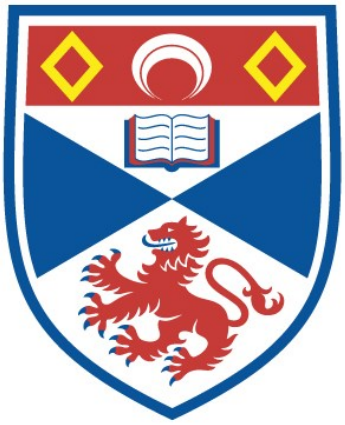
A STUDY OF THE EFFECT OF SUDDEN COOLING ON TENSION DEVELOPMENT BY THE ANTERIOR BYSSUS RETRACTOR MUSCLE OF MYTILUS EDULIS

Catherine Mary Linehan

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



1978

A STUDY OF THE EFFECT OF SUDDEN COOLING ON TENSION DEVELOPMENT BY THE ANTERIOR BYSSUS RETRACTOR MUSCLE OF MYTILUS EDULIS

A thesis submitted to the University of St Andrews for the degree of Doctor of Philosophy

by

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ABSTRACT

The effect of ambient temperature on the ACh-induced tension response of the ABRM of Mytilus edulis was examined. The latter was found to have many temperature dependent variables, these included the latent period, the rate of tension development, maximum tension and the relaxation rate. P_{max} was found to show a negative temperature co-efficent. K^+ contractures also showed a negative temperature co-efficent, P_{max} approximately doubling for a 20°C decrease in temperature.

The application of a cold shock during an ACh-induced contraction-relaxation cycle resulted in a transient increase in tension, the CIC. The production of a CIC was found to be dependent on the immediate presence of a stimulant, the time of application of the cold shock after the addition of stimulant, muscle length and temperature difference. As the temperature difference (ΔT) between the initial and cold shock solutions increased so the size of the CIC increased. The production of a CIC was found not to be directly related to the ACh or to the active state level yet it did not appear to be a passive phenomenon. A CIC was not produced when cold shock was applied to a muscle at rest or during catch, however, when catch was abolished by the application of relaxant a CIC could once again be elicited.

Kinetic analysis of the CIC showed that, however, complex the mechanism two steps appear to be rate limiting, and the increase in tension with increasing ΔT was probably due to an increase in the availability of activator responsible for its production, rather than a differential effect on one of the rate limiting steps.

Although it is conceivable that cold shock may exert a direct effect on the contractile proteins, evidence from the literature, and the experiments reported here, suggest that it is more likely that the CIC results from a transient increase in the level of myoplasmic Ca^{2+}. Pharmacological investigation did not disprove this hypothesis. Of the possible sources of Ca^{2+} responsible for the CIC membrane associated sites seemed the most likely since under conditions which deplete this site no CIC was observed. Also the involvement of cAMP in the production of the CIC was largely excluded.

DEDICATION

I would like to dedicate this thesis to my parents, for continual support and encouragement throughout the years.

CERTIFICATE

I hereby certify that Catherine M Linehan has spent eleven terms engaged in the research work described in this thesis, and that she has fulfilled the conditions of General Ordinance No 12 (Resolution of the University Court No 1, 1967), and that she is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

This research was conducted in the Department of Physiology, United College of St. Salvator and St. Leonard, University of St. Andrews, between January, 1975 and August, 1978.

ACADEMIC RECORD

I graduated as an External student from the University of London in August, 1973. I first matriculated as a research student in the Department of Physiology, University of St. Andrews in October, 1973.

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I would like to extend an especial 'thank you' to Dr R M Pitman for invaluable help and encouragement throughout this work and for giving up time in order to comment upon my thesis at all stages. Also to Mr I Grieve for technical assistance and for an unending supply of glass hooks.

Last, but certainly not least, I must thank Brian, who has given extensive support, advice, assistance and encouragement at all times, and who has, I fear, had much to suffer.

I acknowledge financial support form Action Research For The Crippled Child.

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ABBREVIATIONS

ABRM	=	anterior byssal retractor muscle.
ACh	=	acetylcholine .
AP	=	action potential.
cAMP	=	cyclic 3', 5'-adenosine monophosphate .
CIC	=	cold induced contracture.
dP/dt	=	rate of tension development.
5HT	=	5 - hydroxytraptamine .
k	=	rate constant .
L_o	=	*in situ* muscle length.
LP	=	latent period.
MP	=	membrane potential.
P_{max}, P_o	=	maximum tension .
p	=	probability.
r	=	correlation factor.
RP	=	resting potential.
SR	=	sarcoplasmic reticulum.
T_1	=	upper temperature.
T_2	=	lower temperature.
ΔT	=	temperature difference.
t_o	=	time of addition of stimulant.
SW	=	sea water.
ASW	=	artificial sea water.
RCC	=	rapid cooling contracture.

PART I

SUMMARY

SUMMARY

1). The effect of ambient temperature on the response of the ABRM to ACh was examined. Peak tension was inversely related to temperature and several aspects of the response were temperature sensitive. As temperature decreased latent period increased and the rate of tension development and the time to half relaxation decreased.

2). K-induced contractures also showed a negative temperature coefficent, the tension response approximately doubling in size from 20 ± 2°C.

3). It was found that rapid cooling applied during an ACh-induced tonic response resulted in a tension increment, the CIC. There was no tension increment when cold shock was applied to a muscle at rest or during catch. However, when catch was abolished by the application of relaxant a CIC could once again be elicited.

4). The CIC did not appear to parallel the wash-out contractures described in some smooth muscles, for removal of ACh without a simultaneous temperature shock did not result in a tension response. Furthermore, the possibility that the CIC resulted from a direct interaction between ACh and cold shock was largely discounted by the finding that a CIC could be produced if a cold shock were applied to a muscle during a K contracture.

5). The CIC was not due to an increased sensitivity to ACh at the lower temperature, since cold shock applied to a muscle in a sub-threshold ACh concentration was without effect.

6). With time after the application of ACh active state decayed and cold shock became a progressively less potent stimulus. It was suggested that these properties might be related.

7). The possibility that the CIC was the result of an effect of temperature on some passive component of the muscle was excluded by the observation that the active state level was enchanced by temperature shock.

8). Tension produced in response to cold shock decreased progressively as muscle length was increased from 0.5 - 1.3 L_o.

9). The size of the CIC was found to be dependent on the temperature step (ΔT), showing an exponential increase with increasing ΔT.

10). Kinetic analysis of typical CIC's showed that however complex they may be one forward (Tension producing) and one backward (Relaxation) reaction was always rate-limiting. The increased cold-induced response seen with increasing ΔT could not be attributed to a differential effect on one of the rate limiting steps since they both altered with ΔT in a similar manner. However, the analysis suggested that the increased cold induced response was due to an increase in the availability of activator.

11). The activation energies for the forward and backward reactions were found not to be significantly different, and to be similar to the activation energy for ionic diffusion.

12). The foregoing observations were consistent with the suggestion that Ca^{2+} may be the activator responsible for the CIC. A pharmacological investigation was therefore undertaken in order to test this hypothesis.

13). The CIC appeared to be less dependent on the external calcium concentration than the ACh-induced response:

i) Reducing the $[Ca]_e$ below 4 mM reduced the ACh response, yet left the CIC unaffected.

ii) Verapamil, which blocks Ca-influx in response to excitation was found to abolish the ACh-induced tension response in preference to the CIC. While La^{3+}, which blocks Ca-influx and membrane Ca-movements, reduced the CIC in preference to the ACh-induced response.

14). ACh and cold appear to have different sites of action. Caffeine which is considered to deplete the SR-like elements of Ca^{2+} was found to reduce the ACh-induced response yet leave the CIC largely unaffected. Furthermore, during recovery from caffeine treatment the CIC was observed to increase while the ACh-induced tension was still reduced.

15). On the basis of preliminary experiments using Quinidine, it was tentatively suggested that the CIC was not the result of a release of Ca^{2+} from the mitochondria.

16). Of the possible sources of Ca^{2+} responsible for the CIC, membrane-bound stores seemed the most likely candidate since the others - SR elements, mitochondria, and extracellular Ca^{2+} - had been largely excluded, and, under conditions which deplete this site no CIC was observed.

17). Substances which are thought to alter cAMP levels appeared to be without effect on the CIC, suggesting that alterations in cyclic nucleotide levels were not essential for its production.

PART II

INTRODUCTION

INTRODUCTION

The ability to move is a fundamental property of all animals and some plants. The way in which movement is brought about is, however, related to the individual needs of the organism concerned and although the mechanism will be similar in most cases i.e. the transduction of chemical to mechanical energy via a system of contractile filaments; fundamental differences between species will be apparent.

The mechanism of energy transduction has been largely established in the striated muscle of the 'higher' animals. These have been most widely studied, since they have yielded simpler answers to the fundamental problems of movement than the superficially less complex systems of smooth muscle or of primitative unicellular and multicellular organisms.

The mechanism of muscular contraction has been investigated to such an extent that it can now be considered in molecular terms. Actin and myosin, the contractile proteins, both exist in the form of filaments which interdigitate longitudinally, and contraction occurs by a relative sliding of these filaments past each other - the so-called sliding filament theory (A.F. Huxley, 1957). Actin filaments (F-actin) are formed by a linear aggregation of globular sub-units (G-actin), while myosin filaments consist of rod-shaped molecules each with a globular portion at one end. Light (LMM) meromyosin constitutes the rod or backbone and heavy meromyosin (HMM)

constitutes the globular portion. This latter can be further divided into two sub-fragments $HMMS_1$ and $HMMS_2$, the former of which has ATPase properties. The HMM portion of the molecule is the so-called cross-bridge which extends from the LMM backbone across to active sites on the actin filament.

Actin and myosin, prepared as pure solutions, react together to form a complex, acto-myosin. In skeletal muscle in vivo this reaction is prevented by the presence, on the actin filament, of regulatory proteins, the troponin-tropomyosin complex. In the absence of Ca^{2+}, troponin, in collaboration with tropomyosin, inhibits the interaction of myosin with actin, while in the presence of Ca^{2+} this depressant action is overcome and actin and myosin interact.

All muscles so far studied are thought to contract by a sliding filament mechanism, but the manner in which excitation-contraction coupling is brought about differs between muscle types. For instance, while vertebrate striated muscles rely mainly on Ca^{2+} released from intracellular storage sites smooth muscles (so called because of the absence of the cross striations), rely on external sources and/or intracellular organelles for the Ca^{2+} necessary for myofibrillar contraction. Some smooth muscles also differ from skeletal in that fibrillar myosin is often only visible in the contracted state, and many possess a third filament - the intermediate filament - the function of which is unknown. They differ too, in the size and time course of the tension response they

can produce smooth muscles often being able to hold tension for prolonged periods of time (tonic response). In this respect some invertebrate smooth muscles have become particularly well adapted, being able to hold tension for hours or even days. One such example is the Anterior Byssal Retractor Muscle (ABRM) of Mytilus edulis which is one of the strongest muscles known; it can generate a force of 15 Kg/cm^2 in contrast to the strongest vertebrate muscle which can produce a tension of about 3 - 4 Kg/cm^2 (Twarog, 1976). The requirement for such a contractile system may be understood from an examination of the life style of the animal.

Mytilus edulis, is a sessile bivalve, usually found in littoral and shallow sub-littoral waters. It is gregarious and mainly found in large numbers attached to a variety of sub-strata such as rocks, stones, shingle, shells and even compressed sand or mud which provide a secure anchorage. The attachment is by the way of fine threads of tanned protein, the so-called byssal threads, which are secreted by a collection of glands, the byssal apparatus, located in the foot of the animal. Threads are attached and detached in the process of locomotion, but their primary function is to secure the animal firmly to the substratum. The byssal threads are attached to the byssal retractor muscles which have become modified for resisting the strains imposed by mechanical forces e.g. severe wave action, to which these animals are exposed (Bayne, 1976). This specialization - the ability to hold high levels of tension for prolonged periods of time

with relatively little energy expenditure - is reflected in the structure and physiological properties of the byssal muscles.

Ultrastructure of the ABRM

Structural studies of the ABRM have shown that it shares many of the organizational features of smooth muscles. It is composed of spindle-shaped uninucleate cells, 5 µm in diameter and 1.2 - 1.8 mm in length. These cells, which are electrically connected via nexal junctions, are organized into bundles 100 - 200 µm in diameter which are encased in collagen. Thick and thin filaments are apparent, but there is no transverse alignment of these (Twarog, Dewey and Hidaka, 1973; Twarog, 1976).

The two types of filament differ in diameter and fine structure, (Plate I). The thinner filaments are 5.0 nm across, 11 - 12 µm in length and are similar to vertebrate actin filaments. The thicker ones, which are tapered at each end and are discontinuous along the fibre length, have a maximum diameter of 125 nm (100 - 75 nm), and a length of 25 µm (Plate II). These thick filaments have been found to consist of a paramyosin core with a myosin surround. The ratio of paramyosin to actin filaments is constant at 1:1 (Sobieszek, 1973).

In transverse sections (TS), the paramyosin filaments are further surrounded by a 'halo' of semi-dense material. This is thought to consist of cross-bridges since in some instances

Plate I

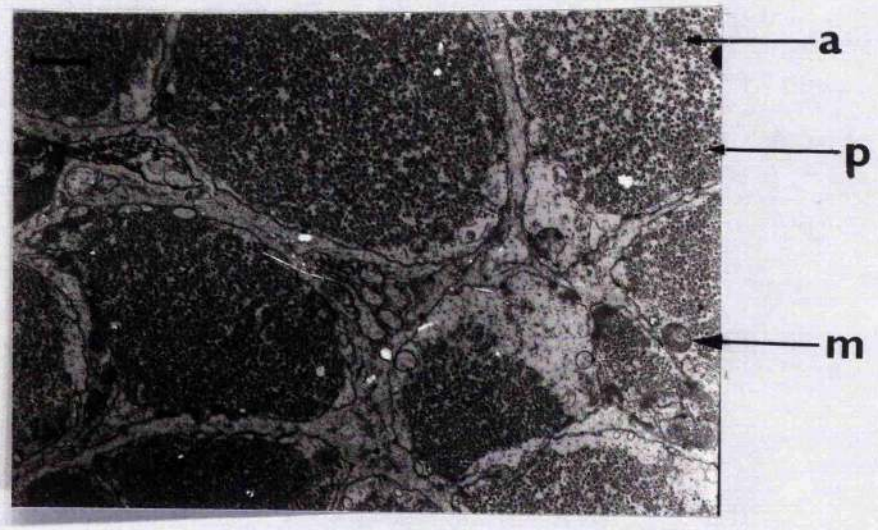
Electron micrograph of resting ABRM of Mytilus. Pre-fixed in 6% gluteraldehyde, and post-fixed in 1% OsO_4. Stained with uranyl acetate and lead citrate.

(A) Cross section showing paramyosin (p), actin filaments (a) and mitochondria (m).

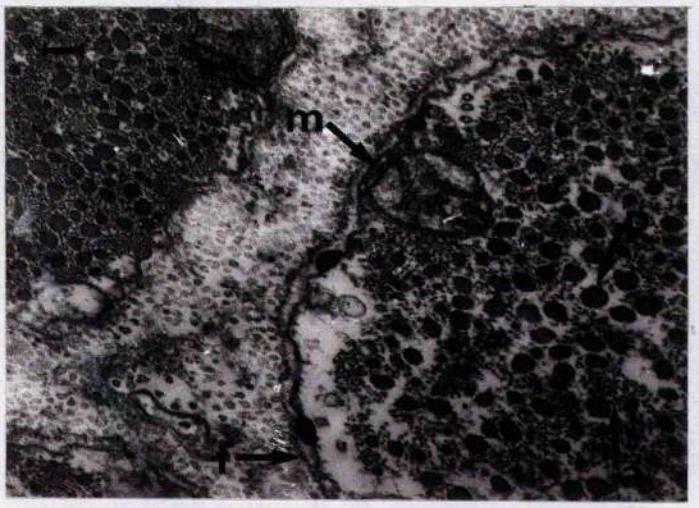
Calibration line 0.5 μm (x 17,483)

(B) Cross section at higher magnification showing paramyosin (p), actin filaments (a), mitochondria (m) and fibre membrane (f).

Calibration line 0.1 μm (x 51,616)



A



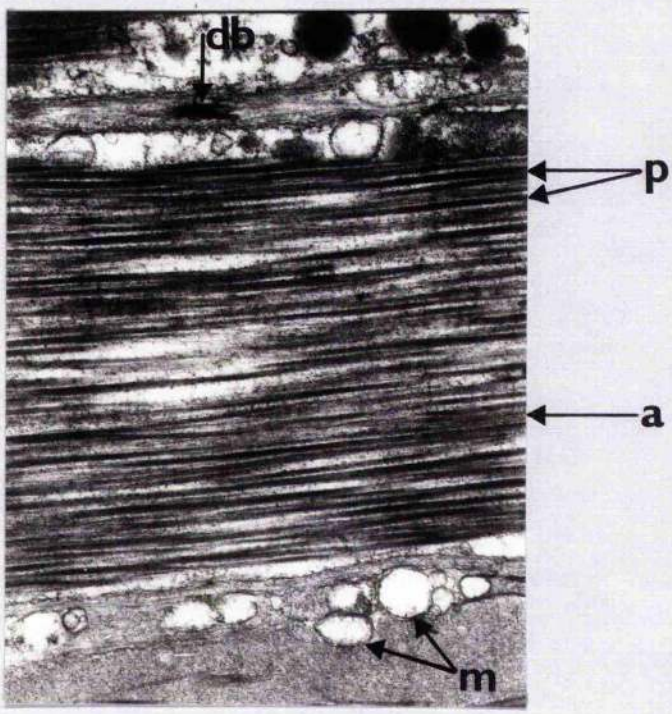
B

Plate II

Electron micrograph of resting ABRM of Mytilus. Pre-fixed in 6% gluteraldehyde and post-fixed in 1% OsO_4. Stained with uranyl acetate and lead citrate.

Longitudinal section showing arrangment of paramyosin (p) and actin filaments (a), a peripheral dense body (db) and membranous vesicles (m).

Calibration line 0.5 μm (x 17,483)


db
p
a
m

connections between this region and actin filaments can be clearly seen.

Although electron microscope investigation showed that the cross-bridges arose in pairs from the paramyosin filament surface as a two stranded helix with a repeat unit of 72 nm and a pitch of 2 x 72 nm with ten residues per turn, it was not possible to tell whether they originated from the paramyosin core or the myosin surround.

Examination of the muscle in the shortened and contracted state shows that the paramyosin filament diameter is constant, and the number of filaments per unit cross-sectional area is also unchanged (Hanson & Lowy, 1960). So that although regularly aligned sarcomeres are not present, a sliding filament mechanism apparently operates, (Gilloteaux and Baguet, 1977).

Actin filaments are found attached to dense bodies, structures which are a consistent feature of all vertebrate and invertebrate smooth muscles. In the ABRM, the dense bodies which are analagous to the Z-lines of skeletal muscle fibres, are on average 1.8 nm long and 0.12 nm in diameter, and act as thin filament attachment points, 60 - 80 thin filaments of opposite polarities being arranged on each side. Although, dense bodies usually have filaments attached at both sides about 7.5% of the total number have thin filaments attached at only one side . These one-sided dense bodies are invariably attached to the cell membrane. They are particularly

numerous at cell apexes, where they are found apposed to dense bodies in neighbouring cells (Sobieszek, 1973). // In a recent electron microscope study, Gilloteaux (1976) showed that the sarcolemma was invaginated at intervals. These invaginations formed dyad-like connections with sub-membrane bound vesicles and were suggested to facilitate the transport of Ca^{2+} into and out of the fibres.

Contractile Responses of the ABRM

Winton(1937) was the first worker to look at mechanical responses in the ABRM. He found that this muscle had the ability to alter its response according to the stimulus applied. In response to an alternating current (AC) stimulus the muscle would produce a short-lasting twitch-like contraction, or after direct current (DC) stimulus a long-lasting tonic response.

It was later found that the muscle could also be activated by other stimuli. Chemical stimulation with a high K^+ saline or, the application of acetylcholine (ACh) together with 5-hydroxytraptamine (5HT), would elicit a phasic response (Singh, 1937; Twarog, 1954); as would electrical or mechanical stimulation of the nervous elements supplying the muscle (Cambridge, Holgate & Sharp, 1959). In contrast, ACh applied alone evoked a tonic response, as did mechanical trauma. 5HT promptly relaxed tonic contractions by increasing the relaxation rate in the absence of any measurable change in membrane potential. Dopamine and lysergic acid diethylamide (LSD) could also be used to relax the tonic response, but their effectiveness was

much less than 5HT (Twarog, 1954).

Since ACh and 5HT are present in the muscle, and the activities of cholinesterase and monoamine oxidase have been demonstrated it is likely that an excitatory cholinergic system and an inhibitory tryptaminergic system exist (Twarog 1954; Cambridge et al, 1959). The phasic response was thought to be due to simultaneous activation of both the excitatory and inhibitory mechanisms, the tonic response being initiated by the excitatory mechanism alone (Jewell, 1959, Cambridge et al, 1959). This has been further corroborated by ultrastructural work in which two types of nerve terminal have been described; one containing clear vesicles (400 Å in diameter) that resemble those found in cholinergic terminals, and others containing dense core vesicles, (600 - 800 Å in diameter), typical of those generally associated with biogenic amines (McKenna & Rosenbluth, 1973).

At first, the tension-holding ability of these molluscan muscles was considered to be a specialization of the tonic mechanism, observed in smooth and striated muscles, under conditions in which repolarization was prevented. It was found, however, that while tonus in the latter depended on a continuous supply of energy, molluscan tonic contraction required little energy expenditure. It was therefore proposed that these muscles possessed a unique mechanism for passively maintaining full activation of the contractile system and early investigators, looking at bivalve shell adductor

muscles, suggested that the fibres hook-up or catch in some way, by an arrangement similar to a ratchet (Grutzner, 1904; Parnas, 1910, and von Uexhull, 1912).

Although Jewell (1959), had shown that the energy expenditure during catch exceeded the basal level, he did not undertake any biochemical studies and evidence that the tonic (catch) contraction required comparatively little energy expenditure was not firmly established until 1966. Nauss and Davies in this year, measured the levels of the high energy phosphate compound arginine phosphate, , and found that, provided the work done was the same, the amount of arginene liberated was identical in both phasic and tonic contractions. During catch there was no increase in the arginene or inorganic phosphate level, in fact there was a recovery of the initial breakdown which had occurred during the active contraction. The application of 5HT resulted in an increase in AgP breakdown associated with an increased efflux of Ca^{2+}.

Baguet and Gillis (1968), measured both heat production and oxygen consumption during phasic and tonic contractions. They found that phasic contractions induced an initial high rate of oxygen consumption which decreased exponentially as the tension decayed and fell to resting levels within 100 mins. However, during the tonic response there was an initial increase in oxygen consumption which was comparable to that seen during phasic contraction, but as long as catch remained there was an excess consumption which was proportional to the remaining tension.

When a muscle during catch was released, the oxygen consumption decreased, and on restretching, the reverse was true. The effect of length changes on the oxygen consumption during a phasic contraction were found to be the converse of this i.e., a release increased the rate of oxygen consumption and a stretch decreased it. These experiments show that the maintainance of tonic tension is an energy dependant process. However, since metabolism is only slightly increased above the basal level, it is likely that this tension can be maintained without incurring any oxygen debt, by the hydrolysis of ATP which is later rephosphorylated from stores of AgP, and ultimately by oxidative phosphorylation. However, it is worth noting that Nauss and Davies (1966), did not find any breakdown of AgP in their studies; this was perhaps due to inadequate measurement techniques. Baguet and Gillis (1968) concluded that the oxygen consumption of the tonically contracted muscle was eleven times less than during a phasic contraction and suggested that catch is the result of a slow but continuous interaction of actin and myosin, with a turnover rate of the interfilamentary cross-bridges, 2,750 times slower than in a tetanic contraction of a vertebrate fast muscle.

The characteristics of phasic and tonic contractions are summarised below:-

Phasic	Stimulus
Short-lasting, twitch-like:	AC
An active contractile state,	K^+ high
associated with a high energy	ACh + 5HT
expenditure present during	Nervous excitation
stimulation: Relaxation	Short duration DC pulses
rapid.	

Tonic	
Long-lasting: slow relaxation:	ACh
A "fused" state associated with	DC
a low energy expenditure	Mechanical stimulation (dissection damage)
occurring after stimulation	
has ended.	Adrenalin

Provided with a preparation which gave both phasic and tonic responses, workers examined these contractions with the intention of elucidating their mechanisms. The phasic state was thought to be due to actin-myosin interaction similar to that which occurs in vertebrate striated muscle. There are, however, two schools of thought on the mechanism of catch. One implicates actin and myosin only (the linkage hypothesis), and the other proposes paramyosin involvement also (independent parallel hypothesis). The former view is supported by morphological evidence which shows that each

paramyosin filament has a myosin surround and consequently is not in direct contact with actin, this suggests that tension results from acto-myosin interaction as in striated muscle. It has been proposed by Baguet and Gillis (1968) and others, that catch results from a slowing of the rate of breakage of these cross links. According to this hypothesis 5HT and/or nerve stimulation act on this system to increase the rate of detachment of the cross links and so cause relaxation. Paramyosin is not directly involved (Lowy and Millman, 1963; Hanson and Lowy, 1957; Nonomura, 1974; Tameyasu and Sugi, 1976).

The second hypothesis proposes that tension is initiated by the acto-myosin system but, after removal of the stimulant a change occurs; the paramyosin filaments which are in parallel with the contractile elements set or catch, and paramyosin takes over the tension maintaining role (Twarog and Johnson, 1960; Johnson, Kahn and Szent-Györgi, 1959; Robinson and Johnson, 1974; Gilloteaux and Baguet, 1977). That these opposing hypotheses have not yet been reconciled is partly a result of the difficulties associated with distinguishing between the two types of linkage experimentally. Lowy and Millman (1963), stretched the muscle in the phasic and tonic condition and compared the decay of tension from the new muscle length. Analysis of the tension decay showed that it could be resolved into two, or three exponential components, in the case of

the phasic and tonic response respectively. The first two components, observed in both types of contraction, were similar to those present in the decay curves of vertebrate skeletal muscle and, therefore, appeared to be related to an acto-myosin system. The third component, however, was only found during the tonic contraction and it was absent after the application of 5HT or following stimulation of the inhibitory nerves. Lowy and Millman, therefore, proposed that the third component was a property of paramyosin. These observations suggested that the phasic and tonic mechanisms were separate and independent and that the behaviour of the ABRM could be explained if the muscle had two types of tension retaining linkage.

Jewell (1959), confirmed this hypothesis. Using Ritchies' (1954), quick release method, he found that during an isometric contraction, induced by AC stimulation, the relationship between the tension response and active state was similar to that seen in vertebrate striated muscle. However, during catch, induced by ACh, tension remained high while the tension redeveloped, after a quick release, was only 25% of that obtained after a quick release from a similar tension level during a phasic response.

Further evidence for the mechanically passive nature of the tonic mechanism was obtained from studies on glycerinated muscle (Johnson, Kahn and Szent-Györgi, 1959). It was found that a catch-like state could be induced by altering the pH and ionic strength

of the bathing solution, and it was suggested that if this occurred in the natural state it could explain the catch mechanism. This conclusion however, has since been questioned by Marchand-Dumont and Baguet (1975) who induced a catch-like state by the method of Johnson et al, in EDTA and Triton X-100-treated fibres, and found that 5HT had no relaxing effect. Since a tonically contracted, untreated, ABRM relaxes immediately after the application of 5HT, it was thought unlikely that such a condition of low ionic strength and pH ever develops in vivo.

That the controversy is still unresolved is illustrated in recent ultrastructural and mechanical studies on the ABRM. Gilloteaux (1977), working on glycerinated and chemically skinned muscle has provided evidence for the direct involvement of paramyosin in the catch state. He observed that during catch the paramyosin filaments appear to increase in diameter and cluster together while attaching themselves to actin filaments. Robinson and Johnson (1974) also working on glycerinated muscle, found that inducing a catch-like state, by altering the pH of the medium increased forward light scattering, and this effect was only slightly reduced by myosin extraction, but completely abolished by the removal of paramyosin. In contrast, Tameyasu and Sugi (1976), working on whole muscles found that in the ABRM the properties of the series elastic component (SEC) depended on the isometric tension level held and not on whether

the muscle was in an active or catch state. A similar dependance of the SEC on isometric tension has been reported in skeletal muscle fibres, where work by Huxley and Simmons (1971), has suggested that a component of the SEC, the short range elastic element, may largely reside in the acto-myosin cross linkages themselves, and in consequence this will be dependant on the isometric tension which is in turn dependant on the number of linkages. Therefore, if a change in linkage occurred, as might happen if paramyosin took over the tension retaining role one might expect to observe a change in the properties of the short range elastic component.

Excitation-contraction Coupling

The first account of the electrical properties of the ABRM was given by Fletcher (1937). Using extracellular electrodes he found that an action potential (AP), following a single shock initiated a brief (phasic) contraction but he was unable to observe electrical activity during prolonged contractions, induced by a DC stimulus, or by dissection trauma. He also observed that short duration DC pulses affected the muscle in a similar manner to AC stimulation. He concluded from this finding that in the phasic contraction of smooth muscle the AP plays the same essential role as in striated muscle contraction, but that a tonic contraction occurs in the

absence of conducted action potentials. Although these observations were confirmed by Twarog (1960), Hoyle and Lowy (1956) found that prolonged contractions were accompanied by increased nervous activity. This controversy was resolved by Johnson and Twarog (1960) who showed that the spike-like potentials seen by Hoyle and Lowy during tonic contractions also occurred in the resting muscle and were not associated with tension retention. They did find, however, that in some preparations the muscle 'slipped' from time to time, and when this occurred nervous activity was observed as the initial tension level was re-attained.

Twarog (1967b), using intracellular micro-electrodes, found that the resting potential (RP) of the ABRM was around 65 mV, and the AP associated with a single stimulus was TTX-resistent, and Ca-dependent, its amplitude being proportional to the log of the external Ca^{2+} concentration (27 mV per ten-fold change in $[Ca_o]$). She also showed that application of ACh caused membrane depolarization and initiated a tonic response, during which repolarization occurred slowly, being complete in about 20 mins., at which time tension was still 70% of maximum. Transferance of the muscle to Na or Ca-free solution at this time was without effect (Twarog and Hidaka, 1971; Twarog, 1967b; Hidaka and Goto, 1973).

There is evidence that the Ca^{2+} entry during ACh-induced depolarization is not solely responsible for the contractile response but rather acts as a "trigger" to stimulate the release of further

Ca^{2+} from intracellular stores. This conclusion follows from a comparison of the contractures induced by K^+ and ACh. K-induced contratures are absent in Ca-free media and almost completely abolished by the addition of Mn ions to the external solution. In contrast, ACh-induced contractures although also abolished in Ca-free media, show less dependance on $[Ca]_e$ e.g. K-contracture tension drops by 50% with a reduction of $[Ca]_e$ from 20 - 1.2 mM, while ACh-induced contractures are largely unaffected by a drop of $[Ca]_e$ from 20 - 0.6 mM. It is suggested that ACh-induced contractures require less $[Ca]_e$ because only a small influx of Ca^{2+} is necessary to trigger the release of the intracellular bound stores. Furthermore, both K^+ and ACh can cause the same degree of depolarization yet the tension response evoked by ACh is always larger, (Sugi and Yamaguchi, 1976; Twarog, 1976).

Additional evidence for the involvement of intracellular Ca^{2+} in the ACh - response comes from cytochemical studies using K-pyro-antimonate, a substance which reacts with Ca^{2+} to produce an electron dense precipitate (Sugi & Atsumi, 1973). In a resting muscle calcium pyroantimonate was observed within vesicles located on the inside of the surface membrane (this was confirmed by the use of X-ray probe analysis). The application of ACh appeared to displace Ca^{2+} from these sites, and disperse it in the myoplasm. Relaxation was associated with a return of the precipitate to the original sites, (Atsumi & Sugi, 1976).

Relaxation

The slow time course of catch relaxation has discouraged extensive investigation of this phenomenon so that general agreement on its mechanism has not been reached. However, Gogjian and Bloomquist (1977), have suggested it is dependent on Ca^{2+} uptake into intracellular vesicles, and Atsumi and Sugi (1976) have shown cytochemically that the appearance of Ca^{2+} deposits during catch are indistinguishable from those at rest.

In contrast 5HT relaxation has been quite extensively studied. Relaxation in response to 5HT is considered to be an energy consuming process since it is associated with a stimulation of Ca-activated ATPase (Nauss and Davies, 1966; Leenders, 1967), an increase in (AgPh) breakdown (Nauss and Davies, 1966) and oxygen consumption (Baguet and Gillis, 1968), and it is inhibited at low temperatures (~2°C) - (Twarog and Muneoka, 1973).

Cole and Twarog (1972), found that a variety of phosphodiesterase inhibitors or agents which stimulate the production of cAMP marginally increased the rate of 5HT-induced relaxation. This was taken to be consistent with the role of cAMP as a mediator of 5HT-induced relaxation, but the evidence was largely circumstantial, and direct application of cAMP had no effect. A later study (Achazi, Dolling and Haakshorst, 1974) provided additional evidence for an increase in the level of cAMP following 5HT treatment. At the end of 5HT relaxation, cAMP had risen to a level some eight to ten

time higher than in the resting state.

In contrast, phasic contractures induced by the simultaneous application of ACh and 5HT to the bathing medium resulted in only a slight rise in the level of cAMP; while, the application of ACh alone had little effect. The involvement of cyclic nucleotides in the relaxation process is in some doubt, since the slight increase in cAMP level seen during phasic contractures together with the 8 - 10 fold increase observed on the application of 5HT to a resting muscle suggests that relaxation may not be dependent on changes in cyclic nucleotide level. In addition, Bloomquist and Curtis (1975), using whole muscle bundles have observed that the application of 5HT to a muscle at rest or in catch results in an efflux of Ca^{2+}. The rate and amount of this efflux is identical in both cases, and again one wonders if this is a specific or general action of 5HT on the muscle.

Scope of the Investigation

Woods (1974) observed that if relaxant (5HT or Dopamine) and a cold shock were simultaneously applied to a muscle in catch a positive increment in tension occurred. She suggested that this was due to the temprature shock "decreasing the rate constants for both the forward (tension producing) and backward (relaxation) reactions, but that the Q_{10} for the backward reaction was greater than for the

forward one" consequently a positive tension response occured. She also suggested that a cold shock response could not be observed in the absence of relaxant such as 5HT or Dopamine. Preliminary experiments conducted in the present investigation showed that this was not the case. A cold induced response (CIC) could also be observed during K and ACh-induced contractures and the latter had the advantage of being produced on a slowly falling base line which facilitated analysis. The principle aim, therefore, of this study was to characterize the CIC produced in this way and determine how it might occur.

PART III

MATERIALS AND METHODS

MATERIALS AND METHODS

Animals. Specimens of Mytilus edulis were obtained from the Gatty Marine Laboratory, University of St. Andrews. They were stored either in aerated sea water (SW), or in a refrigerator at 4°C for up to four days before use.

Dissection. The anterior byssal retractor muscle (ABRM), was dissected out by firstly inserting a sharp scalpel blade between the shells and cutting through the adductor muscles. When the shells were opened a mid-line transection was made through the retractor muscles. The ABRM was stripped of all connective and nervous tissue, and a steel hook was tied around its base, anterior to the foot and the byssal organ. The muscle was freed by a cut just posterior to the byssal organ, and from the foot by cutting through the base of the latter. At its insertion the muscle was removed together with a piece of shell, (Fig. 3.1).

In some experiments the muscle, which is composed of two separate bundles, was carefully trimmed to approximately 1 mm before use, by carefully removing fibres with a fine forceps. Both whole muscles and bundles were used in the experiments. No difference was detected between these preparations.

All the experiments were conducted at a standard muscle length, 80% of the in situ length (L_o).

Fig 3.1

Diagrammatic representation of the internal organs of Mytilus edulis

a : anterior end of shell

b : labial palp

c : the anterior byssal retractor muscles (ABRM)

e : digestive gland

f : ctenidia

g : foot

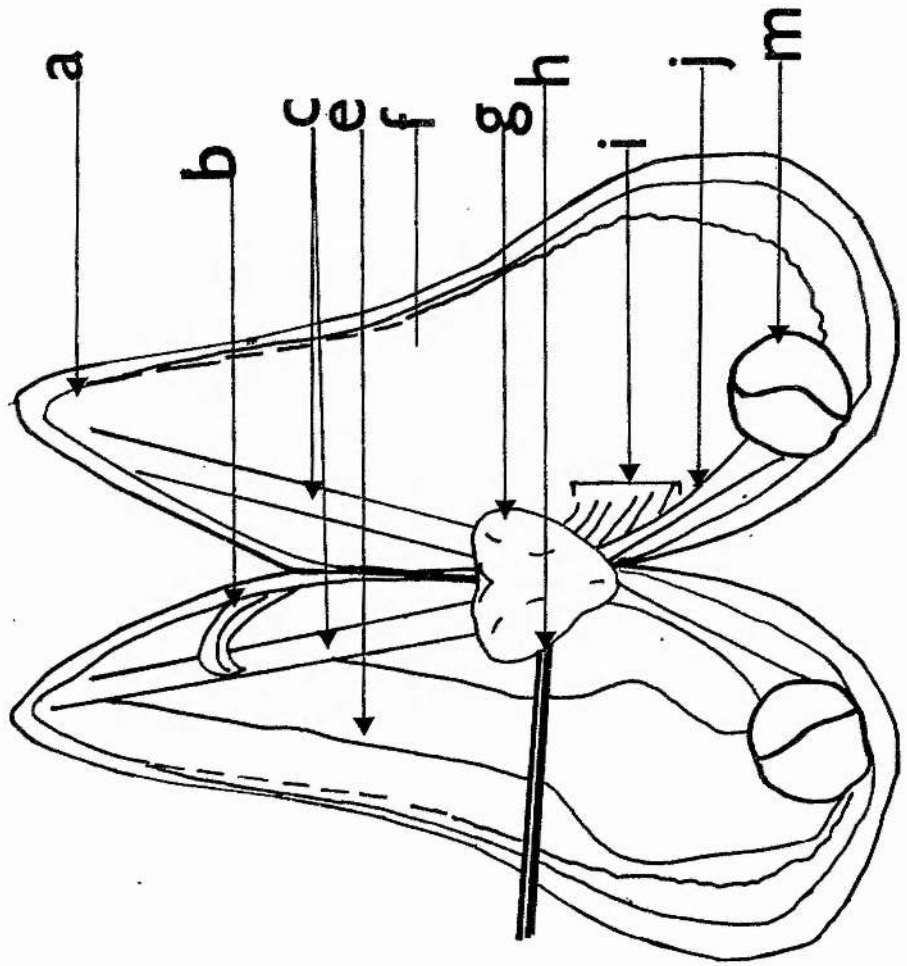
h : byssal threads

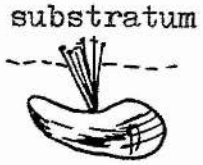
i : pedal retractor muscles

j : posterior byssal retractor muscles (PBRM)

m : shell adductor muscle

Insert shows the method of attachment, using the byssal threads, of Mytilus to the substratum.


a
b
c
e
f
g
h
i
j
m


substratum

Apparatus The apparatus is shown in Fig. 3.2. The muscle was suspended vertically, by passing its upper end through the glass stirrup, and attaching the other end to a silver chain, which was connected to an E & M Linear Core isometric transducer ((range:- 0-1 Kg). This transducer was mounted on an adjustable micrometer, which allowed the muscle length to be varied to within 0.01 mm. A dial guage was used to measure the muscle length. The whole unit was supported on a Palmer stand, enabling it to be raised and lowered into solutions of varying temperatures.

It was essential to maintain the temperature of the test solution to within ± 1°C of the required values between 2°C and 40°C for at least 15 minutes. This was done by placing the containers holding the test solutions into 'wells' cut into a polystyrene block. Polystyrene has a low thermal conductivity and tests showed that the change of temperature was less than 0.01°C per minute even when the initial temperature was as much as 20°C below room temperature. The temperature was monitored by using a thermistor probe.

The muscle was quickly transferred to the various solutions by raising and lowering the Palmer stand. This lifted the muscle out of the container, and by revolving the polystyrene block underneath the preparation the muscle could be replaced in a container of a different solution in 5 - 7 seconds.

Previous work had shown that 90% temperature equilibration occurred throughout the muscle within 7 - 10 seconds of immersion (Woods,

Fig 3.2

Diagrammatic representation of apparatus used.

p : Palmer stand

d : micrometer dial (for monitoring muscle length)

t : tension transducer

mi : micrometer

c : chain

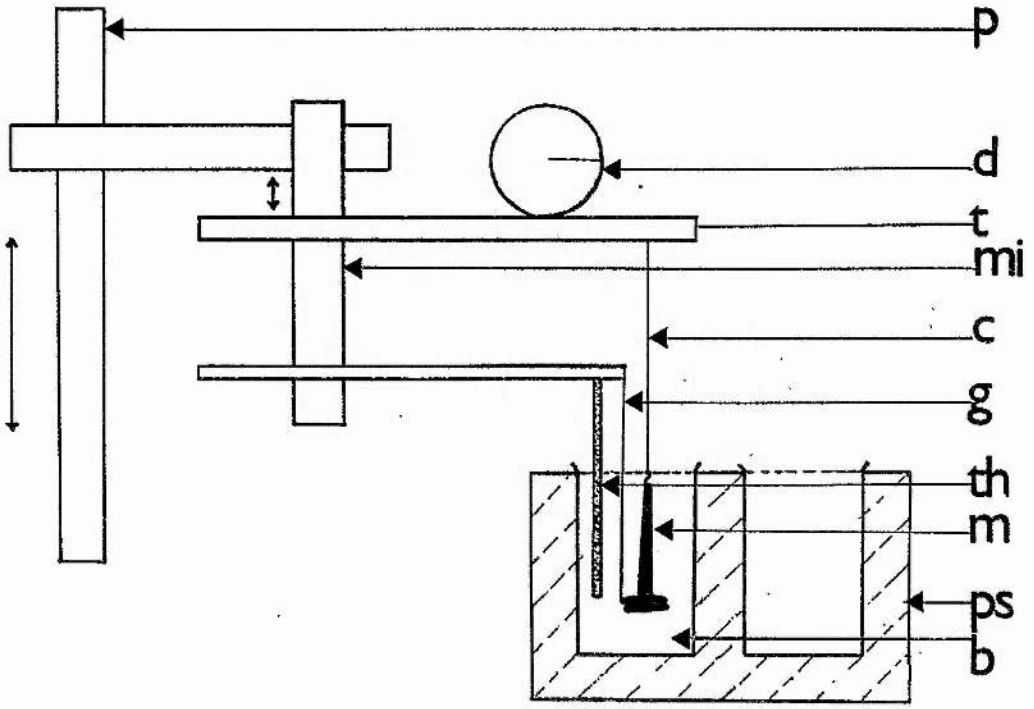
g : glass stirrup

th : thermistor

m : muscle

ps : polystyrene block

b : bathing solution


p
d
t
mi
c
g
th
m
ps
b

1974), however, in this study the ABRM was thinned to permit equilliberation to occur more quickly.

A thermistor bead was implanted into a Frog gastrocnemius muscle and the temperature change monitored. In this system, the delay in conduction of heat throughout the muscle should be the dominant factor. In one experiment, 90% temperature equilibration took 60 seconds. The radius of this muscle was found to be about six times greater than the thinned bundles routinely used in this study. Since diffusion times vary with the square of distance, this means that conduction through the smaller muscle bundle would take about 1½ seconds.

Procedure. The muscle was first placed in a solution containing as a stimulant, either acetylcholine (ACh), or high K^+ sea water (SW), and at some pre-determined time (usually two minutes), after the onset of a contracture, it was quickly transferred to cold sea water. Sea water alone was used when ACh-induced contractures were employed since the continued presence of ACh in the colder solution tended to slow down the natural relaxation rate of the cold induced contracture (CIC). No difference in contracture size was observed when ACh was present, in the cold solution. After complete relaxation at least ten minutes was allowed to elapse before another response was induced.

Quick release experiments. In some experiments the time course of the active state, was determined. This was done by releasing the muscle quickly (approximately 1.5 cm/second), to zero tension and allowing tension to redevelop. The re-developed tension level was compared to the tension that the muscle would be expected from length-tension curves, to exert at the new muscle length. The size of the release was monitored.

Recording. Tension responses were recorded on a Bryans (27000) chart recorder.

Solutions. Fresh or artificial (ASW) sea water was used in all experiments. No difference in result was detected between the two. The ASW used was that of Nagai and Hagiwara (1970):- NaCl 450 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 51 mM, Sigma 7 - 9 50 mM, (pH 7.3-7.4).

Tension was induced by 10^{-3} M ACh, (acetylcholine chloride (MW 181) at this concentration active tension was approximately 90-100% of maximum and repeated contractures could be obtained without any visible adverse effects on the muscle. Tension was abolished with 10^{-6}M 5HT (5-hydroxytraptamine creatine sulphate complex (MW 387.4). These drugs were either made up directly in sea water or as concentrated stock solutions in distilled water and diluted as required in the experimental solution so that the ionic

strength of the SW was not reduced by more than 1%. The concentrations of 5HT and ACh were only varied in some control experiments.

In order to induce catch-tension fully, the method of Twarog (1959) was used, whereby ACh is applied to the muscle for two minutes and then removed. Ten minutes after the first application the muscle is exposed to ACh a second time, again for a period of two minutes. This is done three times in all and after the third time the muscle is deemed to be fully in catch.

In order to initiate phasic contractures, a high K ASW was used, in which 320 mM NaCl is replaced by KCl, (Nagai and Hagiwara, 1970).

Ca-free SW was made up by omitting $CaCl_2$ and adding 1 mM EGTA to the solution. The Ca^{2+} concentration was always less than 0.0025 mM measured by atomic absorption spectrometry.

In SO_4-free SW the $MgSO_4$ was replaced by $MgCl_2$, and where Na-free SW was used the NaCl was replaced by an equal amount of choline chloride.

Pharmacological agents. The effects of a number of pharmacological agents on the CIC were examined.

Verapamil (MW 491.1) - ; 5 N (3,4-dimethoxyphenethyl methylamine -2-(3,4-dimethoxyphenyl(-2- isopylvaleronitrile; - blocks the entry

of external calcium. This drug was used in a concentration of 10^{-4}M, dissolved directly in ASW. After ten minutes in this solution ACh-induced tension was reduced by 40 - 50%.

Lanthanum chloride (MW 371.38) blocks the entry of external calcium ions, and their transmembrane fluxes and induces general membrane stability. $LaCl_3$ was made up in a concentrated form in SO_4 - free SW to avoid the formation of a precipitate of $La_2(SO_4)_3$, and then diluted to the required concentration (10^{-3}M), in normal or ASW.

Caffeine (MW 194.2), is known to deplete the internal stores of Ca^{2+} associated with the sarcoplasmic reticulum (SR) in both skeletal and smooth muscle systems. It is also a phosphodiesterase inhibitor. In this investigation the drug was used in a concentration of 10^{-2} M directly dissolved in ASW or SW.

Theophylline (MW 180.2), another phosphodiesterase inhibitor, was made up in a concentrated form in distilled water and then diluted in SW or ASW to a final concentration of 10^{-2} M.

Nicotinic acid (MW 123.1), has been reported to stimulate phosphodiesterase activity and hence decrease adenyl cyclase levels. This drug was used directly dissolved in SW to a final concentration of 5×10^{-2} M.

Sodium Fluoride (MW 419.9), stimulates adenyl cyclase in broken cell preparations, and was used at a concentration of 10^{-2} M directly dissolved in SW.

Mersalyl Acid (Salygran - MW 483.9), a 5HT-blocker was used in a concentration of 10^{-4} M directly dissolved in SW.

Analysis of tension responses

The size of the CIC was estimated by subtracting the response obtained when the muscle was kept at a constant temperature (A), from that obtained when the temperature was lowered (B). (Fig, 3.3). The tension difference was plotted, and the size of the CIC and its time course were studied. Unless otherwise stated tension is routinely expressed in absolute terms (Kg/cm^2). Muscle length is expressed as a multiple of the in situ length (L_o).

Statistics

A student t-test was used for statistical evaluation of the results ($\bar{x}$ = mean value) and the error at all times is $\pm$ 1 standard error of the mean. Regression analysis was undertaken in some instances in order to estimate the statistical significance of the results.

The statistics used for Part 2 were as follows:-

The curve fitting was done using the Numerical Alogorithms Library (NAG) routine EØ4GAF which follows Marquardt's method to find the least-squares solution of M non-linear equations in N variables.

The correlation factor was obtained from a standard "Goodness of Fit" formula.

$$r = 1 - \left[\frac{|(x - r)|}{|(x + r)|} \right]^2$$

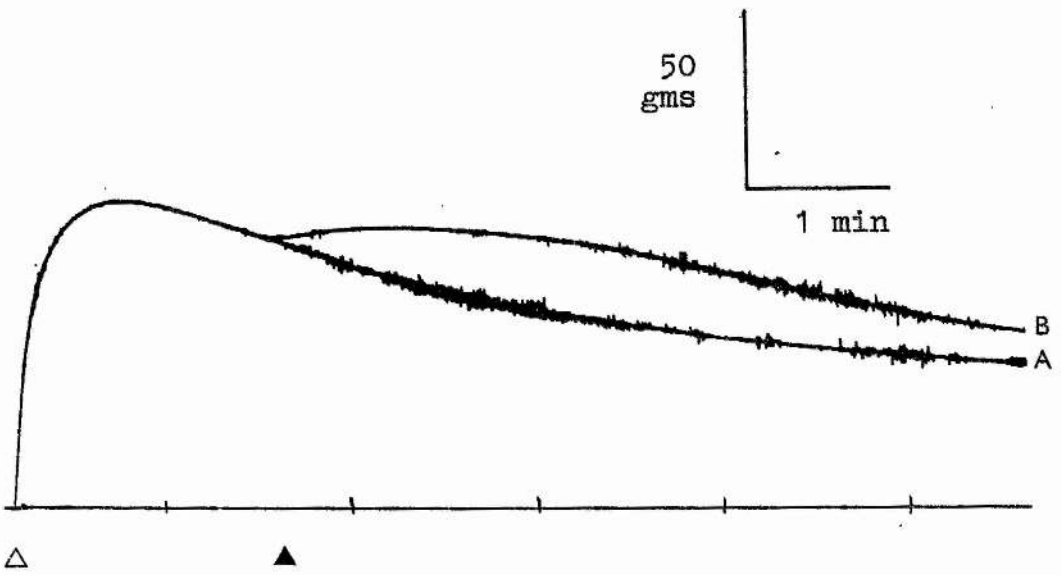
Fig 3.3

Method of estimating the size of the cold induced response.

Trace A shows the response of the ABRM to the application of 10^{-3} M ACh at $20^{o}C$ (indicated by $\triangle$). At two minutes the muscle was transferred to a SW solution at $20^{o}C$ ($\blacktriangle$).

Trace B shows the effect of transferring the muscle, at two minutes, to a SW solution at $2^{o}C$.

The response due to cold shock was taken to be the difference between the two traces.


50
gms
1 min
B
A

Electron Microscopy

Method. Muscle bundles of 1 mm or less were teased out from a whole muscle and allowed to equilibrate at L_o for at least one hour in SW before fixation. In those muscles where tension was monitored during fixation tension decline ceased after 3 - 5 mins., incubation in the fixative.

Control muscles were fixed in a 6% gluteraldehyde solution and post-fixed in OsO_4. They were washed and dehydrated in the usual manner using a series of graded alchols and epoxypropane. Finally, the material was embedded in araldite which was polymerized at 60°C in an oven for 24 - 48 hours.

Muscle sections were cut on a Reichart Ultramicrotome, gold and silver sections only were used, and these were mounted on x 200 copper grids, and viewed on a Philips 301 Electron Microscope, either unstained or stained with uranyl acetate and lead citrate, at an electron beam intensity of 60 - 80 kV.

Cytochemistry

The muscles to be used for the study of intracellular Ca^{2+} deposits were fixed in a 1% osmium tetroxide (OsO_4) solution containing 2% potassium pyroantimonate ($KSbOH_6$) (Sugi and Atsumi, 1976). Before being placed in the $KSbOH_6$ solution the muscles

were washed quickly in zero calcium SW in order to prevent extra-cellular precipitation of $Ca(SbOH_6)_2$. The $OsO_4/KSbOH_6$ solution was made isosmotic by the addition of sucrose.

Membrane Potential Measurements

A modified double sucrose gap technique was used (Stampfli, 1954). The apparatus consisted of: a rectangular perspex container which was divided into three compartments by two partitions. In the centre of each partition there was an aperture which extended to the top of the partition (Fig. 34). The partition was sealed around a bundle of muscle fibres with vaseline. The bundle was secured at either end with small dissecting pins. The compartments were irrigated with either sucrose, ASW or KCl supplied from reservoirs above the set-up. Cooled solutions or drugs could be injected into the ASW inlet. The middle compartment was irrigated with sucrose (1.4 M). In order to keep the three compartments electridally isolated the solutions were channelled separately into three different containers.

Changes in Membrane potential (MP) were monitored by measuring the potential difference between the two outer compartments using a cathode follower (Ceptu teaching unit). Connections were made with each compartment with chloridized silver electrodes inserted into

Fig 3.4

A schematic diagram of the appratus used for membrane potential measurements.

A : inlet for addition of drugs or cooled solutions

E : agar bridge

V : vaseline-filled partition

M : muscle

Pe : perspex chamber

Pi : dissecting pin

C : collecting vessel

CR : chart recorder

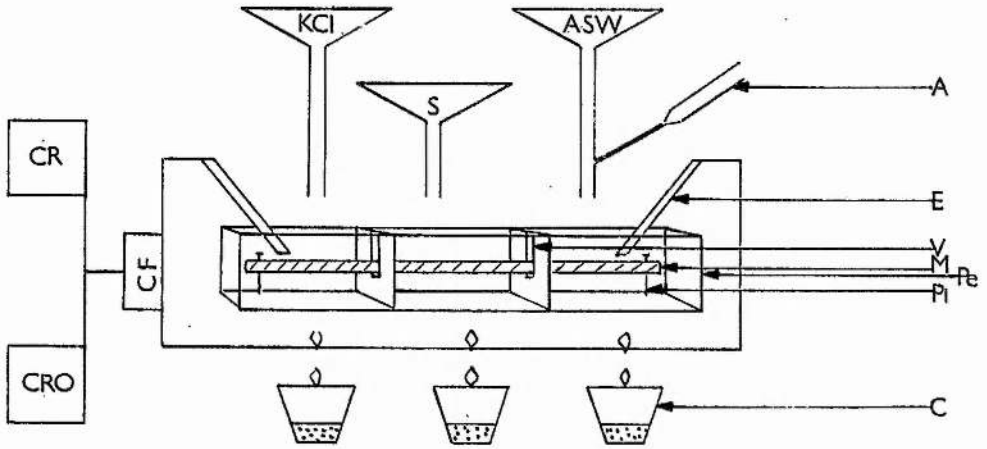
CRO: cathode ray oscilloscope

CF : cathode follower

KCl: 0.45 M potassium chloride

S : 1.4 M sucrose

ASW: artificial sea water


KCl
S
A.SW
A
E
V
M
Pe
Pl
CR
CF
CRO
C

agar bridges. Agar was made-up by dissolving it in hot saturated KCl, and when not in use the bridges were stored in 3M KCl. Reference zero potential was recorded with both electrodes placed in 0.45M KCl.

Permanent recordings of membrane potential were obtained on a Linseis Chart recorder (27000) connected to the output of the cathode follower. Tension records were taken at the same time as electrical potential measurements.

Reagents

The sources of drugs used in this study are as follows:
Lanthanum chloride - BDH Chemicals, Poole, England.
Acetylcholine chloride (ACh); 5 - hydroxytraptamine creatine sulphate complex (5HT); Mersalyl Acid; Nicotinic Acid; Ethyleneglycol-bis (B-amino-ethy-ether) N,N-tetra-acetic acid (EGTA) - Sigma Chemical Company, St. Louis, U.S.A.
Caffeine - Boots Drug Company, Nottingham, England.
Verapamil, kindly supplied by Pflizer Limited, Sandwich, England.
Unless otherwise stated all reagents used were analytical grade.

Notation

The following notation is used in presenting the results:-

ΔT

The size of the temperature step is denoted by ΔT and the upper and lower temperatures by T_1 and T_2 respectively. Thus for a temperature step of 20°C starting at an upper temperature of 30°C and abruptly lowering it to 10°C, the notation is ΔT 20°C (T_1 = 30°).

P_o

The CIC is expressed either as a percentage of the maximum cold-induced tension (P/P_o), or in absolute units of force (Kg/cm^2).

L_o

Muscle length is expressed as a fraction of the *in situ* resting length (L/L_o).

t_o

Time of application of ACh is referred to as t_o, and unless otherwise stated the temperature step is applied two minutes after t_o (t_o + 2 minutes).

dP/dt

The parameter dP/dt was obtained by taking a tangent to the tension increments and calculating the slope. dP/dt, the rate of tension development is expressed in $Kg/cm^2/sec$.

PART IV

EFFECT OF PHYSICAL VARIABLES ON SOME CONTRACTILE RESPONSES OF THE ABRM.

MECHANICAL RESULTS

Responses of the ACh-induced tension to changing temperature

In this investigation an isometric contraction-relaxation cycle was routinely initiated by the application of 10^{-3} M ACh (Fig. 4:1), which was shown to produce 90 - 100% peak tension (P_{max}) at 20°C (Fig. 4:8). Tension development resulting from the addition of ACh is slow, the time to P_{max} is 32.6 ± 9 seconds at 20°C and 142 ± 6 seconds at 2°C; relaxation is not spontaneous but takes hours or even days unless a relaxant, such as 5HT or Dopamine, is applied. These values are several orders of magnitude greater than in frog skeletal muscle where, at 20°C, the time to P_{max} is approximately 50 msec and relaxation is complete in 100 msec.

All aspects of the ACh contraction-relaxation cycle studied here were found to be temperature dependent (Table 4:1). These include the latent period, the rate of tension development, peak tension, the relaxation rate and the 5HT-induced relaxation rate.

The Latent Period

The latent period (LP), deceases from 7.3 ± 1.3 seconds at 2°C to 2 ± 0.1 seconds at 20°C - (Q_{10} 1.73 ± 0.05 - Fig. 4:2). This can be compared to rat portal smooth muscle where the Q_{10} for the LP is 1.63 (Peiper, Lawen and Ehl, 1975).

Table 4.1

Table of temperature variable aspects of the ACh (10^{-3}M) induced tension response.

Temperature °C	Latent Period	Rate of Tension Development	ACh (10^{-3} M) Tension Maximum	Relaxation $\frac{1}{2}$-time	(10^{-6} M) 5HT $\frac{1}{2}$ - time
	Secs.	$Kg/cm^2/sec$	Kg/cm^2	secs.	secs.
2	7.3 ± 1.3	0.135 ± 0.03	5.39 ± 0.12	805 ± 60	240 ± 20
5	6 ± 0.4	0.138 ± 0.02	5.03 ± 0.18	796 ± 83	175 ± 25
10	4.12 ± 0.4	0.136 ± 0.01	4.21 ± 0.18	616 ± 24	130 ± 17
15	2.66 ± 0.3	0.163 ± 0.02	3.85 ± 0.21	570 ± 48	80 ± 10
20	2 ± 0.1	0.196 ± 0.04	3.2 ± 0.17	500 ± 62	43.6 ± 2
25	2 ± 0.2	0.198 ± 0.03	3.0 ± 0.19	177 ± 30	39.5 ± 6
30	1.25 ± 0.25	0.33 ± 0.04	2.6 ± 0.13	46.8 ± 5	43.6 ± 2.6
35	-	0.43 ± 0.03	2.4 ± 0.16	32 ± 4	-
40	-	0.4 ± 0.04	1.96 ± 0.11	19.7 ± 4	10 ± 4
45	-	0.45 ± 0.01	1.2 ± 0.049	9 ± 1.2	-

Fig 4.1

Typical ACh-induced contraction relaxation cycle.

Tension was initiated by the addition of 10^{-3} M ACh (△) for one minute at 20°C; and abolished by the application of 10^{-6} M 5HT (▲) also at 20°C.

This figure also shows that a cold shock (△T 18°C, T_1=20°C) applied to a resting muscle is without effect. ▲ indicates the point of application of the cold shock solution.

Muscle length was 0.8 L_o.

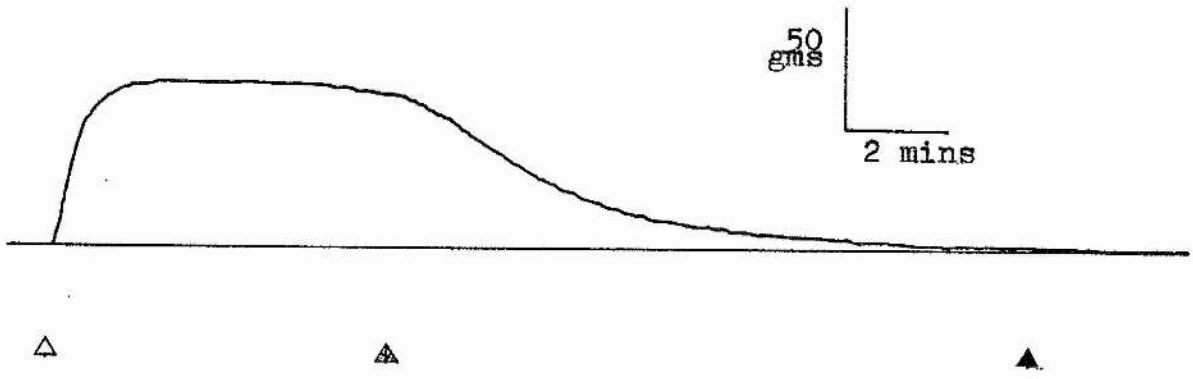
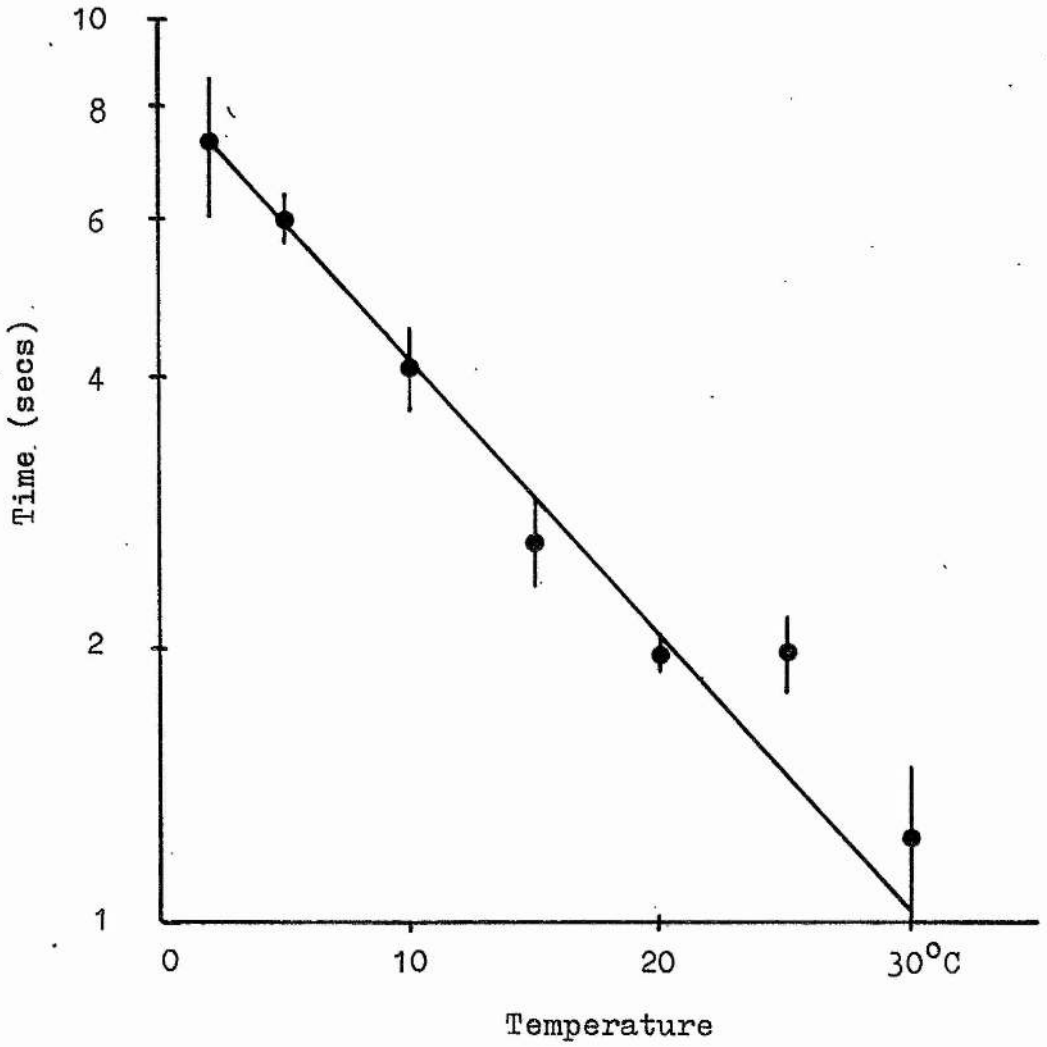

50
gms
2 mins

Fig 4.2

Latent period - temperature relationship.

The log-linear plot of latent period duration with temperature shows that the latent period (LP) after stimulation with 10^{-3}M ACh decreases with increasing temperature, from 7.3 $\pm$ 1.3 secs at 2^{o}C to 1.25 $\pm$ 0.25 secs at 30^{o}C.

Each point represents the mean of seven observations, and the vertical bars the standard error of the mean. The line was fitted by eye.
Muscle length: 0.8 L_{o}.


10
8
6
4
2
1
Time (secs)
0
10
20
30°C
Temperature

Rate of Tension Development (dP/dt)

The log-linear plot of dP/dt against temperature (Fig. 4:3), shows three distinct phases, from 2° - 25°, 25° - 35° and 35° - 45°C, with respective slopes of 0.0032 ± 0.0014; 0.035 ± 0.004 and 0.000 ± 0.0023.

Peak Tension

Peak tension, in response to 10^{-3} M ACh, is inversely related to temperature i.e., the muscle shows a negative temperature coefficient. The reduction in P_{max} associated with an increase in temperature can also be resolved into three portions; The first extends from 2° - 25°C where the reduction in P_{max} is of the order of 0.11 ± 0.003 Kg/°C, the second from 25° - 40°C (0.06 ± 0.006 Kg/°C), and the third above 40°C (0.18 ± 0.002 Kg/°C), (Fig.4:4).

Relaxation

In the absence of 5HT, relaxation from an ACh-induced contracture occurs slowly and shows a positive temperature relationship. At 2°C the mean relaxation half time ($t_{\frac{1}{2}}$) is 805 ± 60 seconds, and at 20°C it decreases to 500 ± 62 seconds. As the temperature of the contracture-relaxation cycle is increased from 20 - 30°C the relaxation half-time decreases ten-fold, from 500 seconds at the lower temperature to 50 seconds at the upper temperature. When the relaxation $t_{\frac{1}{2}}$ becomes faster than approximately 200 seconds the

Fig 4.3

Relationship between temperature and ACh-induced tension development.

The rate of ACh-induced tension development increases with increasing temperature. The log-linear plot shows three distinct phases, between 2 - 25°C, 25 - 35°C and 35 - 45°C.

Each point represents the mean of six observations and the vertical bars the standard error of the mean.

Muscle length was 0.8 L_o.

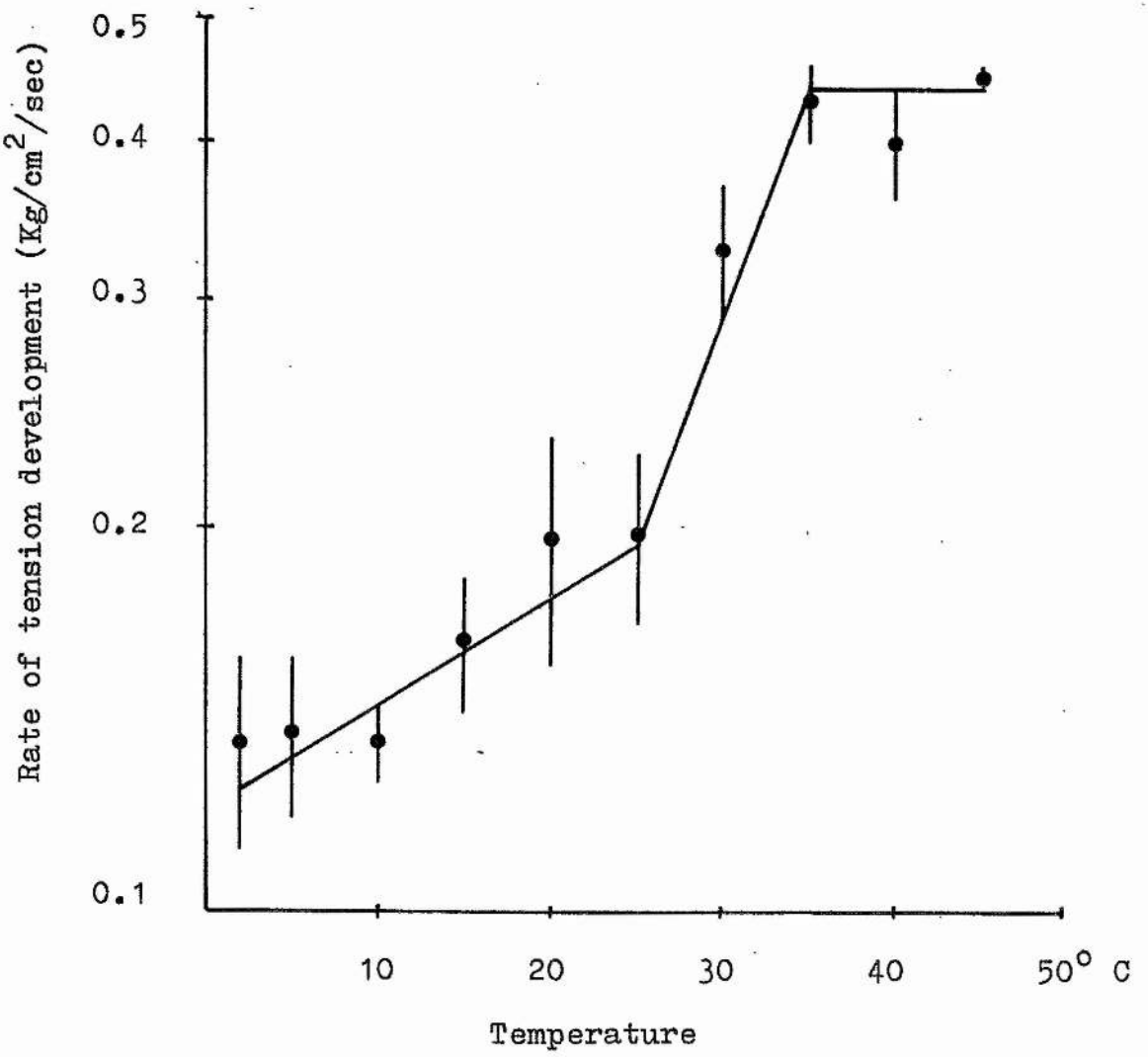

Rate of tension development (Kg/cm^2/sec)
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50° C
Temperature

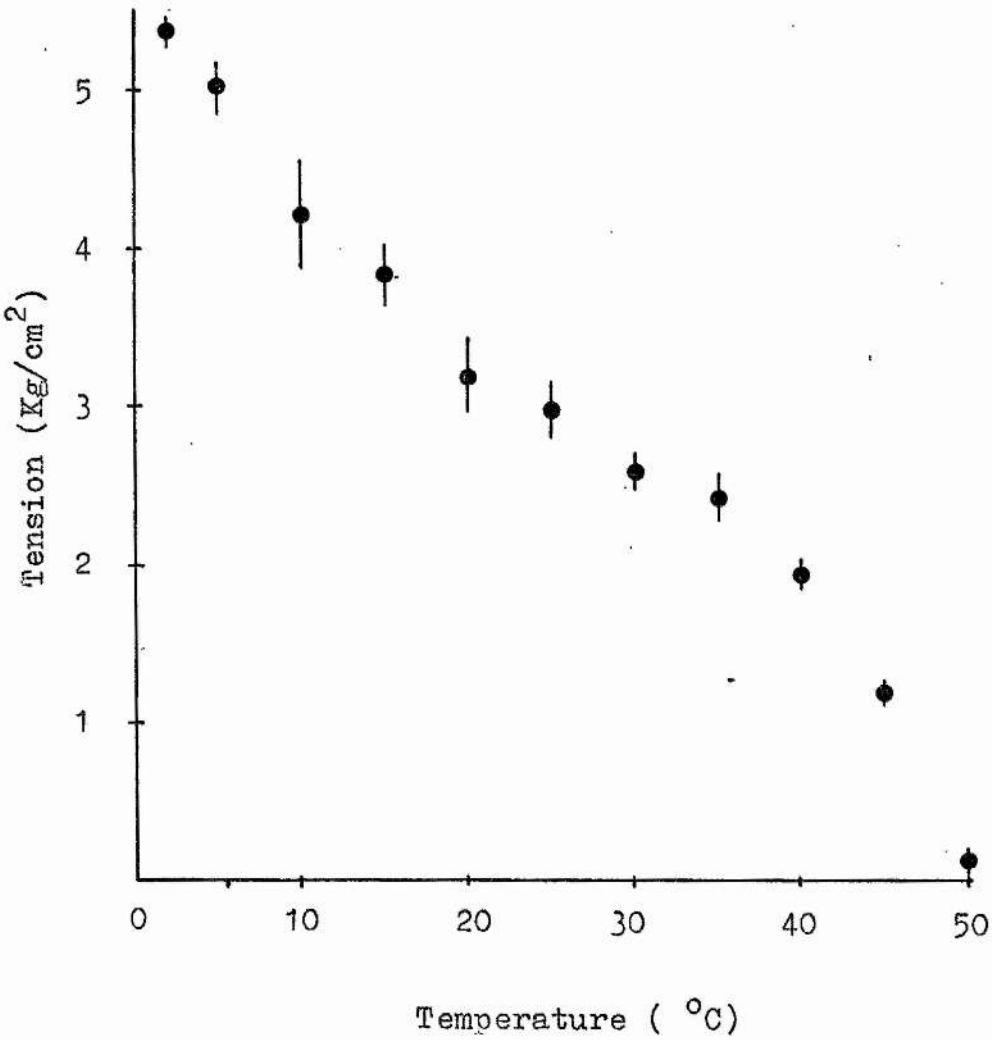
Fig 4.4

Relationship between ACh-induced tension and temperature.

Peak ACh-induced tension decreases as the temperature is increased, from 5.39 ± 0.12 Kg/cm^2 at 2°C to 1.2 ± 0.049 Kg/cm^2 at 45°C.

Each point represents the mean of seven observations and the vertical bars the standard error of the mean.

Muscle length: 0.8 L_o.


Tension (Kg/cm^2)
5
4
3
2
1
0
10
20
30
40
50
Temperature (°C)

muscle is said to relax spontaneously. Above 30°C the relaxation half-time decreases by 2.5 seconds/°C, (Fig. 4:5).

5HT-Induced Relaxation

At temperatures below 30°C, 5HT application markedly increases the relaxation rate; the half-time of 5HT relaxation is 240 ± 20 seconds at 2°C, and 43.6 ± 4 seconds at 20°C (Q_{10} 3.5 2 - 25°C - Fig. 4:6). At temperatures above 27° ± 3°C the relaxation $t_{\frac{1}{2}}$ of 5HT and spontaneous relaxation approach one another (5HT $t_{\frac{1}{2}}$ at 30°C is 43.6 ± 2.6 seconds; spontaneous relaxation $t_{\frac{1}{2}}$ is 48.8 ± 5 seconds).

K-Contractures

It may also be noted here that K-contractures initiated in the ABRM also show a negative temperature co-efficient. Over the range 20 - 2°C the size of the response approximately doubles in association with a decrease in the rate of tension development and relaxation. In contrast to the findings reported here Caputo (1972) found that K-contractures in the frog sartorious were greatly prolonged at lower temperatures, but maximum contracture tension decreased by 15% between 20°C and 3°C.

Fig 4.5

The effect of temperature on the relaxation half-time ($t_{\frac{1}{2}}$) of the ACh-induced tension.

The time to 50% relaxation ($t_{\frac{1}{2}}$) after the addition of 10^{-3}M ACh decreases with increasing temperature, from 805 $\pm$ 60 secs at 2°C to 9 $\pm$ 1.2 secs at 45°C.

Each point represents the mean of six observations and the vertical bars the standard error of the mean.

Muscle length: 0.8 L_o.

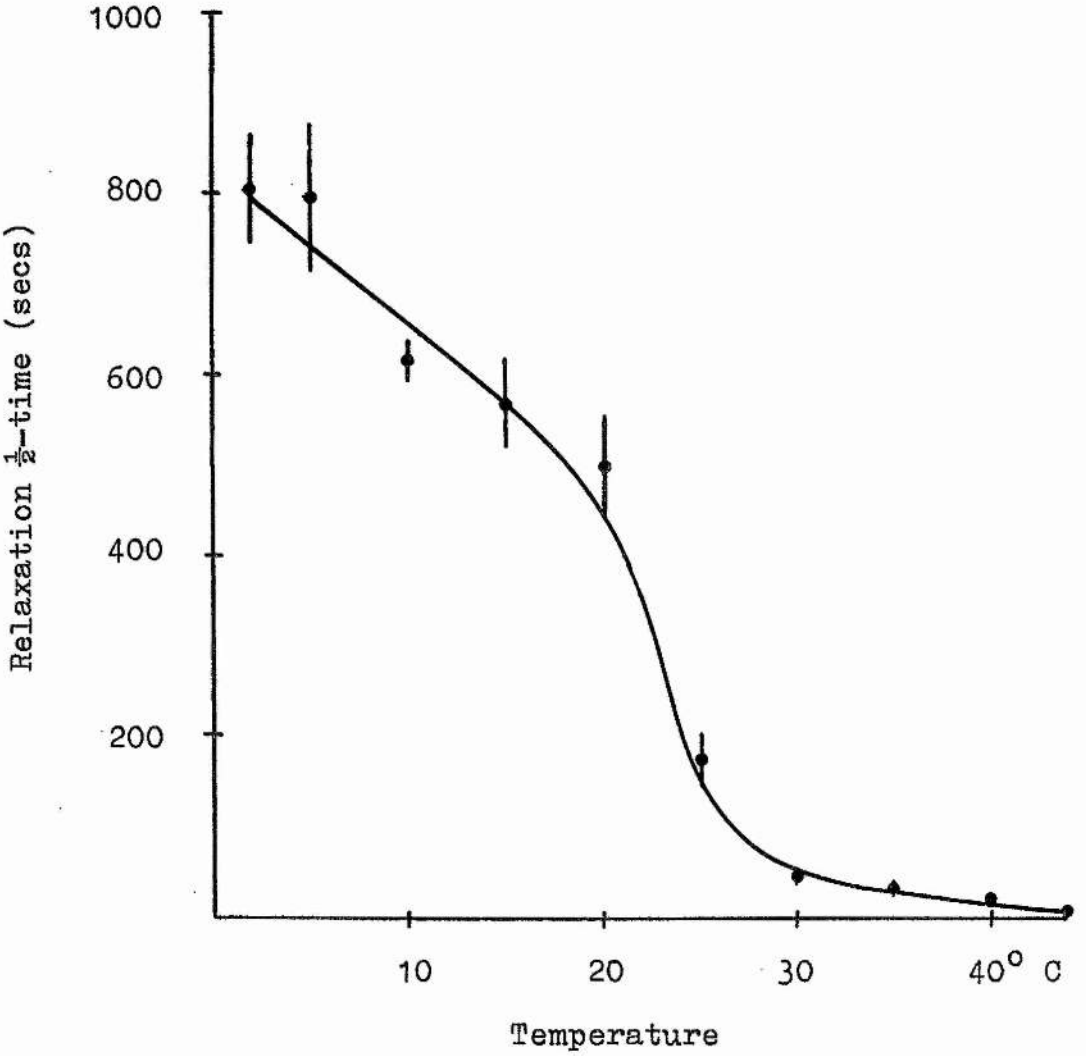

1000
800
600
400
200
Relaxation ½-time (secs)
10
20
30
40° C
Temperature

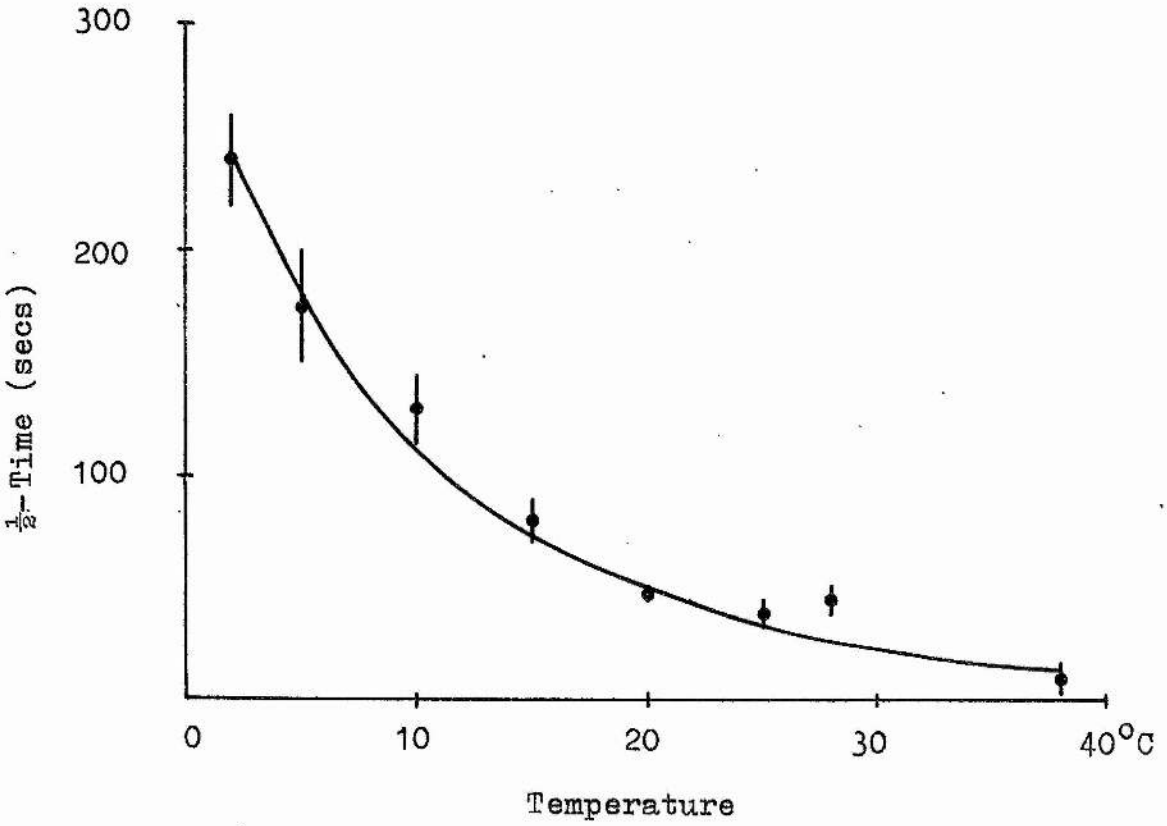
Fig 4.6

The effect of temperature on 5HT-induced relaxation.

The time to 50% relaxation ($t_{\frac{1}{2}}$) in response to 10^{-6} M 5HT decreases as temperature increases, from 240 $\pm$ 20 secs at 2°C to 10 $\pm$ 4 secs at 40°C.

Each point represents the mean of six observations, and the vertical bars the standard error of the mean.

Muscle length: 0.8 L_o.


300
200
100
$\frac{1}{2}$- Time (secs)
0
10
20
30
40°C
Temperature

Effect of a cold shock applied during an ACh-induced contracture-relaxation cycle

If an ABRM, undergoing a normal isometric contraction-relaxation cycle (see below), is suddenly transferred to a colder sea water (SW) solution, in the presence or absence of ACh, an increase in tension occurs; the cold induced contracture (CIC). A normal isometric contraction-relaxation cycle is shown in Fig. 4:1. The muscle shown was treated with 10^{-3} M ACh at 20°C. After two minutes it was transferred to a SW solution also at 20°C, then, after a further 6 minutes 10^{-6} M 5HT was applied and rapid relaxation ensued. In Fig. 4:7, the same initial procedure was adopted, but at 2 minutes a cold shock was applied by transferring the preparation to pre-cooled SW at 2°C. The absolute tension increment was obtained by subtracting the tension response of control contractures from that developed after the application of cold shock (Fig. 3:3). Cold shock solutions which did not contain ACh were used since the presence of stimulant slowed CIC relaxation by approximately 30 ± 5%. In The presence of ACh in the cold shock solution did not alter either the size, or the rate of tension development.

The application of a cold shock does not always result in a CIC:

Fig 4.7

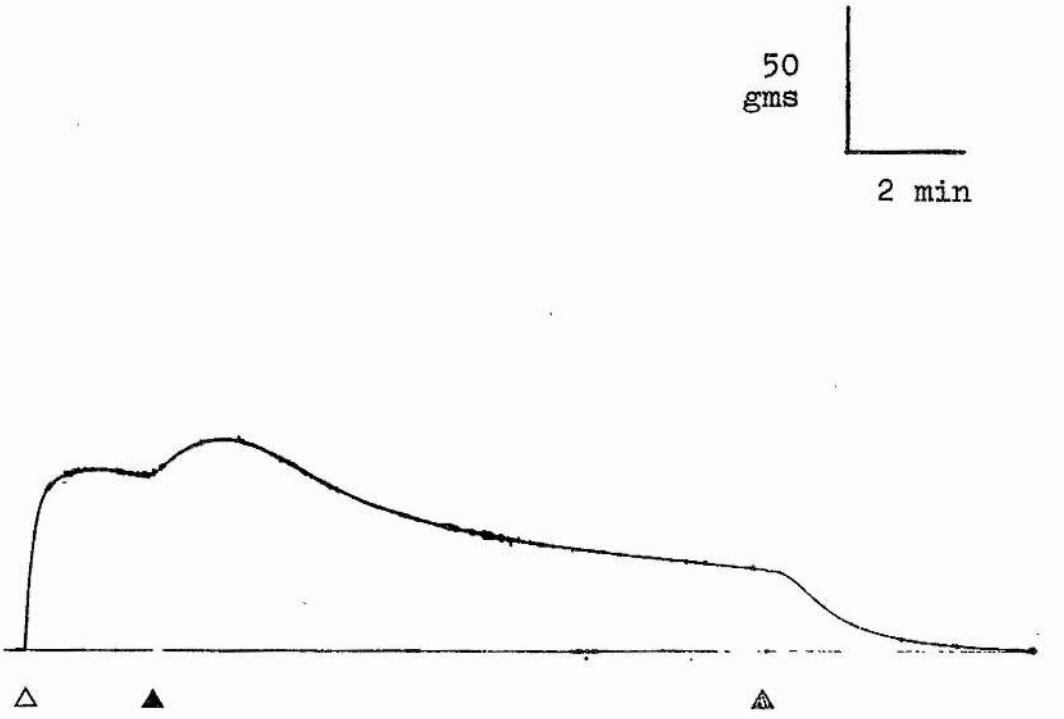
A typical CIC produced by a ΔT of $18^{\circ}C$.

Δ : point of application of 10^{-3}M ACh at $20^{\circ}C$

$\blacktriangle$: point of addition of cold shock solution at $2^{\circ}C$, and removal of ACh

$\blacktriangle$: point of application of 10^{-6}M 5HT

Muscle length: 0.8 L_o.


50
gms
2 min

Firstly, cold shock is without effect on a resting muscle. Secondly, there is no response during a catch contracture, but when a muscle is 'brought out' of catch by the application of 5HT, dopamine or a SW solution above 30°C, cold shock will once again produce a CIC (Fig. 4:8). This is somewhat smaller than that produced after ACh (64 ± 8%), and it relaxes more rapidly. Presumably this is due to the relaxant effect of these substances opposing the reaction/s responsible for the CIC.

Fig 4.8

The effect of cold shock on a muscle undergoing 5HT relaxation.

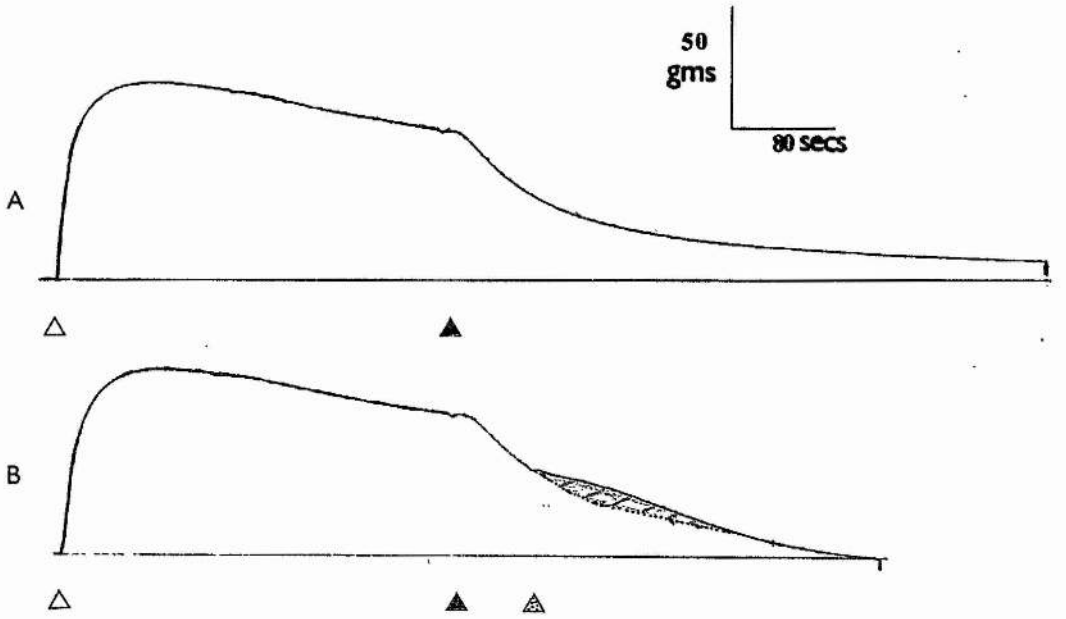
△ : point of addition of 10^{-3}M ACh at 20°C

▲ : point of addition of 10^{-6}M 5HT at 20°C

◬ : point of application of cold shock solution at 2°C (B only).

The shaded area indicates the tension increment in response to cold shock.

Muscle length: 0.8 L_o.


50
gms
80 secs
A
B

PROPERTIES OF THE CIC

Evidence that the CIC is not a wash-out contracture

Ohlin and Stromblad (1963) have described, in the vas deferens a short-lasting contracture, which occurs when stimulant drugs (ACh, Adr or histamine) are removed from the bathing medium. This is, however, different from the CIC for, if ACh is applied to an ABRM at 20°C and the muscle is subsequently transferred to SW at 20°C, no change in tension is observed. Spontaneous wash-out contractures were observed in some preparations but these only occurred when a muscle had been damaged during dissection, over-stimulation, or taken to temperatures above 40°C.

The Dependence of the CIC on ACh

Because initially it appeared that a CIC could not be elicited in the absence of ACh, it was considered possible that the CIC represented an increase in sensitivity to ACh at the lower temperature. In order to test this hypothesis a dose-response curve for ACh was constructed, with the corresponding CIC's produced by a ΔT of 18°C (T_1 = 20°C). It was found that the CIC decreased in size in the same way as the ACh-induced tension suggesting that the former may be dependent on the level of activation of the muscle. The CIC reached its maximum amplitude at 10^{-3} M ACh as did isometric

tension, and it was absent at a concentration of 10^{-5} M ACh at which time the ACh-induced tension was still 10% of maximum (Fig. 4:9). Furthermore, when a muscle was rapidly cooled in the presence of a sub-threshold (5×10^{-5} M) acetylcholine concentration there was no apparent response.

Effect of altering the time of application of the cold shock

The size of the CIC is dependent on the time between t_o and the application of a cold shock. Fig 4:10 shows values for a ΔT of 18°C (20 - 2°C) applied at times ranging from t_o + 5 minutes to t_o + 33.5 minutes. The tension produced during a CIC is inversely related to time after t_o (Fig. 4:11). At the earliest time investigated (t_o + 1 minute) the CIC amounts to approximately 0.3 P_o (1.36 Kg/cm^2), and it decreases to a steady value of 0.11 P_o (0.6 Kg/cm^2) at t_o + 7 minutes. This level remains approximately constant over the range t_o + 7 minutes to t_o + 33.5 minutes (Fig. 4:10).

The usual time of application of the cold shock (indicated by the filled circle in Fig. 4:10) was t_o + 2 minutes. This ensured that the muscle had reached P_{max} (at temperatures from 5° - 45°C), and the CIC was still quite large, approximately 66% of maximum (0.9 Kg/cm^2).

Both dP/dt, and the relaxation rate decrease with increasing time after the stimulus. The former follows a similar pattern to

Fig 4.9

ACh dose-response curve.

• : ACh-induced tension at 20°C

o : CIC tension produced by a ΔT of 18°C (T_1=20°C)

Each point represents the mean of five observations and the vertical bars the standard error of the mean.

The curves were fitted by eye.

Muscle length: 0.8 L_o.

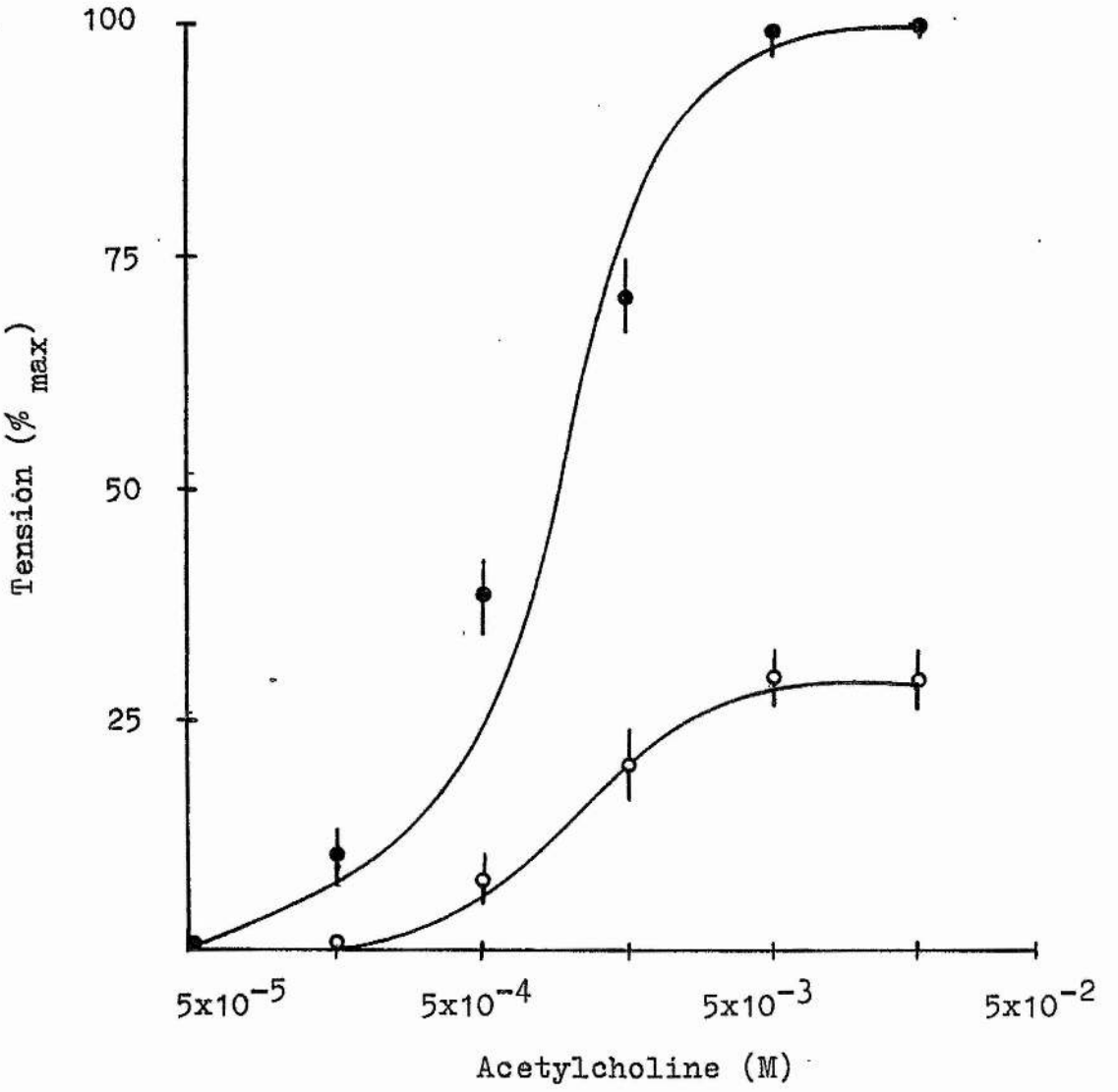

100
75
50
25
Tension (% max)
5x10-5
5x10-4
5x10-3
5x10-2
Acetylcholine (M)

Fig 4.10

CIC tension with respect to the time of application of cold shock after the addition of ACh.

As the time between the addition of 10^{-3}M ACh and the application of the cold shock solution increases the CIC decreases.

CIC tension (P) is expressed in terms of the ACh induced tension (P_o). 0.2 P/P_o is approximately equivalent to 0.9 Kg/cm^2.

The abscissa represents the time of addition of the cold shock solution (2^oC) after the application of ACh at 20^oC.

The ΔT used to elicit the CIC was 18^oC.

In all other experiments the cold shock solution was routinely applied at 120 secs, and this point is indicated by the filled circle.

Each point represents the mean of six experiments and the vertical bars the SE of the mean.

Muscle length: 0.8 L_o.

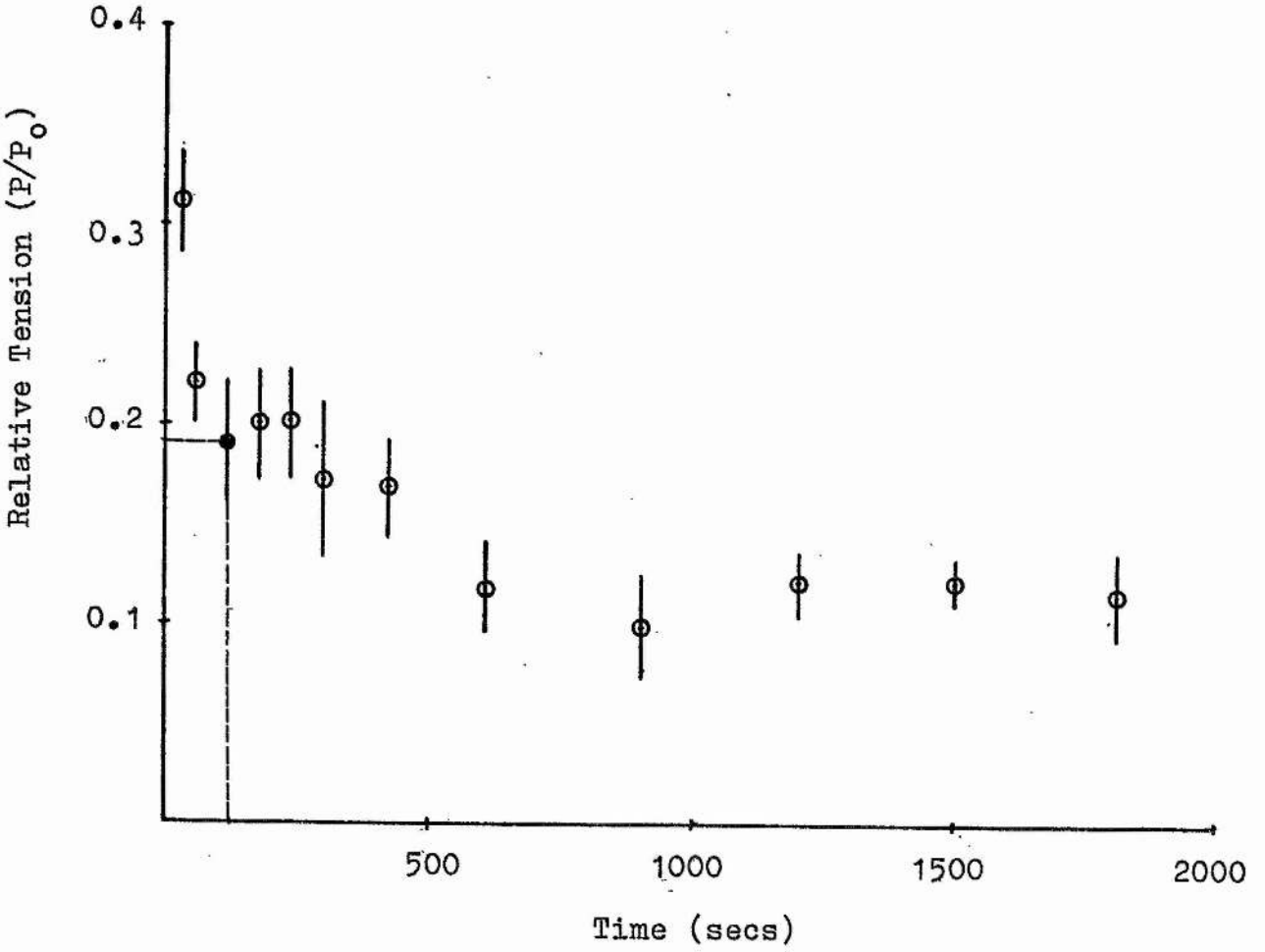

0.4
0.3
0.2
0.1
Relative Tension (P/P$_o$)
500
1000
1500
2000
Time (secs)

Fig 4.11

The effect, on the CIC, of applying a cold shock (ΔT 18°C) at increasing times after the addition of ACh.

△ : point of application of 10^{-3}M ACh at 20°C

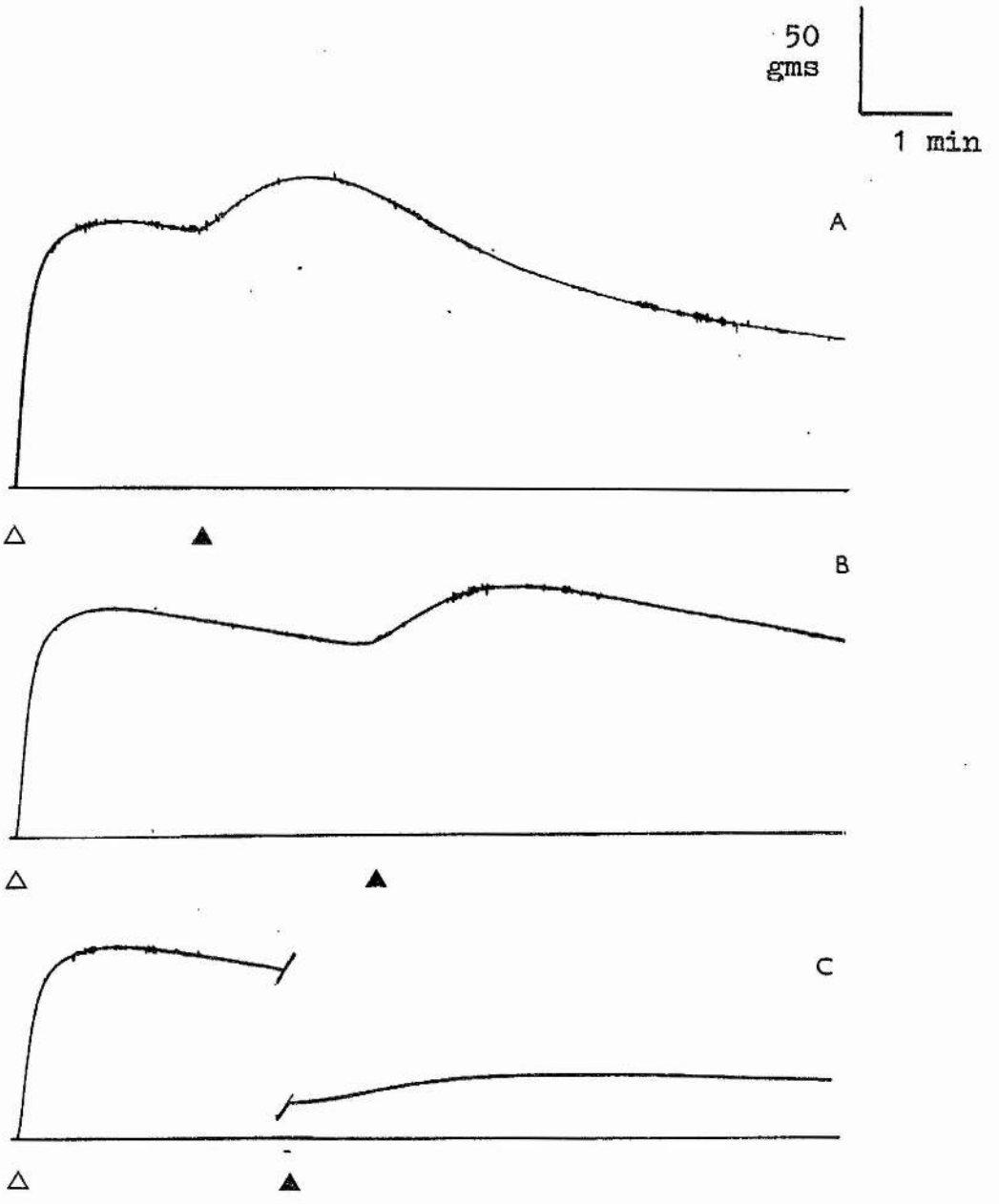
▲ : point of addition of cold shock solution at 2°C, and removal of ACh.

A : cold shock (2°C) applied 2 mins after the application of 10^{-3}M ACh at 20°C

B : cold shock (2°C) applied 4 mins after the application of 10^{-3}M ACh at 20°C

C : cold shock (2°C) applied 30 mins after the application of 10^{-3}M ACh at 20°C

Muscle length. 0.8 L_o.


50
gms
1 min
A
B
C

the absolute size of the CIC; dP/dt is reduced by 70% at t_o + 7 minutes and after this time the rate does not alter significantly ($p < 0.5$) (Fig. 4:12). In contrast, relaxation $t_{\frac{1}{2}}$ shows a progressive four-fold increase over the whole experimental range (Fig. 4:13).

CIC in relation to contractile activity

As active state (activity of the contractile element) and tension produced after cold shock both decrease with time after the stimulus, consideration was given to the possibility that these processes might be related. As the temperature is decreased the time course of the active state is prolonged as is known to occur in skeletal muscle where at 2°C the active state lasts twice as long as at 20°C (MacPherson and Wilkie, 1954). The CIC, therefore, might be produced by a transition of the muscle from the level of contractile activity at the upper temperature to the higher level of contractile activity at the lower temperature. If this were the case then the size of the CIC at each time after the stimulus would be equal to the difference in active state estimations. Active state curves were therefore constructed for 2°C and 20°C, and used to predict the size of the CIC. The method used to estimate the active state level was that of Ritchie (Ritchie 1954 b).

Fig 4.12

The effect of altering the time of application of cold shock on the rate of tension development of the CIC.

The rate of tension development (dP/dt) of the CIC decreases with time after the application of ACh up to approximately 420 secs after which time there is little alteration over the experimental range.

The abscissa indicates time of addition of cold shock after the application of 10^{-3}M ACh.

The CIC was elicited by a ΔT of $18^{o}C$ ($T_1 = 20^{o}$)

Each point represents the mean of five observations and the vertical bars the standard error of the mean.

Muscle length: $0.8\ L_o$.

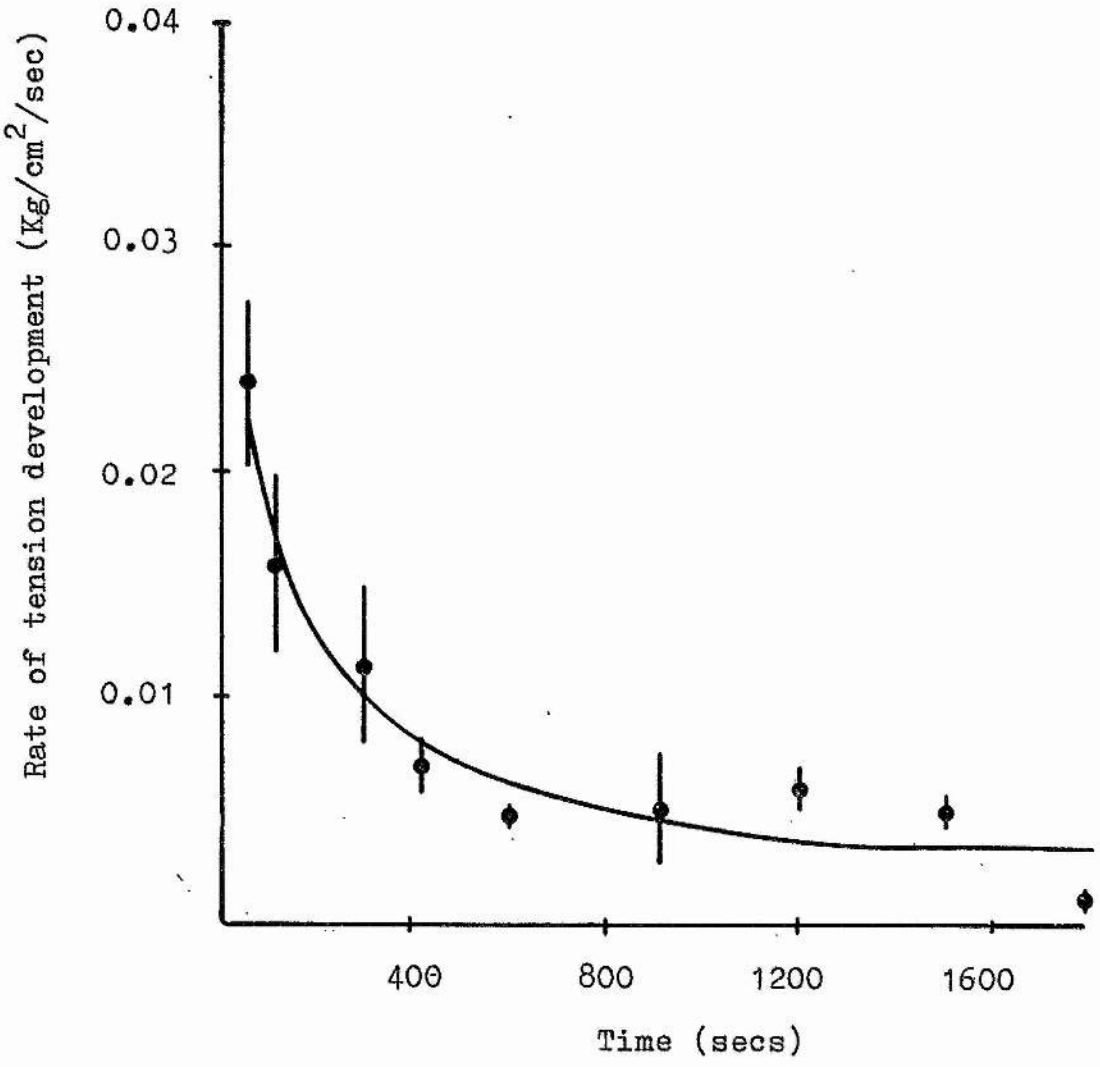

0.04
0.03
0.02
0.01
Rate of tension development (Kg/cm^2/sec)
400
800
1200
1600
Time (secs)

Fig 4.13

The effect on the CIC relaxation $t_{\frac{1}{2}}$ of applying a cold shock at varying times after the addition of 10^{-3}M ACh.

The time to 50% CIC relaxation ($t_{\frac{1}{2}}$) increases four-fold as the time interval between the application of 10^{-3}M ACh and the cold shock increases from 60 secs to 1800 secs.

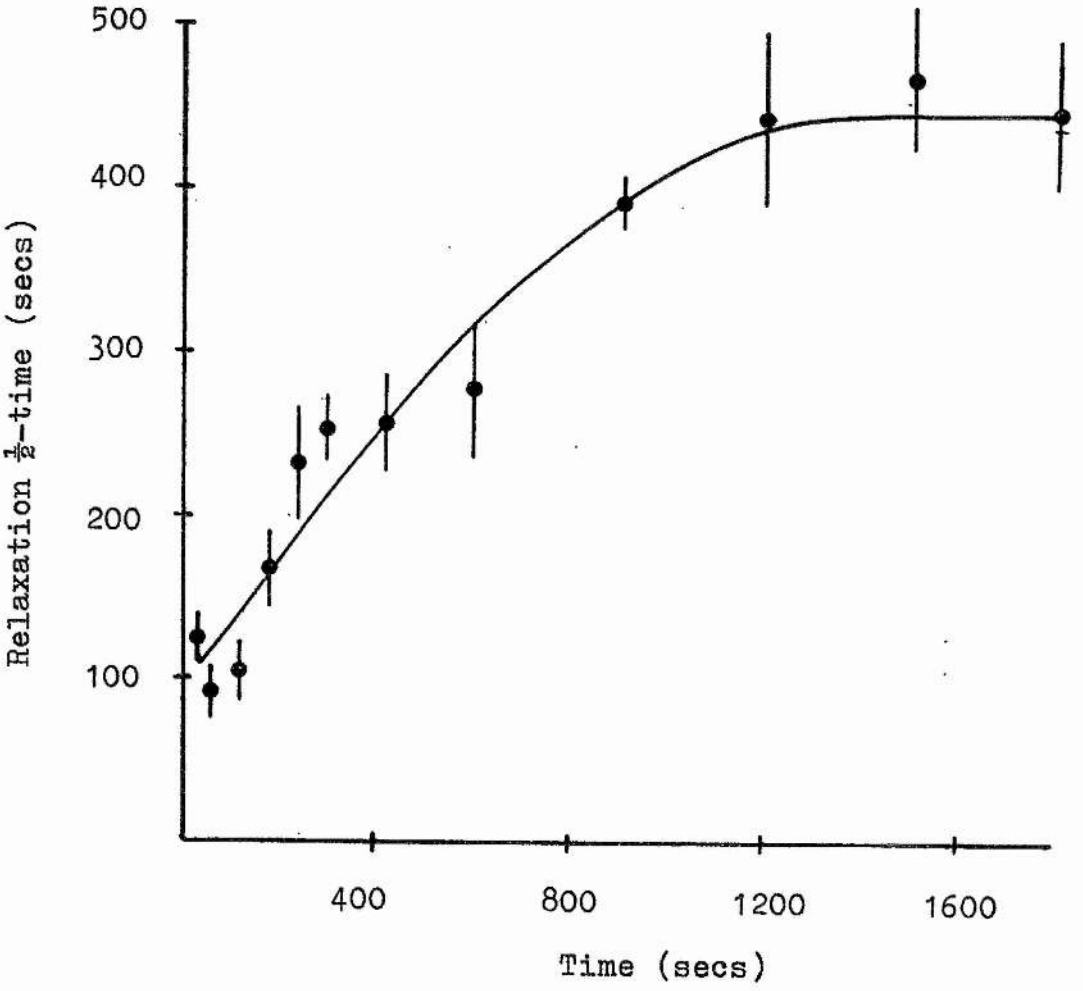
The ordinate shows the $t_{\frac{1}{2}}$ expressed as a % of the initial value obtained at 60 secs (100 % value).

The abscissa indicates time after the addition of ACh.

Each point represents the mean of five observations and the vertical bars represent the standard error of the mean. The curve was fitted by eye.

The CIC was initiated by a ΔT of $18^{o}C$ ($T_1=20^{o}C$).

The muscle length was 0.8 L_o.


500
400
300
200
100
Relaxation ½-time (secs)
400
800
1200
1600
Time (secs)

In this method the intensity of active state, present at any instant after the stimulus, is measured by the tension re-developed after a quick release in which tension falls to zero. In striated muscle, active state usually reaches its full extent immediately after the end of the latent period, while the contractile response, although following the active state curve lags behind the latter because of the presence of the series elastic component. (SEC)

The situation in the ABRM is a little more complicated for here tension held is not always related to active state. When a muscle is in catch the tension held is passive and active state may be either absent, or at a very low level (Baguet and Gillis, 1968). Nevertheless, Ritchies quick release method may be used to observe the decay in contractility of the muscle as it enters the catch state. (Twarog and Johnson, 1960 - Fig. 4:14). It was found that, after 500 seconds at 20°C, the contractile activity had fallen to 10% of maximum, while the same level was reached in approximately twice that time at 2°C (Figs. 4:15 and 4:16). From these graphs values were predicted for the size of the CIC, by subtracting the active state value at 20°C from that at 2°C. It was found that the experimental and calculated values were not linearly related (Fig. 4:17), indicating that the CIC does not represent the difference

Fig 4.14

Tension re-development after a quick release.

△ : point of application of 10^{-3}M ACh at 20°C

▲ : point of addition of 10^{-6}M 5HT at 20°C

A : release 1 min after the additon of 10^{-3}M ACh

B : release 2 min after the addition of 10^{-3}M ACh

C : release 5 min after the addition of 10^{-3}M ACh

D : release 7 min after the addition of 10^{-3}M ACh

Muscle length: 0.8 L_o.

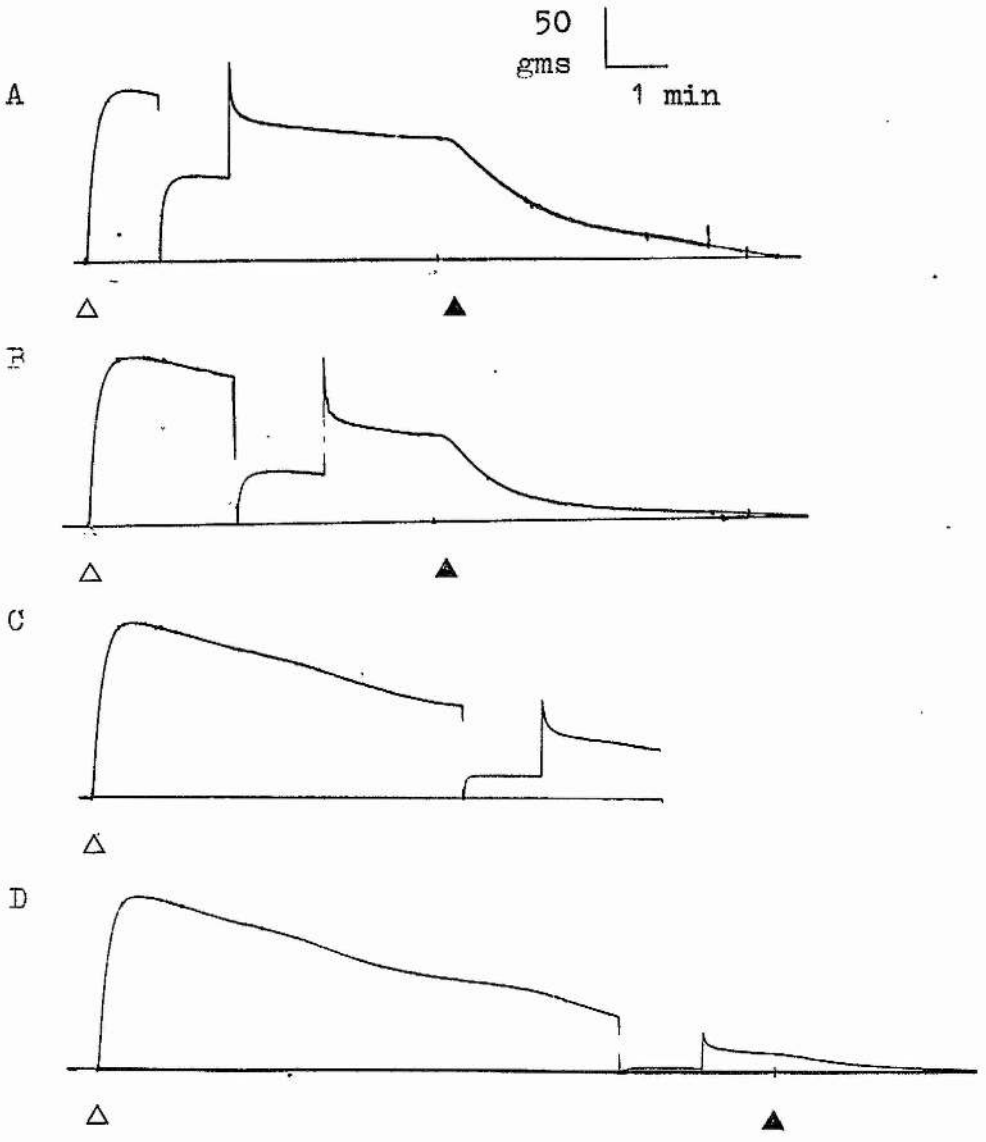

50
gms
1 min
A
B
C
D

Fig 4.15

Decay of contractility of the ABRM at 20°C.

The ordinate shows the tension re-developed at 20°C, after a quick release, as a % of the isometric tension level at the new muscle length (see Materials and Methods P. 30).

The abscissa indicates the time of the release after the addition of 10^{-3}M ACh.

Each point represents one observation. The line was fitted by eye.

Muscle length: 0.8 L_o.

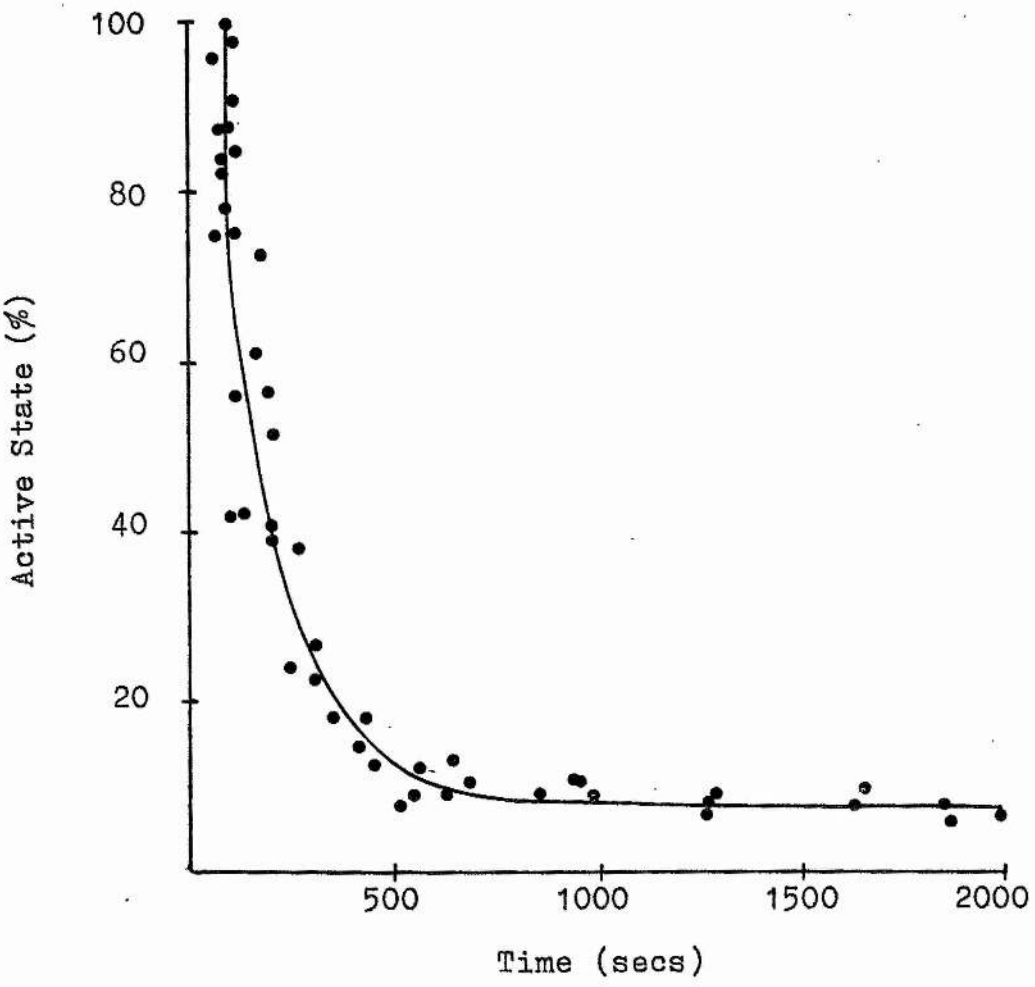

100
80
60
40
20
Active State (%)
500
1000
1500
2000
Time (secs)

Fig 4.16

Decay of contractility of the ABRM at $2^{o}C$.

The ordinate shows the tension redeveloped after a quick release, at $2^{o}C$, as a % of the isometric tension at the new muscle length (see Materials and Methods P. 30).

The abscissa shows the time, after the application of 10^{-3} M ACh, at which the muscle was released.

Each point represents one observation. The line was fitted by eye.

Muscle length: 0.8 L_{o}.

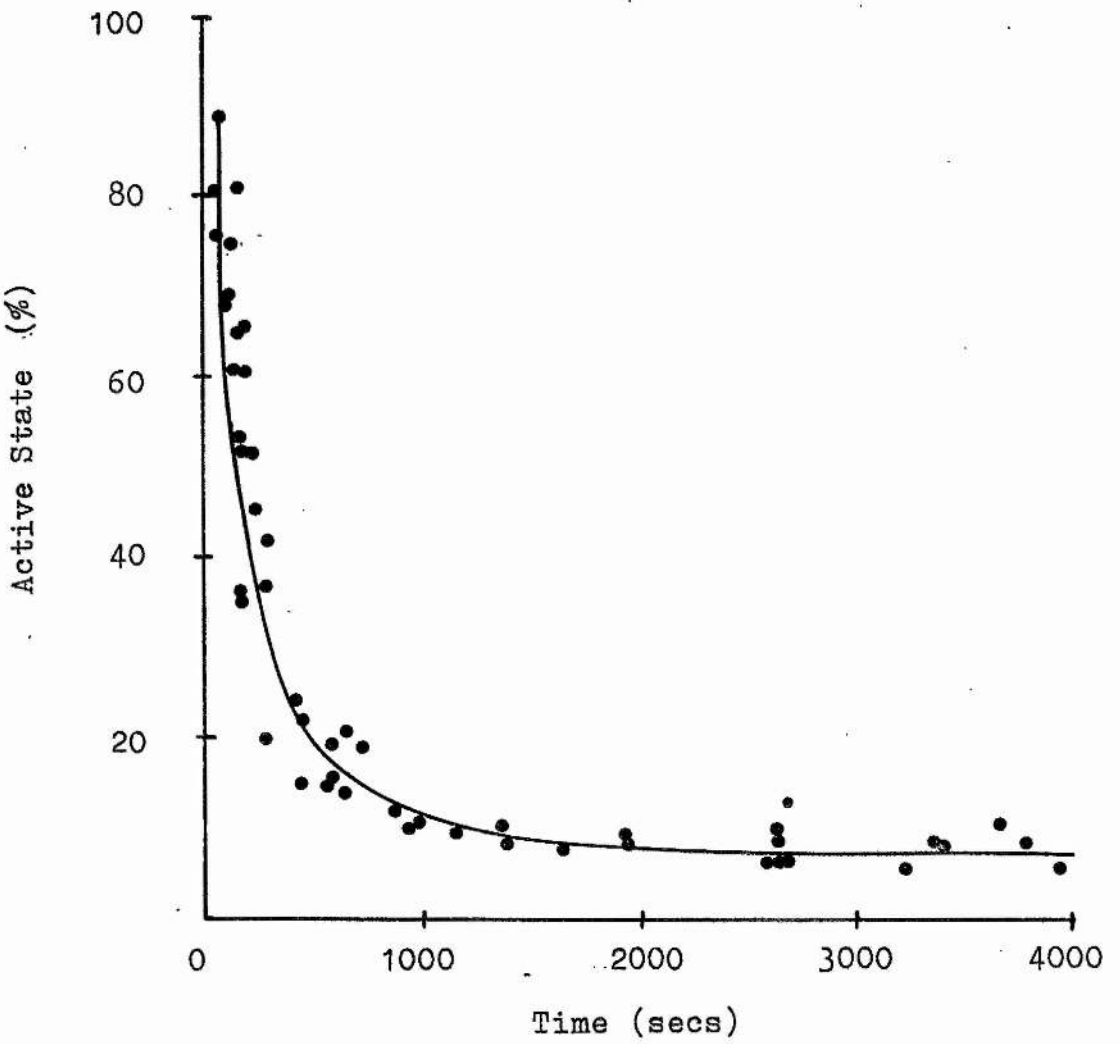

100
80
60
40
20
Active State (%)
0
1000
2000
3000
4000
Time (secs)

Fig 4.17

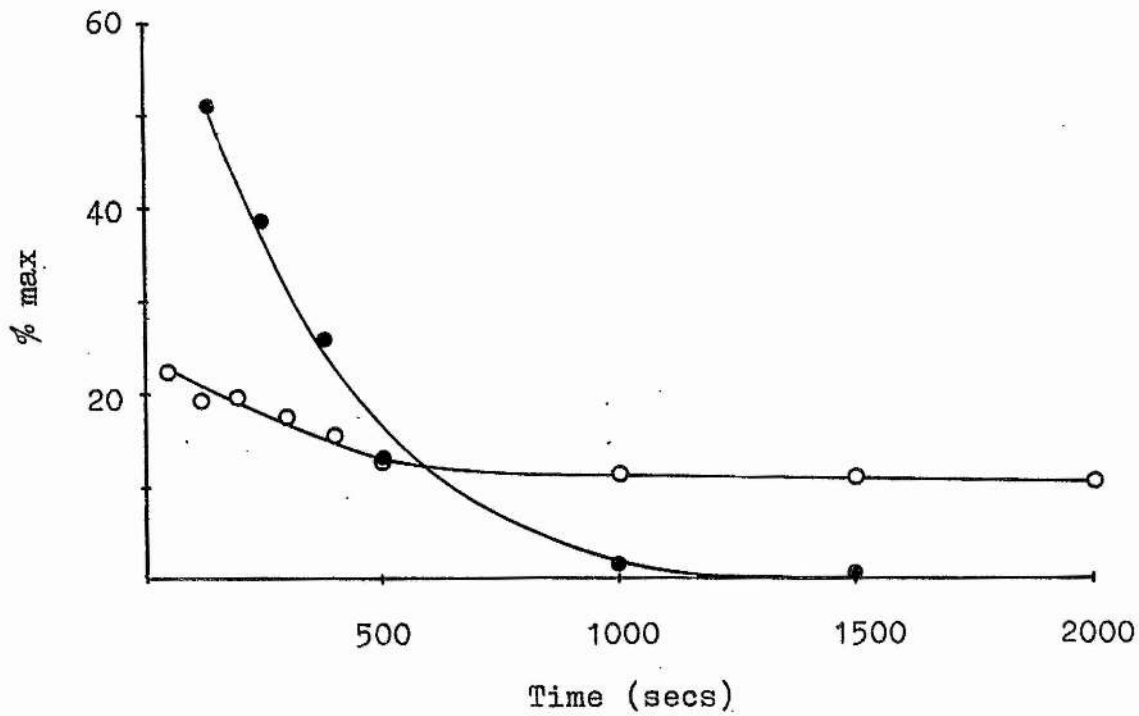
Predicted and actual values for the CIC tension at various times after the stimulus.

- • : difference between active state values at $2^{\circ}C$ and $20^{\circ}C$ (predicted value)
- o : CIC tension

CIC tension is expressed as a % of the ACh-induced tension maximum.

The difference in active state levels is expressed as a % of the maximum active state level.

The abscissa represents time after the application of 10^{-3} M ACh.


60
40
20
% max
500
1000
1500
2000
Time (secs)

in contractility of the muscle, at the two temperatures.

Further evidence against this hypothesis is the observation that the tension observed after a cold shock (ΔT 18°C) does not approach that attained during ACh-induced tension at the lower temperature (T_2 - Fig. 4:18 and 4:19). Presumably, this is because the contractile activity has fallen to 55% of maximum at this time and the increase in active state level at CIC_{max} is only 12 ± 2%.

Effect of Changing Muscle Length on the ACh-induced and CIC tensions

The relationship between muscle length and the size of the CIC was also investigated (Fig. 4:20). The CIC was found not to follow the isometric length-tension relationship. Over the experimental range (0.5 - 1.3 L_o) the CIC shows an inverse tension-length relationship (Fig. 4:21). This is associated with a linear decrease in dP/dt which is reduced from 0.021 Kg/cm^2/sec at 0.5 L_o to 0.002 Kg/cm^2/sec at 1.2 L_o. The rate of relaxation did not alter significantly ($p > 0.5$), over the range 0.5 - 1.0 L_o, while above this length there may have been a slight decrease in relaxation half-time but the small size of the CIC made analysis impossible. This was in contrast to the ACh-induced reponse which exhibited its maximum tension between 0.9 - 1.2 L_o and, on either side of this optimum, deceased almost linearly (Fig. 4:22).

Fig 4.18

A comparison of an ACh induced tension response at $2^{o}C$ with a CIC produced by a cold shock solution at $2^{o}C$ (ΔT $18^{o}C$).

Δ : point of application of $10^{-3}M$ ACh

$\blacktriangle$: point of application of cold shock solution at $2^{o}C$ (B only), and removal of ACh.

A : shows the re-drawn tension response to $10^{-3}M$ ACh at $2^{o}C$.

B : shows the re-drawn tension response to $10^{-3}M$ ACh at $20^{o}C$. Two minutes after the application of ACh a cold shock solution was applied.

Muscle length: $0.8\ L_{o}$.

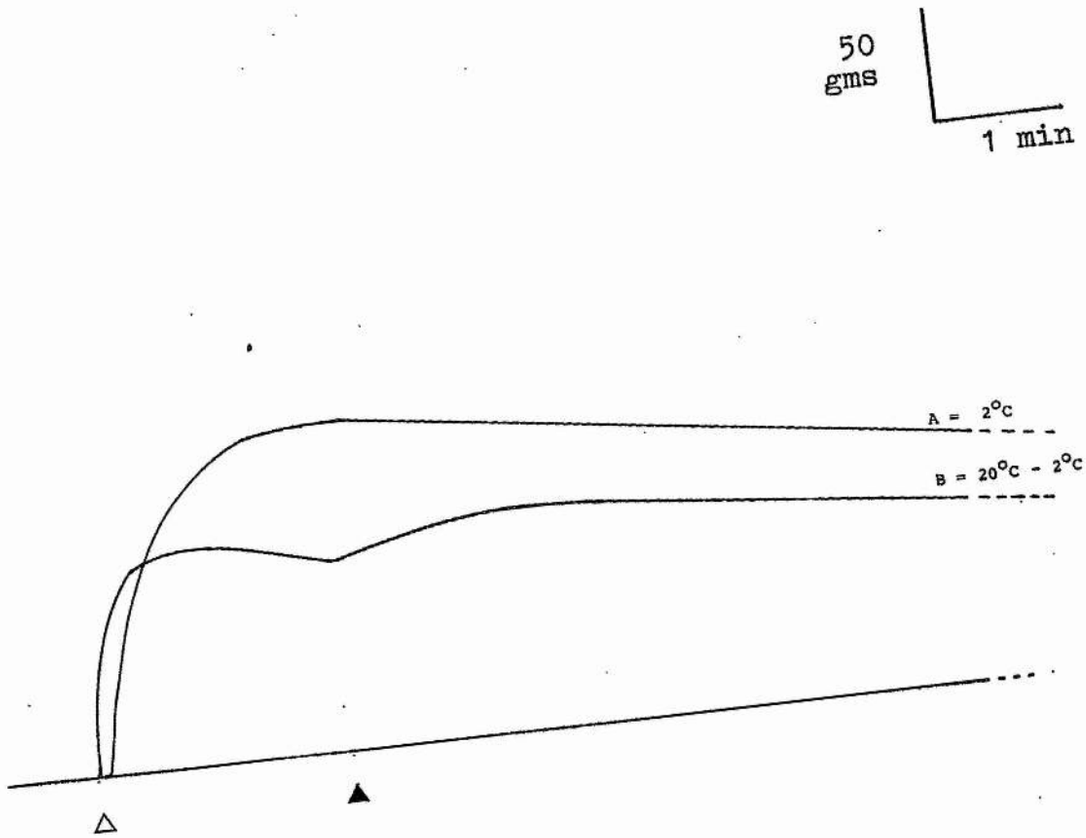

50
gms
1 min
A = 2°C
B = 20°C - 2°C

Fig 4.19

Total tension at CIC_{max} does not attain the isometric tension produced by 10^{-3}M ACh at the same temperature.

The ordinate represents total tension (remaining ACh induced tension plus total CIC tension), as a % of the ACh-induced tension at 2°C.

T_2 remains constant at 2°C, while T_1 varies between 5°C and 45°C.

Each point represents the mean of five observations, and the vertical bars the standard error of the mean.

The line was fitted by eye.

Muscle length: 0.8 L_o.

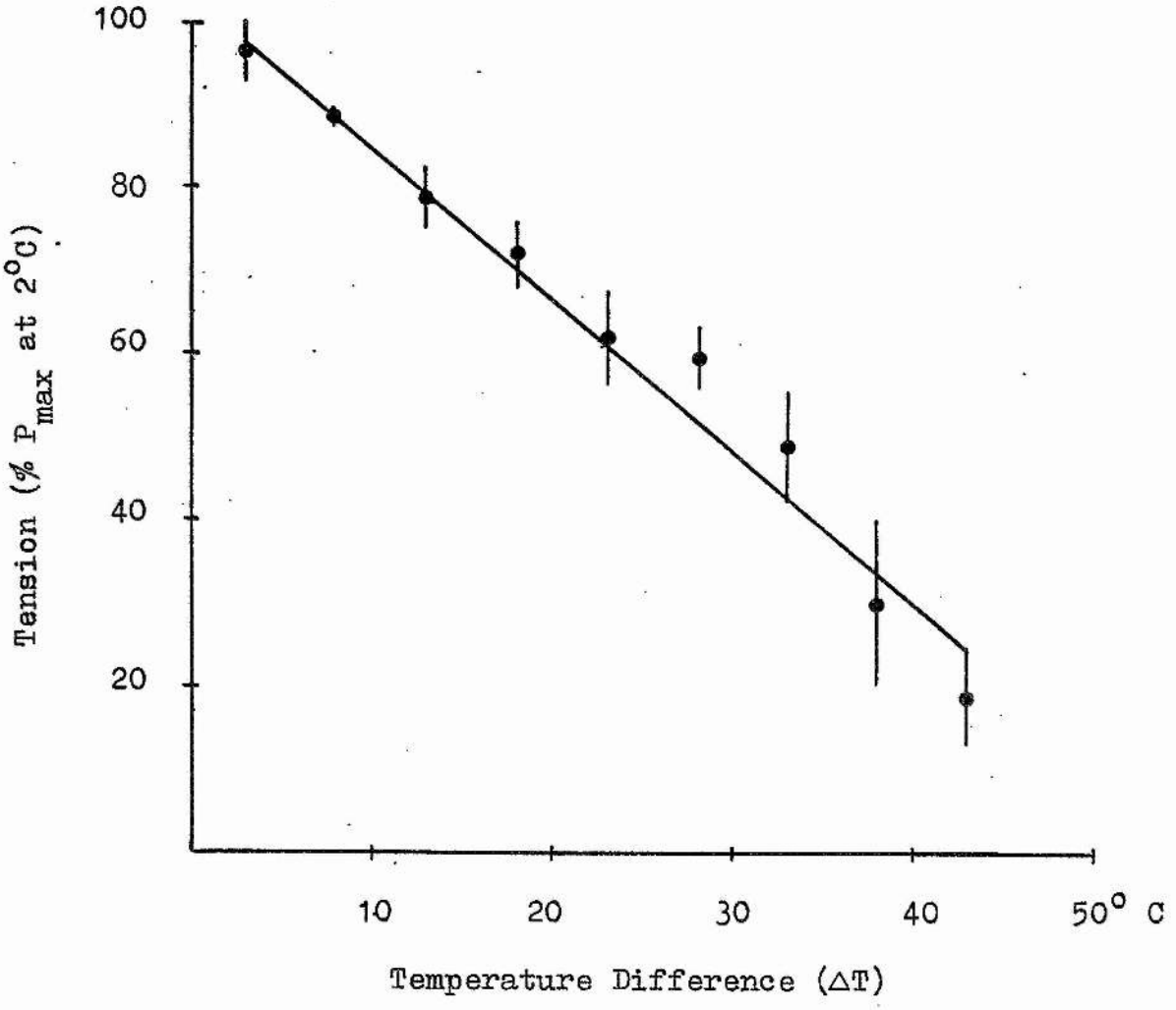

100
80
60
40
20
Tension (% P_{max} at 2°C)
10
20
30
40
50° C
Temperature Difference (ΔT)

Fig 4.20

Effect of muscle length on ACh-induced and CIC tensions.

△ : point of application of ACh (10^{-3}M) at 20°C

▲ : point of addition of cold shock solution at 2°C (ΔT 18°C T_1=20°C), and removal of ACh

A : Muscle length 0.5 L_o

B : Muscle length 0.7 L_o

C : Muscle length 0.8 L_o

D : Muscle length 1.2 L_o (time base is twice that shown in scale).

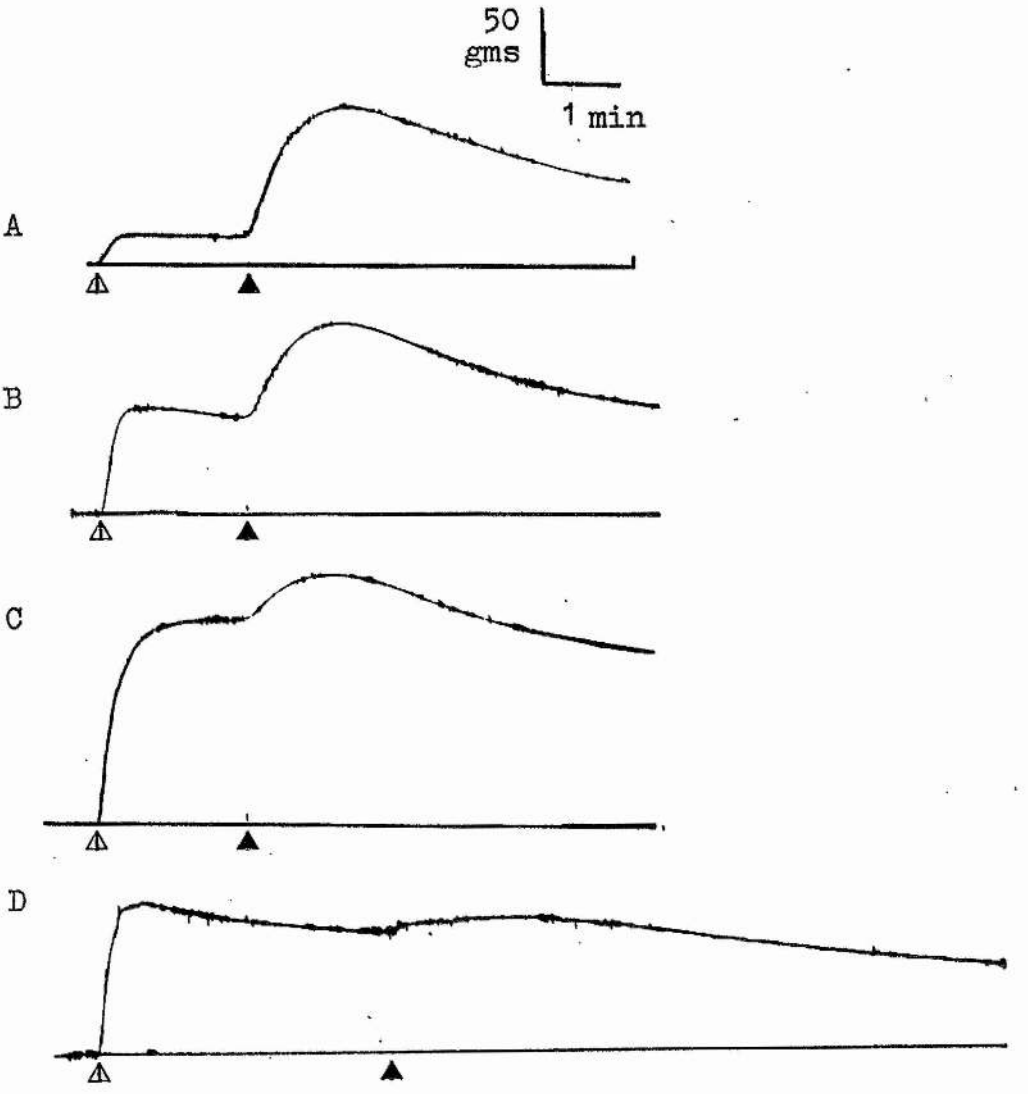

50
gms
1 min
A
B
C
D

Fig 4.21

Relationship between muscle length and CIC tension.

The CIC tension is expressed as a fraction of the maximum CIC tension (P_o). Muscle length is expressed as a multiple of L_o the <u>in situ</u> resting length.

The CIC shows an inverse tension-length relationship over the range 0.5 - 1.2 L_o.

The CIC was produced by a ΔT of 18°C (T_1=20°C).

Each point represents the mean of six experiments and the vertical bars the standard error of the mean.

The line was fitted by eye.

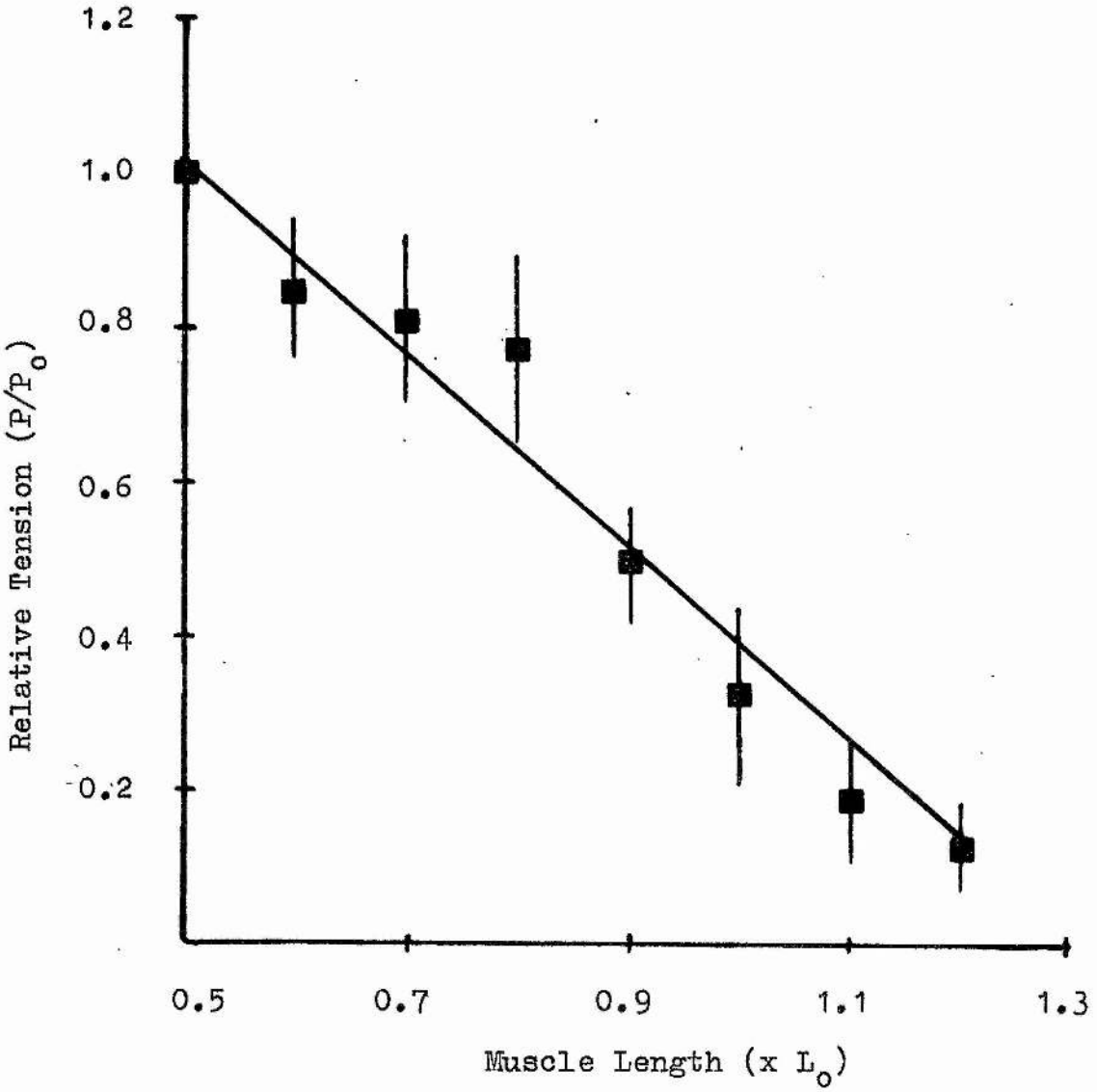

1.2
1.0
0.8
0.6
0.4
0.2
Relative Tension (P/P$_o$)
0.5
0.7
0.9
1.1
1.3
Muscle Length (x L$_o$)

Fig 4.22

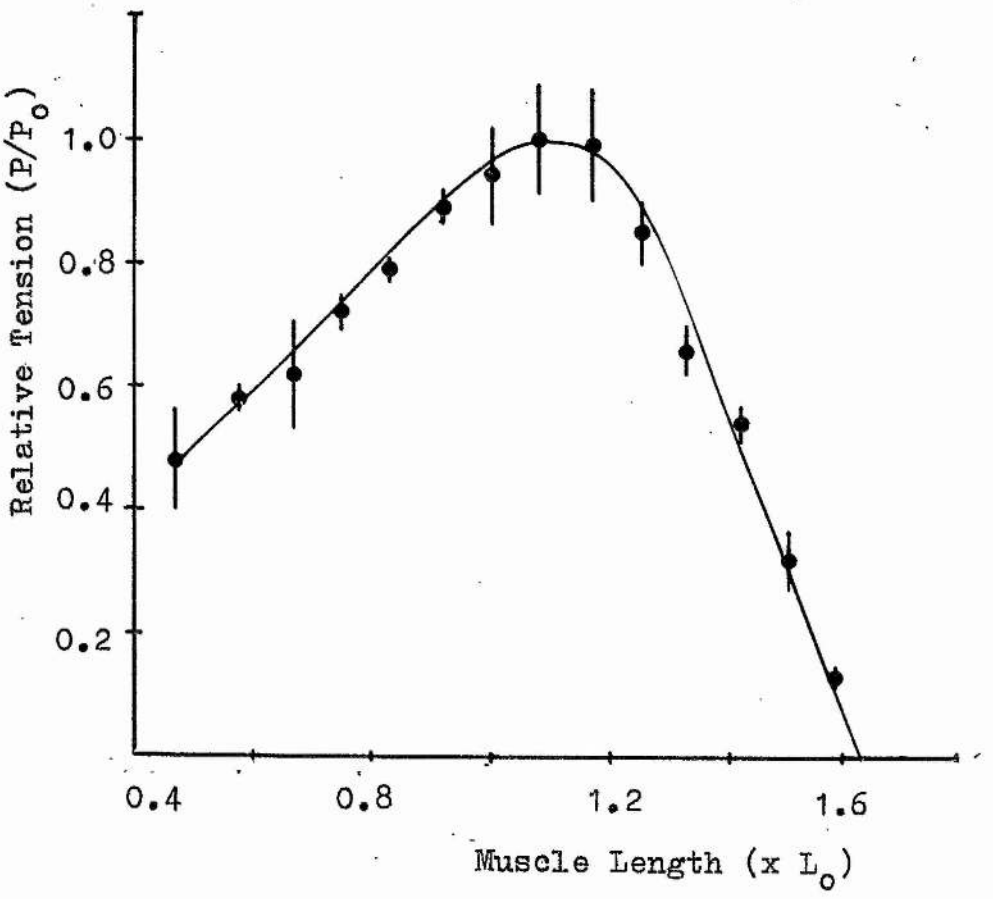
The length dependance of the ACh-induced tension response.

Tension (P) represented on the ordinate is expressed as a fraction of the maximum ACh (10^{-3} M) tension response (P_o).

Muscle length on the abscissa is expressed as a multiple of L_o, the *in situ* resting length.

Each point represents the mean of eight experiments and the vertical bars the SE of the mean.

The experiments were undertaken at a temperature of $20^{o}C$.


Relative Tension (P/P$_o$)
1.0
0.8
0.6
0.4
0.2
0.4
0.8
1.2
1.6
Muscle Length (x L$_o$)

The rate of ACh-induced tension development also altered with muscle length. The relationship bore a resemblance to the length-tension diagram, increasing from 0.5 - 0.9 L_o, and decreasing beyond 1.2 L_o. Over the intermediate range no significant difference was noted. Over the experimental range no significant change in relaxation rate was observed.

Effect on the CIC of altering the temperature step

Method 1 - T_1 constant (20°C)

In this experiment ΔT's of up to 18°C were employed. T_1 was kept at 20°C since at higher temperatures spontaneous relaxation was observed. Fig. 4:23 shows the peak amplitude of the CIC, plotted against ΔT. The data from six muscles show that the CIC deceases exponentially as ΔT decreases. The Log-Linear slope of the relationship is 0.069 $\pm$ 0.01 dP/dt also decreases exponentially as ΔT decreases (Fig. 4:24). Relaxation half-time decreases with increasing T_2 (Fig. 4:25).

Method 2 - T_2 constant (2°C)

An exponential relationship exists between the size of the CIC and ΔT as T_1 is increased from 5 - 35°C (Log-Linear slope 0.044 $\pm$ 0.008); at a T_1 greater than 30°C the tension decreases as T_1 increases (Fig. 4:26 and 4:27). Over the range ΔT = 3 - 23°C the

Fig 4.23

The relationship between CIC tension and ΔT (Method 1).

In this method T_1 remains constant at $20^{\circ}C$, and T_2 is altered from 15 - $2^{\circ}C$.

CIC tension increases with increasing ΔT, from a value of 0.2 ± 0.05 Kg/cm^2 at a ΔT of $5^{\circ}C$ to 0.825 ± 0.2 Kg/cm^2 at a ΔT of $18^{\circ}C$.

Each point is the mean of five experiments and the vertical bars are the SE of the mean. The exponential curve was fitted by eye.

Muscle length: $0.8\ L_o$.

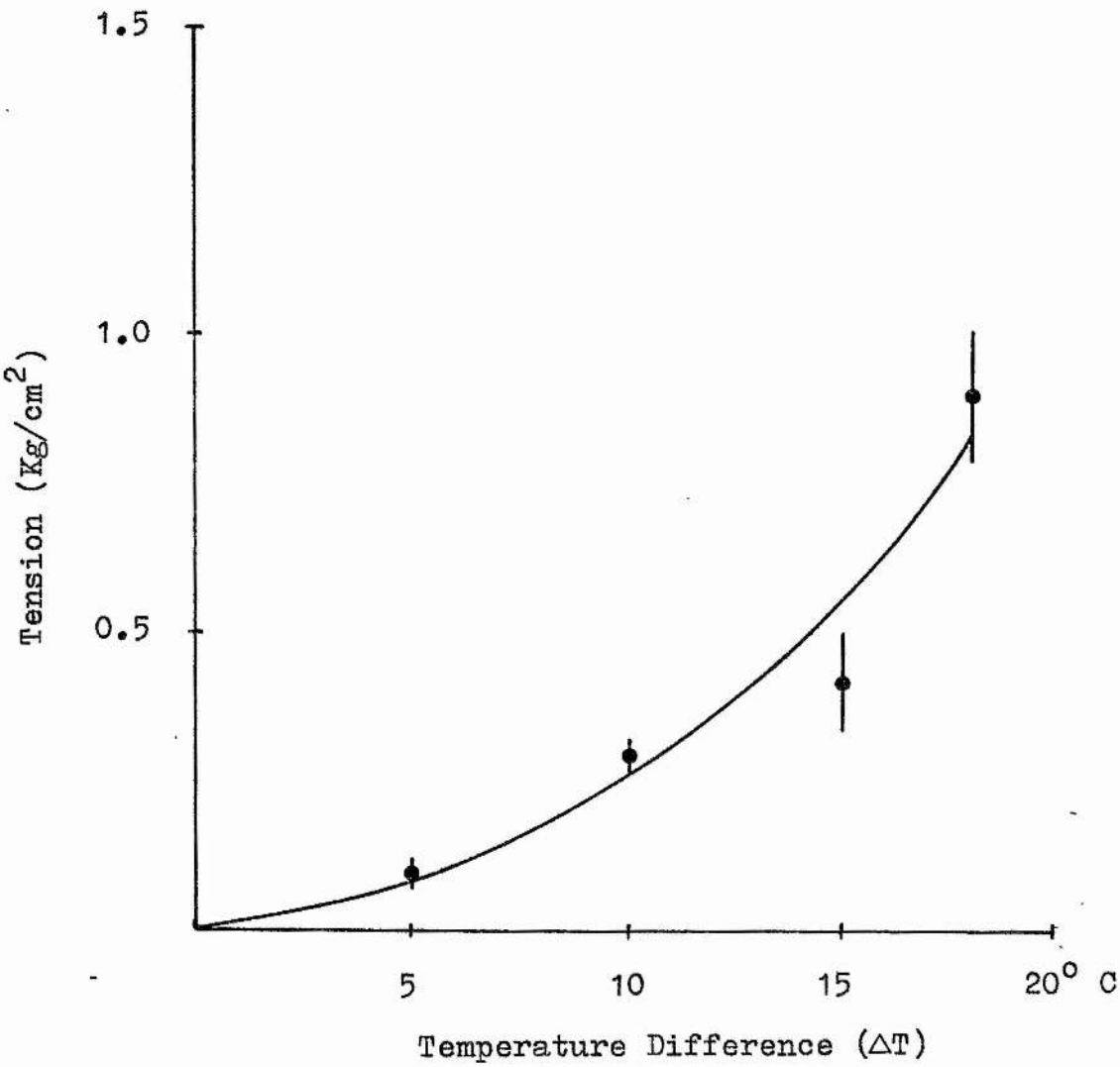

1.5
1.0
0.5
Tension (Kg/cm^2)
5
10
15
20° C
Temperature Difference (ΔT)

Fig 4.24

The effect of ΔT on the rate of tension development of the CIC (Method 1).

In this method T_1 remains constant at $20^{\circ}C$, while T_2 varies between $2^{\circ}C$ and $15^{\circ}C$.

The rate of tension development increases with increasing ΔT, from 0.0177 ± 0.0015 $Kg/cm^2/sec$ at a ΔT of $18^{\circ}C$ to 0.012 ± 0.0017 $Kg/cm^2/sec$ at a ΔT of $5^{\circ}C$.

Each point represents the mean of six experiments and the vertical bars the standard error of the mean. The curve was fitted by eye.
Muscle length was $0.8\ L_o$.

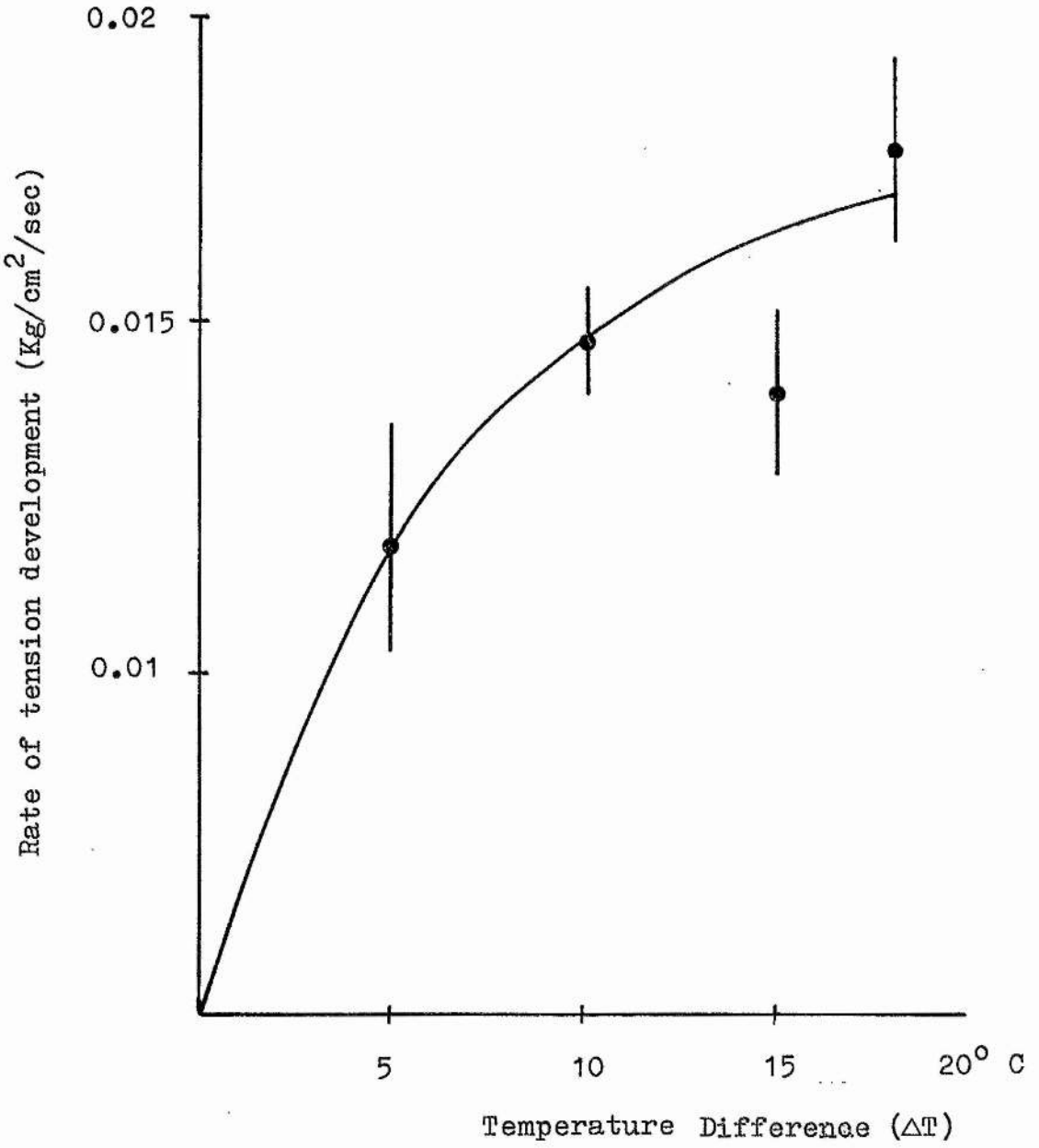

0.02
0.015
0.01
Rate of tension development (Kg/cm^2/sec)
5
10
15
20° C
Temperature Difference (ΔT)

Fig 4.25

The effect of ΔT on the CIC relaxation half-time (Method 1).

In this method T_1 is constant at 20°C, and T_2 varies between 2 - 15°C.

The time to 50% CIC relaxation ($t_{\frac{1}{2}}$) decreases as ΔT decreases, from 760 $\pm$ 70 secs at a ΔT of 18°C (T_2=20°C) to 438 $\pm$ 54 secs at a ΔT of 5°C (T_2=15°C).

The ordinate shows the relaxation $t_{\frac{1}{2}}$ expressed as a % of the value obtained at a ΔT of 18°C.

Each point represents the mean of five observations and the vertical bars the standard error of the mean.
The line was fitted by eye.
Muscle length: 0.8 L_o.

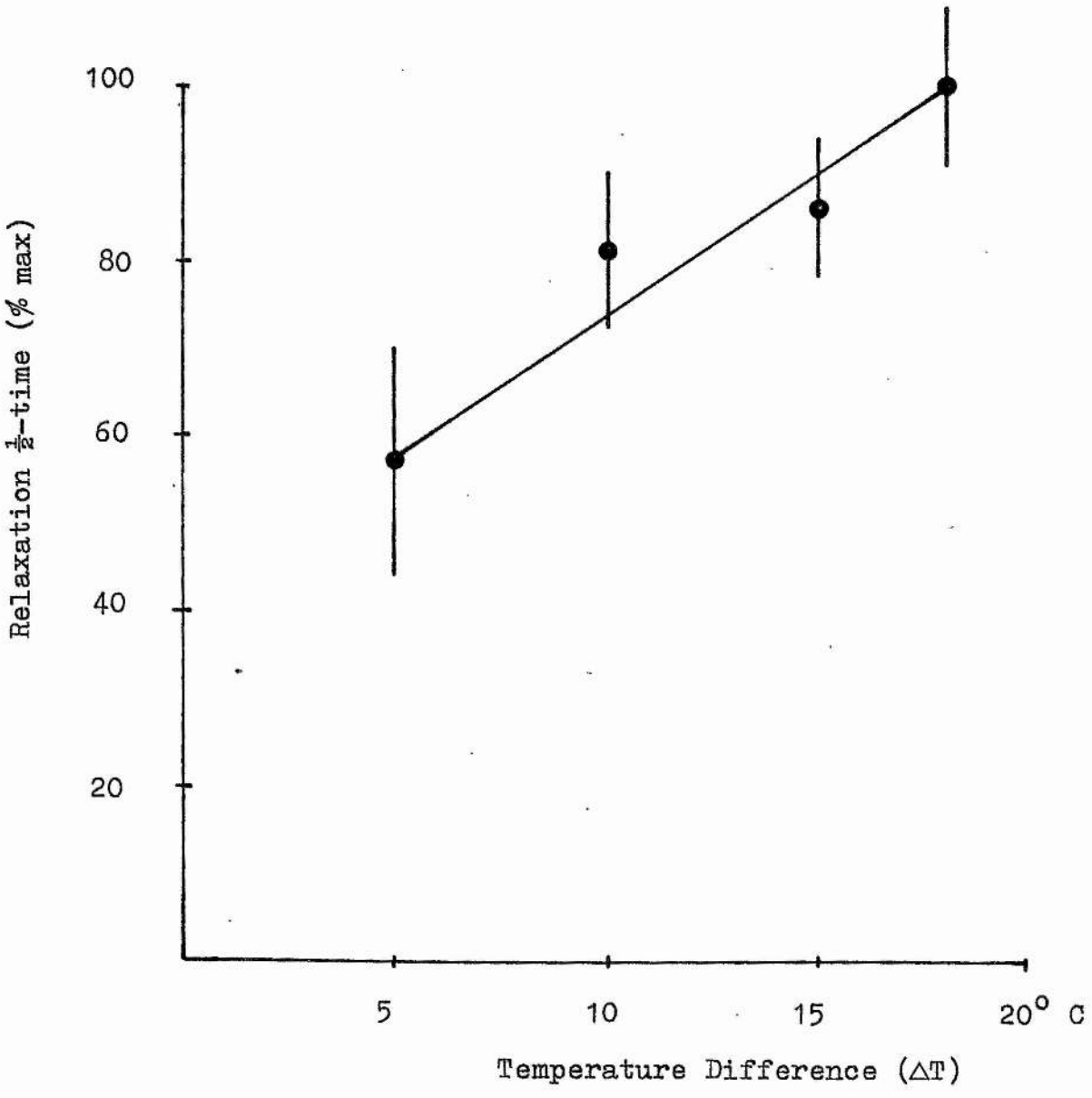

100
80
60
40
20
Relaxation ½-time (% max)
5
10
15
20° C
Temperature Difference (ΔT)

Fig 4.26

The relationship between ΔT and the CIC tension (Method 2).

In this method T_1 varies over the range 5 - 45°C and T_2 remains constant at 2°C.

The size of the CIC increases as ΔT increases, from a minimum of 0.2 ± 0.06 Kg/cm^2 at a ΔT of 3°C, to 3 ∓ 0.25 Kg/cm^2 at a ΔT of 32°C. As the T_1 temperature was increased above 35°C the response declined.

Each point represents the mean of five experiments and the vertical bars the standard error of the mean.

Muscle length was $0.8\ L_o$.

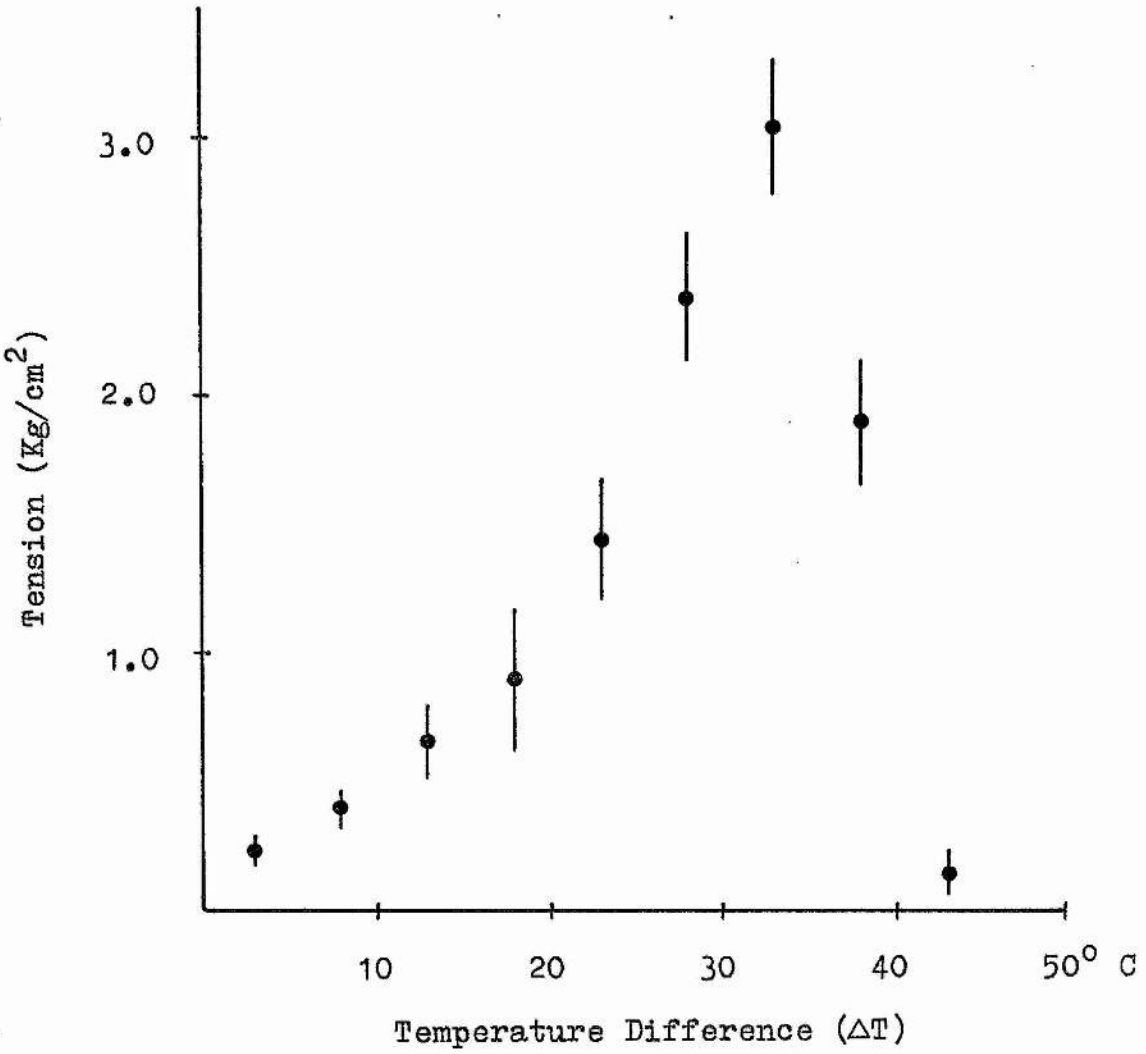

3.0
2.0
1.0
Tension (Kg/cm^2)
10
20
30
40
50° C
Temperature Difference (ΔT)

Fig 4.27

The effect of increasing ΔT on the size and form of the CIC.

Δ : point of application of 10^{-3}M ACh at $10^{\circ}C$ (A); $20^{\circ}C$ (B); $30^{\circ}C$ (C) and $40^{\circ}C$ (D).

▲ : point of application of cold shock solution at $2^{\circ}C$, and removal of ACh.

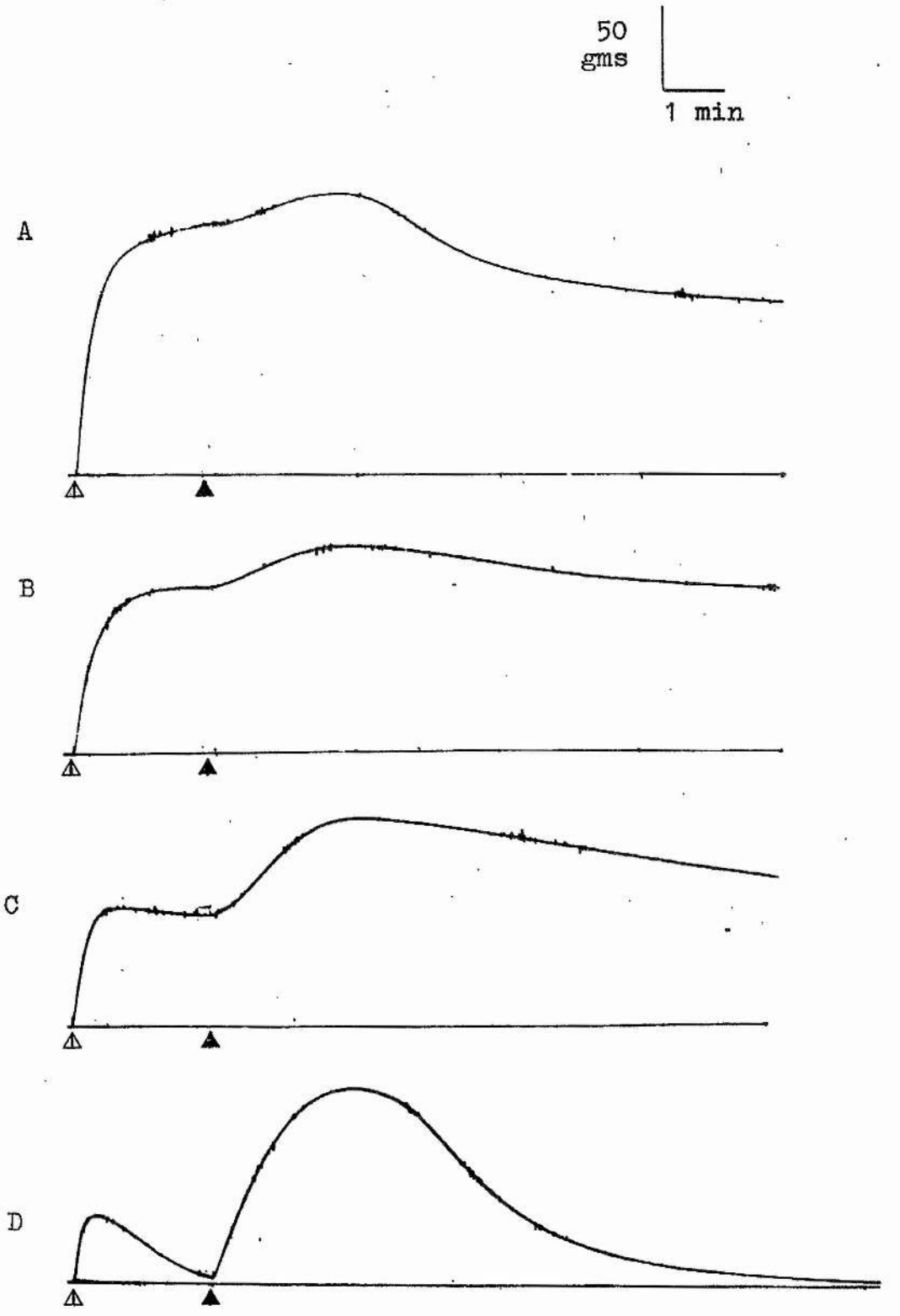
A : ΔT $8^{\circ}C$ (T_1=$10^{\circ}C$)

B : ΔT $18^{\circ}C$ (T_1= $20^{\circ}C$)

C : ΔT $28^{\circ}C$ (T_1=$30^{\circ}C$)

D : ΔT $38^{\circ}C$ (T_1=$40^{\circ}C$)

Muscle length: 0.8 L_o.


50
gms
1 min
A
B
C
D

rate of the CIC tension development also increases exponentially (slope of the log-linear plot 0.00072 ± 0.00023 - (Fig. 4:28). Again, the relaxation half-time decreases with increasing ΔT from 700 ± 60 seconds at a ΔT of 3°C to 188 ± 24 seconds at a ΔT of 38°C. The relationship between ΔT and relaxation $t_{\frac{1}{2}}$ exhibits two linear portions one extending from a ΔT of 3 - 18°C, and another from a ΔT of 18 - 38°C. Over the former range, $t_{\frac{1}{2}}$ decreases by approximately 10% to 630 ± 40 seconds, while over the higher range it is reduced by a further 70% to 140 ± 24 seconds (Fig. 4:29). This relationship is difficult to interpret because it might be expected that, as the CIC is initiated at 2°C, the rate of relaxation would be constant and equal to the rate of relaxation from ACh-induced tension at 2°C. That this is not the case suggests that prior exposure of the muscle to higher temperatures, in some way facilitates the relaxation process.

Fig 4.28

The effect of ΔT on the rate of tension development of the CIC (Method 2).

In this method T_2 remains constant at $2^{\circ}C$ while T_1 varies between $5^{\circ}C$ and $40^{\circ}C$.

dP/dt increases as ΔT increases from a value of 0.0077 ± 0.0006 $Kg/cm^2/sec$ at a ΔT of $3^{\circ}C$ to 0.034 ± 0.004 $Kg/cm^2/sec$ at a ΔT of $28^{\circ}C$.

Each point represents the mean of five observations and the vertical bars the standard error of the mean.

Muscle length: $0.8\ L_o$.

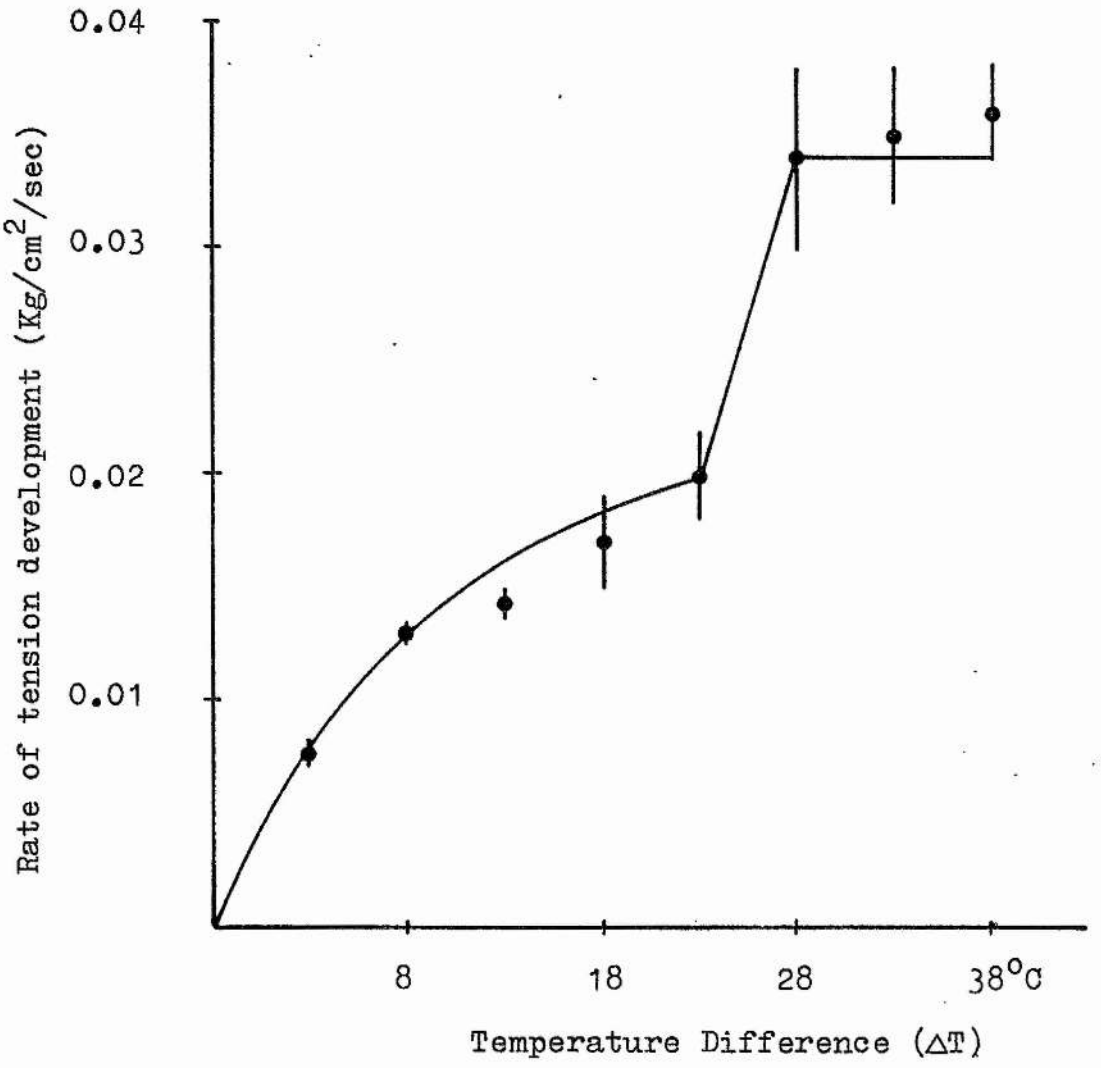

0.04
0.03
0.02
0.01
Rate of tension development (Kg/cm^2/sec)
8
18
28
38°C
Temperature Difference (ΔT)

Fig 4.29

The effect of ΔT on the CIC relaxation half-time (Method 2).

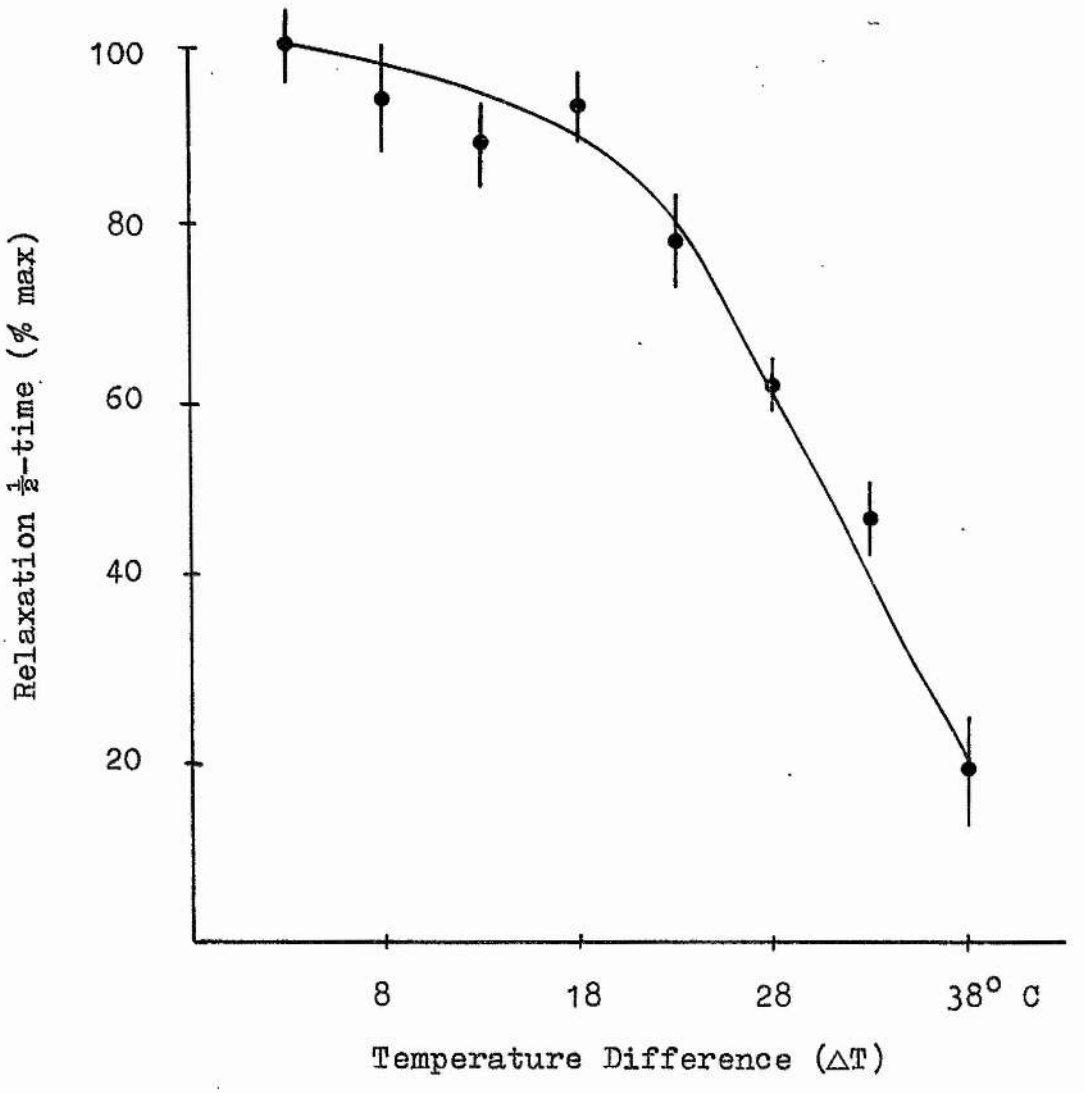
In this method T_2 is constant at $2^{\circ}C$.

The time to 50% CIC relaxation ($t_{\frac{1}{2}}$) decreases as ΔT increases, from 900 ± 63 secs at a ΔT of $3^{\circ}C$ to 180 ± 60 secs at a ΔT of $38^{\circ}C$.

The ordinate shows the relaxation $t_{\frac{1}{2}}$ expressed as a % of the initial value obtained at a ΔT of $3^{\circ}C$.

Each point represents the mean of five observations, and the vertical bars the standard error of the mean. The curve was fitted by eye.

Muscle length: $0.8\ L_o$.


100
80
60
40
20
Relaxation ½-time (% max)
8
18
28
38° C
Temperature Difference (ΔT)

MECHANICAL DISCUSSION

ACh-response in Relation to Temperature

One characteristic feature of the ABRM which has emerged from this study is that, like fast twitch fibres, (Buller, Ranatunga and Smith, 1968), it shows a negative temperature co-efficient i.e. as the temperature is decreased the tension produced during a contracture increases. This property has been noted in other preparations e.g. rat extensor digitorium, tortoise iliotibialis, frog sartorious and gastrocnemius, rat triceps surae, rabbit papillary and ventricular muscle and in cat iris sphincter. (Ranatunga, 1977; Close and Hoh, 1968; Hill, 1951; Doi, 1920; Edman, Mattiaiazzi and Nilson, 1974; Apter, 1972; Mashima and Matsumura, 1964; Kaufmann and Fleckenstein, 1965; Kelly and Fry, 1958). The converse, a positive temperature co-efficient, is observed in slow twitch fibres e.g. soleus (Buller, Ranatunga and Smith, 1968; Close and Hoh, 1968), and in some smooth muscles e.g. uterine (Csapo, 1954).

Various proposals have been put forward to explain the inverse temperature-tension relationship of skeletal muscle. A.V. Hill (1951) suggested that final twitch tension depends on the balance between two opposing reactions, the internal shortening of the contractile element on one hand, and the decay of activity on the other. Normally, the tension produced during a muscle twitch is

less then that of a tetanus, and this has been attributed to the rapid onset of relaxation allowing insufficient time for internal shortening to be completed. Hill suggested that the relaxation process is more temperature sensitive than the activation process, so that, as the temperature is lowered, the former rate is reduced more than the latter and total tension is increased. This hypothesis was based on earlier observations of Hartree and Hill (1921) who found that the temperature co-efficient of the rate of rise of twitch tension was 2.5, while that of relaxation was 3.6. In this report the value for the rate of development of ACh tension in the ABRM was 1.2 from 0 - 25°C and 2.15 from 25 - 35°C; while that for relaxation was 1.2 from 2 - 20°C and 3.5 from 20 - 30°C .

Other theories do exist, however. One suggests that decreasing the temperature prolongs the duration of the action potential (Ward and Thesleff, 1974; Ranatunga, 1977), while another proposes that a decrease in temperature may decrease the rate of removal of activator (Close and Hoh, 1968). In rabbit ventricular and papillary muscle the negative temperature co-efficient is thought to be related to an alteration in excitation-contracture coupling involving an increase in the amount of Ca^{2+} released into the myoplasm in response to the action potential (Kaufmann and Fleckenstein, 1965; Langer and Brady, 1968).

To date the tension-temperature relationship in the ABRM has not been studied in detail. However, results reported in this study have shown that at 2°C the duration of the active state is twice as long as at 20°C suggesting, that more Ca^{2+} may be released, or that its re-uptake may be slowed at the lower temperature (Close and Hoh, 1968). Evidence for the latter is provided by the observations of Gogjian and Bloomquist (1977). These authors showed that Ca^{2+} uptake by vesicular organelles, isolated from the ABRM, was temperature sensitive. However, the rate of uptake was reduced three-fold as the temperature was decreased from 30 - 10°C, while in the present investigation it has been shown that tonic relaxation is reduced thirteen-fold over the same temperature range (Fig. 4:5). A discrepancy, between rate of Ca^{2+} uptake by SR elements and relaxation, has been reported in other preparations; the reason is uncertain but it is suggested that Ca^{2+} may be taken up by a site which differs from its release site (Fuchs, 1974). The other possibility is that the rate of Ca^{2+} uptake by the SR *in vivo* is greater than *in vitro*.

The observation that at temperatures greater than 27 ± 3°C tonic relaxation becomes spontaneous (i.e. catch tension is not held) has been reported previously (Johnson, 1966; Twarog, 1967 a; Bloomquist and Curtis, 1975 b). The reason for the disappearance

of catch at this temperature has not been determined, but Johnson (1966) proposed that it may reflect a phase change in the paramyosin system. Twarog (1967 a) offers two hypotheses; one, that temperature may directly affect the kinetics of an activator substance or, that the temperature effect is mediated by 5HT which may be released from nerve terminals at a critical temperature. The mechanism of this effect has not yet been determined. However, in this study it was found that at, and above 30°C, the half-times for 5HT-induced and spontaneous relaxation approached one another. Additionally both warm and 5HT relaxation are slowed and eventually inhibited by the application of 10^{-5} M Salygran (mersalyl acid). Salygran is an organic mercurial which specifically blocks 5HT action (Twarog and Muneoka, 1973; Preston, Rigter and Strijbos, 1977) by reacting with a sulphydryl group at or near the site where the indole nitrogen binds to the receptor, (Twarog, 1977). These results suggest that above 30°C relaxation is associated with or mediated by 5HT release, or that the requirement for relaxant is lost.

Length/tension relationship of the ACh-induced Response

Most smooth muscles studied contract by a sliding filament mechanism (Peiper et al, 1973) and show an optimal length for tension development. However, it is difficult in these muscles to express this relationship in terms of sarcomere length, as can

be done in striated muscle. Two reasons for this are, firstly, sarcomeres are not usually well defined, and secondly, myosin filaments are often only seen in the contracted state (Schoenberg, 1973; Somlyo and Somlyo, 1973). Although actin and paramysoin filaments are present in both the relaxed and contracted ABRM, and sarcomeres can be defined (Sobieszek, 1973), it is still difficult to relate the length-tension diagram to filament overlap because of the random distribution of dense bodies.

Cornelius and Lowy (1978) found that the length-tension relationship of the ABRM showed a very definate plateau region over the range 0.9 - 1.1 L_o. They suggested that this could be attributed to there being a constant number of cross bridges over this length, due, perhaps to filament re-arrangement which can occur because of the absence of mechanical constraints such M-lines, and because the ratio of actin to paramyosin filaments is 17:1. They also concluded that the presence of 'sharp corners' in their length-tension diagram indicated that the muscle cells act in union, so that throughout the muscle the amount of thick and thin filament overlap is constant at any one time. This result is in contrast to the findings of Hanson and Lowy (1960), and the results reported here, which show a less well defined plateau over the same range. This discrepancy may be due to the fact that Cornelius and Lowy (1978) used small population samples in the construction of their curves.

Obviously, more work needs to be undertaken on the length-tension relationship in this muscle before a definite conclusion can be drawn about Cornelius and Lowys' hypothesis.

Length/Tension Relationship of CIC

It was considered possible that the CIC and the ACh-induced tensions might be related to muscle length in a similar way. The results of the present study show that this is not the case; the size of the CIC decreases with increasing muscle length from 0.5 - 1.3 L_o. At the shortest muscle length used (0.5 L_o) the CIC is equal to or greater than the ACh-induced tension. This finding is difficult to explain solely in terms of the sliding-filament theory. However, recent experimental evidence has suggested that 'the mechanical behaviour of muscle does not always conform to the straight-forward prediction of the sliding filament theory' (Fuchs, 1974), and that alterations in muscle length may affect ofnctors other than the amount of filament overlap.

For example, Edman and Kiesling (1966) reported that the duration of the active state (AS) is shorter at shorter muscle lengths, and that this is not associated with a decreased duration of the AP. They suggested that the degree of muscle extension influences a more intimate step in the excitation-contraction process. Rack and Westbury (1969) showed that in cat soleus muscle a low

stimulus rate produced near-maximum tensions at long muscle lengths, while at shorter lengths only high rates of stimulation would give a near maximal contraction. Close (1972) using frog sartorious and semi-tendinosus, found that the optimal length for twitch and tetanic tension differed. He suggested that this was because the length tension curve for twitch tension is determined not only by the degree of overlap of actin and myosin but also by length-dependent changes in release and removal of an activator. Support for this conclusion was obtained from experiments by Rudel and Taylor (1971) which showed that caffeine had only a slight effect on the plateau region of the curve but caused a marked increase in force development on the ascending part. At a sarcomere length of 1.3 μM where tetanic force development is zero, stimulation in the presence of caffeine caused the fibre to generate as much as 35% of maximum force. Since caffeine is considered to act by facilitating Ca^{2+} release from the SR (Weber, 1968) it appears that the amount of Ca^{2+} normally released at short muscle lengths is insufficient to saturate the myofilaments. In addition, Schoenberg and Podolsky (1972), using skinned fibres of varying sarcomere lengths found that at short sarcomere lengths (1.0 μM) where force production is normally negligible these fibres could develop between 30 - 70% of maximum force provided optimal concentrations of Ca^{2+} were present. Thus the slope of the

ascending limb of the length-tension curve may be determined by a combination of a distorted filament lattice and reduced activation.

In terms of the sliding filament theory, the reduced ACh-tension at short muscle lengths is caused solely by interference between the myofilaments. If this is the case, it is difficult to imagine how cold shock would become a more effective stimulus as muscle length is decreased. However, if, as muscle length is decreased the ACh-tension becomes progessively less due to a decrease in the availability of activator then a process which promotes an increase in activator levels might be more effective as muscle length is reduced.

CIC In Relation to Active State

There appears to be a correlation between the decay of active state and tension produced in response to a cold shock. During the first 7 - 8 minutes after the application of ACh both the active state, at 20°C, and the CIC tension, decay and thereafter level off. In the experiments reported here active state never reached zero, because the stimulant (ACh) was always present (Johnson and Twarog, 1960), but after 8 minutes it had fallen to a level which then remained constant at about 10% of maximum. This suggests that the CIC can manifest itself most easily when the muscle is in a state of activation or flux and its size is related

to the level of this activation. When active state is at very low levels i.e. 10%, the CIC produced is very small, and during catch when contractile activity is very low or absent, no CIC can be produced. The relationship between the size of the CIC and time after ACh application may be compared with the wash-out phenomenon observed in guinea-pig vas deferens by Ohlin and Stromblad (1963). These authors reported that wash-out procedures carried out after the application of ACh, adrenaline or histamine, resulted in a small tension response, the size of which decreased with time after the stimulus. It was considered unlikely, however, that the CIC represented a phenomenon of this type, since a wash-out effect was not seen when the ABRM was transferred from a solutions containing stimulant; and CIC's can be produced during K-contractures when wash-out contractures do not normally occur in smooth muscles (F. Donald personal communication).

Possible Origin of the CIC

There are several ways in which a tension increment might be produced in response to cold shock and any of the following steps in a typical ACh-induced contracture might be affected:

i) The interaction of ACh with a receptor

ii) Membrane depolarization

iii) The influx of Ca_e^{2+}

iv) Ca^{2+} release from intracellular sites

v) Contractile protein interaction

Cold shock is unlikely to increase the ACh sensitivity of the muscle since abrupt cooling in a sub-threshold concentration of ACh does not result in a tension increment, and furthermore, no CIC response is seen when the ACh response is reduced to 10% of maximum.

A direct effect of cold shock on the muscle membrane is also unlikely since the depolarization seen on the application of cold shock to a resting muscle is of the order of 8 - 12 mV which is by itself too small to reach the threshold (35 - 40 mV) (Twarog, 1967) necessary to produce a Ca_e influx and subsequent contracture.

A decreased affinity of the regulatory light chain for Ca^{2+} could result in a CIC. This cannot be considered fully, however, since temperature effects on myosin regulation have not as yet been investigated. However, experimental evidence suggests that the CIC does not arise in this manner. No CIC is observed in a muscle at rest, when a decrease in the Ca^{2+} requirement of the regulatory proteins or a reduction in Ca-sensitivity of the contractile proteins might be expected to produce a tension increment. In addition, there is no evidence to suggest that a cold shock will decrease the Ca-requirement for acto-myosin interaction. In fact,

it has been reported that a decrease in temperature actually increases the requirement for Ca^{2+} in this process (Hartshorne, Barns, Parker, and Fuchs, 1972; Taniguchi and Nagai, 1970).

That the CIC is not a passive phenomenon is suggested by the facts that it is accompanied by an enhancement of active state, and the application of a cold shock to muscles in catch has no effect.

This leaves two possibilities unconsidered i.e. that cold shock increases or initiates an influx of Ca^{2+} from the bathing medium or that it releases Ca^{2+} from intracellular sites. Either of these processes might result in an increase in the myoplasmic Ca^{2+} level; a situation which is thought to mediate the cold induced effects observed in smooth, cardiac and skeletal muscle.

Cooling as a Stimulus

Although cold induced tension increments have been observed in invertebrate (Guttman and Gross, 1956; Guttman and Ross, 1958; Guttman, Dowling and Ross, 1957; Guttman and Katz, 1953); and vertebrate muscle systems, they have routinely been initiated by rapidly cooling a muscle at rest, with or without prior sub-threshold chemical trdatment. Rapid cooling is said to result in the initiation of a tonic contracture in some untreated resting smooth muscles e.g. retractor penis, cat nictitating membrane, pig stomach, and taenia

coli but is without effect on rabbit intestine (Perkins, Mao-Chih Li, Nicholas, Lassen and Gertler, 1950; Evans, 1924; Magaribuchi, Ito and Kuriyama, 1973; Ercoli and Guzzon, 1952). For a cold shock to be an effective stimulus in resting vertebrate skeletal muscle, or in the ABRM, prior treatment with sub-threshold K^+, or caffeine is necessary (Sakai, 1965; Caputo, 1972; Sakai and Kurihara, 1974; Yoshioka, 1976; Guttman et al, 1956, 1957, 1958, 1953; Sakai and Yoshioka, 1973). There are no reports of a CIC occurring when a muscle is rapidly cooled during a contraction-relaxation cycle. This is mainly because the time course of responses in most preparations does not allow this to be investigated.

Guinea-pig stomach, taenia coli and urinary bladder show tonic, cold induced contractures without prior treatment with K^+ or caffeine. These are called rapid cooling contractures and they relax on re-warming. Displacement of the membrane potential (MP), or changing the ionic environment does not modify the amplitude of these contractures. It is postulated that the RCC in these preparations is caused by the release of bound calcium from SR elements, the release of membrane-bound calcium, or by a reduction in the Ca-accumulating ability of the SR (Magaribuchi et al, 1973; Kurihara, Kuriyama and Sakai, 1974).

Sub-threshold caffeine RCC's have been demonstrated in both toad and frog skeletal muscle (Sakai, 1965; Yoshioka, 1976). These are also tonic contractures which relax spontaneously when

the caffeine concentration is low, or, on re-warming when it is high. In this study it was found that sub-threshold caffeine RCC's which relax spontaneously and are unaffected by re-warming, may also be evoked in the ABRM by the application of 3 mM caffeine at 2°C.

Sub-threshold K cold contractures have been observed previously in both frog sartorious and the ABRM (Caputo, 1972; Guttman et al, 1956; 1957; 1958; 1953). In the former case the response is again a tonic contracture which relaxes on re-warming, while in the latter it is a phasic response which is fully relaxed in 10 minutes.

In contrast to the positive tension increments produced on cooling a frog sartorious in the presence of sub-threshold caffeine (Sakai, 1965), or K (Caputo, 1972 a); Hill (1970 a,b), found that rapidly cooling an untreated resting sartorious caused relaxation while warming it induced a contracture. In hypertonic conditions the relaxant effect of cold solutions was preceded by a positive tension increment, the magnitude and time course of which was different from the RCC's observed by Sakai (1965).

Guttman and her collaborators have studied in some detail the sub-threshold K contractures produced by rapid cooling of the ABRM. They characterised the response but offered no real mechanism for it. They found, as in the present investigation, that only rapid cooling was an effective stimulus. Gradual cooling caused the same change in electrical potential but no tension response.

Also, the tension response was dependent on ΔT, as was the RCC in toad and frog sartorious (Sakai, 1965). They observed a ΔT threshold which was between $14 - 23^{\circ}C$ - this is in contrast to the findings presented here which showed that a CIC could be produced with a ΔT as small as $3^{\circ}C$. Sakai (1965) observed a critical lower temperature above which a cold shock solution would not produce a response; this had a value of $10^{\circ}C$ in toad and frog sartorious muscle. In contrast Guttman and Gross (1956) did not observe a critical lower temperature in the ABRM, rapid cooling followed by maintained low temperature resulted in a constant level of depolarization but the tensions response fell in a similar way to K-induced contractures. Guttman, Dowling and Ross (1957) found that the level of depolarization produced by cooling a muscle in a sub-threshold K solution or in normal SW were not significantly different; both were well below threshold yet one caused contracture and the other did not. Altering the $[Na]_e$ or $[Ca]_e$ had no effect. The response persisted after the application of the metabolic poisons cyanide and iodoacetate, or in anaerobic conditions. Anodal repolarizing currents, however, caused the contracture to relax, suggesting that the response was associated in some way with the depolarization process. A later paper (Guttman and Ross, 1958) showed that a sub-threshold concentration of ACh (2.5×10^{-6} M) in SW containing 5X the usual amount of KCl, lowered the threshold for

the initiation of the cold response - in the particular example shown from a ΔT of 25°C to a ΔT of 11°C.

These authors offered no real explanation for their results but in view of work by Heilbrun (1956), they suggested that Ca^{2+} release from the sarcolemma might be a causative factor. Heilbrun (1956), had suggested that membrane rigidity is maintained by the interaction of membrane proteins with divalent cations such as Ca^{2+}. Since the cation-binding power of proteins decreases with decreasing temperature, cold shock may permit the release of Ca^{2+} from membrane-bound sites.

A similar hypothesis has been widely accepted for the mechanism of action of rapid cooling in striated muscle and recent work has confirmed that cold shock releases Ca^{2+} from the SR (Taniguchi and Nagai, 1970; Newbold and Tume, 1977). The latter workers found that sudden temperature shock (23 - 0°C) caused the release of 45% of the Ca^{2+} contained in the SR vesicles isolated from rabbit muscle. They proposed that this occurred because the enzyme-mediated inward transport (Ca-pump ATPase), is more temperature sensitive (Q_{10} 2 -3), than the outwards, passive diffusional, process (Q_{10} 1.4) (Lehninger, 1970). Consequently when the temperature of loaded vesicles is lowered from 23 - 0°C the rate of inward transport is reduced more than the passive outward movement; hence there is a net efflux of Ca^{2+}. 85% of the Ca^{2+} released was dependent on

the temperature step and 15% could be accounted for by the change in pH caused by the step. In addition, they showed that the temperature shock promoted more Ca^{2+} release when the vesicles had become de-stabilized chemically or by storage. If isolation decreased their stability then the Ca^{2+} release from stable vesicles might be too small in vivo to cause a contracture. This is supported by the findings of Taniguchi and Nagai (1970) who calculated that the Ca^{2+} released from isolated SR by a temperature shock of 20 - 0°C would be sufficient to fully activate the muscle, yet Sakai (1965) had previously stated that rapidly cooling a resting muscle to the same extent was without effect. However, cooling by 20°C in the presence of sub-threshold caffeine or K, resulted in activation of the muscle, suggesting that this treatment rendered the muscle more susceptible to cold shock as did storage and chemical de-stabilization. Sakai and Kurihara (1974) suggest that caffeine may facilitate the cooling effect by increasing the rate and amount of Ca^{2+} release from the SR. However, Caputo (1972 a) has proposed that sub-threshold K releases small amounts of Ca^{2+} which at room temperature are quickly taken up by the SR; at lower temperatures however, this uptake is inhibited and a contraction results.

That a similar mechanism might occur in the ABRM is supported by the work of Gogjian and Bloomquist (1977), who have isolated from the ABRM membrane-bound Ca-accumulating vesicles. These structures share many of the properties of SR elements isolated

from skeletal muscle, including ATP dependence, and sensitivity to caffeine and temperature changes. If, as is suggested to be the case for the RCC, the CIC is the result of a transient Ca^{2+} release, then the fact that the CIC is 63 ± 5% larger than a RCC produced in the same preparation, and that a CIC may still be obtained after caffeine treatment suggests that these responses may not depend entirely on the same Ca^{2+} (see Part VI) stores. However, in addition to vesicular Ca^{2+} accumulating elements there is evidence to suggest that the mitochondria and the fibre membrane may act as sources of intracellular Ca^{2+} (Atsumi and Sugi, 1976).

It is therefore conceivable that either or both of these intracellular sites may release Ca^{2+} in response to a temperature shock. This explanation is similar to that proposed by Magaribuchi et al (1973), and Kurihara, Kuriyama and Magaribuchi (1974), to account for the appearance of RCC's insmooth muscle preparations, which also have extensive membrane-bound Ca^{2+} stores.

If Ca^{2+} release is in fact responsible for the CIC, then the increase in the size of the CIC seen with increasing ΔT in Methods I and II, is consistent with the observations that the amount of Ca^{2+} released from isolated SR vesicles is greater for larger temperature jumps. Some of this Ca^{2+} release may result from a change in pH, but as has been previously stated this effect is relatively small - 15% (Newbold and Tume, 1977); and in this

investigation it was found that there was no observable difference in the size of CIC's induced in buffered ASW and those produced in Natural SW. If a pH increase is involved in the production of a CIC its contribution must be minimal.

An explanation such as the above may also explain the increased relaxation rate observed at increasing T_2's for Ca^{2+} re-uptake has been shown to be faster at higher temperatures (Gogjian and Bloomquist, 1977). Interpretation of Method 2, however, poses a problem. In this series of experiments T_2 was always 2°C, while T_1 was varied between 5 - 50°C. In view of the above finding of Gogjian and Bloomquist (1977), it might be supposed that the CIC relaxation rate might be constant at a level near to that expected for 2°C. This was found to be the case over the range of T_1's from 5 - 20°C. Above 20°C, however, this was not so, the relaxation rate appeared to depend to a certain extent on T_1. This may be explained if, as Twarog (1967) suggests that there is a sudden release of 5HT from intramuscular synapses at or near 25°C. While, cold shock may inhibit further release of 5HT, relaxation at the lower temperature may be facilitated by the presence of the relaxant. A similar situation would also arise if a paramyosin phase change occurred (Johnson, 1966). T_1 would presumably decide this and the relaxation at T_2 would be directly affected by it.

In conclusion therefore, it appears possible that the CIC results from an increase in the level of myoplasmic Ca^{2+}, or some other activator. The tension responses were therefore analysed to determine whether the kinetics of the system were consistent with such a hypothesis.

PART V

PRELIMINARY KINETIC ANALYSIS OF THE CIC

PRELIMINARY KINETIC ANALYSIS OF THE COLD-INDUCED CONTRACTURE (CIC)

Sakai and Kurihara (1974), in a review on rapid cooling contractures (RCC's) in both striated and smooth muscle, postulate a model for the effect of cold shock on the contractile apparatus. They envisage a system where the rate constant (k_1) for Ca^{2+} release from the SR is, at rest, very much less than the rate constant for Ca^{2+} uptake, but, on sudden cooling the latter is affected much more than the former, Ca^{2+} is released from the SR and a tension response occurs. Although there are many similarities between RCC's and the CIC (see Part I), the tension response of the latter is obtained at, or near the peak of maximum tetanic tension.

Preliminary analysis of the CIC suggested that it might be considered as a typical bi-exponential, which is the resultant of a forward tension producing reaction, and a backward, relaxation process. The mechanism for the simplest first order case of such a process is:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

so that the reactant A is converted to the product C via an intermediate B. The differential rate equations for the reactions are:

$$d[A]/dt = -k_1 [A] \quad \ldots\ldots\ldots\ldots\ldots 1$$

$$d[B]/dt = k_1 [A] - k_2 [B] \quad \ldots\ldots\ldots\ldots\ldots 2$$

$$d[C]/dt = k_2 [B] \quad \ldots\ldots\ldots\ldots\ldots 3$$

If $[A_o]$ is the initial concentration of A then 1 may be integrated to

give a simple first order equation:

$$[A] = [A_o]\, e^{-k_1 t}$$

and the differential equation for B becomes:

$$d[B]/dt = k_1 [A_o]\, e^{-k_1 t} - k_2 [B]$$

which can be integrated to give the formula for a biexponential equation:

$$[B] = \frac{k_1}{k_2 - k_1} [A_o] \left(e^{-k_1 t} - e^{-k_2 t}\right)$$

A graphical representation of $d[A]/dt$, $d[B]/dt$ and $d[C]/dt$ is shown in Fig. 5.1 (Nicholas, 1976)

Computer analysis of typical CIC tension responses confirmed that they fitted the above equation where [B] is proportional to isometric tension, which in turn is proportional to the number of activated cross bridges. Using the least squares method, values were obtained for k_1, k_2 and $[A_o]$ by considering the tension of the CIC at ten equally spaced intervals of time. A correlation coefficient (r) for the theoretical curves generated by the computer using these values, and the experimental data, was obtained. Values for k_1, k_2 and $[A_o]$ were only used when $r > 0.75$.

The linear relationship between $[B]_{max}$ (the maximum value of [B]) and the peak CIC tension illustrated in Fig. 5.2 a,b shows how well the tension response follows the bi-exponential form (Fig. 5.3) This finding suggests that, although the CIC may involve many intermediate reactions one of the forward and one of the backward reactions

Fig 5.1

A typical bi-exponential reaction.

(from Nicholas, 1976)

The variation with time of reactant A, intermediate B and product C, in a system of two consecutive first order reactions

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where $k_2 = 2k_1$.

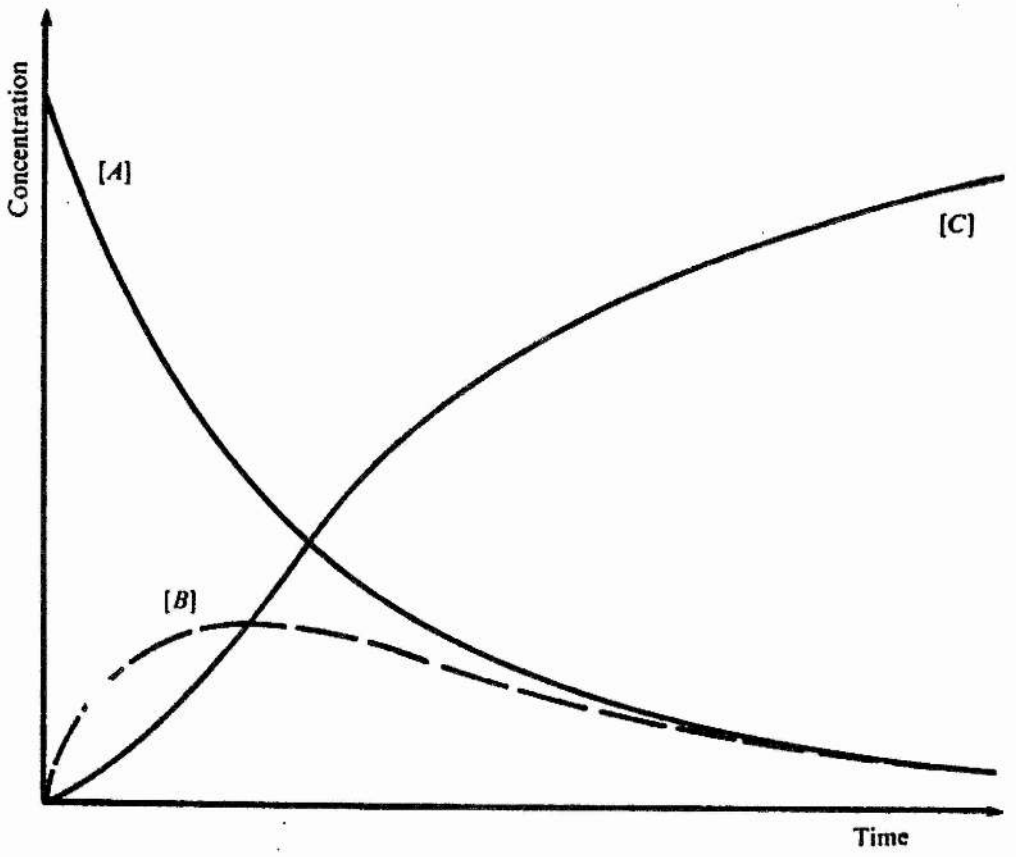

Concentration
[A]
[B]
[C]
Time

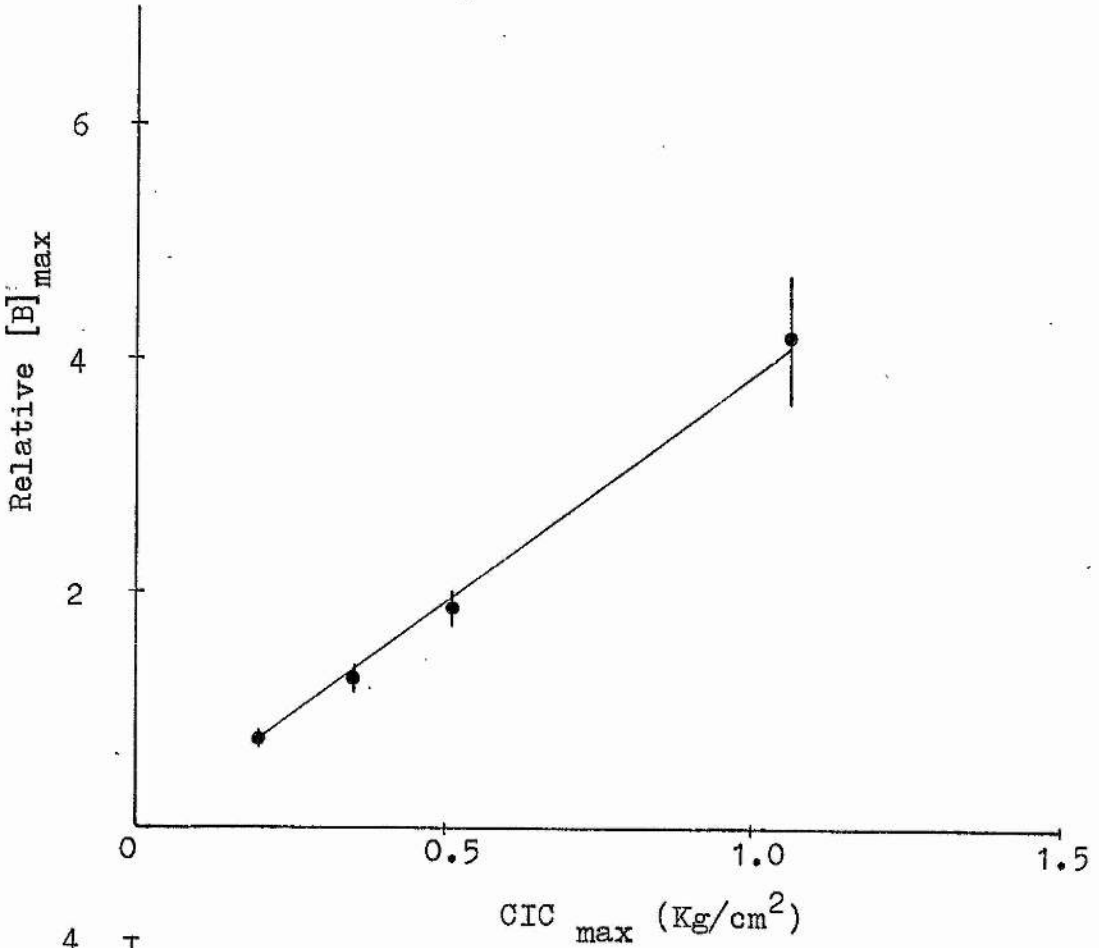
Fig 5.2

CIC_{max} obtained from Method 1(A) and Method 2(B), related to calculated values of $[B]_{max}$.

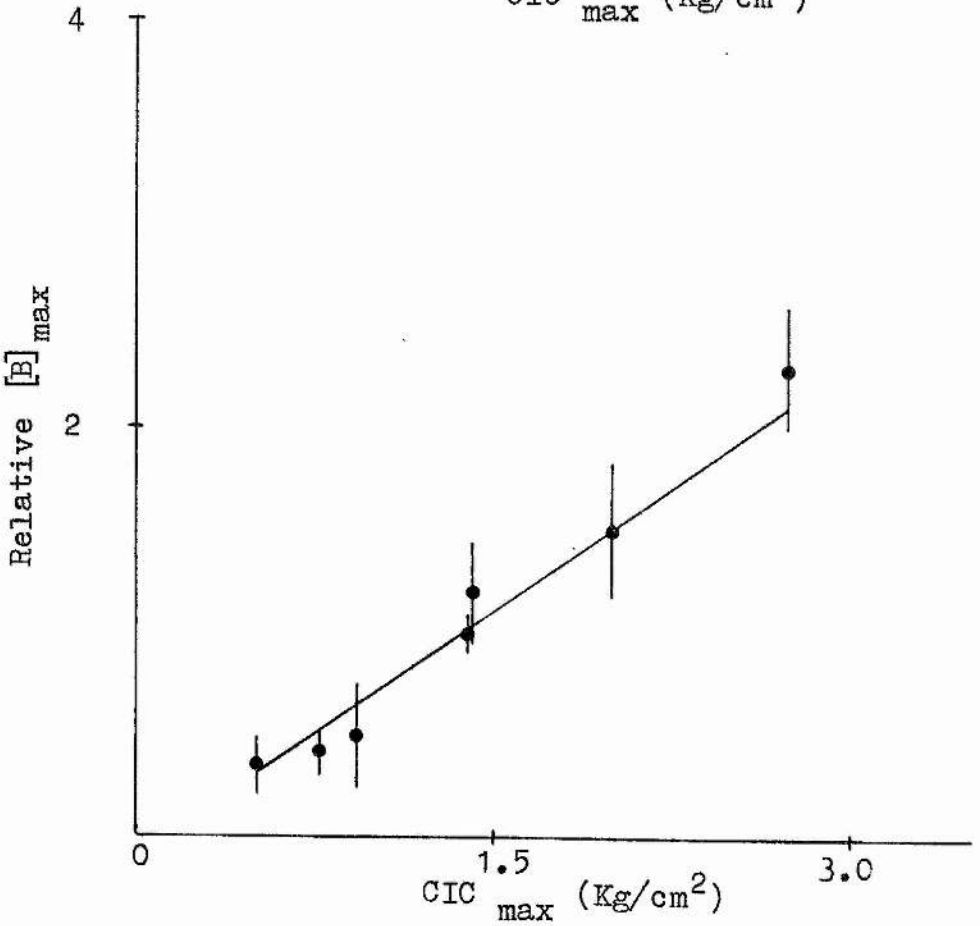
Values for $[B]_{max}$ were obtained using k_1 and k_2 values obtained from computer analysis and substituting these values in the bi-exponentail formula (see text).

Relative $[B]_{max}$ was obtained by dividing each value by one of the calculated values.

Each point represents the mean of three observations and each vertical bar the standard error of the mean. The lines were fitted by eye.


Relative [B]'max
6
4
2
0
0.5
1.0
1.5
CIC max (Kg/cm2)

A


Relative [B]max
4
2
0
1.5
3.0
CIC max (Kg/cm2)

B

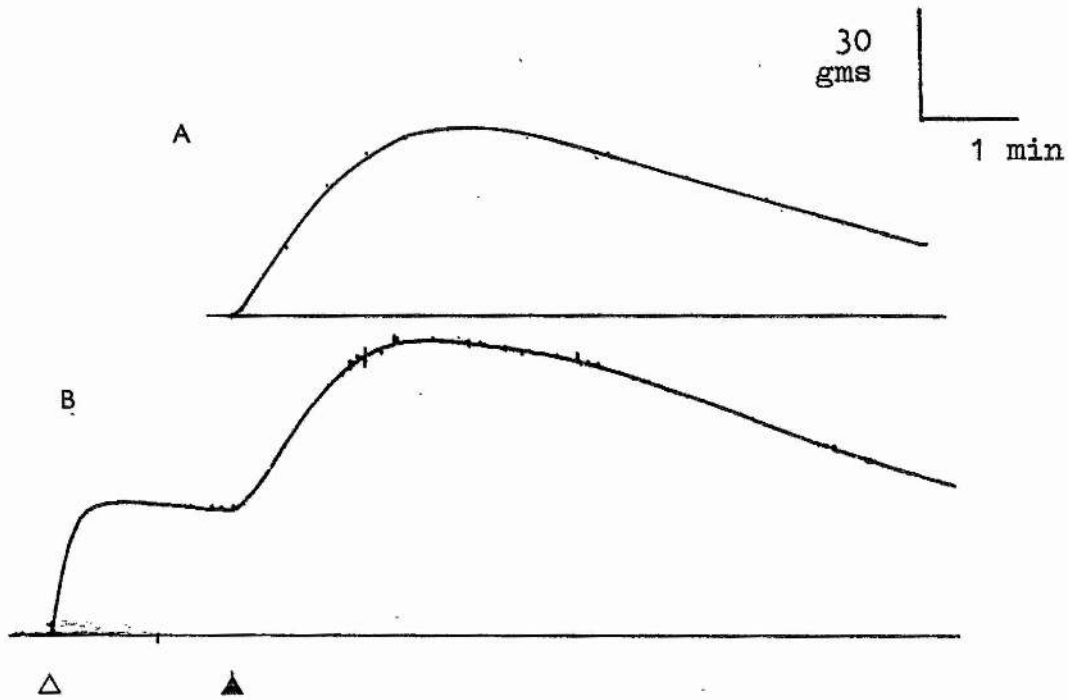
Fig 5.3

Comparison of a bi-exponential curve generated by computer and its respective CIC.

△ : point of application of 10^{-3} M ACh at 20°C

▲ : point of application of the cold shock solution at 2°C (ΔT 18°C), and removal of ACh

B shows the CIC produced in response to a cold shock (ΔT 18°C T_1=20°C). A represents the curve drawn by the computer using k_1 and k_2 values calculated from B.


30
gms
1 min
A
B

must be rate limiting.

In the first set of experiments analysed (Method I - Part 4), ACh-induced tension was initiated at 20°C, and a cold shock was applied at temperatures of 2,5,10 and 15°C. Although the absolute size of the CIC increased with increasing ΔT this cannot be attributed to an increase in k_1 or a decrease in k_2. Fig. 5.4 shows that both k_1 and k_2 decrease as ΔT increases suggesting that these values may be dependant on T_2 (the lower temperature). The increased tension response is attributable to an increase in $[A_o]$ (Fig. 5.5). The physiological significance of $[A_o]$ is discussed below.

In Method II (Part 4), the temperature of the cold shock solution was constant at 2°C and the upper temperature was varied from 5-40°C. Over the experimental range the value of k_1 does not alter significantly ($p=0.1$), but at $T_1 > 20°C$ an increase in k_2 is apparent (Fig. 5.6). This finding is consistent with the view that, at temperatures about 20°C, a relaxant may be released into the muscle (Twarog, 1967) and explains the observation that, above a T_1 of 20°C the rate of relaxation of the CIC is increased although T_2 is constant. As was the case in Method I, the increase in tension B_{max} as ΔT increased is attributable to an increase in $[A_o]$. Fig. 5.7 shows the linear relationship between $[B]_{max}$ and $[A_o]$ over the experimental range.

The suggestion that k_1 and k_2 are dependent on T_2 is partly

Fig 5.4

Variation of k_1 and k_2 with ΔT (Method 1).

● : k_2

▲ : k_1

Both k_1 and k_2 appear to be linearly related to ΔT.

Each point represents the mean of three observations and each vertical line the standard error of the mean. The lines were drawn by eye.

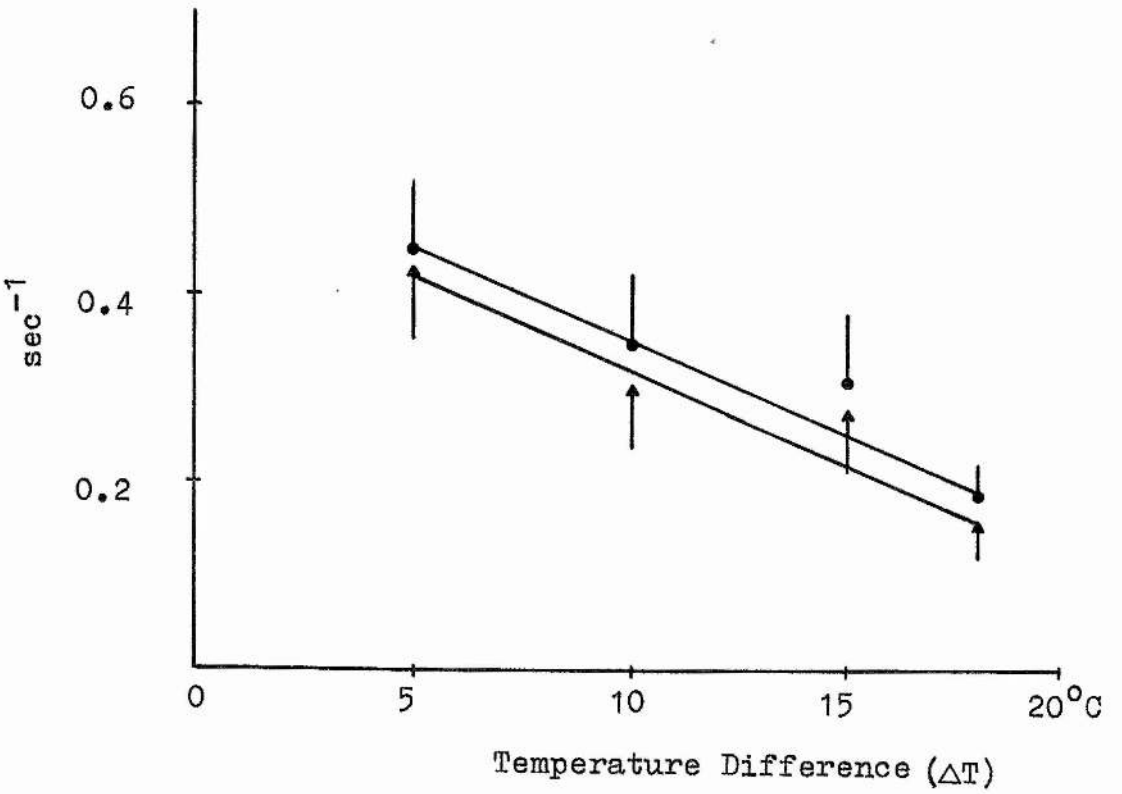

0.6
0.4
0.2
sec^{-1}
0
5
10
15
20°C
Temperature Difference (△T)

Fig 5.5

The relationship between calculated $[A_o]$ and $[B]_{max}$.

$[A_o]$ represented on the ordinate is calculated by substituting values, obtained from computer analysis, for A, k_1 and t in the formula:

$$[A] = [A_o] e^{-k_1 t}.$$

Relative $[B]_{max}$ is calculated as described in the legend to Fig 5.2.

Each point represents the mean of three observations and the vertical and horizontal bars the standard error of the mean. The correlation co-efficent was o.96, and the line was drawn by eye.

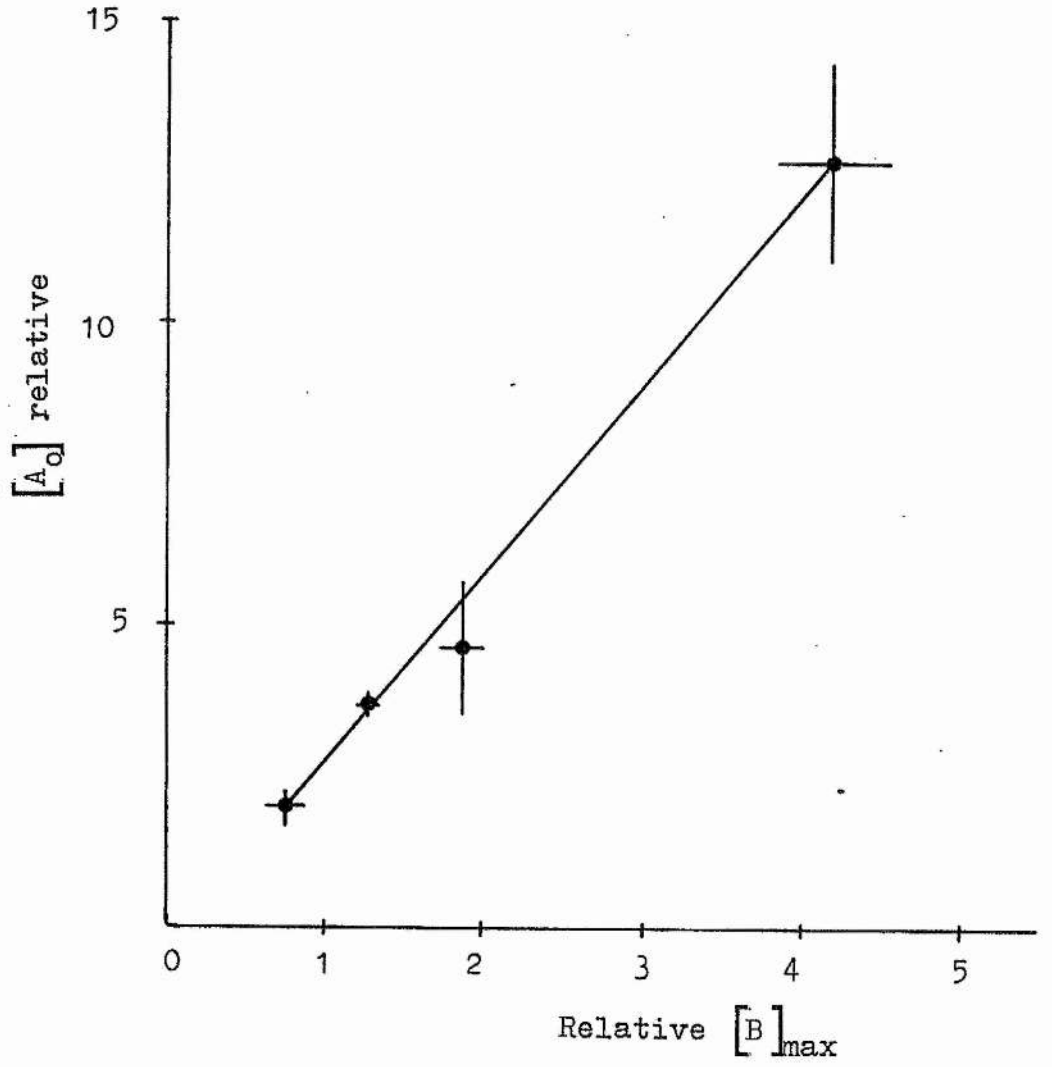

15
10
5
0
1
2
3
4
5
[A_o] relative
Relative [B]max

Fig 5.6

The relationship between ΔT and k_1 and k_2 (Method 2).

● : k_2

▲ : k_1

Over the experimental range k_1 does not alter significantly ($p>0.1$). However, as ΔT is increased above $18^{\circ}C$ k_2 increases significantly ($p=0.02$).

Points shown are the mean of three observations and the vertical bars represent the standard error of the mean.

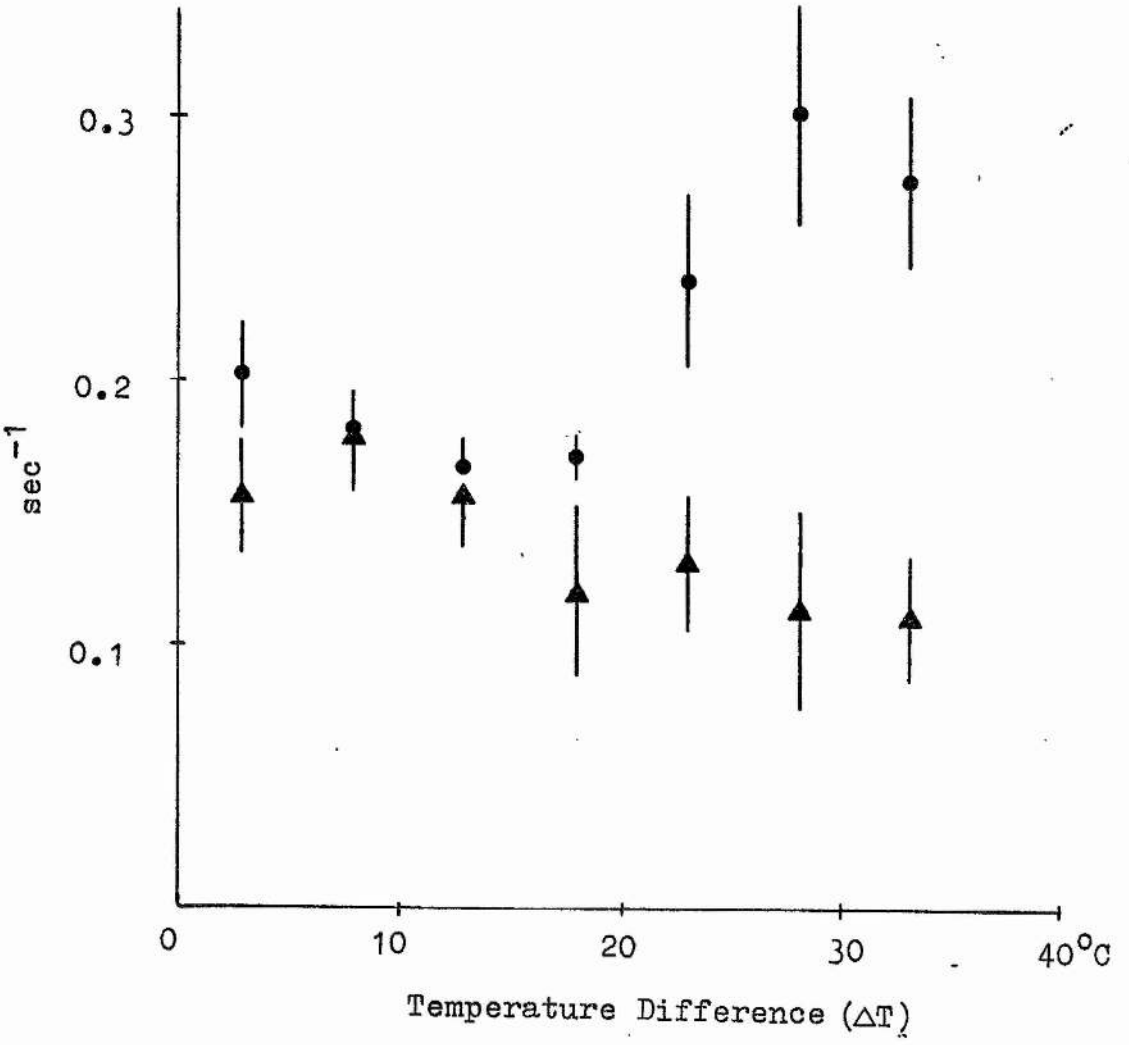
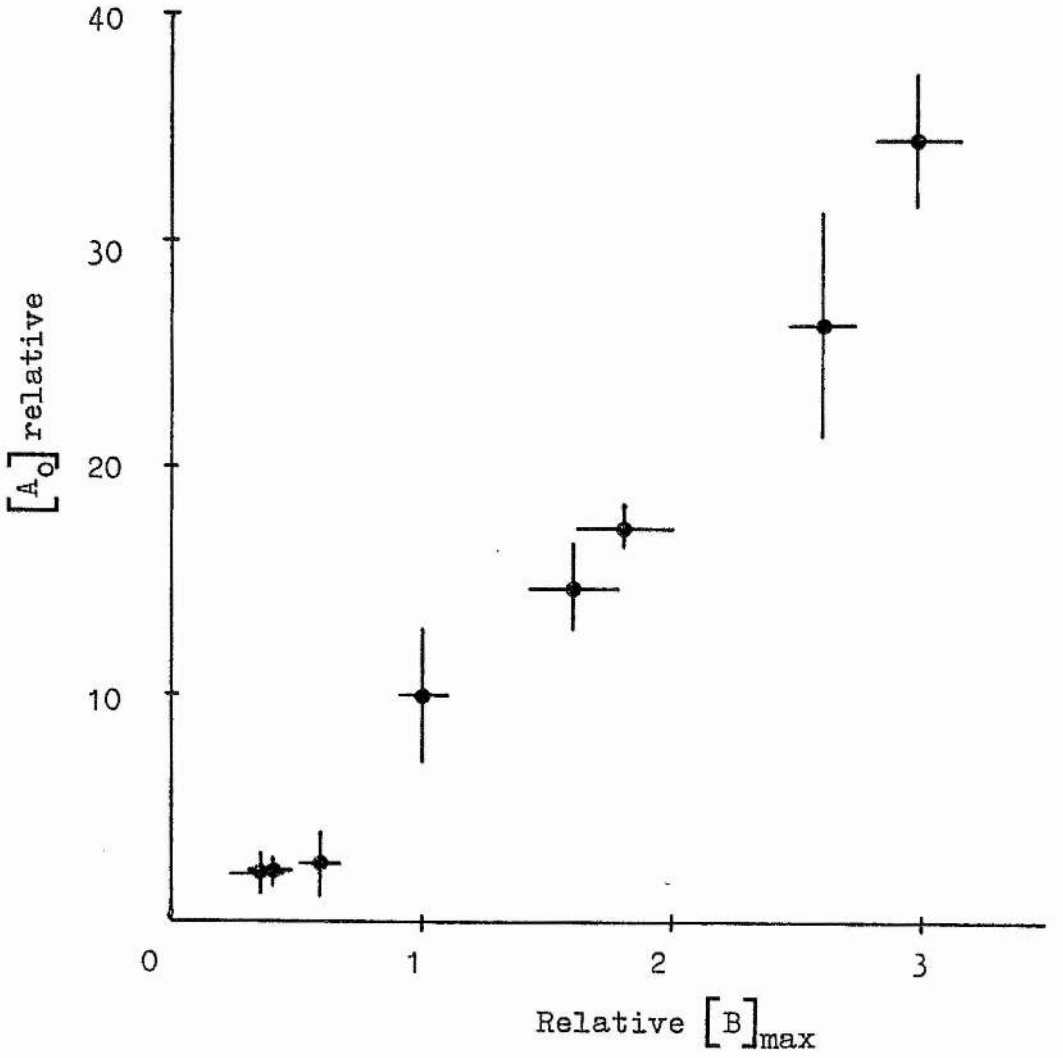

0.3
0.2
0.1
sec^{-1}
0
10
20
30
40°C
Temperature Difference (ΔT)

Fig 5.7

The relationship between calculated $[A_o]$ and $[B]_{max}$.

$[A_o]$ and $[B]$ relative were calculated as described in legends to Figs 5.5 and 5.2 respectively.

Each point represents the mean of three observations and the vertical and horizontal lines the standard errors of the mean. The correlation co-efficient was 0.93.


40
30
20
10
0
1
2
3
$[A_o]$ relative
Relative $[B]_{max}$

confirmed by a comparison of the values obtained in both Method I and II for a T_2 of 2°C. The values obtained for k_1 in either method are not significantly different ($p > 0.9$), this is also the case for k_2 values when $T_1 \leq 20°C$ ($p > 0.5$). However, when $T_1 > 20°C$ this does not apply; k_2 increases significantly ($p = 0.02$) up to a T_1 value of 35°C. Arrhenius plots for the rate constants of the forward (k_1) and the backward reaction (k_2) when T_1 = 20°C yield activation energy values which are not significantly different (49.8 kJ/mole and 53.3 kJ/mole respectively).

Although the tension response of the CIC may approximate to a bi-exponential of the form;

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

it is hazardous to attempt to assign definite physiological identities to A, B and C. However, as Charles Darwin has remarked 'False facts are highly injurious to the progress of science, for they often endure long, but false views, if supported by some evidence, do little harm, for everyone takes a salutatory pleasure in proving their falseness; and when this is done, one path towards error is closed and the road to truth is often at the same time opened'. (The Descent of Man). In view of this statement and what is known about muscle physiology in general it is possible to propose physiological analogues for A, B and C.

As suggested earlier [B] may represent the number of activated cross bridges. [A] is proposed to be proportional to an unspecified

activator responsible for cross bridge reactivation, while, [C] is the product of the backward reaction which can be assumed to correspond either to activator re-uptake or inactivation, and proceed at a rate which is dependant on k_2.

Candidates for [A] include Ca^{2+}, or some unknown substance which also has tension promoting activity. Ca^{2+} is known to be responsible for activating the contractile proteins of both vertebrate and invertebrate systems and is almost certainly responsible for cold-induced effects on resting muscle (Sakai and Kurihara, 1974). Although it is likely that the CIC in the ABRM is also mediated by an increased Ca^{2+} availability, this hypothesis has not been substantiated unequivocally, and cold induced effects may be the result of an increase in the concentration of an as yet unidentified stimulant (activator). An alternative interpretation is that the CIC could result from a decrease in activity, of inhibitors such as the SR or regulatory proteins; this effect would be indistinguishable from the above.

This evidence allows the tentative conclusion that the CIC is the result of an increase in the effective level of an activator, and that this activator is probably Ca^{2+}. If this is so then for a given ΔT, $[A_o]$ represents the amount of Ca^{2+} or activator which is made available to be released from intracellular stores. The rate of release and diffusion into the myoplasm will probably be limiting in comparison with the rate of interaction of Ca^{2+} with the

contractile proteins (I. Johnson personal communication). This would appear to be the case as the activation energy for the rate limiting step of the CIC $\dot{d}s$ of the order usually attributed to ionic diffusion (J. McCallum, personal communication). The fact that $[A_o]$ increases with increasing ΔT is in agreement with the results of Newbold and Tume (1977), and Taniguchi and Nagai (1970), who showed that increasing ΔT resulted in an increase in the amount of Ca^{2+} released from SR elements in vertebrate skeletal muscle. The findings of these workers have been discussed more fully in Part IV.

PART VI

EFFECT OF DRUGS ON THE CIC

PHARMACOLOGY INTRODUCTION

The results reported in Part IV and Part V have partially confirmed the working hypothesis that the CIC is the result of an increase in the level of an activator, and in view of other reported effects of cold shock on muscle it has been suggested that this activator may be Ca^{2+}. It, therefore, seemed worthwhile, to examine the effect on the CIC of substances which are thought to influence Ca^{2+} storage and/or movements.

The possible sources of Ca^{2+} responsible for the CIC are: extracellular, membrane-bound, vesicular or mitochondrial. Therefore, agents which are thought to affect these sources in related contractile systems were used in order to exclude some or all of these possibilities. It was hoped to couple this investigation to a cytochemical study using the K-pyroantimonate technique (Atsumi and Sugi, 1976). Using this technique, Atsumi and Sugi studied Ca^{2+} movements in the ABRM during activation. The principle of this method is that K-pyroantimonate reacts with Ca^{2+} to produce an electron-dense precipitate of Ca-pyroantimonate. These workers showed that the Ca-binding sites in this muscle are associated with the inner surface of the fibre membrane, and with vesicles which are closely apposed to it. Mitochondria subjacent to the fibre surface also contained precipiate. These results were confirmed in this study (Plate 3 a, b). Ach-stimulation of

Plate 3

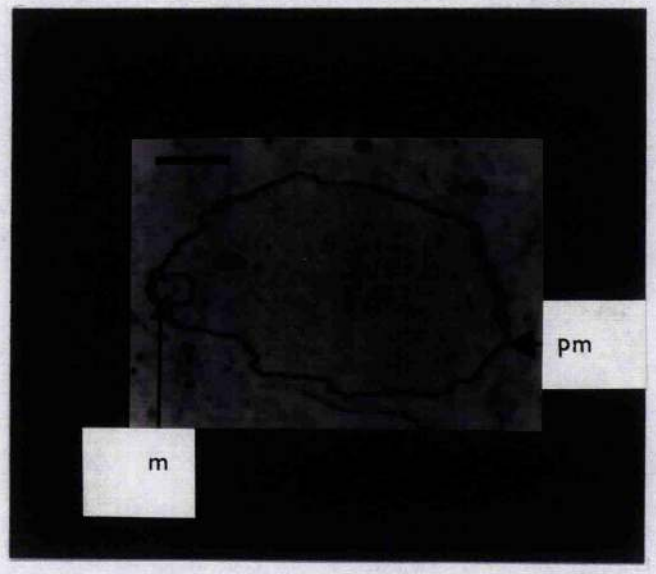
Localization of electron-opaque precipitate in resting ABRM. Fixed in a 1% OsO_4 solution containing 2% potassium pyroantimonate.

(a) Cross section showing localization of precipitate along the plasma membrane (pm) and in the mitochondria (m). Unstained.

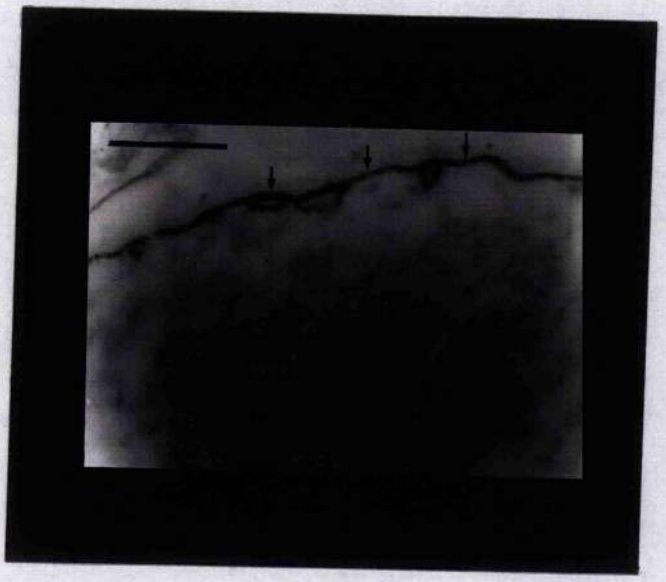
Calibration line 1.0 μm (x 10,138)

(b) Higher magnification view of plasma membrane, illustrating the localization of precipitate in sub-membranous vesicles. Arrow indicates outer surface of plasma membrane. Unstained.

Calibration line 0.5 μm (x 32,706)



a



b

the muscle resulted in a dispersion of Ca^{2+} from these sites into the myoplasm while the situation seen in catch was similar to that at rest (Atsumi and Sugi, 1976).

Unfortunately the pyroantimonate method was found to be unsuitable for studying Ca^{2+} movements during the CIC, primarily because -- the rate of fixation was too slow-acting to allow preservation during the cold-induced response; (this was shown by monitoring tension decay during fixation). Information about the CIC had, therefore, to be obtained solely by the use of pharmacological agents.

K + NTAdr cal.
affected to diff. degrees by
verap. ∴ not direct
effect on actomyosin.
(ha effects both)

PHARMACOLOGY RESULTS

Dependence of the CIC on the $[Ca]_e$

Verapamil

Verapamil, is thought to be a specific Ca^{2+} antagonist in cardiac muscle (Coraboeuf and Vassort, 1968; Kohlhardt, Bauer, Krause and Fleckenstein, 1972) and in a variety of smooth muscle preparations (Haeusler, 1972; Hayashi and Toda, 1977; Peiper, Griebel and Wende, 1971). Its mode of action is not fully understood, but it appears to reversibly interfere with Ca^{2+} influx across the cell membrane, perhaps by inhibiting the interaction between Ca^{2+} and a membrane receptor group. In the present study verapamil (10^{-4} M) was used to estimate the dependence of the CIC on Ca^{2+} influx.

It was observed that verapamil reduced the ACh-response by 40 - 50% of maximum within 10 minutes, and abolished it completely after 100 minutes (Fig. 6:1). The reduction in peak tension was associated with an decrease in dP/dt (Fig. 6:2a), which was reduced by 50 - 60% in 10 minutes.

The effect of verapamil on the cold-induced contracture (CIC) is markedly different. The CIC tension is not reduced until the ACh-induced tension has been abolished. In fact, 70 minutes after the addition of the drug there is a statistically significant increase (30 $\pm$ 5% $p = 0.01$) in CIC tension. From this peak the CIC declines through control levels at 100 minutes to almost zero at

Fig 6.1

The effect of verapamil (10^{-4} M) on the ACh and CIC tensions.

- ● : 10^{-3} M ACh induced tension at 20°C
- ■ : CIC tension produced by a ΔT of 18°C (T_1=20°C)
- o : total tension at CIC_{max}

Each point represents the mean of six experiments and the vertical bars the SE of the mean.

Muscle length: 0.8 L_o.

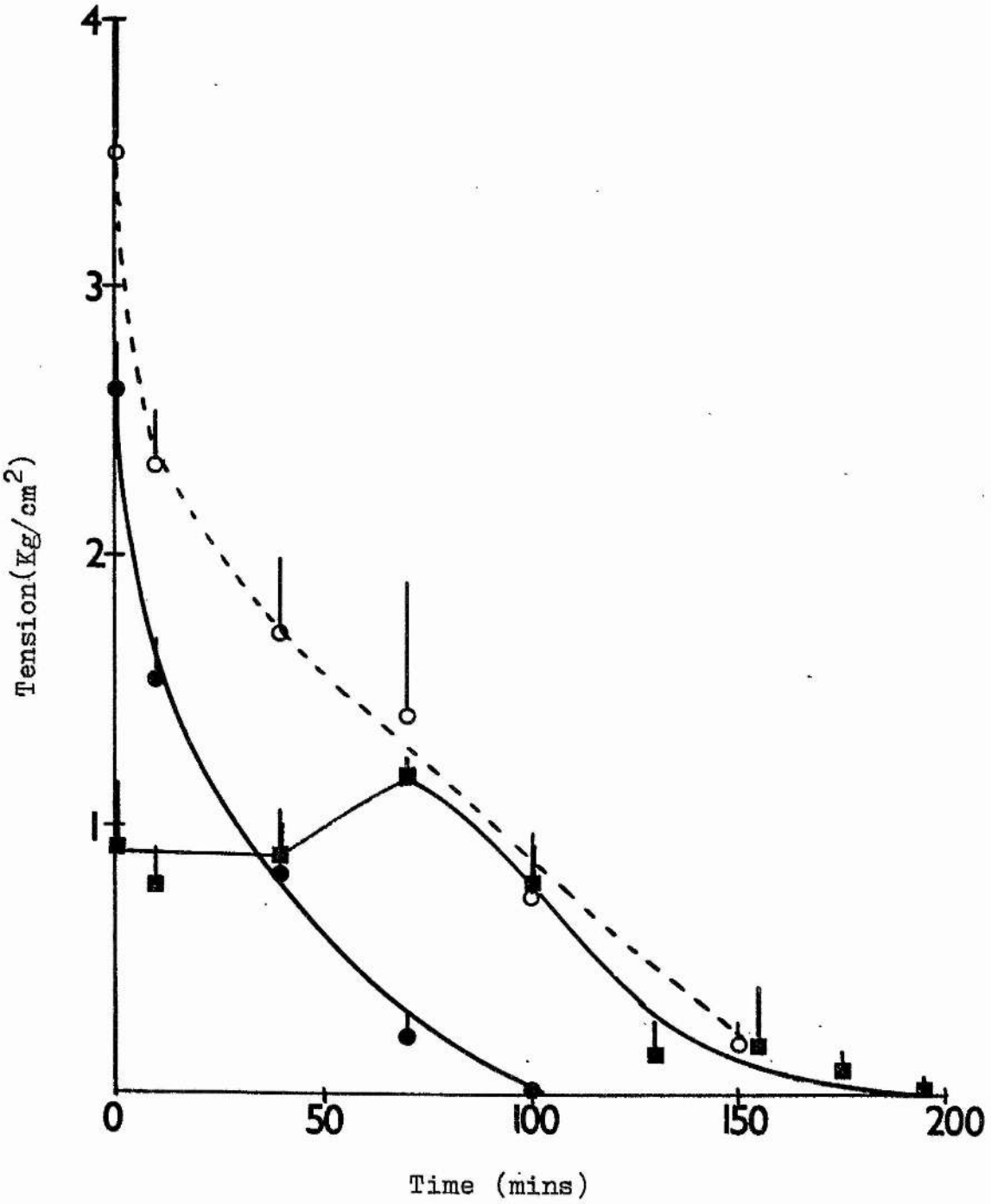

4
3
2
1
0
50
100
150
200
Tension(Kg/cm²)
Time (mins)

Fig 6.2

The effect of verapamil(10^{-4}M) on the rate of tension development of the ACh(A) and the cold induced(B) tension responses of the ABRM.

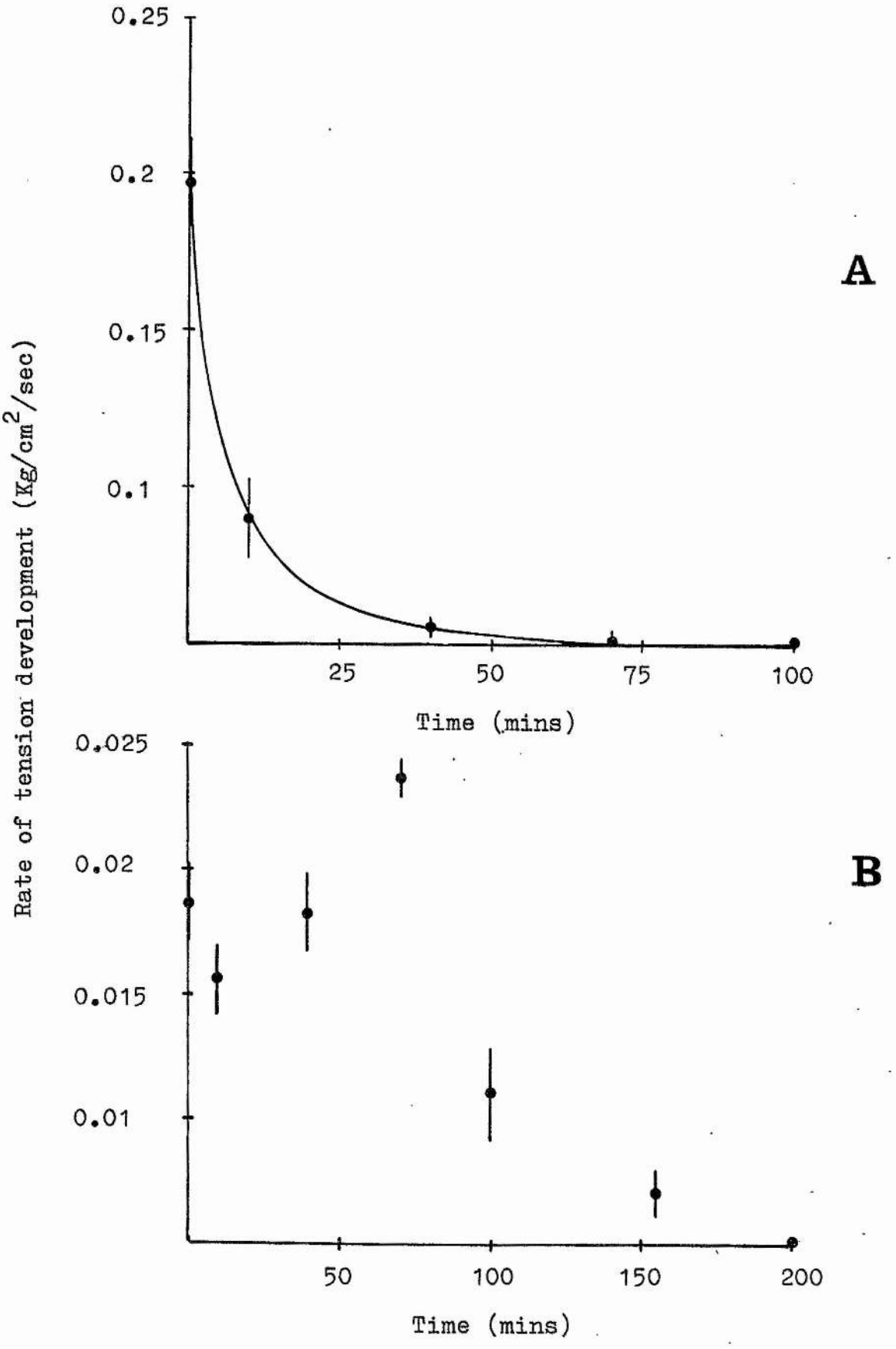
The ACh tension development shows a progressive decrease with time in the drug, from 0.196 ± 0.02 Kg/cm^2/sec in the untreated muscle to 0.0019 ± 0.001 Kg/cm^2/sec after 70 mins incubation in the drug.

The CIC shows an initial increase (18 ± 3%) followed by a progressive decrease.

Note that the range of abscissa values differ in A and B.

Each point represents the mean of six observations and the vertical lines the standard error of the mean.

Muscle length: 0.8 L_o.


0.25
0.2
0.15
0.1
A
Rate of tension development (Kg/cm^2/sec)
25
50
75
100
Time (mins)
0.025
0.02
0.015
0.01
B
50
100
150
200
Time (mins)

200 minutes (Fig. 6:1). The dP/dt relationship shows a similar trend (Fig. 6:2b).

These results show that verapamil has differential effects on the ACh-induced and cold-induced tensions (Fig. 6:3) and suggests that the CIC is less dependent on external Ca^{2+}, than the ACh-induced tension.

Alternatively, the sensitivity of the muscle to verapamil may be reduced at lower temperatures and a higher concentration of the drug may be required in order to completely block Ca^{2+} entry at 20°C. This possibility has not been tested, and there is no evidence in the literature to suggest that this is the case.

$LaCl_3$

Although La^{3+} like verapamil inhibits Ca^{2+} influx it has additional properties. The ion also inhibits Ca^{2+} efflux and interferes with the release of Ca^{2+} from the interior surface of the sarcolemma (Laszlo, 1952). Therefore, if the effects of varapamil and La^{3+} are different then this difference may be attributable to the additional effects of the latter.

In the series of experiments reported in this study, 1 mM $LaCl_3$ was used. As lanthanum sulphate ($La(SO_4)_3$) tended to precipitate from normal sea water, $LaCl_3$ was routinely made up in ASW, (Nagai and Hagiwara, 1971), in which sulphate ions were replaced by chloride ions.

Fig 6.3

Effect of verapamil (10^{-4} M) on the ACh and cold induced tension responses.

△ : point of addition of 10^{-3} M ACh at 20°C

▲ : point of application of cold shock solution (2°C), and removal of ACh.

⚠ : point of addition of 10^{-6} M 5HT

A : untreated control response

B : after 10 mins incubation in 10^{-4} M verapamil

C : after 70 mins incubation in 10^{-4} M verapamil

D : after 100 mins incubation in verapamil(10^{-4}M)

E : after 130 mins incubation in verapamil(10^{-4}M)

Muscle length: 0.8 L_o.

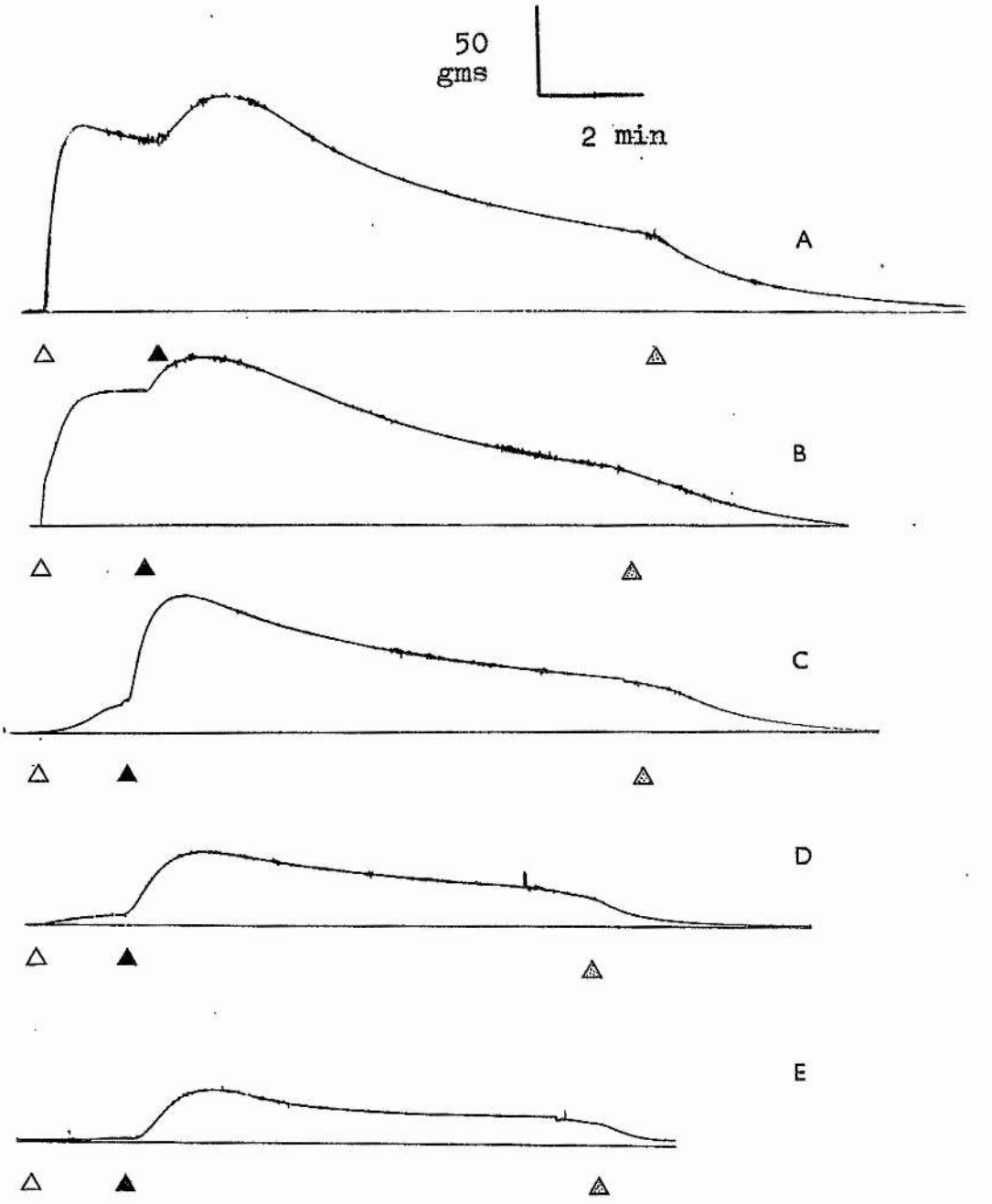

50
gms
2 min
A
B
C
D
E

Fig. 6.4 shows the time-course of the effect of La^{3+} on the ACh-induced and cold-induced tensions. The effect of La^{3+} on both systems is similar i.e. they both decay with time in the drug (Fig. 6.5). However, the rate of reduction of peak tension is greater in the case of the CIC. During the first ten minutes of La^{3+} treatment the peak CIC tension is reduced to 18 $\pm$ 2% ($p = 0.001$) of control values while the ACh-induced tension decreases by only 20% to 80 $\pm$ 10% ($p>0.2$) of its initial level. In addition, the CIC tension falls to near zero after 130 minutes in $LaCl_3$ while a reduced ACh-induced tension may still be observed after 150 minutes. With time in La^{3+} dP/dt for both ACh and cold-induced tensions decreases by approximately 90% in 40 minutes, (ACh: dP/dt 13 $\pm$ 2% ($p<0.001$); CIC: dP/dt 10 $\pm$ 2% ($p<0.001$). However, after 10 minutes the dP/dt relationship of the CIC has decreased to 20 $\pm$ 4% ($p=0.01$) while the ACh tension has declined to only 60 $\pm$ 10% (Fig. 6.6).

The effect of La^{3+} on both the ACh-induced and the CIC tensions is, therefore, quite distinct from that of verapamil and is probably related to the fact that their modes of action are thought to differ.

Ca_e

The preceding results suggest that the ACh response is more sensitive, than the CIC, to a reduction in the influx of Ca^{2+}. If this is the case then the former should also be more sensitive than the latter to a reduction in $[Ca]_e$. A series of experiments

Fig 6.4

The effect of $LaCl_3$ (1 mM) on the ACh and cold induced tension responses of the ABRM.

▽ : ACh (10^{-3}M) induced tension at 20°C

■ : CIC tension produced by a ΔT of 18°C (T_1=20°C)

● : total tension at CIC_{max}

Both the ACh and CIC tensions decrease with time in the drug. The former declines from a control value of 3.1 ± 0.31 Kg/cm^2 to 0.28 ± 0.2 Kg/cm^2 after 130 mins incubation in the drug. The CIC tension was reduced from an untreated control value of 0.763 ± 0.062 Kg/cm^2 to 0.099 ± 0.6 Kg/cm^2 after 110 mins treatment with $LaCl_3$.

Each point represents the mean of six observations and the vertical bars the standard error of the mean.

Muscle length: 0.8 L_o.

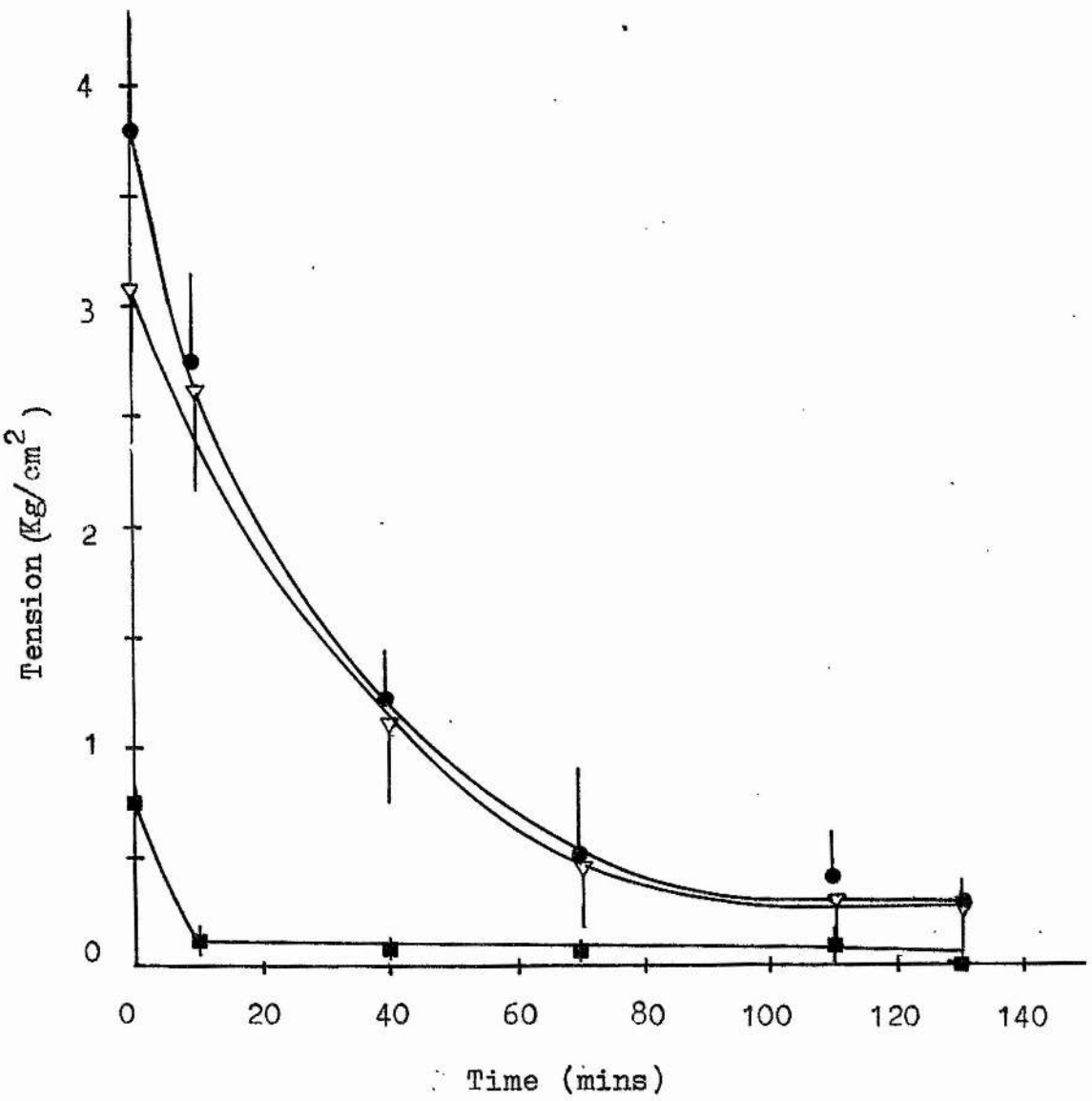

4
3
2
1
0
Tension (Kg/cm^2)
0
20
40
60
80
100
120
140
Time (mins)

Fig 6.5

The effect of $LaCl_3$ (1 mM) on the ACh and CIC tension responses of the ABRM.

△ : point of application of 10^{-3} M ACh at 20°C

▲ : point of addition of cold shock solution at 2°C (△T 18°C T_1=20°C), and removal of ACh

A : untreated control

B : after 10 mins incubation in $LaCl_3$

C : after 70 mins incubation in $LaCl_3$

Muscle length: 0.8 L_o

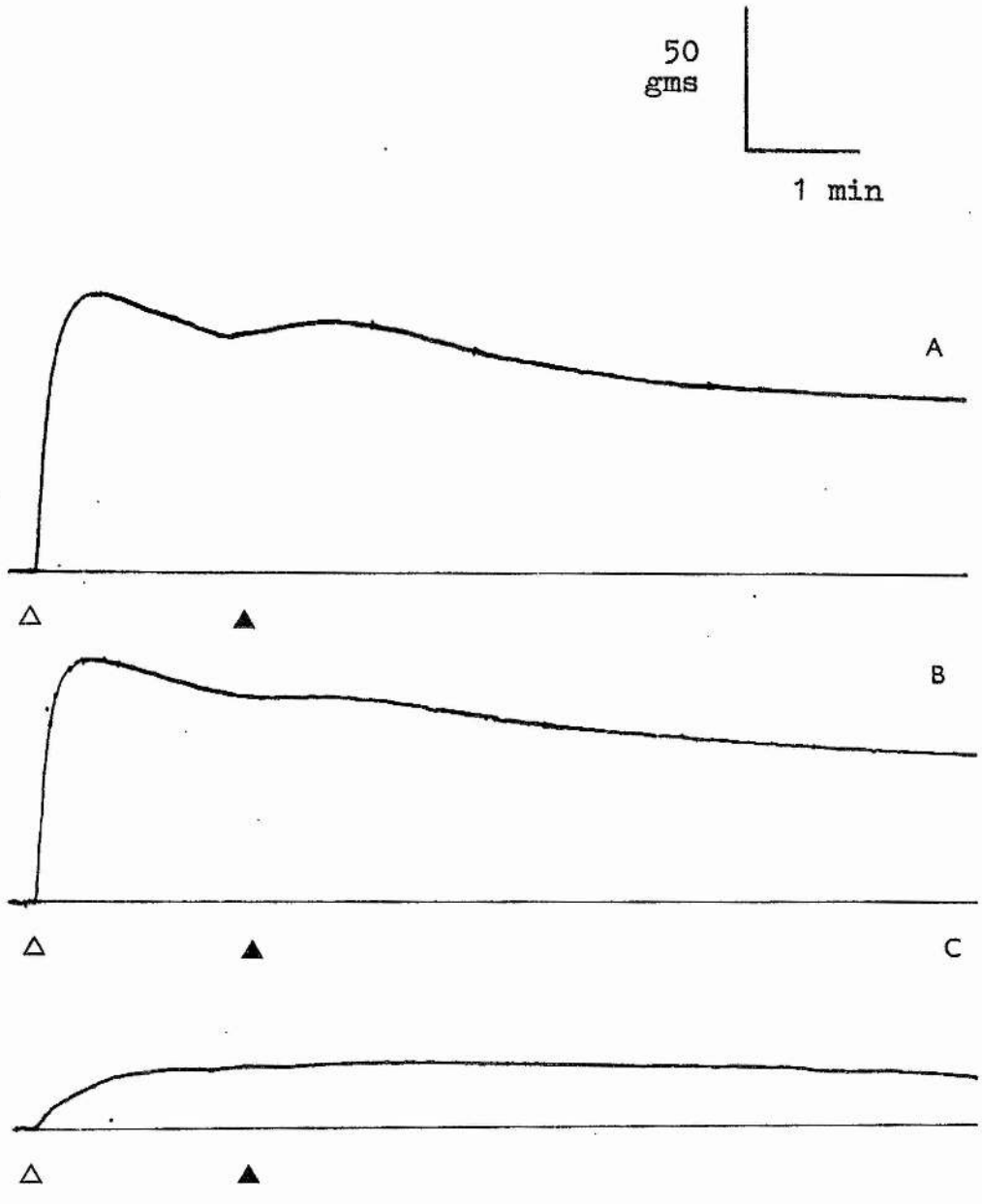

50
gms
1 min
A
B
C

Fig 6.6

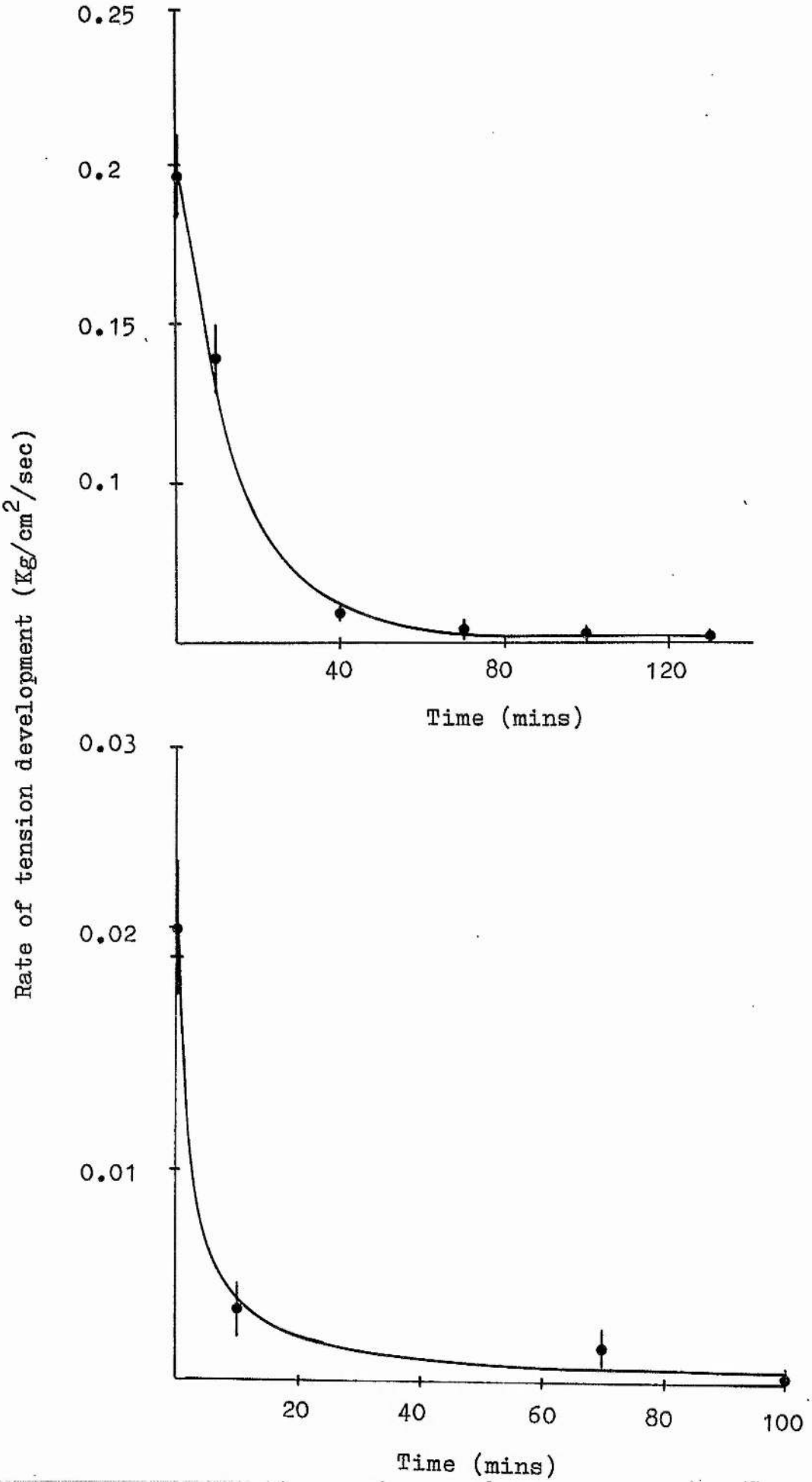
The effect of $LaCl_3$ (1 mM) on the rate of tension development of the ACh (A) and CIC (B) tensions.

Both responses show a progressive decrease in dP/dt with time in the drug.

Note that the range of abscissa values differ in A and B.

Each point represents the mean of six observations and the vertical bars the SE of the mean.

Muscle length: $0.8\ L_o$.


0.25
0.2
0.15
0.1
40
80
120
Time (mins)
A
Rate of tension development (Kg/cm^2/sec)
0.03
0.02
0.01
20
40
60
80
100
Time (mins)
B

was, therefore, undertaken in which the $[Ca]_e$ was altered over the range 20 - 0 mM.

These experiments proved technically difficult since muscles differed in their sensitivity to changing $[Ca]_e$. Reducing the extracellular Ca^{2+} level to 5 mM and below, (ASW usually contains 10 mM Ca^{2+}), very often resulted in the production of spontaneous and irregular contractures. However, maximal ACh-induced tension was obtain at a $[Ca]_e$ of 4 - 20 mM; while maximal CIC tension was observed between 2 - 20 mM $[Ca]_e$. This result does not invalidate the hypothesis that the CIC may be less dependent than the ACh-induced tension on the extracellular [Ca].

The effect, on the CIC, of Depletion of Intracellular Ca^{2+} stores

Caffeine

When caffeine is applied to a skeletal muscle it produces a contracture, which is not accompanied by membrane depolarization (Axelsson and Thesleff, 1958; Bianchi, 1961), and which is thought to be due solely to a release of Ca^{2+} from the SR. Caffeine also produces a tension response in the ABRM (Twarog & Muneoka, 1973; Muneoka & Mizoshi, 1975), the SR-like component of which has been shown to be caffeine sensitive (Gilloteaux, 1976; Gogjian & Bloomquist, 1977), and moderately well-developed. It is likely, therefore, that caffeine acts on the ABRM in a way that is comparable to its mode of action in skeletal muscle. However, Stossel & Zebe, (1968), have shown that the Ca-binding ability of SR-like vesicles isolated from the ABRM is relatively slow, one-ninth that of vertebrate skeletal muscle. As a result several hours are required for full re-accumulation after caffeine depletion (Twarog personal communication).

In this study caffeine depletion of the SR-like elements was performed in order to determine whether these sites were involved in the production of a CIC. In order to produce a caffeine contracture the muscle was placed in Ca-free ASW containing 10 mM caffeine. This procedure was repeated two or three times until the muscle failed to respond, when the caffeine-sensitive Ca^{2+} stores were deemed to be fully depleted. The caffeine contracture usually

consisted of a tonic response alone (Fig. 6:7), but was sometimes preceded by an initial phasic component (Fig. 6.8b).

The effect of depletion of the caffeine-sensitive Ca^{2+} sites on the CIC was then tested in the following way. The muscle was placed in normal SW for 2 - 3 minutes and then transferred to Ca-free ASW. This pre-treatment allowed an apparently normal, though reduced, ACh-induced contracture to be obtained without replenishing to any great extent the internal stores of Ca^{2+}. Under these conditions an apparently normal CIC results. However, if the muscle is incubated for 60 minutes in normal SW then subjected to a cold shock an increased CIC results (Fig. 6:8; 6:9) (this is discussed more fully in the section on cyclic nucteotide metabolism). Fig. 6:10 shows that depletion of the caffeine-sensitive Ca^{2+} stores affects the rate of development of both the ACh and cold-induced contracture tensions. The former is initially reduced to 63 ± 4% ($p > 0.02$) of control values and is still reduced after 100 minutes recovery in normal Ca^{2+} SW. In contrast dP/dt of the CIC is not significantly altered immediately after caffeine depletion but shows a 2.7 fold increase ($p = 0.001$) after 60 minutes recovery in normal SW, which remains even after a recovery period of 100 minutes.

Thus, a prodedure which is reported to deplete the SR of its Ca^{2+} (Twarog and Muneoka, 1973) does not significantly affect the

Fig 6.7

Caffeine contracture.

△ : point of application of 10 mM caffeine at 20°C

The tension produced in this typical example is 12 gms.

Muscle length: 0.8 L_o

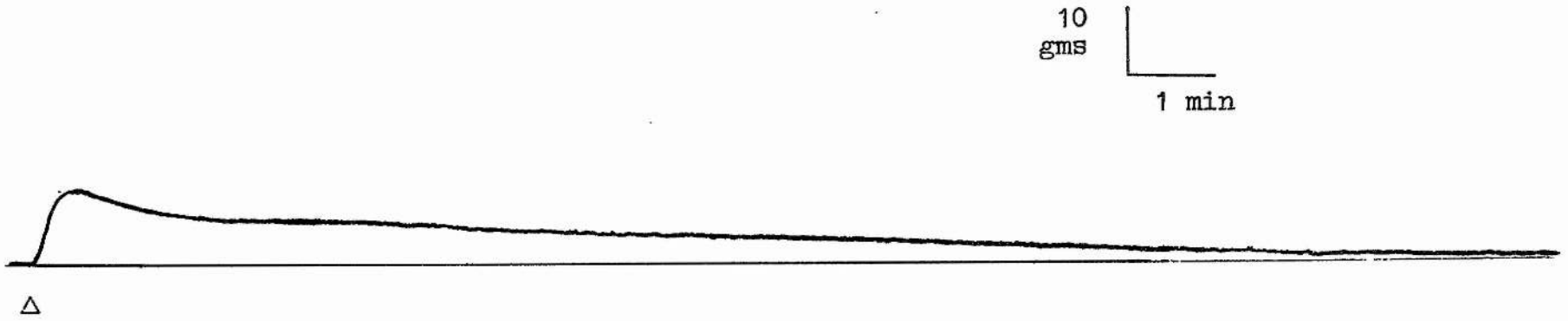

10
gms
1 min
Δ

Fig 6.8

The effect of 10 mM caffeine on the ACh (1) and cold induced (2) tension responses of the ABRM.

a : untreated control values

b : tension response in zero Ca^{2+} SW immediately following a caffeine contracture

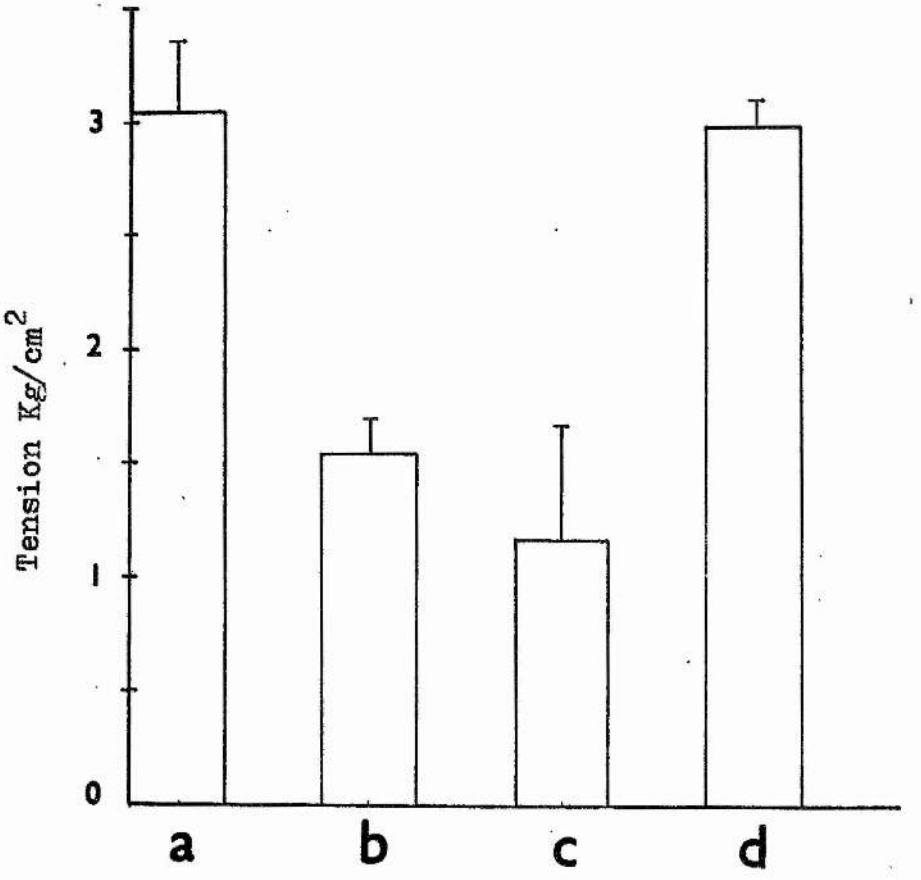
c : tension response after 60 mins recovery in normal Ca^{2+} SW

d : tension response after 100 mins recovery in normal Ca^{2+} SW

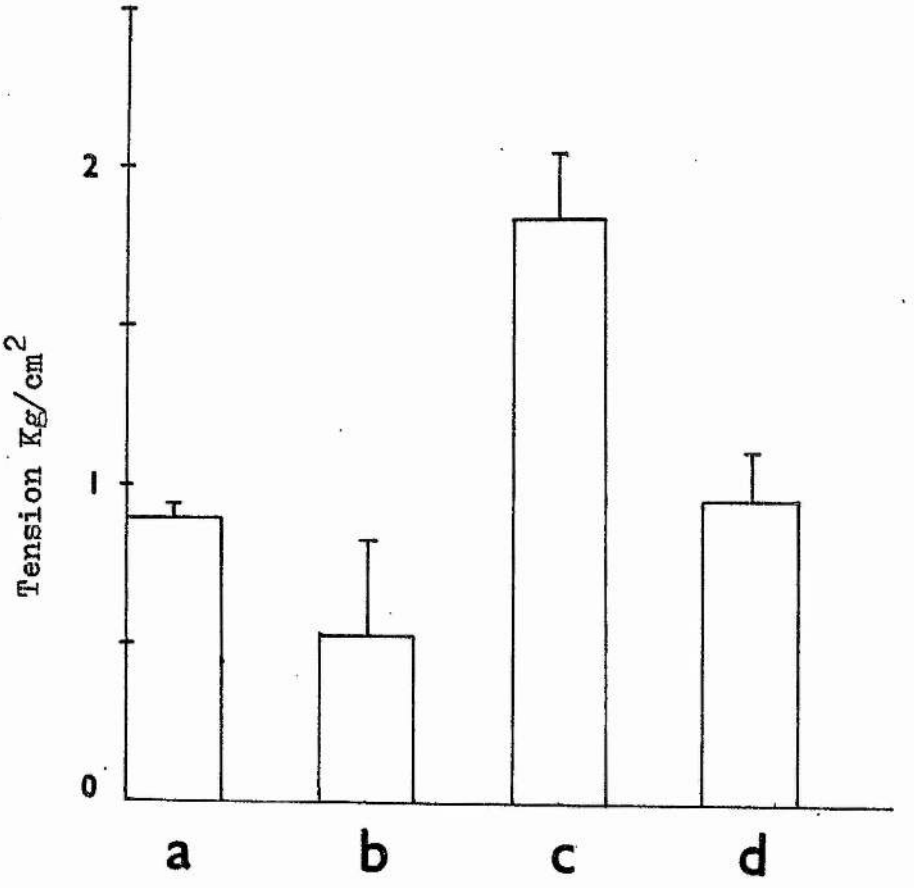
ACh tension was induced by the application of 10^{-3}M ACh at 20°C. A CIC was induced by the addition of a cold shock solution (2°C) two mins after the application of ACh.

Each column shows the mean value for five observations $\pm$ SEM.

Muscle length: 0.8 L_o.



1



2

Fig 6.9

The effect of 10 mM caffeine on the size and form of the ACh and CIC tensions.

△ : point of application of 10^{-3}M ACh at 20°C

▲ : point of application of cold shock solution at 2°C in A,C and D. ACh removed.

▲ : point of application of 10 mM caffeine (B)

A : control response (10^{-3}M ACh at 20°C)

B : bi-phasic response to 10 mM caffeine

C : response to ACh and cold shock after 60 min recovery from caffeine treatment.

D : response after 100 min. recovery

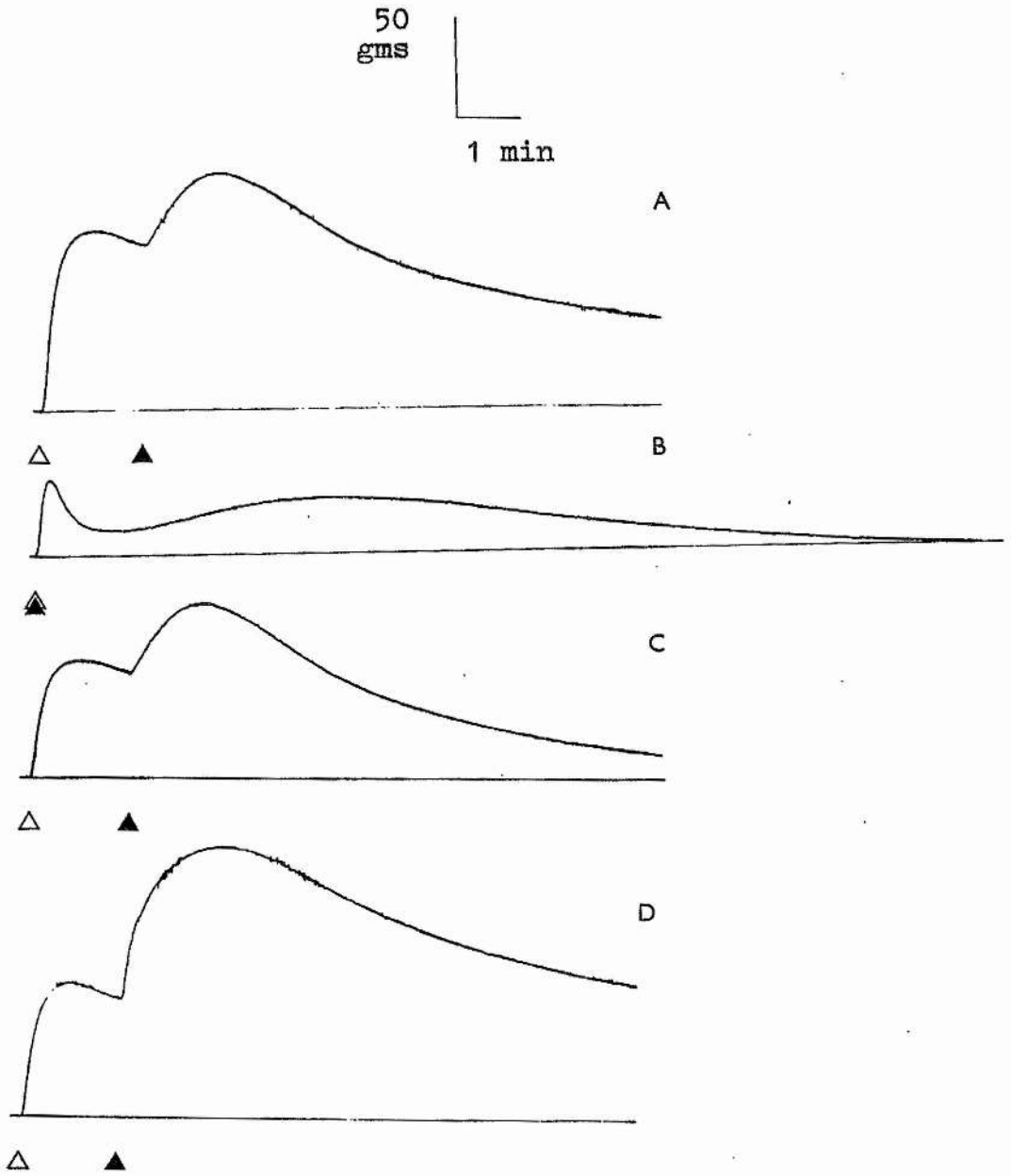

50
gms
1 min
A
B
C
D

Fig 6.10

The effect of caffeine treatment on the rate of tension development of the ACh (1) and cold induced (2) tensions.

a : untreated control

b : response in zero Ca^{2+} SW immediately following a caffeine contracture (10 mM) at 20°C

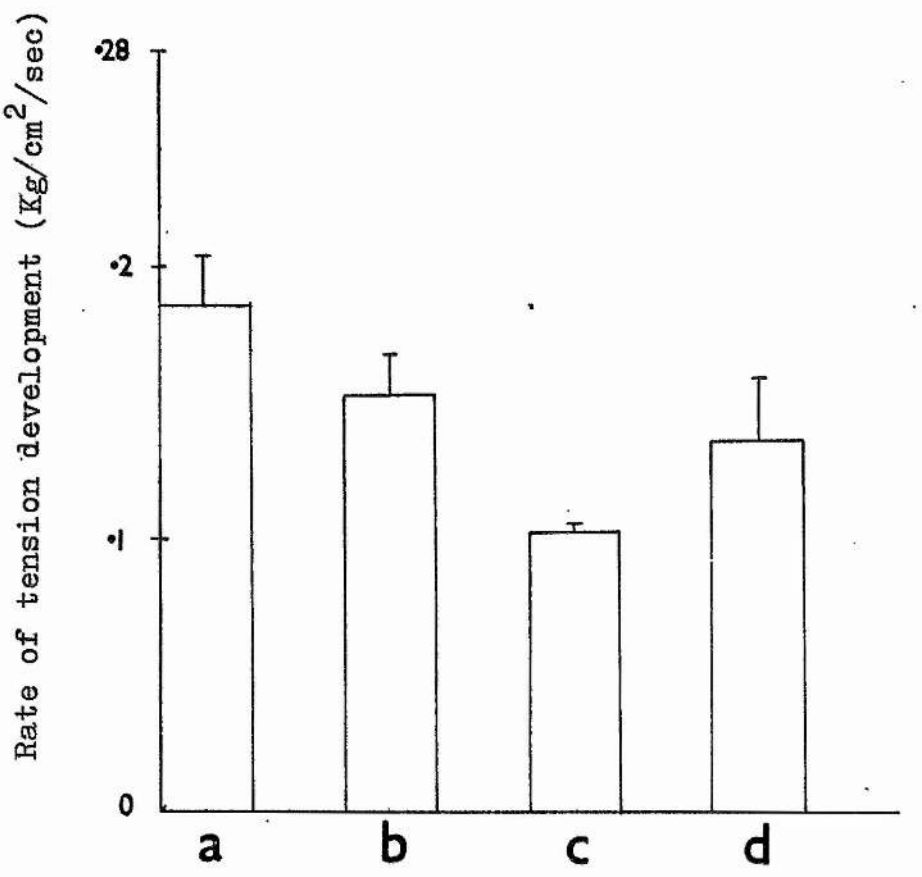
c : response after 60 mins in normal Ca^{2+} SW

d : response after 100 mins in normal Ca^{2+} SW

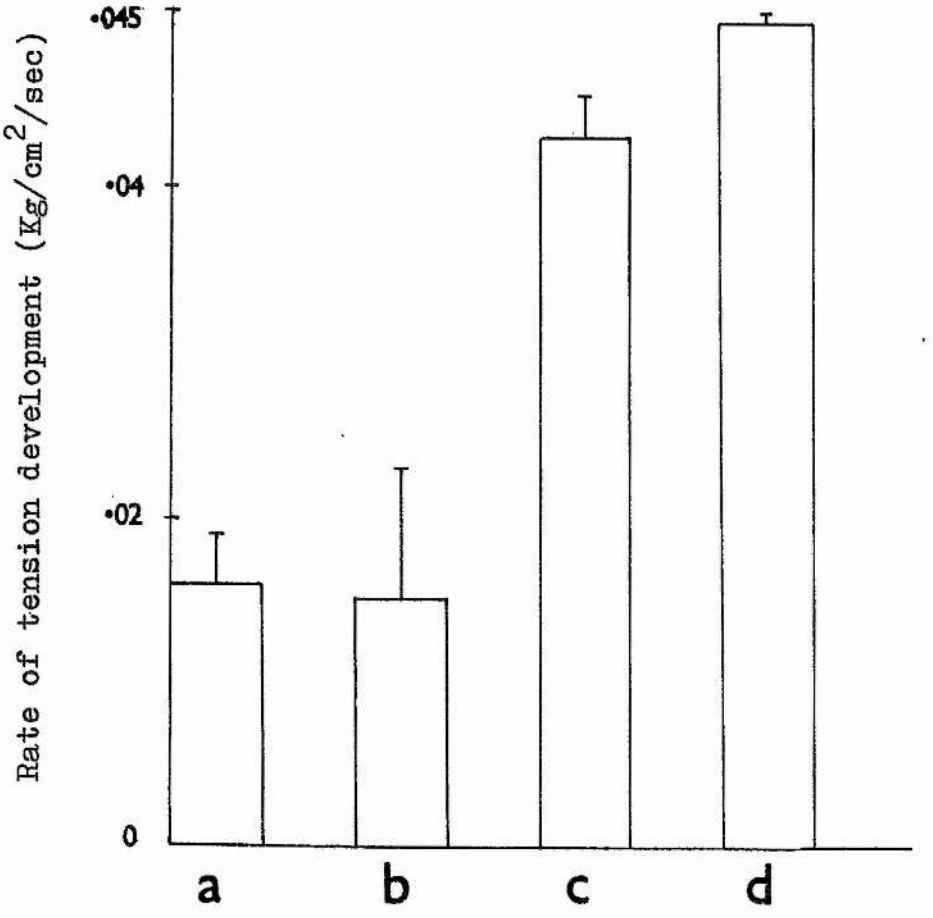
ACh induced tension was initiated by the application of 10^{-3}M ACh at 20°C, and a CIC was induced by the application of a cold shock solution (2°C) two minutes after the application of ACh.

Each column represents the mean of five observations and the vertical bars the standard error of the mean.

Muscle length: 0.8 L_o.


Rate of tension development (Kg/cm2/sec)
·28
·2
·1
0
a
b
c
d

1


Rate of tension development (Kg/cm2/sec)
·045
·04
·02
0
a
b
c
d

2

the CIC. This observation suggests that the origin of the Ca^{2+} thought to be responsible for the CIC is not that which is caffeine sensitive; some other internal source of Ca^{2+} may be involved such as that stored on the inner surface of the fibre membrane or in the mitochondria. In this connection Huddart, Hunt and Oates (1977) have shown, in the smooth pharangeal and columella muscles of Buccinum and Neptunia that the mitochondria are the most likely storage sites for intracellular Ca^{2+}, since an SR is apparently absent.

During the course of these experiments it was found that the sensitivity of the ABRM to caffeine is temperature dependent. This has been noted in many preparations (Blinks, Olson, Jewell and Braveny, 1973; Sakai and Kurihara, 1974 Review), and it has been discussed more fully in Part IV. The application of 3 mM caffeine which is without effect at 20°C can produce a contracture when applied at 2°C. This 'temperature assisted caffeine contracture' is smaller than the contracture produced by 10 mM caffeine at 20°C and can usually only be repeated once or twice.

In addition, a cold shock applied to a muscle undergoing a caffeine contracture at 20°C (ΔT 18°C) elicits a CIC which is similar to, though smaller than, that produced during an ACh-induced contracture (Fig. 6:15). This may be accounted for by the above observations, that a muscle appears to have a higher sensitivity to caffeine at lower temperature.

Fig 6.15

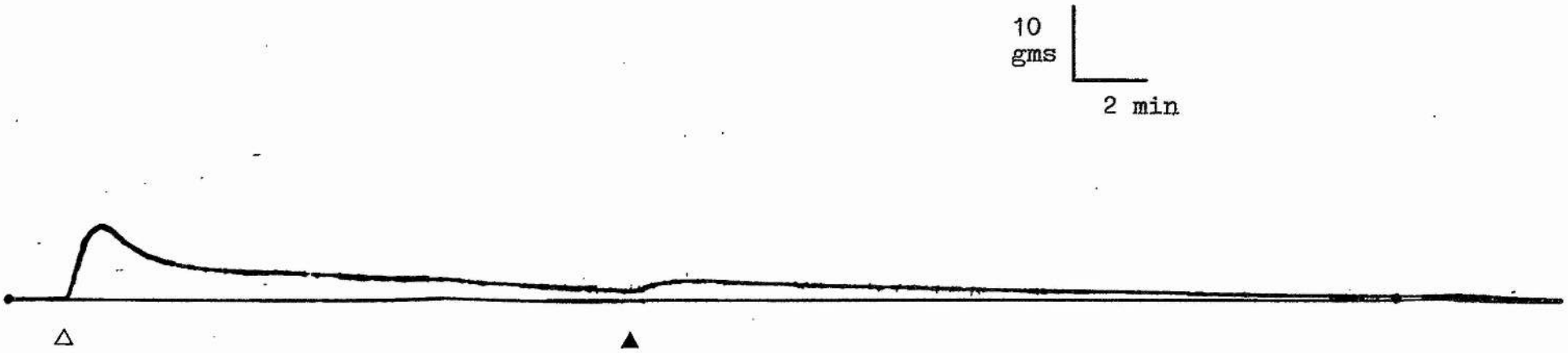
Effect of cold shock on a muscle undergoing a caffeine contracture.

△ : point of application of 10 mM caffeine at 20°C

▲ : point of application of cold shock solution at 2°C. The cold shock solution did not contain caffeine.

In this example the caffeine contracture tension was 10 gms and the CIC tension was 1.5 gms.

Muscle length: 0.8 L_o.


10
gms
2 min

Verapamil appears to have no effect on caffeine contractures; this is similar to the findings of Chiarandini and Bentley (1973), in toad gastrocnemius, and shows, that while verapamil has a significant effect on the action of isolated SR (Balzer, 1972) in whole muscle preparations it appears to be unable to penetrate the cell membrane.

The role of cyclic nucleotides in the production of a CIC

Although caffeine has already been shown to affect ACh-induced tension in preference to the CIC, interpretation of this result is complicated by the fact that the drug both inhibits phosphodiesterase activity (Twarog and Cole, 1972) and causes the release of Ca^{2+} from SR-like elements. It was, therefore, necessary to compare the direct effects of caffeine with those of other substances which alter cyclic nucleotide levels. The level of cAMP may be increased by methyl xanthines such as caffeine and theophylline which inhibit phosphodiesterase activity. Alternatively, a reduction in cAMP may be obtained using substances such as nicotinic acid which stimulates phosphodiesterase, (Twarog and Cole, 1972), and sodium fluoride which inhibits adenyl cyclase (Robison, Schmidt and Sutherland, 1970).

In the preceding section it was noted that the CIC is not markedly affected by caffeine pre-treatment, but the ACh-induced tension is reduced by on average, 50%. In addition, it was found that if a muscle was returned to normal SW for 60 minutes, then stimulated with ACh and subjected to a cold shock, the resulting CIC was substantially greater (x 1.6) than the control value. At this time the ACh tension was still 33% of its control value. There are two possible explanations for this observation. Firstly, Ca^{2+} normally taken up by the ACh-sensitive stores may be diverted to sites which are susceptible to cold shock. Secondly, the potentiating effect

of caffeine may be due to a change in the level of cAMP. It has been shown that relaxation in the ABRM, in response to 5HT, is accompanied by an increase in the level of cAMP (Achazi et al, 1974). It therefore seems unlikely that caffeine exerts its effect on the CIC by altering cyclic nucleotide levels. The results obtained with theophylline support this conclusion.

Theophylline

Theophylline, inhibits phosphodiesterase but its potency in this respect is six times greater than caffeine (Bourne and Sutherland, 1962).

In the present investigation it was found that exposure for long periods (up to 120 minutes) to a relatively high dose of theophylline (10^{-2} M); had little or no affect ($p > 0.5$) on the ability of the muscle to respond to ACh or cold shock (Fig. 6:11; 6:12). It, therefore, seems unlikely that the CIC is effected by a change in the level of cAMP.

Nicotinic Acid/Sodium Fluoride

Nicotinic acid, an agent which has a stimulating affect on phosphodiesterase activity (Twarog and Cole, 1972) has no effect on the ACh-induced tension or the CIC.

Fig 6.11

Effect of theophylline (10^{-2}M) on the ACh and cold induced tension responses of the ABRM.

● : ACh (10^{-3}M) induced tension at 20°C

■ : CIC tension (ΔT 18°C, T_1=20°C)

Over the experimental range there is no significant alteration in either the ACh or cold induced tension values (see text).

The abscissa indicates the time of incubation in the drug.

Each point represents the mean of five observations and the vertical bars the standard error of the mean.

Muscle length: 0.8 L_o.

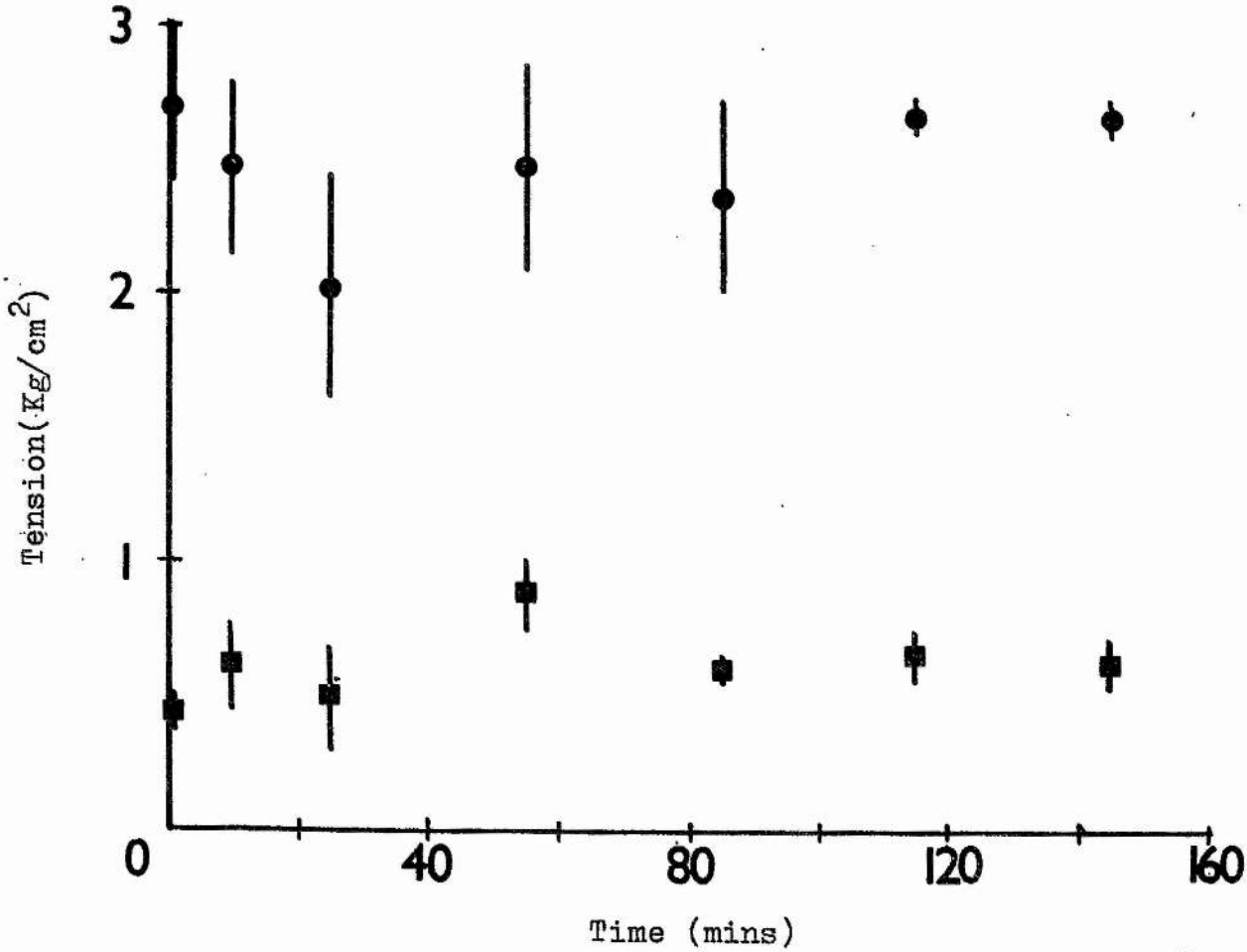

3
2
1
0
40
80
120
160
Tension(Kg/cm2)
Time (mins)

Fig 6.12

The effect of theophylline (10^{-2} M) on the ACh and cold induced tension levels.

△ : time of application of 10^{-3} M ACh at 20°C

▲ : point of application of cold shock solution (ΔT 18°C T_1 = 20°C), and removal of ACh

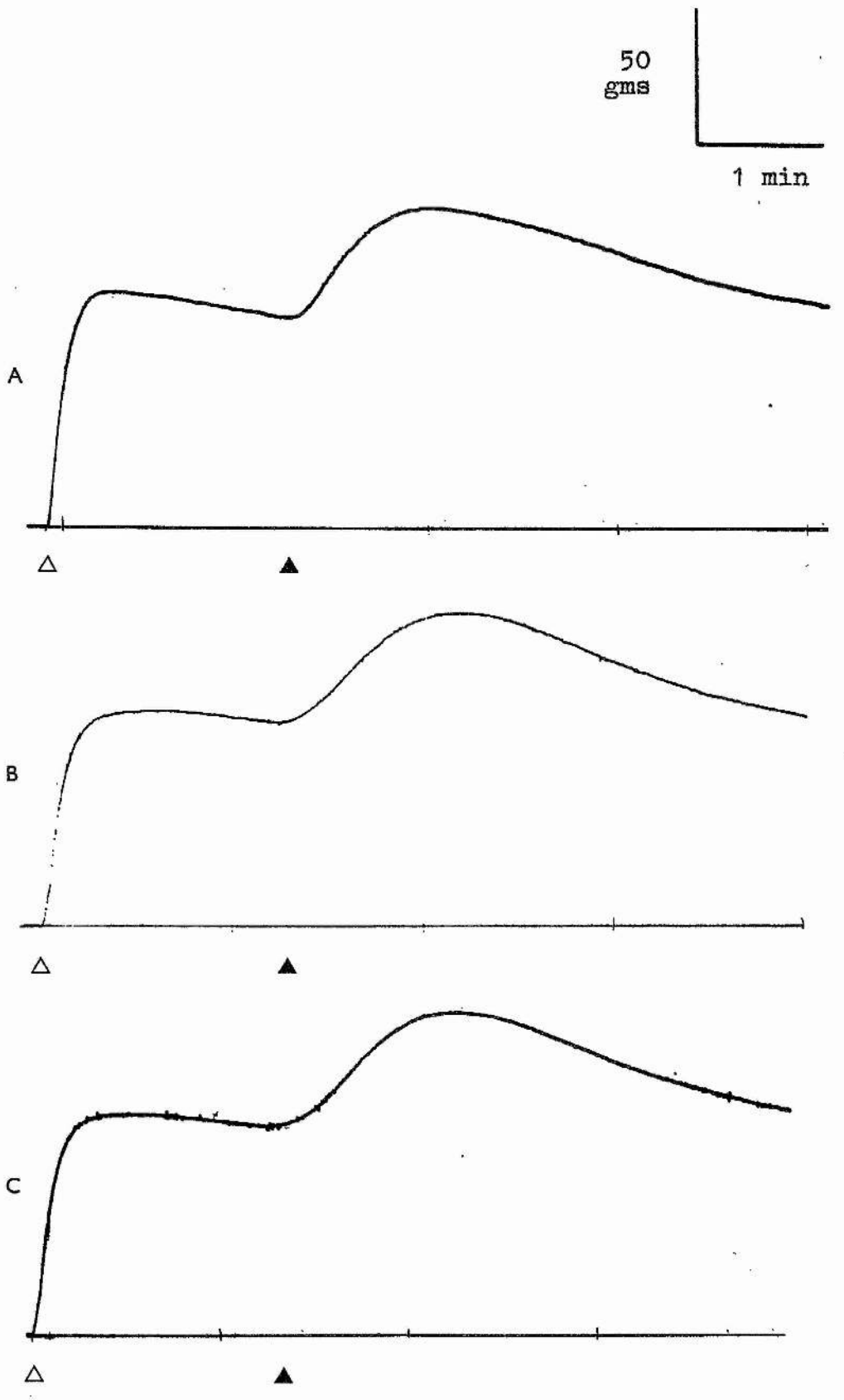
A : untreated control

B : after 55 mins incubation in theophylline

C : after 115 mins incubation in the drug

No significant difference in tension values for either the ACh or CIC tensions was observed.

Muscle length: 0.8 L_o.


50
gms
1 min
A
B
C

Similarily, sodium fluoride, which is known to stimulate adenyl cyclase in mammalian broken cell preparations (Robison, Schmidt and Sutherland, 1970), was also without affect.

Taken together the observations presented in this section strongly suggest that in the ABRM the tensions elicited by ACh or cold shock are not significantly affected by alterations in cyclic nucleotide levels.

Potassium Cold Induced Contractures

The possibility that the CIC resulted from a direct interaction between ACh and the temperature shock was excluded by the use of K-contractures. These were induced by depolarizing the muscle in ASW containing 330 mM K^+ (Fig. 6:13), and are affected by cold shock as shown in Fig. 6:14.

In a series of fifteen experiments, the cold-induced contracture obtained by cooling the muscle (ΔT = 18°C) 2 minutes after immersion in high K^+ SW was 0.88 $\pm$ 0.11 Kg/cm^2, compared to a value of 0.94 $\pm$ 0.23 Kg/cm^2 obtained by abruptly cooling an ACh-induced response. These values are not significantly different ($p = 0.5$) and support the conclusion that the CIC may not be explained in terms of an effect of temperature on, either, ACh-receptor interaction, or ACh breakdown by cholinesterase.

Fig 6.13

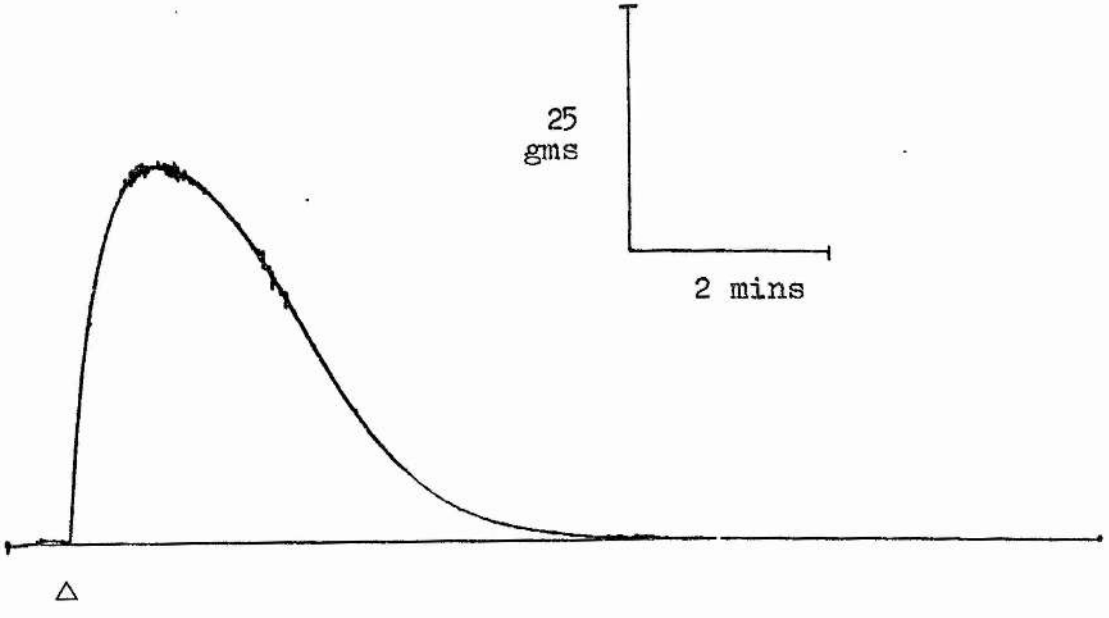
Potassium contracture.

△ : point of application of high K^+ ASW at 20°C

In the typical response shown the tension exerted was 38 gms.

The high K^+ ASW contained 330 mM KCl.

Muscle length: 0.8 L_o.


25
gms
2 mins
△

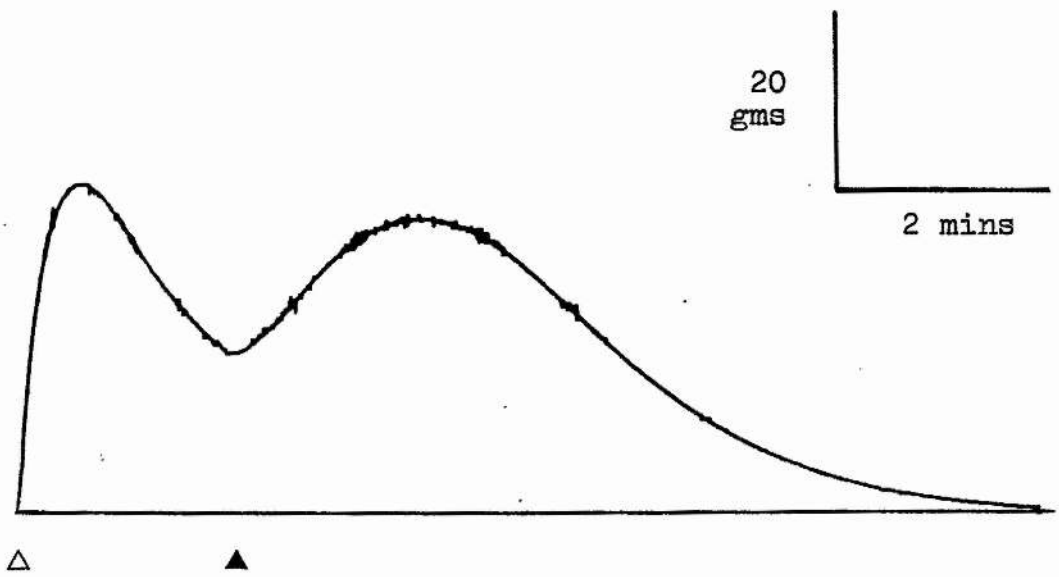

20
gms
2 mins
△
▲

Fig 6.14

The effect of cold shock on a muscle undergoing a K-contracture.

△ : point of application of high K^+ ASW (330mM KCl) at 20°C

▲ : point of application of cold shock solution (ASW) at 2°C

In the typical example shown, the K^+contracture tension is 36 gms and the CIC tension is 28 gms.

No apparent difference was observed in the responses to cold shock initiated by high or normal K^+ ASW solutions.

Muscle length: 0.8 L_o

PHARMACOLOGY DISCUSSION

Several hypothesis have been proposed for the mode of action of cold shock on the ABRM, these include; membrane effects, an increasing sensitivity to ACh, a change in Ca^{2+} affinity of regulatory proteins and a transient increase in the level of an activator. Each of these hypotheses were considered and in the light of the experimental data presented here, and observations made by previous workers on temperature effects on whole (Sakai and Kurihara, 1974) and disrupted muscle (Newbold and Tume, 1977; Taniguchi and Nagai, 1970); only the suggestion of a transient increase in the myoplasmic level of an activator (probably Ca^{2+}), seems likely. This hypothesis is further supported by the fact that substances which are known to directly affect Ca^{2+} movements alter the response of the muscle to cold shock. Although these agents could directly affect the contractile proteins, there is no evidence in the literature to suggest that this may be the case. Also, their influence if any on Na^{+} movements may be largely disregarded, for their is evidence that the Na^{+} influx associated with ACh-induced depolarization in the ABRM is not necessary for the initiation of a tension response (Twarog and Muneoka, 1973), and a CIC can be produced in Na-free SW.

In the ABRM there are three possible sites of Ca^{2+} which alone or together might be involved in the production of the CIC;

these are; extracellular, membrane-bound or intracellular stores. In the present report these possibilities were investigated by the use of agents which are known to affect the availability of one or more, of these Ca^{2+} sources in related contractile systems. Four main conclusions were drawn from the experimental results obtained:

i) The CIC appears to be relatively independent of extracellular Ca^{2+} influx.

ii) The CIC appears to be ultimately dependent on Ca^{2+} movements.

iii) If the CIC is induced by the release of intracellular Ca^{2+} then the site of its release is pharmacologically distinct from the caffeine and ACh-sensitive sites.

iv) The CIC does not appear to result from alterations in intracellular concentrations of cyclic nucleotides.

Evidence that the CIC is relatively independent of Ca_e influx

ACh-induced tension is known to be dependent on the external Ca^{2+} concentration. Entry of a small quantity of "trigger" Ca^{2+} is thought to cause the release of Ca^{2+} from membrane-bound stores (Twarog and Muneoka, 1973; Sugi and Yamaguchi, 1976; Twarog, 1976). This situation is similar to that seen in some smooth muscles where the mechanism is termed pharmamechanical coupling (Somylo and

Somylo, 1968). Evidence for pharmamechanical coupling in the ABRM is derived from work by Sugi and Yamaguchi (1976), who showed that both K and ACh could cause the same degree of depolarization, yet the tension response to the former was smaller. Also, K-contractures were reduced by 30-50% at a $[Ca]_e$ of 1.2 mM, while the ACh contractures were unaffected at a $[Ca]_e$ concentration as low as 0.6 mM.

In this study, the ACh-induced tension appeared to be more sensitive to the $[Ca]_e$ than was reported by Sugi and Yamaguchi (1976) since the ACh-induced response was reduced at extracellular Ca^{2+} concentrations below 4mM . The CIC, however, was found to be insensitive to the $[Ca]_e$ over a wider range, a tension reduction only being observed at a $[Ca]_e$ of less than 1 mM.

The insensitivity of the CIC to Ca_e is further emphasized by the finding that a CIC may be initiated by the application of cold, Ca-free SW, both during ACh and K-induced tensions and after caffeine pre-treatment. Since it is difficult to determine the [Ca] on the external fibre surface quantitative interpretation is not possible and some Ca_e influx may be involved. The only firm conclusion which can be drawn from these observations is that CIC and ACh-induced tensions appear to differ in their sensitivity to the $[Ca]_e$.

The results obtained using verapamil, a drug which is thought to selectively block the influx of Ca^{2+} into heart and smooth muscle cells (Kohlardt, Bauer, Krause and Fleckenstein, 1972; Haeusler,

1972; Hayashi and Toda, 1977; Peiper, Griebel and Wende, 1971), also suggest that the CIC is less dependent on the entry of Ca_e than the ACh-induced tension. This conclusion is arrived at because both peak ACh-tension and the rate at which this tension develops are reduced during initial exposure to the drug, yet the absolute CIC tension is not reduced during 100 minutes in verapamil, and actually shows a significant increase (20% $p = 0.01$) at 70 minutes. It is, however, eventually abolished after 200 minutes.

In accordance with its presumed action, verapamil causes a gradual reduction and eventual abolition of the ACh response within 100 minutes. This reduction may be explained if the Ca^{2+} released from the internal stores is related to the quantity of trigger Ca^{2+} which can enter the muscle during depolarization. Some such relationship appears to hold since ACh applied after incubation in zero Ca SW produces no tension response, and the correlation between ACh-induced tension and the $[Ca]_c$ shows a gradual rather than a sudden decline.

Since verapamil does not significantly alter the CIC tension response until after the ACh tension has reached zero, it may be proposed that the level of activation necessary for a CIC is lower than that required for an ACh-induced contracture. A difference in susceptibility of different responses to the action of verapamil has been noted previously. In vascular smooth muscle K-responses,

Fig 7.1

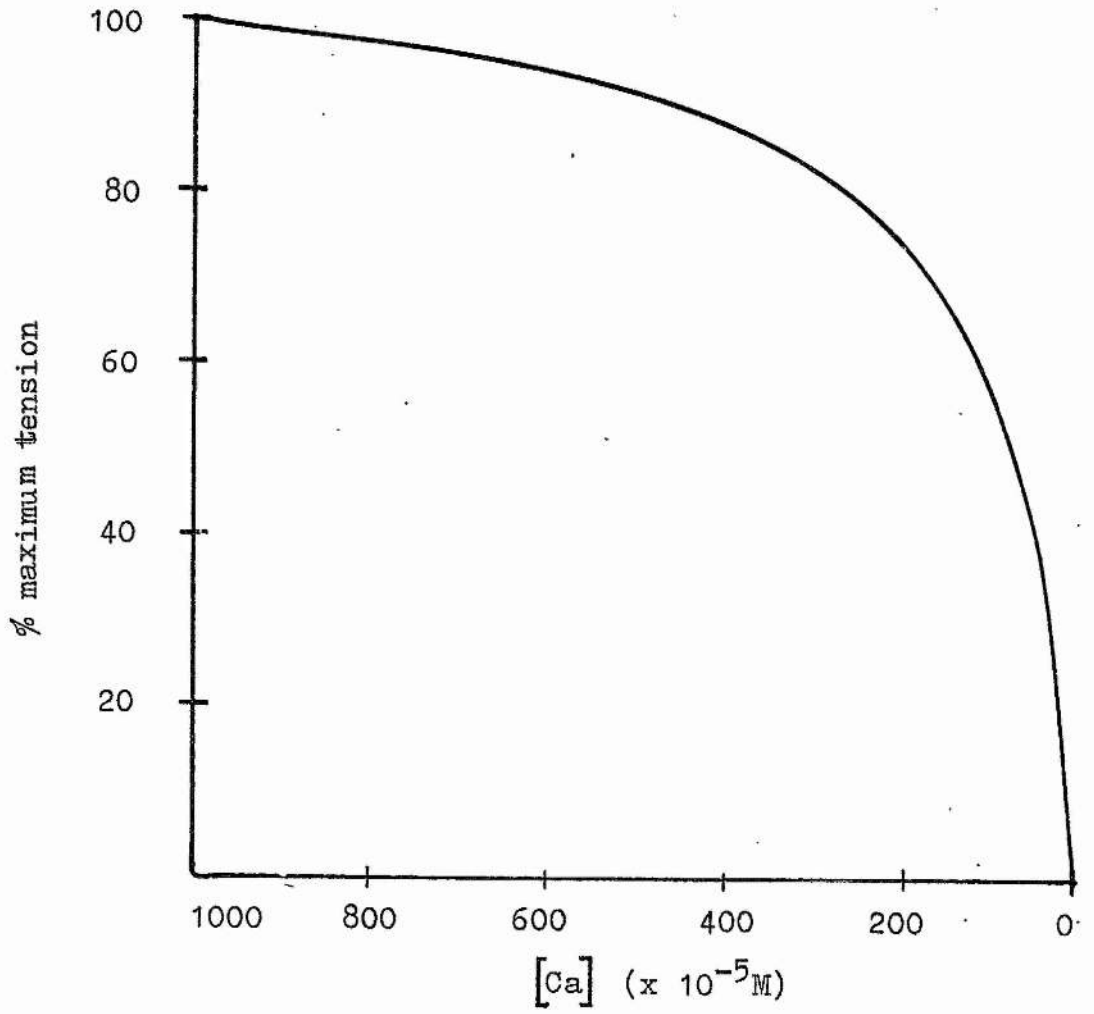
The activation / [Ca] relationship in highly permeable fibres of the ABRM.

This relationship was derived from data given by Twarog & Muneoka (1973).

Highly permeable fibres are fibres treated with 540 mM KCl-EDTA.

The ordinate represents the tension level as a % of maximum. The abscissa shows the Ca^{2+} concentration.

Maximum tension occurs at a Ca^{2+} concentration of 10^{-2}M.


100
80
60
40
20
% maximum tension
1000
800
600
400
200
0
[Ca] (x 10^{-5}M)

which are thought to rely mainly on the entry of Ca_e, are more susceptible to the action of verapamil than nor-adrenaline contractures which are thought to arise from a direct release of membrane bound stores (Haeusler, 1972; Peipel et al, 1971).

The observation that the absolute size of the CIC is initially unaffected by verapamil treatment has been taken to suggest that this drug does not immediately reduce the cold induced alteration in activator levels. However, if as suggested, the activator is Ca^{2+} then the hyperbolic relationship which is thought to exist in the ABRM between Ca^{2+} and tension must be considered (Twarog and Muneoka, 1973). The tension response of the CIC in relation to a given release of Ca^{2+} may depend on the underlying level of activation of the muscle. For example, the same quantity of Ca^{2+} which will cause an increase in tension equal to 40% Pmax, when the muscle is 10% activated, will induce only a 15% increase at 60% activation (Fig. 7:1). However, these values were obtained by Twarog and Muneoka (1973), using skinned and permeable muscles (EDTA treated), and they may not be a true representation of the relationship in vivo. In the case of the results obtained in this study, the finding that there is little variation in the size of the CIC up to 100 minutes in verapamil might suggest that during this time the amount of activator made available during cold shock is reduced, since the level of activation produced by 10^{-3} M ACh is

decreasing. At the present time there is no explanation for the significant increase ($p = 0.01$) in CIC tension seen after 70 minutes in verapamil.

Evidence that the CIC appears to be ultimately dependent on Ca^{2+} movements

Evidence that the CIC is ultimately dependent on Ca^{2+} movements comes from experiments using $LaCl_3$. La^{3+} has been widely used in the study of smooth muscle function. It has been shown by radioautographic and electron microscope techniques not to penetrate the smooth muscle membrane (Laszlo, 1952; van Breemen, 1969), yet it appears to block both Ca^{2+}-influx and efflux (van Breemen et al 1973). By binding at the cell surface it replaces Ca^{2+} at superficial sites and stabilizes the membrane (Weiss and Goodman, 1969). La^{3+} also appears to prevent inward release of Ca^{2+} from membrane-bound sites (laszlo, 1952), and at least in aortic smooth muscle to prevent re-occupation of these sites (Deth and Van Breemen, 1974). In disrupted muscle, La^{3+} inhibits uptake of Ca^{2+} by the isolated SR but it does not cause Ca^{2+} release; it has a similar effect on mitochondria (Batra, 1973).

In many respects the action of La^{3+} in these experiments appears to be similar to that described in other smooth muscles

since those contractures which depend to a certain extent on Ca_g may be almost completely blocked. However, in the ABRM, La^{3+} appears to be able to penetrate the external membrane, because caffeine contractures which depend on intracellular Ca^{2+} release are abolished if the muscle is depolarized in the presence of the ion (Twarog and Muneoka, 1973).

Although in the present investigation La^{3+} blocks both the ACh and CIC tensions, the sensitivity of the two responses to the ion differ. Within ten minutes La^{3+} reduces the CIC by 82% of maximum while the ACh tension is reduced by only 20%. Furthermore, the CIC is abolished at or near 130 minutes incubation in the presence of the ion while the ACh-induced tension is still present after 150 minutes. The rate of tension development in both cases progressively decreases suggesting that La^{3+} reduces the availability of activator.

As previously stated verapamil is thought to act on the surface of smooth muscles to specifically inhibit Ca^{2+} influx. If this is also the case in the ABRM then the reduction in CIC tension, induced by La^{3+}, cannot be explained simply in terms of a decrease in the entry of Ca^{2+}. Although La^{3+} is also considered to reduce Ca^{2+} influx its potency in this respect appears to be less than verapamil, as the latter blocks ACh tension more markedly. This difference in potency was also reflected in the action of these substances on K-contractures. Preliminary investigation showed that

K-contractures were abolished after approximately 30 minutes incubation in verapamil while the presence of $LaCl_3$ was required for at least 60 minutes. Also a direct effect of La^{3+} on the contractile proteins may be excluded as Baguet (1972), using glycerinated taenia-coli fibres found that the ion was without effect. Furthermore, the finding that La^{3+} does not reduce the ACh tension as markedly as the CIC, suggests that its action cannot be attributed to an effect on Ca^{2+}/regulatory protein interaction unless inhibition is increased at low temperatures. This has not been investigated.

Intracellular Source Of Activator Responsible For the CIC

If the CIC and ACh tensions were dependent on the same Ca^{2+} store then depletion of these sites would affect both responses to the same extent. To test this hypothesis caffeine was used to reduce the Ca^{2+} available for release by ACh. As previously stated caffeine, applied to a vertebrate striated muscle releases Ca^{2+} from the SR (Axelsson and Thesleff, 1958; Bianchi, 1961), but does not cause the release of mitochondrial Ca^{2+} in disrupted frog or rabbit muscle (Batra, 1974).

The site of action of caffeine in some smooth muscles is reported to be different from that in vertebrate striated muscle. It has been suggested that in guinea-pig taenia coli, caffeine acts by changing the ionic permeability of the smooth muscle membrane

(which is equivalent to skeletal muscle SR) and causing it to release Ca^{2+} (Ito and Kurihara, 1971). In addition, caffeine appears to act on rat ileal muscle to inhibit Ca^{2+} efflux and promote Ca^{2+} binding to the plasma membrane fraction (Huddart and Syson, 1975). In contrast the mode of action of caffeine in the ABRM is considered to be similar to that in vertebrate striated muscle (see Caffeine Results Section).

The results reported in this study show that the ACh-induced tension is reduced after caffeine depletion while the CIC tension is initially unaffected. This suggests that the sites of action of ACh and cold shock may be different. The finding that caffeine pre-treatment partially reduces the ACh-response corroborates the findings of Twarog and Muneoka (1973), and Sugi and Yamaguchi (1976).

The increase in rate of rise and final CIC tension observed after 60 minutes recovery from caffeine depletion suggests that caffeine pre-treatment may promote an increase in the Ca^{2+} content of the CIC stores. If, under normal conditions, the CIC and ACh stores compete with each other for free Ca^{2+} and as has been suggested (Gogjian and Bloomquist, 1977) caffeine decreases the rate of uptake into the membrane-bound SR-like vesicles, caffeine could cause Ca^{2+} normally taken up by the latter to be diverted into the former. Another possibility, is that Ca^{2+}, unable to enter ACh-stores becomes loosely intracellularly-bound and in this

state can be released by cold shock. This may follow from the observation of Huddart and Syson (1975) who, using rat ileal muscle found that caffeine increased Ca-binding to the isolated muscle membrane.

This data, however, may be interpreted differently. As stated previously, the Ca^{2+} - tension relationship in this muscle may be hyperbolic. Consequently, the initial, but not statistically significant, decrease in CIC tension observed after caffeine depletion may actually represent a decrease in the availability of activator, since this treatment reduces the ACh-induced tension level to 50% of maximum. Similarily, the increased CIC tension observed after recovery in normal Ca^{2+} SW, might occur in response to levels of activator which produce a control response when the ACh tension is normal. If this interpretation is valid then the hypothesis that the CIC is unaffected by caffeine depletion is not strictly correct. However, it does not invalidate the hypothesis that the sites of action of cold shock and ACh are distinct.

Moreover, during K or ACh-contractures a given cold shock produces the same tension response irrespective of the fact that K-contractures are thought to rely solely on the entry of Ca_e and are considerably smaller than ACh contractures which are thought to rely on both Ca_e and Ca_i. This suggests that the CIC may be

initiated to the same extent when the muscle is activated by different mechanisms. If the cold shock were able to release Ca^{2+} from ACh-sensitive stores then the CIC produced during a K-contracture might be larger than that produced during an ACh-response. Since this is not the case, cold shock appears to be unable to release Ca^{2+} from these sites. In addition, if the tension-Ca^{2+} relationship holds, then the CIC produced during a K-contracture will require less activator than a CIC of similar size produced during an ACh contracture.

Evidence that the CIC does not appear to result from alterations in cyclic nucleotide levels .

It has been shown, that in the ABRM, the application of 5HT results in an increase in the level of cAMP (Achazi et al, 1974). cAMP has therefore been implicated as a mediator of relaxation, a role it is suggested to play in vascular smooth muscle (van Breemen, 1977). Theophylline and caffeine, which may be expected to elevate the intracellular level of cyclic nucleotides by inhibiting phosphodiesterase have been reported by Twarog and Cole (1972) to have limited relaxing action. However, these results could not be repeated in this present investigation and drugs, such as theophylline, nicotinic acid and sodium fluoride, which are

thought to affect cAMP levels, appeared to have no significant effect on the level of tension produced during either ACh or cold-induced responses. These experiments, therefore, offer no evidence to suggest that cAMP production has a direct effect on tension activation or relaxation in the ABRM.

Possible role of mitochondria in the production of a CIC

Batra (1973), found that quinidine (1-2 mM) applied to disrupted striated muscle induced the release of Ca^{2+} from mitochondria in preference to the SR. In addition, the application of quinidine to whole muscle resulted in a contracture which was not accompanied by depolarization and which was assumed to be due to Ca^{2+} release from mitochondria. If the mitochondria of the ABRM act as a source of Ca^{2+} then quinidine depletion might be expected to reduce the effectiveness of cold shock. However, preliminary experiments showed it was not possible, either at 2°C or 20°C, to elicit a quinidine contracture in the ABRM even at a concentration as high as 10^{-2} M or to observe any significant effect on the tension levels produced by ACh or cold shock. There may be several reasons for the insensitivity of this muscle to quinidine:

a) quinidine does not penetrate the muscle membrane

b) insufficient Ca^{2+} is stored in mitochondria of this muscle to activate the contractile apparatus.

a) is unlikely since Isaacson and Sandow (1967) stated that both caffeine and quinidine pass through muscle membranes readily, while b) appears to follow from what is known about the relative numbers of mitochondria in skeletal and smooth muscle, the latter having ten times less cytochrome c (a marker for mitochondria) per gram of tissue (Needham, 1970). Also, some smooth muscles become quinidine-sensitive in association with an increase in the number of mitochondria e.g. quinine (an l-optical isomer of quindine), is without effect on non-gravid uterine smooth muscle, but as pregnancy proceeds and the number of mitochondria progressively increases (Burnstock, 1970), its action becomes more noticeable (oxytocic action - Goodman and Gillman, 1975).

In conclusion, therefore, these results have largely excluded three possible sources of activator which might have been responsible for the initiation of the CIC.

i) Ca_e appears only to be required in minimal amounts, and this may only be necessary for the initiation of the active tension, for the CIC appears to be dependent on activation to manifest itself.

ii) caffeine depletion reduces the response of the muscle to ACh but has little initial effect on the CIC; indicating that the CIC is largely independent of the caffeine sensitive store.

Caffeine depletion still get
Ca | Mg - free cont 1

iii) quinidine is similarily ineffective in reducing the CIC, thus tending to exclude the mitochondria as the CIC Ca^{2+} source.

In view of the results reported in this section, it appears most likely that the CIC results from a release of the membrane associated Ca^{2+} which remains even after stimulation with ACh. Furthermore, preliminary investigations suggest that when this source of Ca^{2+} is depleted a CIC may not be produced. Sugi (1971), described in the ABRM, a tonic response which occurs on exposing the muscle to Ca/Mg-free SW containing EDTA, and has shown cytochemically that during this contracture membrane-bound Ca-sites are fully depleted (Atsumi and Sugi, 1976). When these contratures were repeated in the present investigation the application of cold shock had no apparent effect. This hypothesis is corrobrated by the results obtained using $LaCl_3$ which was the only agent to inhibit the CIC in preference to the ACh response. The abolition of the CIC by La^{3+} may be associated with the membrane stabilizing effect of this ion, since under some conditions such as at rest, or in the catch state, no CIC may be produced even though cytochemical evidence suggests that membrane bound Ca^{2+} levels are high (Atsumi and Sugi, 1976). However, on the addition of substances e.g. ACh, K, 5HT or caffeine, which promote Ca^{2+} fluxes a cold induced effect can be observed. This requirement for chemical destabilization is mirrored in other preperations, where,

in the absence of sub-threshold K^+ or caffeine, cold shock applied to a resting muscle does not effect an increase in tension (for review see Sakai and Kuffihara, 1974 - review).

PART VII

CONCLUSION AND SCOPE FOR FURTHER STUDY

CONCLUSIONS AND SCOPE FOR FURTHER STUDY

The present study has been concerned with the elucidation of the mechanism whereby rapid cooling of the ABRM, during a contracture-relaxation cycle, results in a tension increment. Although the precise details have not been established, the experimental evidence reported is consistent with the hypothesis proposed for RCC's that cold shock increases myoplasmic levels of Ca^{2+}. However, it is possible that cooling contractures result from an increase in the availability of some as yet, unidentified activator which has tension promoting properties.

Of the possible mechanisms proposed in Part IV, all were tentatively excluded except for the suggestion that the CIC results from a sudden transient increase in the level of myoplasmic Ca^{2+}. The kinetic model developed in Part V supported this working hypothesis and furthermore suggested that the rate limiting step in the production of a CIC was ionic diffusion. Since the principle ion involved in the regulation of contractile systems is Ca^{2+}, Part VI examines the possibility that this ion is responsible for the CIC and attempts to localize its source. In this the study was only partially successful, but it did suggest that extracellular, mitochondrial and caffeine sensitive Ca^{2+} sites were not the main sources of activator responsible for the production of a CIC. However, this investigation leaves several questions unanswered, and the scope for further study is extensive.

Ca^{2+} Contractile Protein Interaction

A direct effect of cold shock on the contractile proteins has not been conclusively eliminated. The use of glycerol extracted muscles might be used to test this directly, since glycerol extraction disrupts both surface and internal membrane systems leaving a lattice of contractile protein. If cold shock produces a tension response in glycerol-extracted muscles then it might be supposed that it exerts a direct effect on the contractile proteins.

Ca^{2+} Release from Intracellular Organelles

If no direct effect on the contractile proteins were observed then the involvement of the surface membrane and intracellular organelles could be investigated by the use of skinned and chemically treated fibres, and by isolation procedures. If a CIC is observed in a skinned fibre then it might be assumed that the surface membrane is not essential for the response. Alternatively, possible sources of Ca^{2+} such as mitochondria and SR-like vesicles could be isolated by differential centrifugation and subjected to cold shock.

Cytochemical investigation of Ca-movements during cold shock

The use of isolated organelles is liable to criticism on the basis that their properties and function may become altered or impaired during isolation. Cytochemical investigation coupled

to X-ray probe analysis would not be liable to such criticism, and might prove useful in determining the source of Ca^{2+} responsible for the CIC and for monitoring the effect of drugs on Ca^{2+} movements.

Kinetic Analysis of RCC's

Methods developed in this study could be used to investigate the validity of the hypothesis proposed by Sakai and Kurihara (1974), for the production of RCC's. These authors envisaged a system of Ca^{2+} release and re-uptake involving two rate limiting steps, and suggested that the backward reaction was more temperature sensitive than the forward one so that, as ΔT increased, the increased size of the RCC was due mainly to an effect on the backward reaction. This contrasts with the findings of the present report (Part V) where computer analysis shows that the increase in size of the CIC on increasing ΔT is probably due to an increase in the availability of activator rather than alteration of either of the rate constants responsible for tension production and relaxation.

Another interesting point which arose from kinetic analysis was that the ACh-induced tension initiated from 2 - 12°C, followed very closely the bi-exponential form. However, above 12°C this correlation was lost suggesting that the response became kinetically more complex, although no obvious change was apparent. Further analysis

could perhaps be used to propose a model which would fit the ACh-induced tension above 12°C and this could then be related to known physiological properties of the muscle.

PART VIII

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