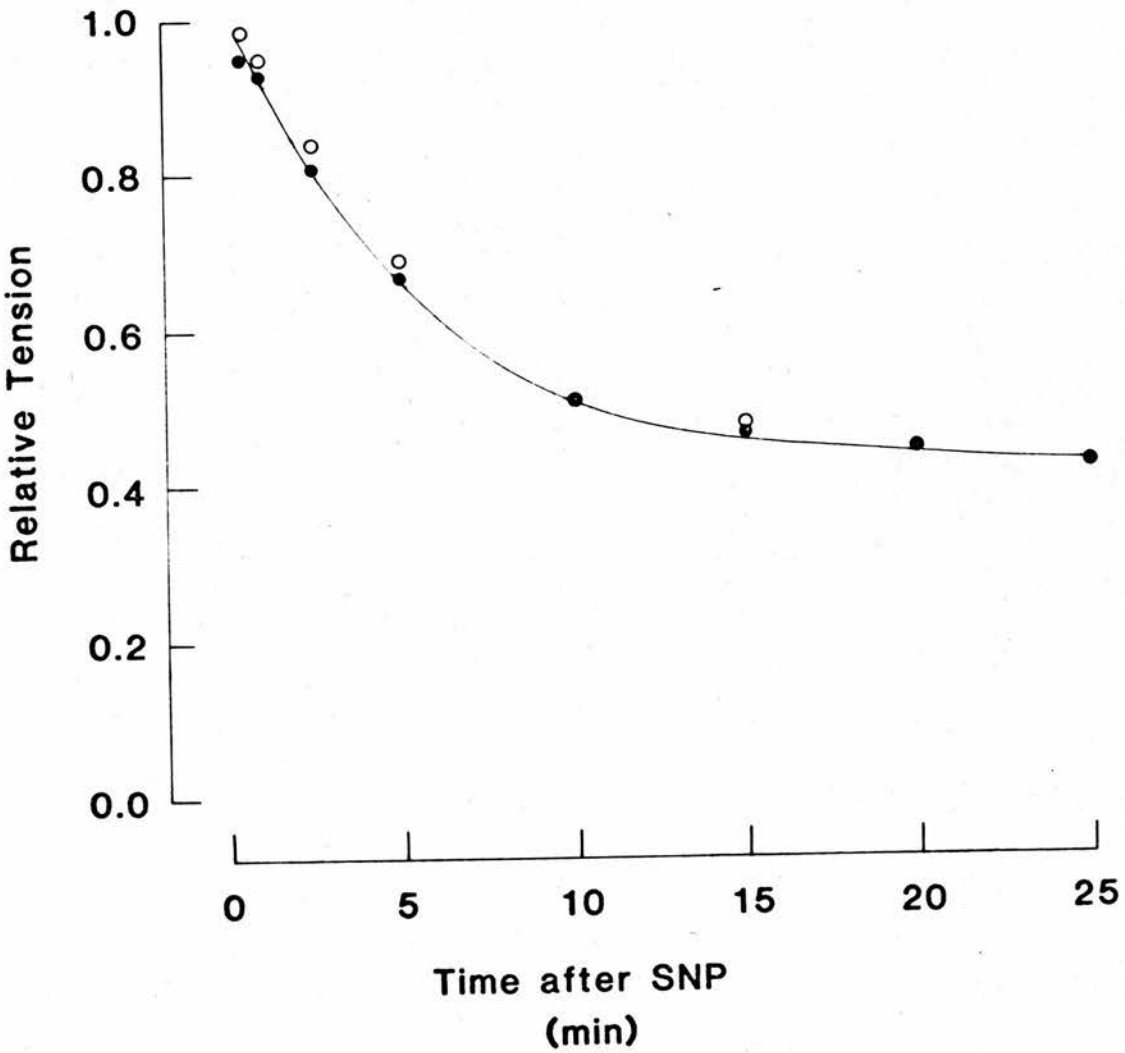

12
10
8
6
4
2
0
(T1/2) min
-7 -6 -5 -4 -3 -2
10
Concentration of SNP (M)

Fig. 3.III.7

Effects of cholinergic & adrenergic blockers

The graph shows the result of a series of experiments in which the controls (open circles) were exposed to 10^{-4} SNP, while the tests (closed circles) were in addition to 10^{-4}M SNP treated with 10^{-6}M Atropine, 10^{-6}M Phentolamine and 10^{-7}M Propanolol. The results show no significant difference between presence and the absence of these blockers.


1.0
0.8
0.6
0.4
0.2
0.0
Relative Tension
0
5
10
15
20
25
Time after SNP
(min)

Effects of the phosphodiesterase inhibitor, theophylline. In a similar manner to the above, experiments were conducted in which the ventricle was divided into two halves; one half (control) was superfused with 10^{-3}M SNP alone, and the other (test) was first pretreated for 20-30 minutes with 10^{-4}M theophylline, and then exposed to 10^{-3}M SNP, in the continuing presence of theophylline.

The results obtained in 8 experiments using 'paired' half-ventricles are shown in Fig. 3.III.8. The open circles show mean value ± S.E for the decrease in contractile force with time for SNP and theophylline. The outcome of these experiments is clear: theophylline antagonises the SNP induced response. Thus, after 50 minutes the steady state tension reached in the presence of SNP alone was 33% of the initial value, but with theophylline present, the twitch only declined to 65% of its initial level. Statistically, these differences are highly significant ($p < 0.001$ two tailed, paired t-test).

These results provide additional, circumstantial evidence that cyclic GMP may be acting to stimulate cyclic AMP-phosphodiesterase activity, a point which is taken up again later (discussion, page 45).

Effects of SNP on electrical activity

Effect of SNP on action potential. Fig. 3.III.9.A, shows the time course of changes in twitch tension on first and second applications of 5 x 10^{-3}M SNP, using a small strip of ventricle. Intracellular micro-electrode recordings show that SNP has marked effects on the shape of the action potential. Fig. 3.III.9B, shows superimposed oscilloscope recordings of twitch tension and action potentials, before the addition of SNP (Ba), 20 seconds after application of SNP (Bb), and on reaching steady state (Bc). There are several points of interest:

(1) there is an immediate decline in twitch amplitude, with a decrease in the time to peak tension.

Fig. 3.III.8

Effect of 10^{-4}M Theophylline on the ventricular response to 10^{-3}M SNP

The test half-ventricles were treated with 10^{-4} Theophylline for a period of 20-30 minutes prior to application of SNP. The diagram shows that prior treatment with Theophylline partially antagonises the effect of 10^{-3} SNP.

The results are expressed as mean ± S.E, and (n) number of experiments = 8. The results show that theophylline treated half ventricles are significantly different from the corresponding SNP alone value ($P<0.001$).

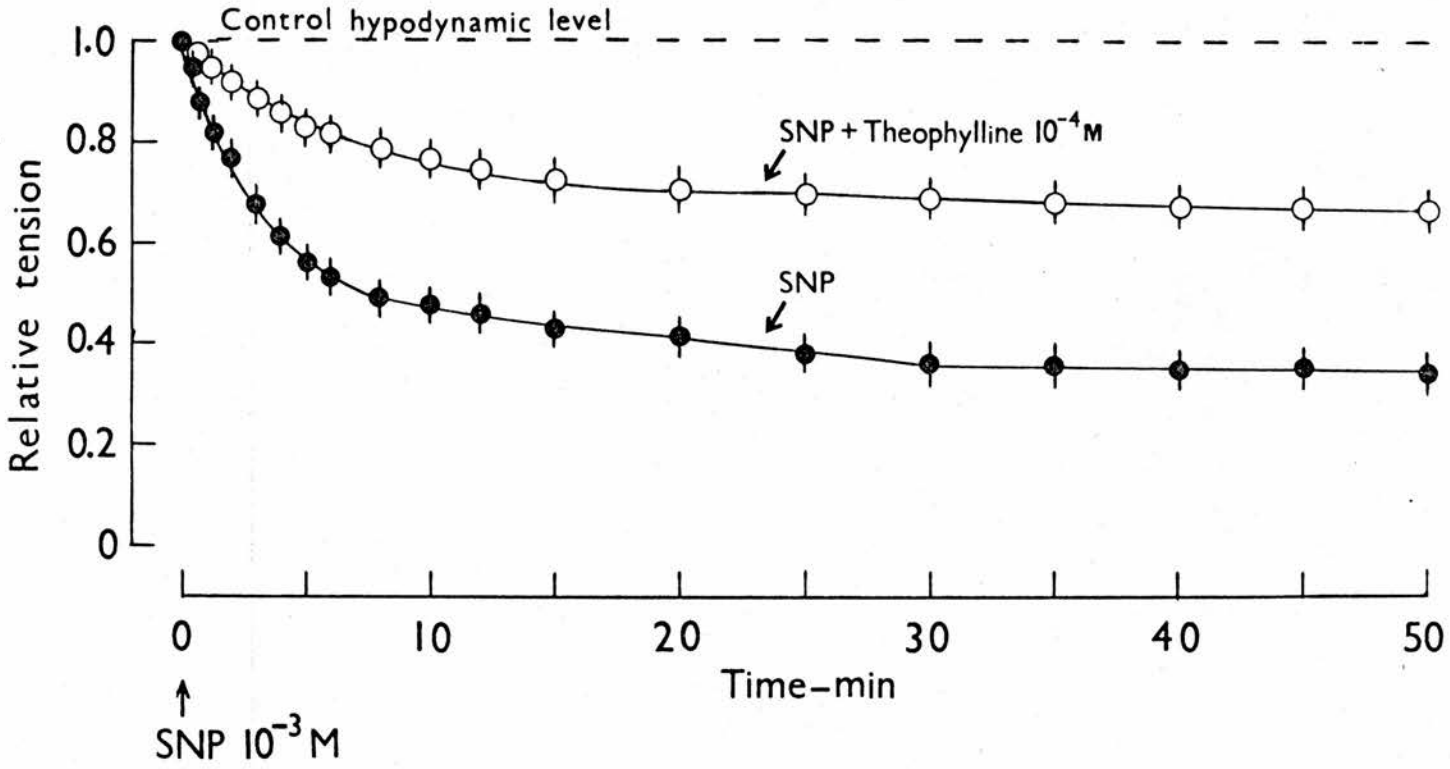

Control hypodynamic level
SNP + Theophylline 10^{-4} M
SNP
Relative tension
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Time–min
SNP 10^{-3} M

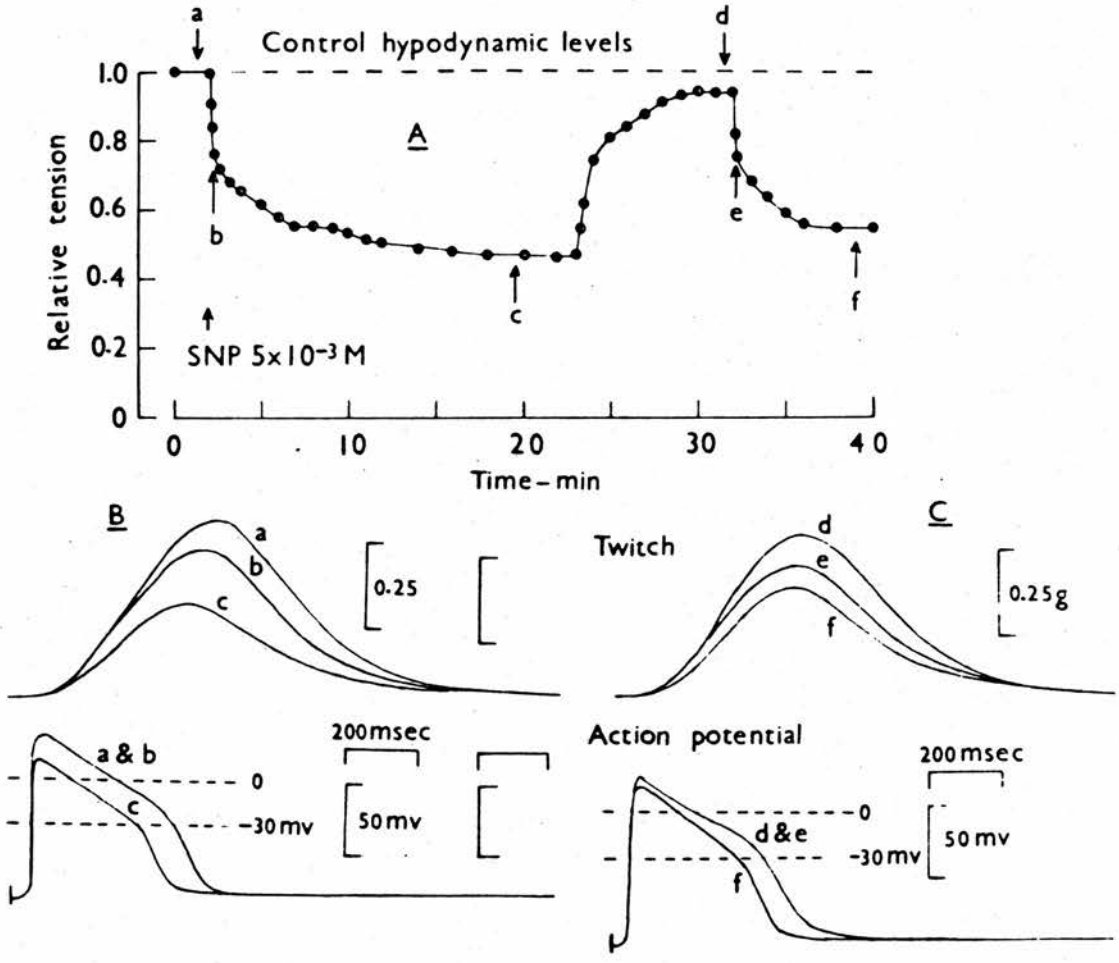
Fig. 3.III.9

The effects of 5×10^{-3}M SNP on ventricular contractility and the shape of the action potential

A) Shows the decline in contractility upon application of 5×10^{-3}M SNP. The ventricle was then superfused with fresh Ringer solution to allow recovery. Subsequently, it was re-exposed to SNP.

B) Shows the effect of SNP on ventricular twitch tension and the size of the action potential. With 20% reduction in contractility, there is no change in action potential shape (a,b). However, after 20 minutes (at steady state c), there is 26% reduction in the duration of action potential (D-30mV).

C) Shows the effect of 2nd application of 5×10^{-3}M SNP. Again, there is no change in the shape of the action potential initially (d,e), but at (f) there is a reduction in the action potential duration and overshoot.


a
Control hypodynamic levels
d
1.0
0.8
0.6
0.4
0.2
0
Relative tension
A
b
c
e
f
SNP 5x10⁻³ M
0
10
20
30
40
Time – min
B
a
b
c
0.25
Twitch
C
d
e
f
0.25g
200msec
a & b
0
c
-30mv
50mv
Action potential
200msec
0
d & e
-30mv
f
50 mv

(2) for up to 26% reduction in twitch tension, there is no change in the shape of the action potential.

(3) after the initial stage, there is a decline in positive overshoot, and a decrease in action potential duration. The change in action potential duration correlates closely with the observed decrease in isometric twitch tension.

The latter two points are illustrated more fully in Fig. 3.III.10, and below.

Relationship between changes in the duration of the action potential and the decrease in contractile force. The relationship between changes in action potential duration (ΔD-30mV) and isometric twitch tension (ΔP) for 5 x 10^{-3}M SNP are shown in Fig. 3.III.11. The results were obtained from two series of experiments (open and closed circles).

In each case there is no change in the action potential duration to begin with (represented by points which lie on the dotted line, depicting zero change in the action potential duration). However, after a period of 1.5 minutes there is a parallel decrease in D-30mV and in twitch amplitude (slope of regression line = 0.78, correlation for values other than those of (x = o) is = 0.76).

Effect of SNP on endogenous 3',5'-cyclic nucleotide levels

The effect of SNP on the metabolism of endogenous 3',5'-cyclic nucleotide levels was investigated. The experiments were carried out using 10^{-3}M SNP. Previous authors have reported little or no effects on the levels of 3',5'-cyclic AMP [26], and it was thought that the use of a relatively high concentration of SNP would maximise the chances of observing any effect on 3',5'-cyclic AMP levels.

Fig. 3.III.10

Effect of 5 x 10^{-3}M SNP on ventricular contractility and the action potential duration (D-30mV)

The histogram shows the changes in contractility and the D-30mV action potential duration which follow application of 5 x 10^{-3}M SNP to a strip of ventricular tissue. The outer histogram (error bars up) represents the action potential duration (APD), while the inner histogram (error bars down) is that of twitch tension.

For the first 2 minutes there is no change in A.P.D., while the twitch declines by 26% of control value. However, after this initial stage, there is a parallel decline in both A.P.D. and twitch tension.

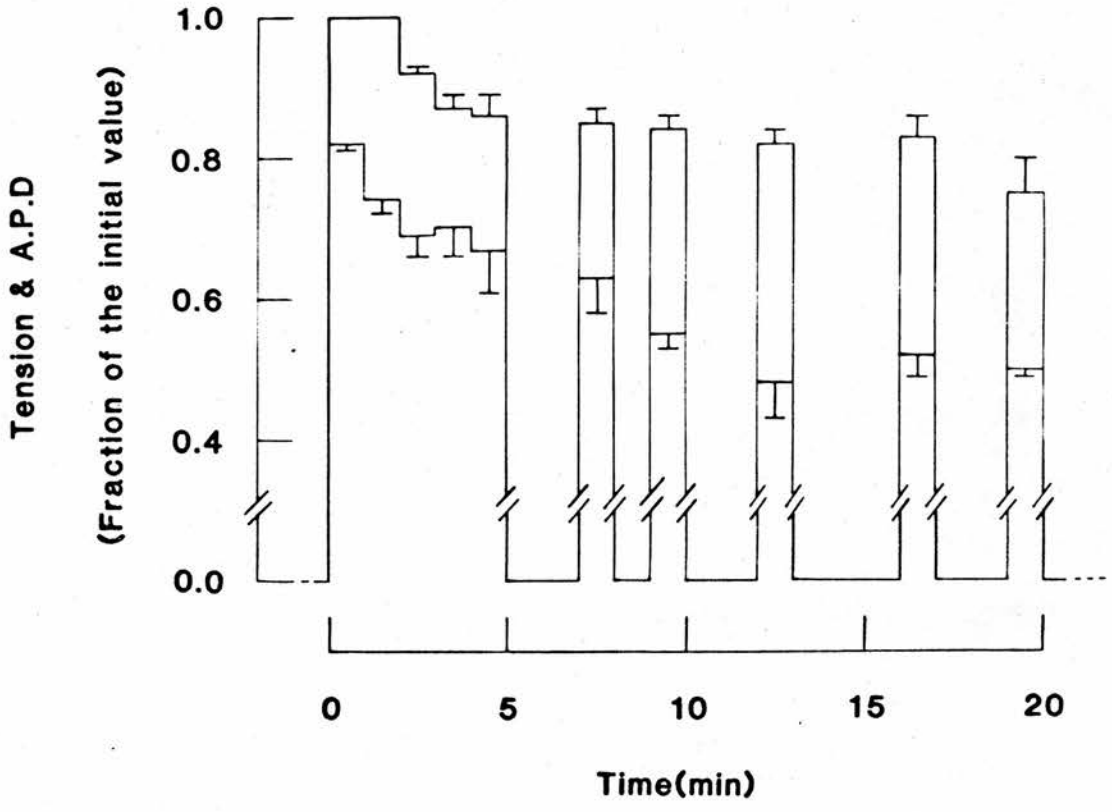

Tension & A.P.D
(Fraction of the initial value)
1.0
0.8
0.6
0.4
0.0
0
5
10
15
20
Time(min)

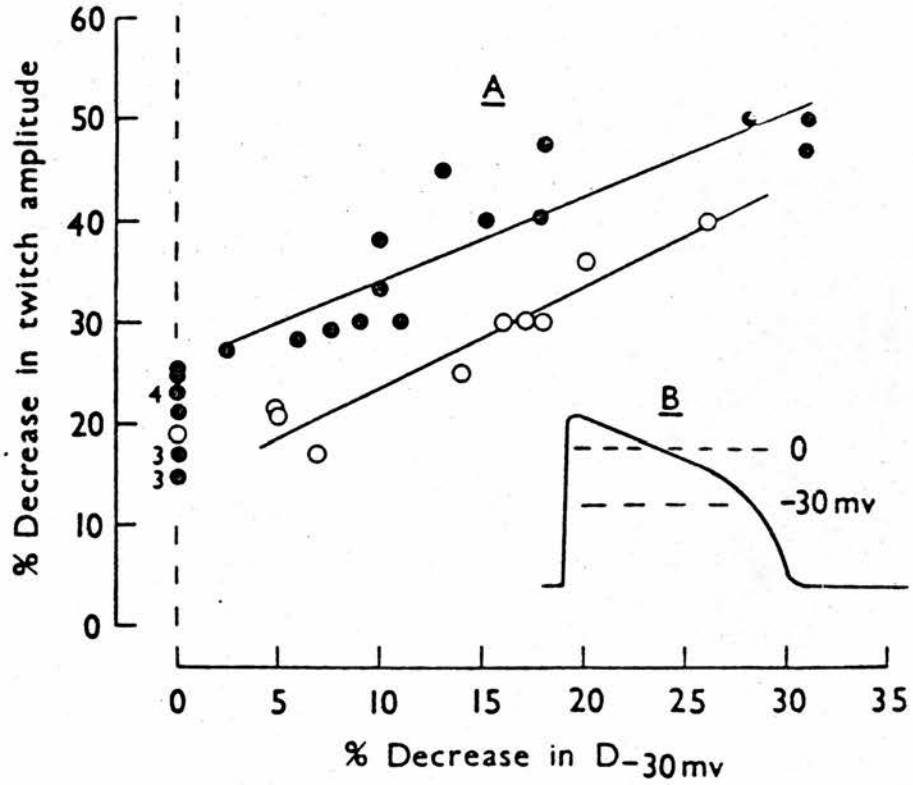
Fig. 3.III.11

Relationship between twitch tension and the action potential duration (D-30mV)

Regression line represents the correlation between the % decrease in twitch amplitude and the % decrease in D-30mV. (The points which lie on the ordinate are excluded from the regression analysis.) Data from two preparations (indicated by open and closed symbols).

Open circles: slope of regression line = 0.99; intercept on ordinate = 13.77; correlation coefficient = 0.95.

Closed circles: slope of regression line = 0.78; intercept on ordinate = 26.14; correlation coefficient = 0.76.


% Decrease in twitch amplitude
60
50
40
30
20
10
0
A
B
0
-30 mv
4
3
3
0
5
10
15
20
25
30
35
% Decrease in D−30 mv

Time course of metabolism of the endogenous 3',5'-cyclic nucleotides.

The experimental procedure is described in detail under 'Methods'. Fig. 3. III.12. summarises the results from a series of preparations (n = 15); the cyclic nucleotide levels are all expressed as multiples of those present in the untreated (control) half-ventricle. The raw data from these experiments are tabulated in Table 3.III.13. There are two points to emphasise:

(1) SNP stimulates the metabolism of both cyclic nucleotides. Initially (during the first 90 seconds) cyclic AMP and cGMP decrease abruptly to around 50% of their control levels. This early decrease is then followed by an increase in the levels of both cyclic nucleotides, cyclic AMP reaching a peak value of 4X its control value and cyclic GMP of 8X its control value after 4 minutes. Thereafter, cyclic AMP returns slowly to its initial level, whereas after an early fall, cyclic GMP levels remain elevated at 4X the control value for up to 50 minutes.

(2) in absolute terms, the mean velocity of the increase in cyclic AMP levels is about 7X greater than that of cyclic GMP (see below).

> The control levels of cyclic AMP and cGMP were 5.06 $\pm$ 0.8 p.mol. mg protein^{-1} and 0.36 $\pm$ 0.06 p.mol. mg protein^{-1} respectively. During the early decreases, cyclic AMP fell to 2.5 p.mol. mg protein^{-1}, and cyclic GMP to 0.18 p.mol. mg protein^{-1}. In the subsequent 3.5 minutes period, during which time both cyclic nucleotides increased, cyclic AMP rose to 4 x 5.06 = 20.24 p.mol. mg protein^{-1}, cyclic GMP to 8 x 0.36 = 2.88 p.mol. mg protein^{-1}. The differences were therefore 20.24 - 2.50 = 17.74 p.mol.mg protein^{-1} for cyclic AMP and 2.88 - 0.18 = 2.70 p.mol. mg protein^{-1} for cyclic GMP. Hence the mean velocities, averaged over the 3.5 minute period, were 17.73/3.5 = 5.07 p.mol. mg protein^{-1} min^{-1} for cyclic AMP and 2.70/3.5 ; 0.77 p.mol. mg protein^{-1} min^{-1} for cyclic GMP. The rate of increase of cyclic AMP therefore exceeded that of cyclic GMP by a factor of around 6.7 times.

Relationship between contractility and the cyclic nucleotide levels. The decrease in isometric twitch tension, (closed square Fig. 3.III.14.A) is

Fig. 3.III.12

Effect of SNP on metabolism of cAMP and cGMP

The graph shows the variation in the level of both cyclic nucleotides upon application of 10^{-3}M SNP. There is an initial decrease followed by an increase in the levels of cAMP and cGMP. cAMP (closed circles) rising to 4X and cGMP (open circles) rising to 8X their respective control levels. cAMP returns to normal after 17 minutes, while cGMP remains elevated at 4-5X its control value for up to 51 minutes.

All cyclic nucleotide levels are expressed as a fraction of the control.

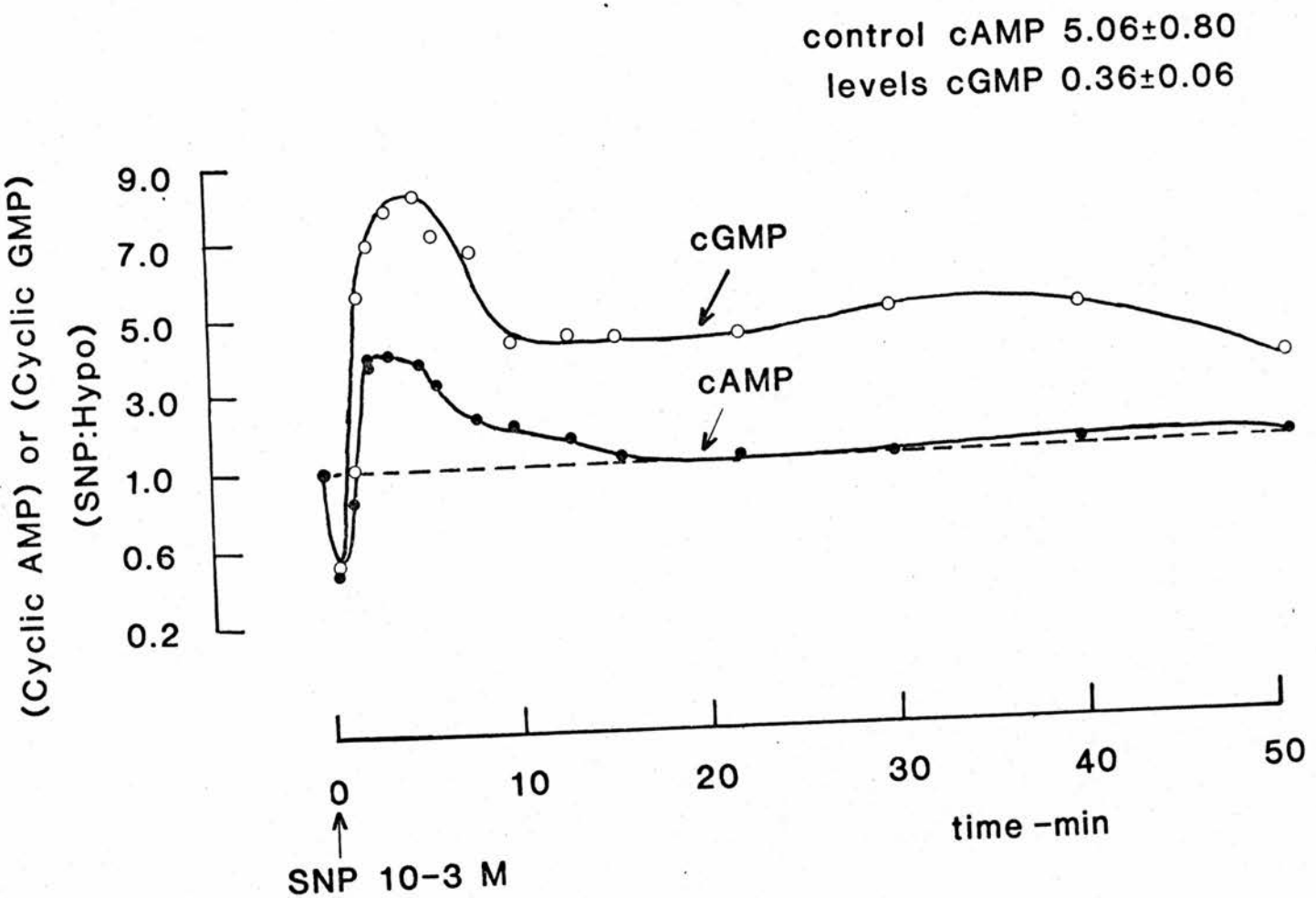

control cAMP 5.06±0.80
levels cGMP 0.36±0.06
(Cyclic AMP) or (Cyclic GMP)
(SNP:Hypo)
9.0
7.0
5.0
3.0
1.0
0.6
0.2
cGMP
cAMP
0
10
20
30
40
50
time -min
SNP 10-3 M

Table 3.III.13

Effect of 10^{-3}M SNP on endogenous cyclic nucleotide levels and ventricular contractility

The table shows the changes in absolute and relative amounts of cyclic AMP and cyclic GMP following exposure of the half ventricles to 10^{-3}M SNP.

Attention is drawn to columns 8 and 9 showing the parallel decline in R and P_R.

$$R = \frac{\text{cAMP/cGMP (test half ventricle)}}{\text{cAMP/cGMP (control half ventricle)}}$$

$$P_R = \frac{\text{P test (twitch amplitude of test half ventricle)}}{\text{P control (twitch amplitude of control half ventricle)}}$$

Control cAMP (mean 5.06 ± 0.80), cGMP (mean 0.36 ± 0.06).

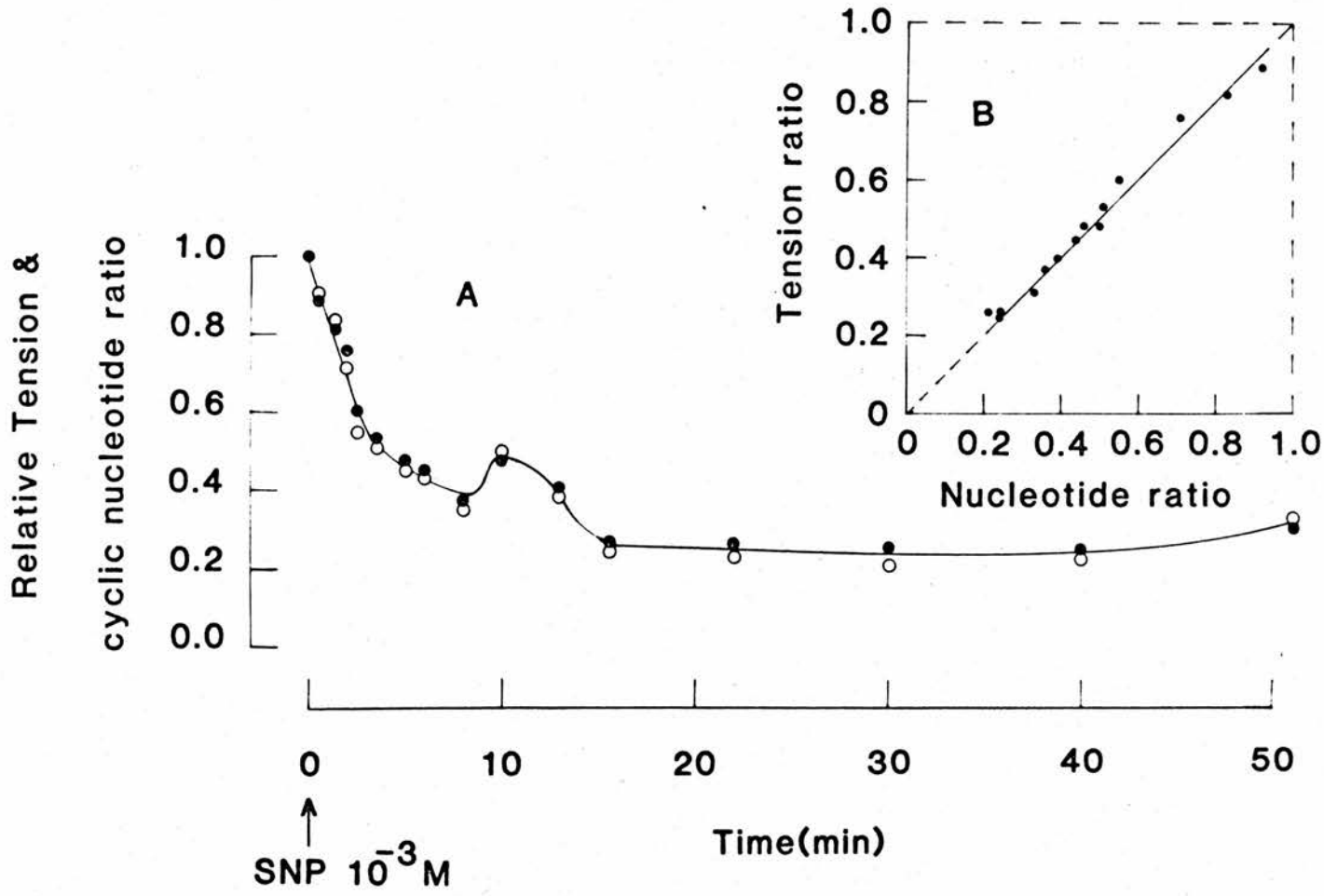
Time (min)	cAMP			cGMP			R	P_R
	SNP P mol. mg^{-1} Protein	Cont. Protein	SNP/Cont	SNP P mol mg^{-1} Protein	Cont. Protein	SNP/Cont.		
0.5	5.20	11.68	0.45	0.20	0.41	0.49	0.92	0.89
1.4	3.90	4.68	0.83	0.37	0.37	1.00	0.83	0.82
2.0	16.70	4.28	3.90	1.10	0.20	5.50	0.71	0.76
2.5	18.51	4.95	3.74	3.36	0.49	6.86	0.55	0.60
3.5	16.67	4.20	3.97	2.10	0.27	7.78	0.51	0.53
5.0	4.50	1.20	3.75	1.13	0.14	8.07	0.46	0.48
6.0	4.48	1.43	3.13	3.04	0.43	7.07	0.44	0.45
8.1	6.90	2.92	2.36	3.65	0.55	6.64	0.36	0.37
10.0	6.18	3.00	2.06	0.71	0.17	4.16	0.50	0.48
13.0	4.55	2.69	1.69	0.92	0.21	4.38	0.39	0.40
15.5	4.06	3.78	1.07	2.44	0.57	4.28	0.25	0.27
22.0	3.23	3.20	1.01	0.76	0.18	4.22	0.24	0.27
30.0	6.86	6.66	1.03	0.78	0.16	4.88	0.21	0.26
40.0	7.03	6.30	1.12	0.90	0.19	4.74	0.24	0.25
51.0	16.00	15.06	1.06	3.21	1.00	3.21	0.33	0.31

Fig. 3.III.14

Relationship between changes in isometric twitch tension (ΔP) and cyclic nucletoide ratio cAMP/cGMP (P_R)

A) Shows the decline in relative tension (closed squares), and the corresponding decline in the nucleotide ratio (open squares).

B) Shows the correlation between the reduction in ventricular contractility and the cyclic nucleotide ratio.
All cyclic nucleotide levels and twitch tensions are expressed as a fraction of the control.


Relative Tension &
cyclic nucleotide ratio
1.0
0.8
0.6
0.4
0.2
0.0
A
0
10
20
30
40
50
Time(min)
SNP 10^{-3}M
Tension ratio
B
Nucleotide ratio

paralleled by a corresponding reduction in the relative proportion of cyclic AMP to cyclic GMP present in the fibres, (open squares).

i.e.) $\Delta P_R \propto \Delta R$

where, $\Delta P_R = \frac{\text{tension decrease in SNP treated ventricle}}{\text{tension decrease in control ventricle}}$

and, $\Delta R = \frac{\text{cyclic AMP/cyclic GMP in SNP treated ventricle}}{\text{cyclic AMP/cyclic GMP in control ventricle}}$

This relationship is more clearly shown in Fig. 3.III.14.B, where ΔP is plotted as a function of ΔR.

This correlation is also seen during responses evoked by a variety of other agents (including ATP, UTP, isoprenaline, adenosine, dibutyryl cyclic AMP, 8-bromo-cyclic GMP, adenaline and acetylcholine [39-49 & 110-112], and it provides further evidence in support of the hypothesis that the contractile performance of the heart is regulated by cyclic AMP and cyclic GMP acting in an antagonistic fashion. The possible nature of the cyclic AMP:cyclic GMP antagonism is discussed in more detail later.

In addition, the transient increase of tension seen between 8-12 minutes Fig. 3.III.14.A, corresponds closely with fall in cyclic GMP from its peak value of 8X to 4X control levels. It should be emphasised that this increase in contractility is not uncommon feature; it was almost always present as can be seen from Fig. 3.III.2. & Fig. 3.III.4, which show accumulated results from 10 experiments.

CHAPTER IV

DISCUSSION

The results summarised in this thesis show :

(1) that the decline in twitch tension during the development of the hypodynamic state is accompanied by a reduction in action potential duration.

(2) TFP and SNP have a negative inotropic effect on the ventricle. Initially both TFP and SNP tend to stabilize the action potential at a time when the twitch is declining. The initial 'stabilization' period is followed by a parallel decline in both the twitch amplitude and action potential duration (APD).

(3) The response to SNP is accompanied by changes in the levels of both cyclic nucleotides. The above observations are discussed in more detail below.

1. Relationship between the decline in twitch tension and A.P.D. during the development of the hypodynamic condition

The method of vertical perfusion was not suitable for making micro-electrode recordings of the action potential. It was necessary to perfuse small strips of ventricle at a lower flow rate and in the horizontal direction in order to secure long-lasting impalements. Such preparations took on average 3X longer to reach a stable (hypodynamic) condition. This observation confirms previous results obtained in this laboratory, that flow rate is one of many factors which influence the time course of the decline in twitch tension.

It was found that the sensitivity of vertically perfused preparations to both SNP and TFP was significantly greater than that of horizontally perfused ones: the rate at which the twitch became depressed and the degree of depression were substantially less with horizontally as compared to vertically perfused hearts. The reason for this difference is not known.

Microelectrode recordings showed a gradual reduction in the duration of the action potential (APD) which paralleled the decline in peak twitch tension. The relationship was approximately linear (Fig. 3.I.3.B). It has been known for some years that there is a progressive decrease in intracellular calcium [9; 78], and a diminished rate of uptake of calcium [13; 14], into the fibres during the development of the hypodynamic state. Both observations can be explained by a reduction in the amount of calcium entering the fibres during membrane excitation; this would tend to abbreviate the action potential.

2. Inotropic responses to TFP & SNP

Both TFP and SNP depress the twitch in a dose dependent manner, with ED_{50} values of 1.2×10^{-6} M ($T_{\frac{1}{2}}$ = 7 mins) and 2×10^{-5} M ($T_{\frac{1}{2}}$ = 9 mins) respectively. 2×10^{-5} M TFP totally abolished the twitch, and this was not reversible, even after prolonged washing with Ringer solution. In contrast, SNP at 10^{-2}M depressed the twitch to 18% of its control level and this effect was almost fully reversible. The negative inotropic effect of SNP is particularly interesting, since it has been reported that the twitch amplitude in the cat atria was not depressed when superfused with 10^{-4}M SNP; in fact, a small increase was recorded [26]. This difference in response can only be attributed to species and tissue differences.

The responses to both TFP and SNP were unaffected by atropine, propranolol and phentolamine, which demonstrates the absence of any cholinergic or adrenergic receptor involvement in the actions of either of these agents. Furthermore, theophylline antagonised the depressant effects of SNP, but had no effect on the TFP response, (Fig. 3.II.5.). The antagonistic effect of theophylline on the SNP response may be due to its inhibitory effect on cAMP and cGMP phosphodiesterase (see later page 45).

3. Effects of TFP and SNP on electrical activity

Effects on Ca^{++}-influx mechanism. Inward Ca^{++} current during the plateau of the cardiac action potential is the major route of trans-sarcolemmal influx of Ca^{++} for the beat to beat Ca^{++} regulation [103]. In addition to this Ca^{++} current, a carrier mediated Na^{+}:Ca^{++} exchange mechanism contributes to the beat to beat contraction [76; 2]. Experimentally, it has been established that the carrier exchange mechanism involves two Na^{+} for one Ca^{++} [36]. Anderson, Hirsch & Kavaler [2] provide evidence that the contractile activation of the frog ventricle is brought about by a trans-sarcolemmal net influx of calcium, which is enhanced by increased extracellular [Ca^{++}], by decreased extracellular [Na^{+}] or by hyperpolarising the membrane.

In so far as TFP is concerned, previous authors [79] have demonstrated that calmodulin possesses a high affinity TFP binding site which is Ca^{++} dependent [79; 96]. SNP on the other hand, does not require Ca^{++} in the extracellular medium in order to stimulate production of cGMP [90]. Although no experiments were carried out in the absence of extracellular Ca^{++} during these experiments, with either of the above agents, the observations on the changes in action potential induced by TFP & SNP are interesting. On application of either agent to the superfusate there was an immediate decrease in contractility, but reduction in action potential was not observed until the twitch had decreased to 85% (during the first 10 minutes after TFP) and 75% (during the first 2 minutes after SNP) of their respective control levels. After this initial phase however, there appeared a correlation between the decline in contractility and reduction in the action potential duration. This indicates that both TFP and SNP have an initial stabilizing effect on the action potential.

4. Relationship between changes in cyclic nucleotide levels and twitch tension

One of the aims of the present study was to establish whether there exists a relationship between changes in the ratio of the two cyclic nucleotides (cAMP:cGMP) and the observed reduction in ventricular contractility. Several agents studied by Flitney & Singh [39-49 & 110-112] have produced inotropic responses which are directly correlated to changes in the ratio of cAMP:cGMP. These observations indicate that either both cyclic nucleotides are important in regulating ventricular contractility, or that changes in intracellular cyclic nucleotide levels are only incidental to the effect on the twitch.

In the light of the evidence presented here on the effect of SNP an intracellular cyclic nucleotide levels and those of the agents studied previously [39-49 & 110-112], it is difficult to dismiss the theory that both cyclic nucleotides are in some way involved in regulation of myocardial contractility. For such a relationship to be true, the regulatory effects of cAMP and cGMP must be antagonistic, the former increasing and the latter reducing contractility.

Evidence in support of this concept has been presented by a number of authors. Several β-agonists increase the level of cAMP [122], while responses to acetylcholine are accompanied by an increase in cGMP [51; 58]. On the other hand, a number of cardioactive agents, while causing an increase in contractility, appear to have no effect on cAMP levels [122]. Similarly, some recent studies have shown a 'dissociation' between the levels of cGMP and contractility evoked by other negative inotropic agents [26; 10]. Brooker [10], for example observed that 100X more carbachol was required to change cGMP levels than was needed to bring about a 90% depression of the twitch. Diamond et al [26] observed

17X increase in cGMP levels with 10^{-4}M SNP without a decline in contractility (these measurements were made at 15 and 60 seconds after the application of SNP). These observations of Diamond et al [26] are interesting. During the course of these studies, SNP, even at very low concentrations, always produced a negative inotropic response.

The weakness in the so called 'dissociation' experiments is that in some instances only one (and not both) cyclic nucleotide was measured; and in addition in those experiments where both cAMP and cGMP were monitored, the measurements were made at only one or a few time points during the response. The design of such experiments is defective for two reasons. First, if the action of cyclic AMP and cyclic GMP are important, then their relative proportions will be the major determinant factor in regulating contractility. Secondly, the time course of changes in cyclic nucleotide levels is often complex, as can be seen in Figs. 3.III.13. & 14. and in the studies of Flitney and Singh [39-49 & 110-112]. Interesting changes might therefore be missed if only a few time points are selected for study.

Antagonistic effects of cAMP and cGMP

So far the evidence presented supports the idea that cAMP and cGMP are in some way involved in regulating myocardial contractility.

Action of cAMP on intracellular proteins. It is well established that in most cases cAMP's actions are mediated via phosphorylating enzymes called 3',5'-cyclic AMP-dependent protein kinase. Several substrates for the enzyme have been shown to be important in the regulation of cardiac contractility. These include i) Troponin I (TnI) which is a subunit of the regulatory protein complex [22; 33]; ii) phosphalamban, a constituent of the S.R. [68; 118]; and iii) a surface membrane bound

protein (SMBP), which is thought to be a structural component of the slow inward Ca^{++} current channel [134].

Phosphalamban and SMBP regulate the distribution of $[Ca^{++}]$, between the S.R and myoplasm, and between the interior of the fibre and the extracellular fluid, and as such they could influence contractility by controlling the Ca^{++} pool. TnI has an inhibitory effect upon the interaction of actin and myosin, an effect which is suppressed when TnC interacts with Ca^{++} upon stimulation. Recent studies have suggested that it may also have an important part to play in regulating the Ca^{++} sensitivity of the contractile apparatus. Rubio et al [106] found that phosphorylation of TnI in guinea-pig cardiac muscle increased sensitivity of actomyosin ATP-ase to Ca^{++} while Ray & England [101] and England [34] found the reverse to be true in bovine and rat heart. More recent studies by Mope, McClellan & Winegrad [88] substantiates the latter finding. They show that Ca^{++} sensitivity of cardiac myofibrils is inversely related to the degree of phosphorylation of TnI, and that the latter is stimulated by cAMP and inhibited by cGMP. It is also known that increase myocardial contractility is associated with increased phosphorylation of TnI in vivo [34; 113].

Possible action of cGMP on protein phosphatases. It seems likely that phosphorylation of TnI is controlled by the amount of cAMP and cGMP within the myocardium. This is based on two series of studies. First, the relationship between cAMP and cGMP observed in the study of the SNP and in the previous studies [39-49 & 110-112]. Secondly, the variation between myocardial contractility and parallel changes in incorporation of ^{32}P into TnI [33; 34; 113]. If these observations are correct, then a possible site of action of cGMP can be suggested. The antagonism implied by the relationship between the ratio of the cyclic nucleotides

and contractility suggests that cGMP may promote de-phosphorylation of regulatory proteins, by stimulating protein phosphatase activity.

Primary site of action of TFP & SNP on ventricular myocardium

Action of TFP on calmodulin. The current view concerning the mode of action of TFP is that it interferes with the cyclic nucleotide system [79], by initiating calmodulin (calcium dependent regulatory protein). It is believed that in cardiac muscle, calmodulin is involved in Ca^{++}-dpendent activation of cyclic nucleotide phosphodiesterase and of adenylate cyclase. Cardiac myosin light chain kinase is also Ca^{++}-calmodulin dependent [124]. In addition calmodulin is involved in the control of the phosphorylation of the S.R-Ca^{++}pump activator (phosphalamban) thereby modulating the rate of Ca^{++} uptake [124; 79].

Thus, although no experiments were carried out to investigate the possible effects of TFP on intracellular cAMP and cGMP, it is possible that the depressant effect of TFP is due to a decrease in the ratio of cAMP:cGMP. This would involve inhibition of adenylate cyclase as well as both cAMP and cGMP phosphodiesterases [17], with a greater inhibitory effect on cGMP-phosphodiesterase [130]. The above hypothesis is interesting in the light of the observations made in the presence of theophylline, where TFP effect was unaltered initially. This is possibly due to the fact that TFP inactivates the phosphodiesterase activator protein, and since its binding is irreversible there can be no further inhibition of the phosphodiesterase by theophylline. However, to confirm the above statements, and to provide a possible explanation for the lack of alternate beats, which appeared 15 minutes after addition of TFP in the presence of theophylline, one must continue this line of investigation.

Action of SNP on guanylate cyclase. It is well established that SNP stimulates guanylate cyclase, resulting in increased cGMP production [90]. This activation of the guanylate cyclase is achieved by modification of the cationic requirement of the enzyme. At the same time, there is biochemical evidence that guanylate cyclase can catalyze the formation of cAMP from ATP following activation by SNP [90]. This observation further substantiates the results of this study that the intracellular levels of cAMP are also affected by SNP. Indeed it was observed that the rate of increase of cAMP (in absolute terms) was 7X greater than that of cGMP following exposure to SNP. This observation reinforces the need to make measurements of both cAMP and cGMP during exposure of the myocardium to any cardioactive agent. Furthermore, theophylline antagonised the depressant effects of SNP. This is presumably the result of the inhibitory effect of theophylline on cAMP & cGMP phosphodiesterases. These results are similar to those obtained by Flitney & Singh on the effect of theophylline upon application of 8-bromo-cyclic GMP [47]. The observation is consistent with the idea that cGMP suppresses cAMP levels by stimulating cAMP-phosphodiesterase activity, as postulated recently by the above authors [47]. Thus although theophylline is an inhibitor of both cAMP and cGMP-phosphodiesterases, it's inhibitory effect upon cAMP-phosphodiesterases may be offset by activation of cAMP-phosphodiesterase due to an increased cGMP level. Thus, overall there will be smaller decrease in the ratio of cAMP:cGMP and consequently a smaller decrease in force. However, in order to substantiate the above hypothesis, it is necessary that experiments be carried out in which the intracellular levels of cyclic nucleotides are also measured. So far, this has not been done.

Conclusion & scope for future studies

The experiments described in this thesis provide evidence to substantiate the already established direct correlation between changes in the ratio of the cyclic nucleotides and that of contractility.

As it was pointed out earlier (p. 15), these observations do not rule out the possibility that the correlation between changes in twitch amplitude (ΔP) and the ratio of cAMP:cGMP (ΔR) could both arise as the result of a common cause, namely involvement of Ca^{++} in both regualting contractility and the metabolism of intracellular cyclic nucleotides via calmodulin. Indeed this point reinforces the need to establish a definitive role for intracellular activities of calmodulin by use of such compounds as TFP. Nevertheless in the case of SNP a general increase in cGMP and a short lasting increase in cAMP was observed. It is the ratio of the two cyclic nucleotides which most closely parallels the change in contractile activity of the ventricle.

There is an important consequence of this conclusion. The control levels of cAMP and cGMP were 5.06 ± 0.8 p.mol.mg^{-1} protein, and 0.36 ± 0.006 p.mol.mg^{-1} protein respectively, which gives a ratio of cAMP:cGMP of 14:1. Thus, small alterations to the absolute levels of cGMP can lead to a large change in the ratio, whereas much larger variations in the absolute levels of cAMP are required in order to bring about the same change. It follows that substances which directly or indirectly influence cGMP metabolism may turn out to be particularly effective agents in the treatment of cardiac disorders. The present work forms a framework for the study of TFP and SNP as cardioactive agents. However, further studies are required to establish the possible role of cyclic nucleotides (in particular that of cGMP) in mediating the effects of TFP & SNP on the heart.

CHAPTER V

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CHAPTER VI

APPENDIX & TABLES

Fig. 3.I.2

Comparison of the development of hypodynamic state in Horizontally and Vertically perfused ventricles

Horizontally perfused			Vertically perfused		
Time (min)		relative tension	Time (min)		relative tension
Pmax	0	1.00	Pmax	0	1.00
	2.5	0.98 ± 0.01		1	0.96 ± 0.01
	5	0.96 ± 0.01		2	0.93 ± 0.01
	7.5	0.94 ± 0.01		3	0.90 ± 0.01
	10	0.93 ± 0.01		4	0.88 ± 0.01
	15	0.90 ± 0.01		5	0.84 ± 0.01
	20	0.88 ± 0.01		7.5	0.80 ± 0.02
	25	0.85 ± 0.01		10	0.75 ± 0.03
	30	0.82 ± 0.02		12.5	0.71 ± 0.03
	40	0.78 ± 0.02		15	0.67 ± 0.03
	50	0.74 ± 0.02		20	0.61 ± 0.03
	60	0.70 ± 0.02		25	0 .57 ± 0.03
	70	0.67 ± 0.02		30	0.55 ± 0.03
	80	0.64 ± 0.02		35	0.53 ± 0.02
	100	0.59 ± 0.02		40	0.51 ± 0.02
	120	0.54 ± 0.02		45	0.49 ± 0.02
	140	0.50 ± 0.02			

Fig. 3.I.3.A.

Decline in isometric twitch tension and the corresponding reduction in D-30mV action potential duration

Time (min)	relative tension	relative A.P.D.$_{-30mV}$
0	1.00	1.00
2.5	0.98 ± 0.01	0.99 ± 0.01
5	0.96 ± 0.01	0.97 ± 0.01
7.5	0.94 ± 0.01	0.95 ± 0.02
10	0.93 ± 0.01	0.94 ± 0.01
15	0.90 ± 0.01	0.93 ± 0.01
20	0.88 ± 0.01	0.92 ± 0.02
25	0.85 ± 0.01	0.91 ± 0.02
30	0.82 ± 0.02	0.89 ± 0.02
40	0.78 ± 0.02	0.88 ± 0.01
50	0.74 ± 0.02	0.83 ± 0.02
60	0.70 ± 0.02	0.81 ± 0.01
70	0.67 ± 0.02	0.81 ± 0.01
80	0.64 ± 0.02	0.80 ± 0.02
100	0.59 ± 0.02	0.78 ± 0.02
120	0.54 ± 0.02	0.76 ± 0.02
140	0.50 ± 0.02	0.75 ± 0.01

Initial A.P.D. = 730 ± 30 m sec
Initial tension = 960 ± 100 mg

N.B.: The amount of force produced depends largely on the size of tissue strip used.

Fig. 3.I.3.B

Comparison of the relative decline in isometric twitch tension and the corresponding decline in A.P.D.

$$(1 - \frac{x}{\text{maximum}}) \times 100\%$$

Tension	D-30mV A.P.D.
0 ± 0	0 ± 0
2 ± 1	1 ± 1
4 ± 1	3 ± 1
6 ± 1	5 ± 2
7 ± 1	6 ± 1
10 ± 1	7 ± 1
12 ± 1	8 ± 2
15 ± 1	9 ± 2
18 ± 2	11 ± 2
22 ± 2	12 ± 1
26 ± 2	17 ± 2
30 ± 2	19 ± 1
33 ± 2	19 ± 1
36 ± 2	20 ± 2
41 ± 2	22 ± 2
46 ± 2	24 ± 2
50 ± 2	25 ± 1

Fig. 3.II.1.

Effect of 5×10^{-5}M TFP on isometric twitch tension during vertical perfusion

Prior to TFP		After application of TFP		
Time (min)	relative tension	Time (min)	Relative tension 5×10^{-5}M	10^{-6}M
Pmax	1.00	45.00	0.49 ± 0.02	0.49 ± 0.02
1	0.96 ± 0.01	45.17	0.39 ± 0.03	
2	0.93 ± 0.01	45.33	0.26 ± 0.06	
3	0.90 ± 0.01	45.50	0.19 ± 0.06	
4	0.88 ± 0.01	45.67	0.11 ± 0.05	
5	0.84 ± 0.01	45.83	0.07 ± 0.04	
7.5	0.80 ± 0.02	46.00	0.05 ± 0.03	0.47 ± 0.02
10	0.75 ± 0.03	47.50	0.02 ± 0.01	0.44 ± 0.03
12.5	0.71 ± 0.03	50.00	0.01 ± 0.01	0.38 ± 0.03
15	0.67 ± 0.03	52.50	0.005	0.34 ± 0.02
20	0.61 ± 0.03	55.00	0.00	0.32 ± 0.02
25	0.57 ± 0.03	60.00		0.30 ± 0.03
30	0.55 ± 0.03	65.00		0.29 ± 0.03
35	0.53 ± 0.02	70.00		0.27 ± 0.02
40	0.51 ± 0.02	75.00		0.26 ± 0.02
45	0.49 ± 0.02	80.00		0.26 ± 0.01

Fig. 3.II.2

Decline in isometric twitch tension 30 minutes after application of TFP during vertical perfusion

TFP concn. (M)	Tension: (Fraction of the initial value)									mean ± S.E
10^{-8}	0.94	0.93	0.98							0.95 ± 0.01
2×10^{-8}	1.00									1.0 ± 0.0
4×10^{-8}	0.96	1.00								0.98 ± 0.01
10^{-7}	0.88	0.89	0.78	1.00						0.89 ± 0.03
2×10^{-7}	0.65	0.75	0.93	0.77	0.58					0.74 ± 0.04
4×10^{-7}	0.76	0.74	0.95	0.74	0.69					0.77 ± 0.03
5×10^{-7}	0.89									0.89 ± 0.0
8×10^{-7}	0.69	0.70	0.68	0.62	0.56					0.65 ± 0.02
10^{-6}	0.52	0.65	0.64	0.41	0.36	0.80	0.63	0.72	0.50	0.58 ± 0.03
1.5×10^{-6}	0.55	0.55								0.55 ± 0.0
2×10^{-6}	0.14	0.50	0.50	0.48	0.38	0.38	0.22	0.64		0.41 ± 0.05
3×10^{-6}	0.19	0.30	0.30							0.26 ± 0.03
4×10^{-6}	0.29	0.18	0.23	0.28						0.26 ± 0.04
8×10^{-6}	0.14	0.18								0.16 ± 0.02
10^{-5}	0.10	0.21	0.11	0.12	0.18					0.14 ± 0.04
2×10^{-5}	0	0	0							0.0 ± 0.0
5×10^{-5}	0	0	0	0						0.0 ± 0.0
10^{-4}	0									0.0 ± 0.0

Fig. 3.II.2

Decline in isometric twitch tension 30 minutes after application of TFP during later al perfusion

TFP concn. (M)	Tension: (Fraction of the initial value)									
8×10^{-7}	0.99	1.00								
10^{-6}	0.97	0.93	0.98	0.91						
4×10^{-6}	0.88	0.85	0.93	0.90						
10^{-5}	0.88	0.76	0.77	0.80	0.84	0.75	0.85	0.78		
5×10^{-5}	0.53	0.55	0.50	0.50	0.52	0.50	0.38	0.52	0.57	0.55
10^{-4}	0.17	0.25	0.32							
5×10^{-4}	0	0								

Fig. 3.II.3

Half maximum response time for different concentrations of TFP superfused vertically

Concn. of TFP (M)	Time taken to reach 50% of max response $T_{\frac{1}{2}}$
10^{-8}	11.0 ± 0.25
10^{-7}	10.0 ± 0.5
8×10^{-7}	9.0 ± 1.0
10^{-6}	7.5 ± 0.25
1.5×10^{-6}	6.5 ± 0.5
2×10^{-6}	5.0 ± 0.25
3×10^{-6}	3.5 ± 0.5
4×10^{-6}	3.0 ± 0.5
10^{-5}	0.75 ± 0.5
5×10^{-5}	0.33 ± 0.25

Fig. 3.II.4

Comparison of the effect of the TFP on twitch tension and that of TFP + Propranolol, Phentolamine and Atropine

Time after TFP (min)	Tension: (Fraction of Hypodynamic) TFP 5×10^{-6}M alone	5×10^{-6}M TFP Propranolol 10^{-7}M Phentolamine 10^{-6}M Atropine 10^{-6}M
0	1.00	1.00
1.0	0.84	0.80
1.5	-	0.67
2.5	0.49	0.43
5	0.29	0.27
10	-	0.20
15	0.16	0.18
20	0.14	0.16
25	0.14	0.16
30	0.14	0.12

Fig. 3.II.6

Effects of Theophylline on TFP (10^{-6}M) response

Time (min)	Test 10^{-4} Theo + 10^{-6} TFP	Control 10^{-6} TFP
0	100 ± 0	100 ± 0
1	74 ± 8	71 ± 6
2	64 ± 9	62 ± 8
3	59 ± 7	57 ± 7
4	54 ± 7	54 ± 7
5	52 ± 7	52 ± 9
7½	49 ± 8	49 ± 8
10	47 ± 8	48 ± 8
15	47 ± 8	48 ± 8

Fig. 3.II.8

Effect of 5×10^{-5} M TFP on isometric twitch tension and the corresponding changes in D-30mV action potential duration

Time (min)	relative tension	relative A.P.D.
0	1.00	1.00
2	0.997	1.00
4	0.988	1.00
6	0.93 ± 0.02	1.00
8	0.88 ± 0.03	1.00
10	0.86 ± 0.02	1.00
12	0.80 ± 0.02	0.988 ± 0.01
14	0.79 ± 0.03	0.970 ± 0.01
16	0.76 ± 0.04	0.966 ± 0.01
18	0.74 ± 0.02	0.960 ± 0.01
20	0.72 ± 0.04	0.956 ± 0.01
26	0.70 ± 0.05	0.946 ± 0.03
28	0.67 ± 0.08	0.927 ± 0.02
30	0.63 ± 0.11	0.920 ± 0.02
32	0.59 ± 0.06	0.915 ± 0.02
46	0.52 ± 0.02	0.890 ± 0.02
58	0.48 ± 0.01	0.870 ± 0.02

Fig. 3.II.9

Relationship between twitch tension and the action potential duration (D-30mV)

% Decrease Tension	D-30mV A.P.D.
0 ± 0	0 ± 0
0.3 ± 0	0 ± 0
1.2 ± 0	0 ± 0
7 ± 2	0 ± 0
12 ± 3	0 ± 0
14 ± 2	0 ± 0
20 ± 2	1.2 ± 1
21 ± 3	3 ± 1
24 ± 4	3.4 ± 1
26 ± 2	4 ± 1
28 ± 4	4.4 ± 1
30 ± 5	5.4 ± 3
33 ± 8	7.3 ± 2
37 ± 11	8 ± 2
41 ± 6	8.5 ± 2
48 ± 2	11 ± 2
52 ± 1	13 ± 2

Fig. 3.III.5

Decline in isometric twitch tension 25 minutes after application of SNP

SNP (M)	Relative Tension mean ± S.E
10^{-7}	0.91 ± 0.02
10^{-6}	0.82 ± 0.02
10^{-5}	0.68 ± 0.03
5×10^{-5}	0.64 ± 0.03
10^{-4}	0.40 ± 0.03
10^{-3}	0.30 ± 0.03
*10^{-2}	0.22 ± 0.03

Results are expressed as mean ± S.E, (N) number of samples = 8 (except 5×10^{-5}M, where N = 6).

* $[Na^+]$ adjusted to keep at 118mM (with choline chloride).

Fig. 3.III.6

Half maximum response time for different concentrations of SNP superfused vertically

Concn. of SNP (M)	$T_{\frac{1}{2}}$
10^{-7}	11 ± 0.5
10^{-6}	11.25 ± 1.0
10^{-5}	10 ± 0.5
5×10^{-5}	9 ± 1.0
10^{-4}	4.5 ± 1.0
10^{-3}	2.5 ± 0.5
10^{-2}	0.5 ± 0.25

Fig. 3.III.7

Comparison of the effect of SNP on twitch tension and that of SNP, propranolol, phentolamine and atropine

Time (min)	Relative-Tension	
	SNP alone	SNP, propran, phentol & atrop.
0.0	1.00	1.00
0.5	0.98 ± 0.01	0.95 ± 0.02
1.0	0.95 ± 0.02	0.93 ± 0.02
2.0	0.84 ± 0.03	0.81 ± 0.01
5.0	0.69 ± 0.04	0.67 ± 0.01
10.0	0.51 ± 0.08	0.51 ± 0.02
15.0	0.48 ± 0.06	0.47 ± 0.02
20.0	0.45 ± 0.07	0.45 ± 0.03
25.0	0.43 ± 0.07	0.43 ± 0.03

Concentration of drugs used are as follows.

SNP	10^{-4}M
Propranolol	10^{-7}M
Phentolamine	10^{-6}M
Atropine	10^{-6}M

Fig. 3.III.8

Effects of Theophylline on SNP (10^{-3}M) response

Time (min) after addn. of SNP	Tension % of Hypodynamic state		
	SNP alone	SNP + 10^{-4} Theoph	
0	100 ± 0	100 ± 0	
0.5	95 ± 1	97 ± 1	
1.0	89 ± 2	95 ± 1	*
1.5	84 ± 3.2	94 ± 1.5	*
2.0	77 ± 4	92 ± 2	**
3.0	68 ± 4	89 ± 2.5	***
4.0	61 ± 3.7	86 ± 2.8	***
5.0	56 ± 3.3	83 ± 2.6	***
6.0	53 ± 3.3	82 ± 3	***
8.0	49 ± 3.5	79 ± 2.7	***
10	48 ± 3.5	77 ± 2.5	***
12	46 ± 3.6	75 ± 2.4	***
15	43 ± 3	73 ± 2.5	***
20	41 ± 3	71 ± 2.4	***
25	38 ± 3	70 ± 2.6	***
30	36 ± 3.3	69 ± 2.7	***
40	35 ± 3.3	67 ± 3	***
50	33 ± 3.3	65 ± 3.3	***

Results are expressed as mean ± SE, (N) number of samples = 8

* Significantly different from the corresponding SNP alone value ($P < 0.05$)
** Significantly different from the corresponding SNP alone value ($P < 0.01$)
*** Significantly different from the corresponding SNP alone value ($P < 0.001$)

Fig. 3.III.9

1st & 2nd application of 5×10^{-3}M SNP to a strip of ventricular tissue

Time (min)	Tension % age of Hypodynamic state		
	1st application	Recovery	2nd application
0	100	47	94
0.1	91	51	85
0.2	84	56	80
0.3	76	59	76
0.5	72	62	72
1.0	67	75	69
1.5	66	77	65
2.0	65	80	63
3.0	61	85	57
4.0	58	87	55
5.0	57	91	55
6.0	56	93	55
7.0	56	94	55
8.0	54	94	55
9.0	51	94	
12.0	49		
14.0	47		
21.0	47		

Fig. 3.III.10

Effect of 5 x 10^{-3}M SNP on isometric twitch tension and the corresponding changes in D-30mV A.P.D.

$Time_{(min)}$	Relative Tension	Relative A.P.D.
0.25	0.90 ± 0.01	1.00
0.5	0.85 ± 0.02	1.00
1.0	0.82 ± 0.02	1.00
1.5	0.76 ± 0.02	1.00
2.0	0.74 ± 0.03	1.00
3.0	0.69 ± 0.04	0.92 ± 0.01
4.0	0.70 ± 0.06	0.87 ± 0.02
5.0	0.67 ± 0.03	0.86 ± 0.03
8.0	0.63 ± 0.05	0.85 ± 0.02
10.0	0.55 ± 0.02	0.84 ± 0.02
13.0	0.48 ± 0.05	0.82 ± 0.02
17.0	0.52 ± 0.03	0.83 ± 0.03
20.0	0.50 ± 0.01	0.75 ± 0.05

Appendix I

Effect of 5×10^{-3} M SNP on twitch amplitude and the action potential duration of a ventricular strip

Time (min)	% decrease in Twitch amplitude	% decrease in D-30mv duration	
0.1	15	0	1st application
0.1	17	0	
0.1	15	0	
0.1	15	0	
0.2	17	0	
0.5	23	0	
1.0	23	0	
1.5	23	0	
1.5	25	0	
1.5	23	0	
1.5	25	0	
2.0	27	2.6	
2.5	29	7.7	
3 .0	33	10	
4.0	38	10	
4.5	40	15	
5.5	40	18	
8.0	44	31	
12.5	52	18	
17.5	48	13	
18.5	50	31	
19.5	50	28	
1.0	17	0	2nd application
2.0	21	0	
3.5	28	6	
5.5	30	9	
6.0	30	11	
9.0	30	17	

Correlation coefficient for values other than those of (x = 0) is = 0.76.

Slope of regression line = 0.78

Intercept on ordinate = 26.14

Appendix II

Effect of 5×10^{-3} M SNP on twitch amplitude and the action potential duration of a ventricular strip

Time (min)	% decrease in Twitch amplitude	% decrease in D-30 mv duration	
2.0	17	7	1st application
4.0	21	5	
5.0	21	5	
9.0	19	0	
1.5	25	14	2nd
2.0	30	16	
4.0	30	18	
13.0	40	26	
9.0	36	20	3rd

Correlation coefficient = 0.95

Slope of regression line = 0.99

Intercept on ordinate = 13.77

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Effects of sodium nitroprusside on isolated frog ventricle

By F. W. Flitney, M. Moshiri and J. Singh. *Department of Physiology, University of St Andrews, Fife, Scotland*

Sodium nitroprusside (SNP) is a potent stimulator of guanylate cyclase activity *in vitro*, and in a variety of intact cell systems (Murad, Arnold, Mittal & Braughler, 1979). Its effects on the frog ventricle have therefore been studied, to investigate further the nature of the involvement of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) in regulating myocardial contractility. Isolated ventricles were superfused with Ringer solution containing SNP (10^{-7} to 10^{-3} M) and its effects on the isometric twitch and on endogenous cyclic nucleotide levels were measured. The results obtained show: (1) SNP depresses the twitch, in a dose dependent and reversible manner. Second (or subsequent) applications depress contractility more rapidly and to a greater extent than first applications. (2) SNP (10^{-3} M) stimulates the metabolism of adenosine 3′5′ cyclic monophosphate (cyclic AMP) and cyclic GMP. The levels of both decrease initially (first 9 sec), and later increase, cyclic AMP rising to 4× and cyclic GMP to 8× control levels. Cyclic AMP returns to normal after 17 min, but cyclic GMP remains elevated (at 4–5× its control value) for up to 52 min. (3) The degree of depression of the twitch is paralleled by equivalent reductions in the ratio cyclic AMP: cyclic GMP.

These results are consistent with the idea that *both* cyclic nucleotides are involved in regulating contraction, and that they function antagonistically, cyclic AMP potentiating the twitch and cyclic GMP depressing it. Evidence that cyclic GMP may exert its antagonistic effect by stimulating cyclic AMP phosphodiesterase activity, thereby depressing intracellular cyclic AMP levels, was presented in an earlier communication (Flitney & Singh, 1980). The role of cyclic GMP in *mammalian* heart is less clear. Early experiments by George, Polson, O'Toole & Goldberg (1970) led to the suggestion that it functions as a 'second messenger', mediating the negative inotropic responses induced by cholinergic agonists, but more recent studies (Diamond, Ten Eick & Trapani, 1977; Brooker, 1977) have raised doubts concerning the interpretation of these findings. The latter authors have demonstrated a clear 'dissociation' between changes in contractility and in cyclic GMP levels. There is no obvious explanation that can account for these disparate results at the present time, other than the possibility of species-related differences.

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Effect of sodium nitroprusside on action potentials in frog ventricle

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Sodium nitroprusside (SNP) has a negative inotropic effect on the frog ventricle which is accompanied by changes in cyclic nucleotide levels (Flitney, Moshiri & Singh, 1980). The experiments described establish that the action potential (AP) is unaffected by SNP initially (during the first 45–60 sec), but that both its duration and overshoot are progressively reduced at later times. The experimental procedure is described elsewhere (Flitney & Singh, 1980).

Fig. 1*A* shows the response of the ventricle to 5×10^{-3} M-SNP. Typically, isometric tension declines abruptly during the first 45–60 sec, and thereafter more slowly, approaching a steady state after 25 min. Oscilloscope recordings of APs and twitches are shown in Fig. 1*B*, *C* (at times indicated in Fig. 1*A*). APs recorded during the first 1·5 min ($a \rightarrow b$ and $d \rightarrow e$) were indistinguishable, even though twitch tension fell by up to 25 %. However, at later times (c, f) both the positive overshoot and duration were reduced. Multiple impalement during the interval $b \rightarrow c$ reveal that the decrease in AP duration correlates closely with the reduction in peak tension.

The lack of correlation between AP parameters and peak tension initially contrasts

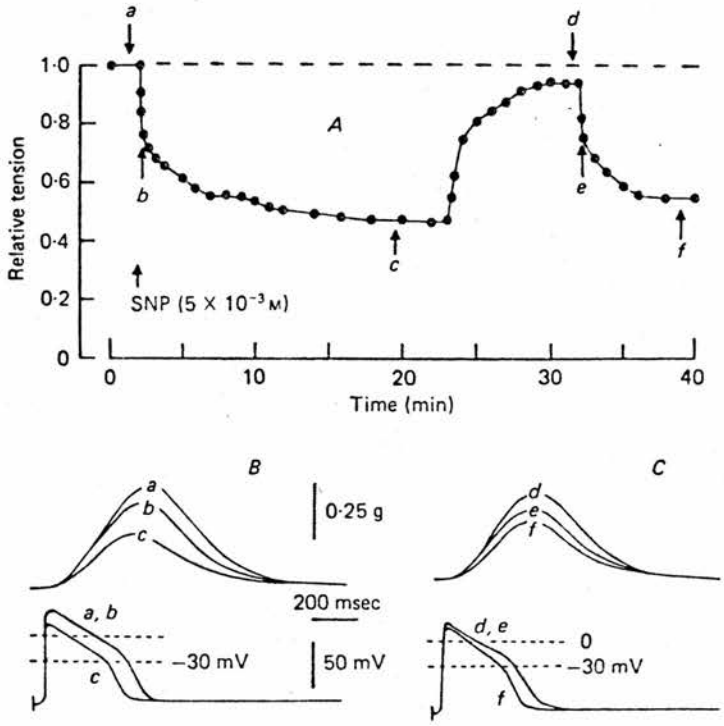

Fig. 1. Effect of SNP on contractility and cardiac action potentials.

[P.T.O.

with the *parallel* decline in contractility and in the ratio cyclic AMP:cyclic GMP, which holds throughout the entire time course of the response (Flitney *et al.* 1980).

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Effects of trifluoperazine on the frog ventricle

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The response of the frog ventricle to trifluoperazine (TFP), a potent inhibitor of the Ca^{2+}-dependent regulator protein calmodulin (Levin & Weiss, 1977), has been investigated. Isolated half-ventricles (from specimens of *Rana temporaria* and *R. pipiens*) were superfused with Ringer's solution (flow rate: 100 ml. min^{-1}; temp.: 19–21 °C) and stimulated electrically through Ag wire electrodes (frequency: 0·5 Hz; 10 V (4 mA); 5 msec duration). Measurements of peak isometric tension (P) and membrane potential (using 3 M-KCl-filled 'floating' electrodes; tip resistances: 20–50 MΩ) were made. TFP (10^{-7} to 10^{-4} M) produced a dose-related, negative inotropic response, which was half maximal at a concentration of $1{\cdot}2 \times 10^{-6}$ M. Its depressant effect on the twitch was not affected by either phentolamine (10^{-6} M), propranolol (10^{-7} M) or atropine (10^{-6} M). [TFP]'s > 10^{-4} M abolished the twitch and often produced persistent contractures. Micro-electrode recordings show that TFP has a transient stabilizing effect on the action potential (AP). It halted temporarily (for 10 min) the gradual decline in AP duration which accompanied the development of the hypodynamic condition (unpublished observations), although the decline in P was markedly accelerated and fell to around 80% of its pre-TFP value during this period. Thereafter, P and AP duration showed a parallel decline.

The Ca^{2+}-activated form of calmodulin is an allosteric effector of a cyclic nucleotide phosphodiesterase isoenzyme and of adenylate cyclase (Cheung, Lynch & Wallace, 1978). It is not yet clear whether the effects of TFP on intact ventricular cells can be attributed to changes in cyclic nucleotide levels resulting from inhibition of phosphodiesterase activity. The fact that TFP stabilizes (rather than attenuates) the action potential makes it seem unlikely that its effects are mediated by depressing transmembrane Ca^{2+} entry, at least during the initial phase of a response. Experiments are now in progress to measure endogenous cyclic AMP and cyclic GMP during TFP-induced responses.

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