J. exp. Biol. **107**, 73–93 (1983)
Printed in Great Britain © *The Company of Biologists Limited 1983*

INFLUENCE OF SOME COMMON METHYLXANTHINES ON CONTRACTILE RESPONSES AND CALCIUM MOBILIZATION OF ILEAL, VAS DEFERENS AND BLADDER SMOOTH MUSCLE

BY H. HUDDART, E. BAYTON AND J. SHANKLIN
Department of Biological Sciences, University of Lancaster, Lancaster LA1 4YQ, England

(*Received 4 November 1982 – Accepted 23 March 1983*)

SUMMARY

1. Caffeine and theophylline (0·1–5·0 mM) relaxed rat ileal muscle and reduced spontaneous rhythmicity. They inhibited the K-induced tonic contractures of rat vas deferens and bladder muscle strips but were without significant effect on the phasic responses to K.

2. Theobromine (1·0–2·5 mM) induced contractures in ileal muscle and enhanced both the phasic and tonic components of K-induced contractures in vas deferens and bladder muscle strips.

3. Theophylline and caffeine inhibited by varying degrees the ^{45}Ca efflux from ileal, vas deferens and bladder muscle strips during the slow intracellular phase, but theobromine significantly stimulated ^{45}Ca slow compartment efflux in all three types of muscle.

4. Caffeine and theophylline both depressed, to varying degrees, the ^{45}Ca influx into all three muscles while theobromine stimulated ^{45}Ca influx in all cases.

5. Caffeine and theophylline were either without much effect or slightly stimulated calcium binding by microsomes and mitochondria isolated from ileum, vas deferens and bladder, while theobromine significantly inhibited calcium binding by both sub-cellular fractions in all three muscles.

6. The inhibitory action of caffeine and theophylline on these muscles appears to be due to inhibition of calcium influx coupled with some stimulation of intracellular binding.

7. Theobromine's excitatory action appears to be related to stimulation of calcium influx and inhibition of cellular calcium binding.

INTRODUCTION

The naturally-occurring methylated xanthine alkaloids caffeine, theophylline and theobromine are widely consumed in beverages and employed as stimulants of the central nervous system and low toxicity diuretics. A number of early studies have shown that methylxanthines influence vertebrate cardiac muscle. Bennett (1873) observed that caffeine, theophylline and theobromine accelerated cardiac muscle of

Key words: Calcium fluxes, methylxanthines, smooth muscle.

both the frog and the rabbit and Pickering (1893) found that while xanthine depressed chick embryonic heart, caffeine and theobromine increased heartbeat frequency.

Caffeine has been shown to have a wide variety of actions on skeletal muscle of vertebrates and arthropods which include lowering of the mechanical threshold (Sandow, 1965; Huddart, 1969; Huddart & Abram, 1969), stimulation of the ^{45}Ca efflux from the intracellular compartment (Bianchi, 1961; Isaacson & Sandow, 1967*a,b*; Chen, Bittar, Tong & Danielson, 1972; Huddart & Syson, 1975) and inhibition of intracellular calcium binding by the sarcoplasmic reticulum (Herz & Weber, 1965; Carvalho, 1968; Huddart & Williams, 1974). This latter action is presumably the basis for caffeine-induced contracture in skeletal muscle (Weber & Herz, 1968; Isaacson, 1969; Hill, 1980) and in the myocardium (Chapman & Leoty, 1976).

Recently there has been a tendency to ascribe some of the actions of methylxanthines on muscle tissue to actions on cyclic nucleotide phosphodiesterase. Some studies on smooth muscle have suggested that caffeine may influence contraction by inhibition of cyclic AMP phosphodiesterase, altering the cyclic AMP/cyclic GMP balance (Kukovetz & Poch, 1970; Andersson, 1973; Poch & Umfahrer, 1976; Theobald, Syson & Burrin, 1978) and Beavo *et al.* (1970) have shown that some xanthine derivatives alter lipolytic activity in rat epididymal fat cells, the lipolytic activity being correlated with inhibition of cyclic AMP phosphodiesterase. More recently it has been shown that some methylxanthines can antagonize adenosine receptors (Fredholm, 1980; Fredholm & Hedquist, 1980; Daly, Bruns & Snyder, 1981; Fredholm & Persson, 1982). This may prove to be a more likely mode of action of methylxanthines than their rather weak inhibition of phosphodiesterase, since adenosine is now known to be implicated as a modulator of some responses to neurotransmitters (Schubert, Reddington & Kreutzberg, 1979; Burnstock, 1981).

Smooth muscles are varied in their response to caffeine. Among mammals, caffeine usually has a relaxing effect on smooth muscle (McFarland & Pfaffman, 1972; Sakai & Iizuka, 1972; Wixted & Zimskind, 1977), and in the spontaneously active mammalian ileum this activity may be explained by observed effects of caffeine upon the cells: to inhibit ^{45}Ca efflux from the intracellular compartment, to promote cellular calcium binding and to inhibit spontaneous membrane spikes (Huddart & Syson, 1975; Syson & Huddart, 1976). In some mammalian smooth muscles, caffeine induces contractures (Ito & Kuriyama, 1971; Siegman & Gordon, 1972) which appears inconsistent with the effects of caffeine upon calcium mobilization in the ileum.

In the flounder, caffeine induces contractions of intestinal smooth muscle, in agreement with an observed increase in ^{45}Ca influx, but with little observed effect on ^{45}Ca efflux from the cellular compartment (Barratt & Huddart, 1979). It should be noted that an effect on the cellular calcium pool would be difficult to distinguish because of the small size of this compartment in this tissue (see Alohan & Huddart, 1979). In invertebrate visceral muscles caffeine has a clearly excitatory role (Alohan & Huddart, 1979; Hill, 1980).

Recently, attempts have been made to deduce a structural basis for methylxanine actions. Bianchi (1968) examined caffeine interactions with skeletal muscle sarcolemma while Chapman & Miller (1974) and Johnson & Inesi (1969) examined caffeine inhibition of ATP-promoted calcium transport in the sarcoplasmic reticulum of cardiac and skeletal muscles. While no clear picture emerges, it seems possible that

methylxanthines may interact with cellular receptors which in turn modify secondary messengers. If this were to be the case, structural differences between various methylxanthines may be important in explaining differences in observed actions on smooth muscle.

In this paper we compare the effects of caffeine with those of two other common methylxanthines, in different muscles from the same animal: the spontaneously active ileum and non-spontaneous vas deferens and bladder smooth muscle of the rat. Differences in the effect upon contraction were related to differences in effect on cellular calcium mobilization.

MATERIALS AND METHODS

Male Wistar strain albino rats were used throughout this study. The animals were killed by a blow to the head and smooth muscle strips were dissected from the ileum and the bladder. The vasa deferentia were divided into two portions and longitudinally bissected. All preparations were maintained in Krebs' solution containing (in mM) NaCl, 120·7; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; NaH_2PO_4, 1·2; $NaHCO_3$, 15·5 and glucose, 11·5 at pH 7·3, continuously aerated and kept at 37 °C.

To record tension, preparations were mounted vertically in a series of jacketed 25 ml organ baths whose contents were continuously aerated and could be rapidly changed. Preparations were connected to either a Grass Instruments force displacement transducer or a Washington Instruments isotonic strain gauge. The output was displayed and recorded on either a Grass model 79 polygraph or a Washington MD1 oscillograph. High K salines were prepared by substitution of Na by equimolar amounts of K. The reduced Na level has been shown to be without effect in itself on these visceral muscles (Huddart & Syson, 1975). Caffeine, theophylline and theobromine (Sigma) were prepared as stock solutions (10 mM) in normal Krebs' solution. In our Krebs' solution at 37 °C, 10 mM was saturation level for theobromine. The appropriate quantity of methylxanthine was added directly to the organ bath. After each experiment preparations were flushed with fresh Krebs' solution and each preparation was rested for a standard time of 15 min.

Techniques for estimating ^{45}Ca efflux from smooth muscle strips were as described previously (Huddart & Syson, 1975; Huddart & Saad, 1977). Briefly small strips of muscle (2 mm × 10 mm) were loaded for 1 h in ^{45}Ca Krebs' solution (4 μCi ml^{-1}). After washing and blotting to remove surface label each preparation was placed in an Eppendorf pipette tip connected to a continuous flow pump delivering Krebs' solution at 0·5 ml min^{-1}. The muscle effluent was collected on a conventional fraction collector at 5-min intervals and counted in a Packard TriCarb scintillation counter using a home made scintillation/emulsifier cocktail. The ^{45}Ca efflux of visceral muscles is readily resolvable into two major components: an initial fast component (lasting for about 1 h) related to calcium loss from the extracellular compartment, followed by a small sustained slow component related to loss from the intracellular compartment (see Huddart & Syson, 1975; Saad & Huddart, 1980). It is this latter component which was of interest in this study. After estimating activity remaining in the muscle following tissue solubilization, the efflux curve was expressed as a rate coefficient. In the experiments involving methylxanthines, the washing solution was switched to one

containing the appropriate drug during the slow intracellular efflux phase (see Huddart & Syson, 1975) as indicated in each group of experiments.

Influx of ^{45}Ca into smooth muscle strips was determined as described elsewhere (Huddart & Saad, 1977). Briefly, small muscle strips were exposed to Krebs' solution (with and without methylxanthines) containing ^{45}Ca ($1{\cdot}0\,\mu Ci\,ml^{-1}$). At 5-min intervals sample strips were removed, cleaned of any mucosal material, washed, weighed and ashed. The ash was taken up in 0·1 M-HCl, then neutralized, and the activity in the sample was estimated by conventional scintillation counting. Calcium uptake by microsomes and mitochondria was measured as previously described (see Saad & Huddart, 1981).

RESULTS

Tension studies

The effect of caffeine

Resting tonus of the spontaneously active ileal longitudinal smooth muscle fell with increasing caffeine concentration over the range 0·5 to 5·0 mM, and spontaneous contractions were abolished at 2 to 5 mM (Figs 1, 2).

The effect of caffeine upon tension in smooth muscle prepared from the vas deferens and bladder was studied by examining effects upon the contraction produced by raising the external K concentration (Syson & Huddart, 1973; Saad & Huddart, 1981). The contraction consists of an initial large phasic response followed by a smaller but longer protracted tonic response which persists with only slight decline over the period of K application in both vas deferens (Fig. 3A) and bladder (Fig. 3D). The phasic response was unaffected by 1·0 to 6·0 mM caffeine whereas the tonic response was markedly reduced by concentrations from 3·0 to 6·0 mM (Figs 3, 4).

In several instances, repeated exposure of vas deferens (but not bladder) muscle to caffeine resulted in the development of abnormal spontaneous phasic contractions, even up to 1 h after return to normal Krebs' solution (Fig. 3G). This might be due to an effect on plasma membrane adenylate cyclase, permitting pulsatile signals of extracellular calcium to enter the cells, analogous to the situation following

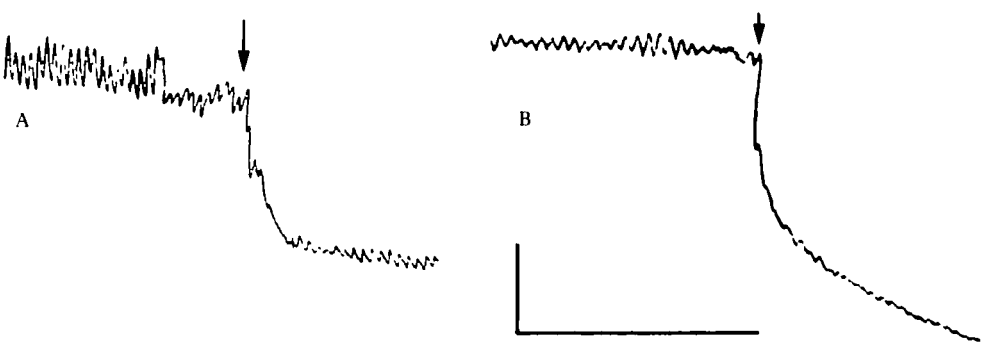

Fig. 1. The effect of caffeine (added at arrow) on resting tension and spontaneous activity of rat ileal longitudinal smooth muscle. (A) 1 mM, (B) 4 mM caffeine. Time and tension calibrations (1 min and 0·5 g respectively) apply to both traces.

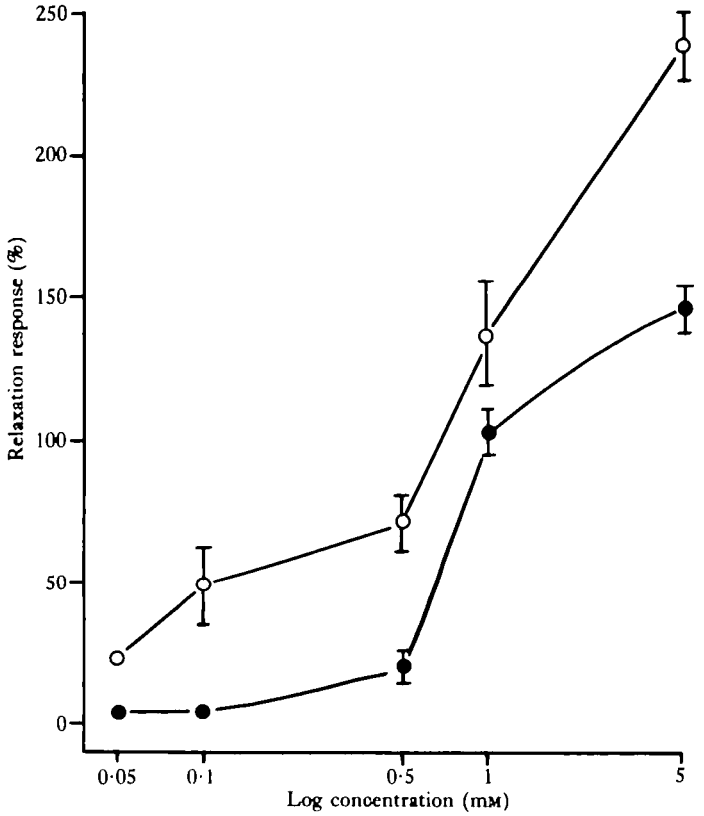

Fig. 2. Dose-response curve showing the relaxatory effect of caffeine (filled circles) and theophylline (open circles) on isolated ileal longitudinal smooth muscle of the rat. Each point is the mean of five experiments ±S.E. of mean.

noradrenaline exposure (Saad & Huddart, 1981). In addition, such adenylate cyclase activity may have affected cyclic nucleotide levels, thus affecting phosphorylation of the contractile proteins.

The effect of theophylline

Rat ileal smooth muscle was found to be far more sensitive to theophylline than to caffeine. Resting tension was reduced by concentrations as low as 0·1 mM (Figs 2, 5) and spontaneous contractions, both the minute rhythm and individual responses, were abolished at 1 mM and above (Fig. 5). The effects at all concentrations were fully reversible.

Theophylline, like caffeine had no significant effect on the K-induced phasic contractures of either vas deferens or bladder smooth muscle strips but the tonic response was progressively reduced over the range 1·0 to 5·0 mM (Fig. 6).

The effect of theobromine

In complete contrast to caffeine and theophylline, theobromine was found to have an excitatory effect on ileal smooth muscle at quite low concentrations (Fig. 7). At

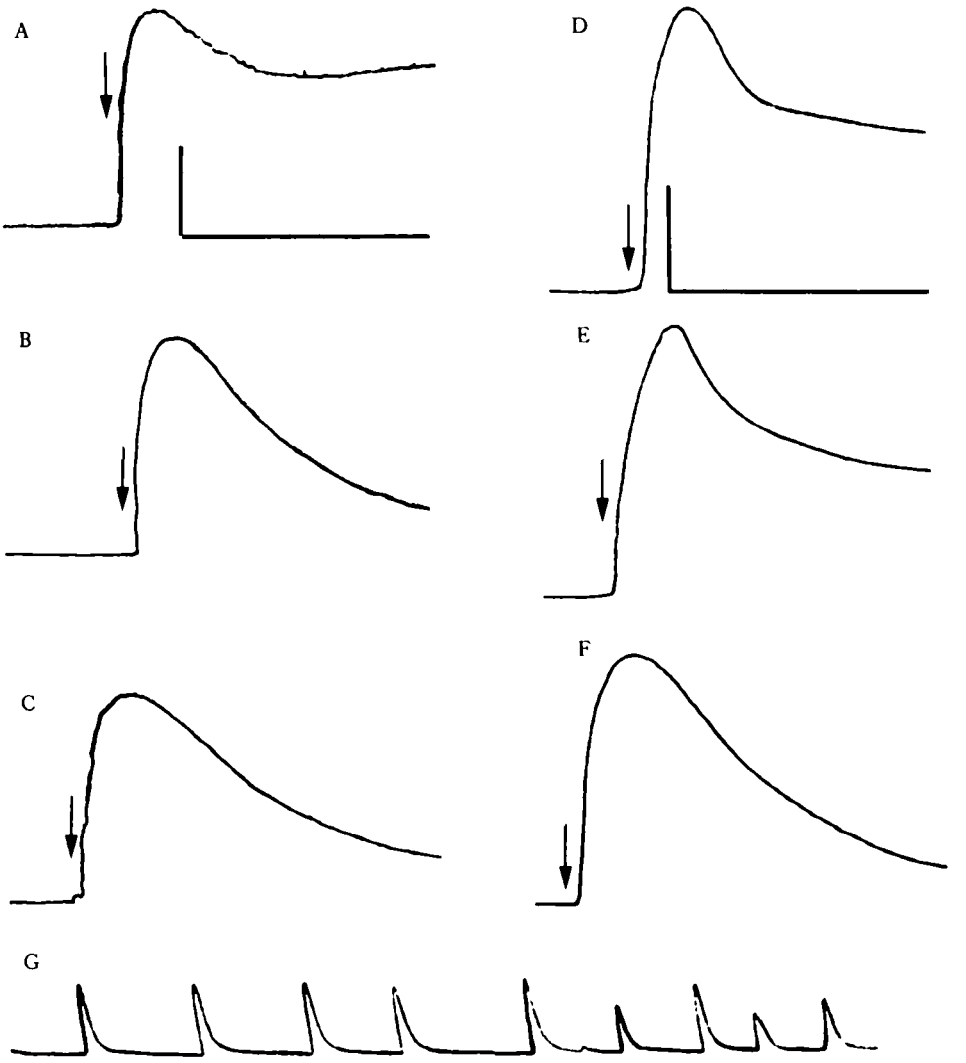

Fig. 3. The effect of caffeine on rat vas deferens smooth muscle strips (A–C) and on rat bladder smooth muscle strips (D–F). (A) Control contracture response to 80 mM-K Krebs' solution, showing clear phasic and tonic components, (B) response to 80 mM-K Krebs' solution plus 4 mM caffeine, (C) response to 80 mM-K Krebs' solution plus 6 mM caffeine, (D) control contracture response of bladder to 80 mM-K Krebs' solution, (E) response to 80 mM-K Krebs' solution plus 1 mM caffeine, (F) response to 80 mM-K Krebs' solution plus 3 mM caffeine, (G) spontaneous contractions of vas deferens after repeated exposures to caffeine containing salines. Time and tension calibrations (1 min and 0·5 g respectively) in (A) apply to A, B, C and G, and (D) apply to D, E and F. The addition of high K solutions or high K and caffeine solutions is indicated by arrows.

only 1 mM, theobromine increased muscle resting tonus by over 100%, while maintaining spontaneous rhythmic contractions. With high concentrations, such as 2·5 mM, the increase in resting tension was so high (over 250%) that spontaneous contractions were prevented. These effects were fully reversible.

Theobromine also exerted an excitatory effect on the non-spontaneous smooth muscles of the rat vas deferens and bladder. In the concentration range 1·0 to

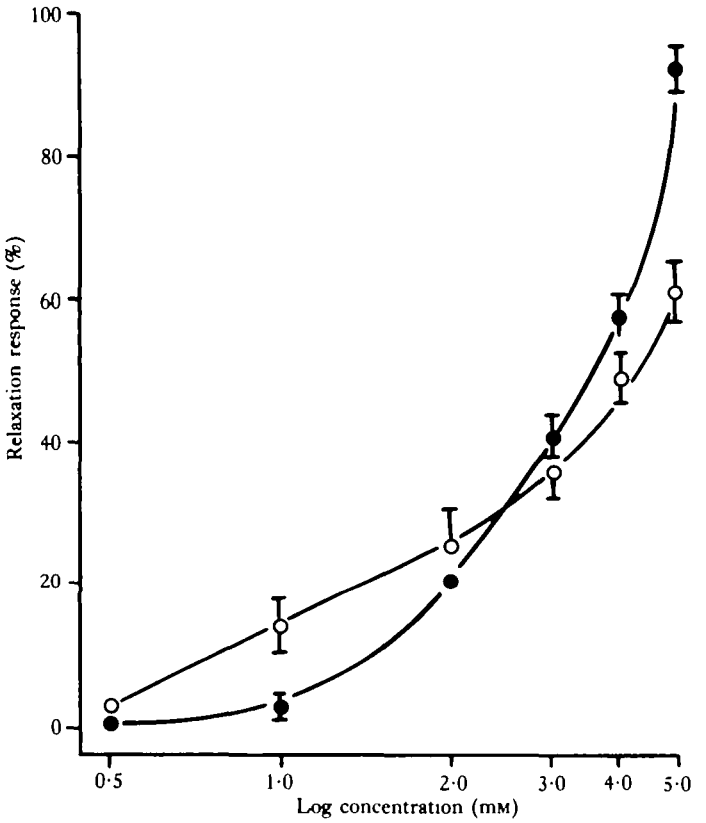

Fig. 4. Dose-response curve showing the relaxatory effect of caffeine on 80 mM K-induced tonic contractures of vas deferens (closed circles) and bladder (open circles) smooth muscle strips. The relaxation of the normal tonic contracture response was estimated at a standard time of 1 min after drug addition. Each point is the mean of five estimations ±S.E. of mean.

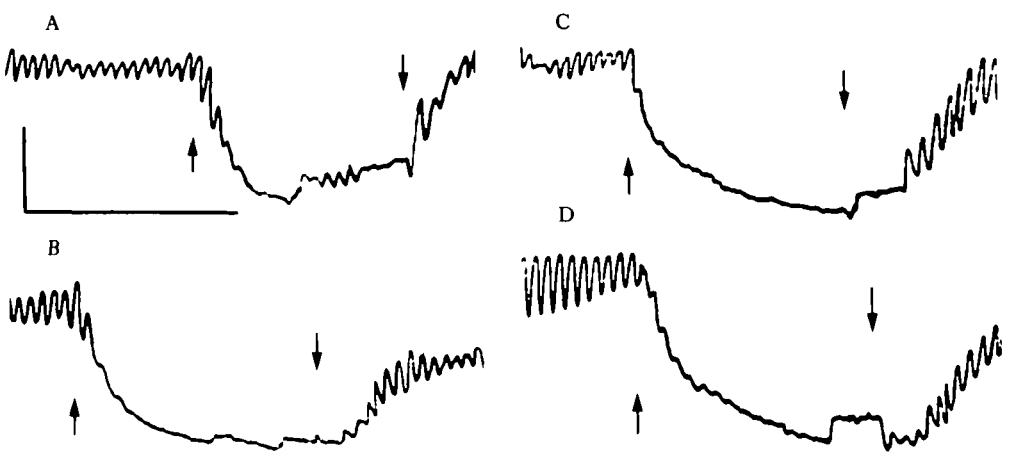

Fig. 5. The effect of theophylline on tension and spontaneous activity of rat ileal longitudinal smooth muscle strips. Theophylline-containing solution was added at the first arrow and removed at the second arrow. Concentrations were as follows (A) 0·5 mM, (B) 1·0 mM (C) 1·5 mM and (D) 2·0 mM. Calibrations (1 min and 0·5 g) apply to all traces.

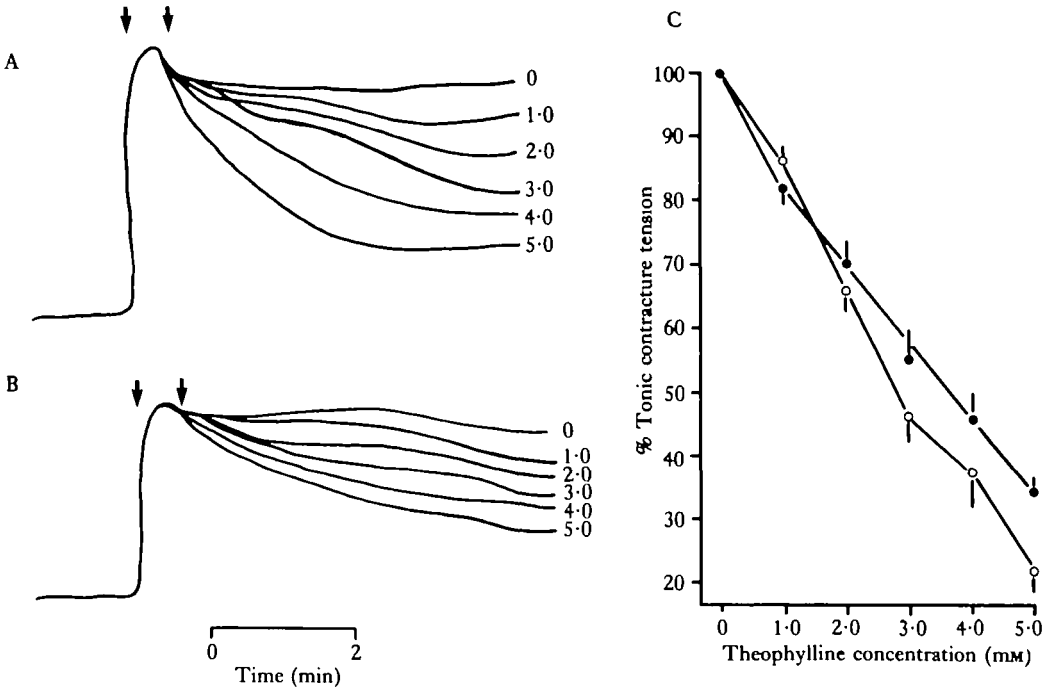

Fig. 6. The effect of theophylline on K-induced contracture tension in non-spontaneous rat smooth muscle strips. (A) and (B) show superimposed traces of contracture responses of vas deferens and bladder respectively, when exposed to 100 mM-K solution (first arrow). When the tonic response was established, theophylline was added (second arrow) at the concentrations stated (mM). Time calibration in min. (C) Summary dose-response curve of inhibition by theophylline of the tonic contracture response in vas deferens (open circles) and bladder (closed circles) smooth muscle strips. Each point is the mean of six responses ±S.E. of mean.

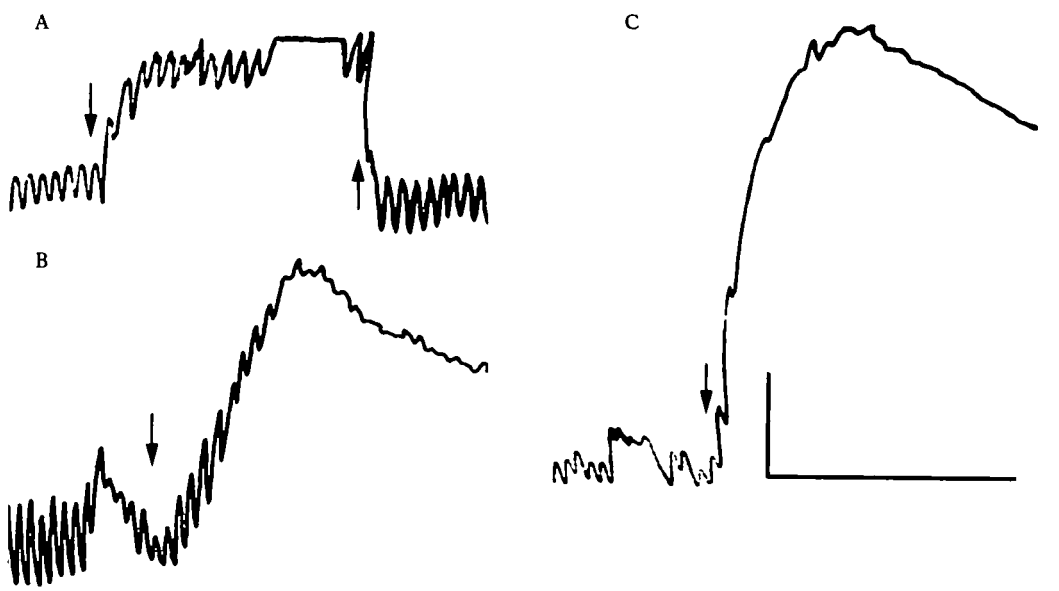

Fig. 7. The effect of theobromine (added at the arrows) on tension in rat ileal smooth muscle strips. Concentrations were as follows (A) 1·0 mM, (B) 2·0 mM and (C) 2·5 mM. Note the very clear stimulatory effect. Calibrations (1 min and 0·5 g) apply to all traces.

2·5 mM, theobromine enhanced K-induced contracture tension in a dose-dependent manner up to 100%, although the vas deferens was consistently more sensitive to theobromine than bladder muscle strips (Fig. 8). On return to normal Krebs' solution normal tension was restored and the contractures were consistently repeatable.

The effect of methylxanthines on calcium mobilization

It is now universally accepted that tension changes in muscle fibres are a result of changes in the level of myoplasmic free calcium made available to the contractile apparatus of the cell. There are two experimentally separable aspects of cell calcium dynamics which influence myoplasmic free calcium levels. Firstly there is the influx of extracellular calcium down its electrochemical gradient entering passively or induced by depolarization or receptor-carrier mediation coupled with counter-exchange extrusion (Blaustein, 1976; Huddart & Latham, 1981). Secondly there is the activity of calcium-sequestering organelles which over a long term basis maintain intracellular calcium homeostasis (see Saad & Huddart, 1981). These views largely correspond to the various models produced by Blaustein (1974), Blaustein, Ratzlaff & Kendrick (1978) and Brinley (1978) to explain calcium regulation in nerve terminals.

Experimentally we can investigate calcium mobilization by examining calcium influx into muscles and calcium binding by subcellular fractions, using calcium efflux as a measure of the level of cellular free calcium (see Deth & Van Breemen, 1977).

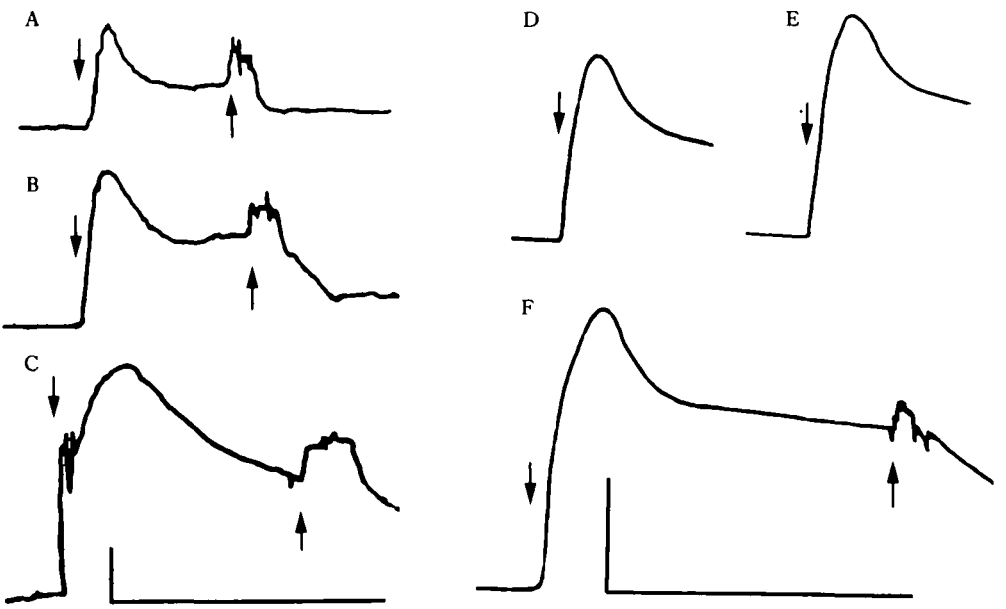

Fig. 8. The effect of theobromine on rat vas deferens (A–C) and bladder (D–F) smooth muscle strips. Treatments were as follows: (A) control 80 mM-K contracture, (B) response to 2 mM theobromine-80 mM-K solution and (C) response to 2·5 mM theobromine-80 mM-K solution. Calibration in (C) (1 min and 0·5 g) applies to A, B and C. (D) Control contracture response of bladder strip to 80 mM-K solution (E) response to 1 mM theobromine-80 mM-K solution and (F) response to 2 mM theobromine-80 mM-K solution. Calibration in (F) (1 min and 0·5 g) applies to D, E and F.

Calcium efflux

Pre-loaded strips of ileal vas deferens and bladder smooth muscle were allowed to efflux into Krebs' solution for 1 h so that the efflux consisted of the slow sustained efflux from the intracellular compartment (see Isaacson & Sandow, 1967*a,b*; Huddart & Syson, 1975). In this situation, the efflux rate coefficient mirrors the level of myoplasmic free calcium. The efflux rate coefficient of ileum was reduced in the presence of caffeine and theophylline but rose considerably in the presence of theobromine, indicating a steep rise in myoplasmic free calcium (Fig. 9). All effects were reversible.

For muscle strips of vas deferens (Fig. 10) and bladder (Fig. 11) caffeine and theophylline depressed slow compartment calcium efflux, an indication of a fall in myoplasmic free calcium. Theobromine had a somewhat unstable stimulatory effect on vas deferens muscle (Fig. 10) and a massive stimulatory effect on bladder muscle (Fig. 11), which can only be interpreted as representing a rise in myoplasmic free calcium.

These experiments indicate that caffeine and theophylline depress calcium availability to the contractile apparatus while theobromine application results in an increase. This could arise from effects on calcium influx and/or cellular binding.

Calcium influx

Calcium uptake was unaffected by 4 mM caffeine but was significantly depressed by 4 mM theophylline in all preparations (Fig. 12). With 2 mM theobromine there was a marked stimulation of influx in all cases, amounting to as much as a 50 % stimulation over the time course of these experiments (Fig. 12).

Calcium binding by subcellular fractions

In smooth muscle, the microsomes and mitochondria appear to play the main role in calcium regulation (Heumann, 1976; Uchida, 1976; Deth & Casteels, 1977; Janis & Daniel, 1977; Raemaekers, Wuytack, Batra & Casteels, 1977; Nilsson, Andersson, Mohme-Lundholm & Lundholm, 1978; Huddart, 1981). Microsomal and mitochondrial fractions were prepared from ileal, vas deferens and bladder smooth muscle and their capacity to take up ^{45}Ca from standard incubation media (containing 2 mM-ATP, 4 mM-$MgCl_2$ and 2·5 mM-$CaCl_2$) was examined. Parallel incubation runs were carried out with this medium, but containing in addition either 4 mM caffeine, 4 mM theophylline or 2 mM theobromine. Theobromine inhibited calcium binding in all cases while caffeine and theophylline were either without much effect or enhanced the binding (Table 1).

Fig. 9. The slow intracellular ^{45}Ca efflux from ileal smooth muscle strips as influenced by caffeine, theophylline and theobromine. The calcium loss from the tissue is expressed as a rate coefficient 1 h after commencement of efflux when the slow compartment was established. On switching the effluxing medium to one containing caffeine and theophylline, a fall in efflux rate coefficient is seen. With theobromine, a considerable stimulation of efflux is seen. Each point on all curves represents the mean of four separate experiments ±S.E. of mean.

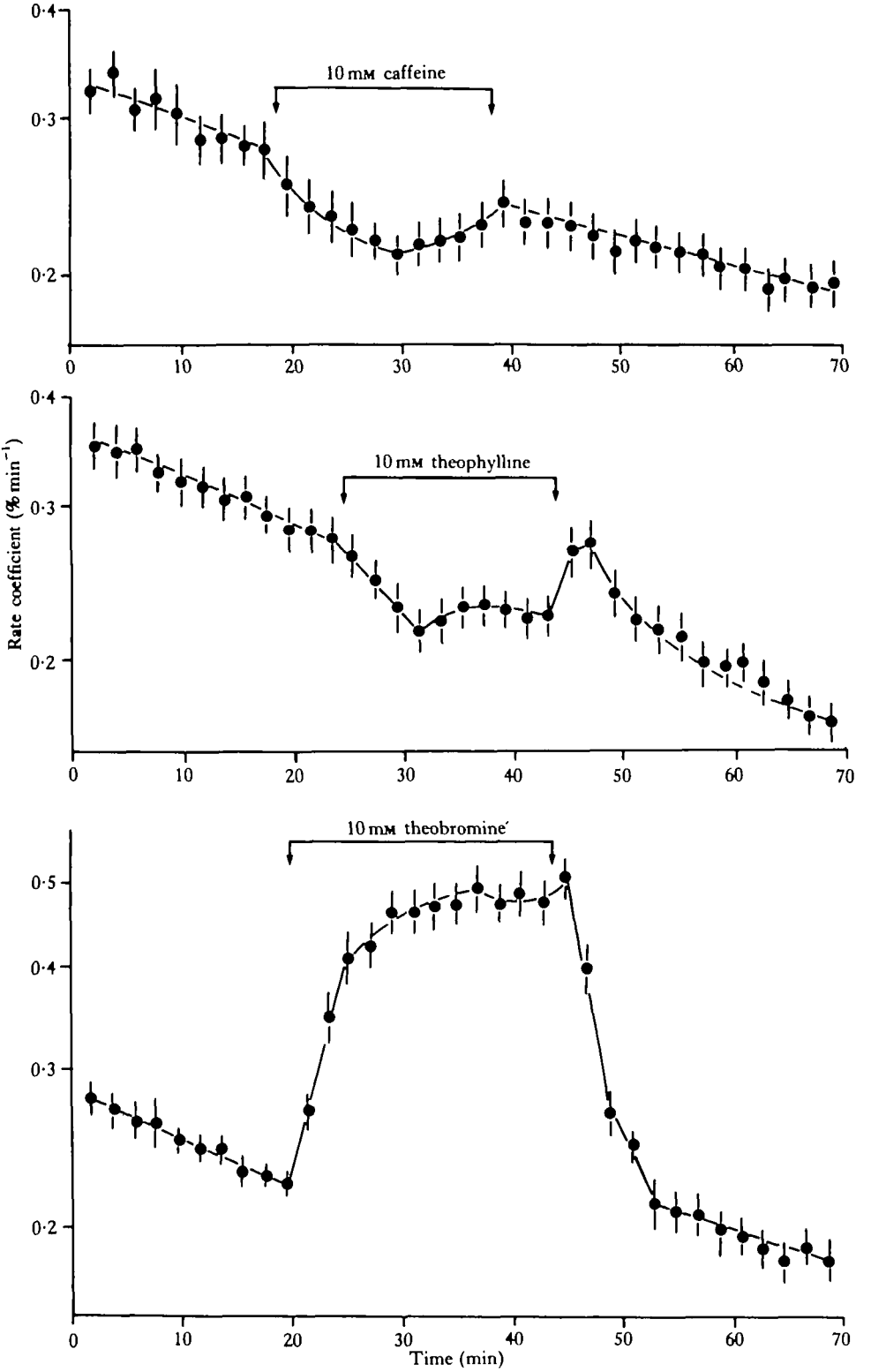
10 mM caffeine
10 mM theophylline
10 mM theobromine
Rate coefficient (% min⁻¹)
Time (min)
0·4
0·3
0·2
0·5
0
10
20
30
40
50
60
70

Fig. 9

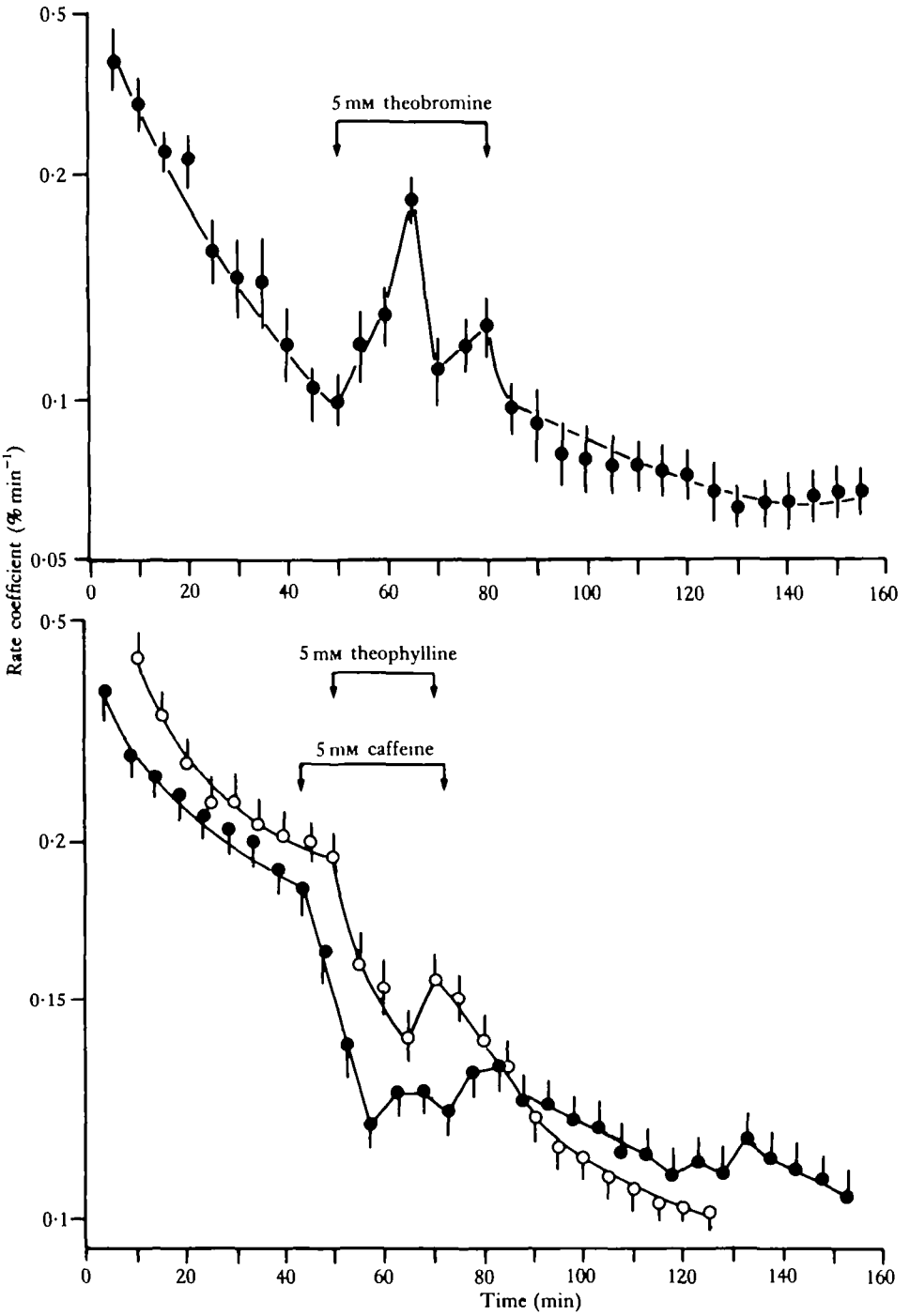

Fig. 10. The slow intracellular ^{45}Ca efflux from vas deferens smooth muscle strips expressed as a rate coefficient. On switching the effluxing medium to one containing caffeine or theophylline a fall in calcium efflux is seen, while theobromine causes a significant stimulation of efflux. These curves are the mean of four separate experiments ±S.E. of mean.

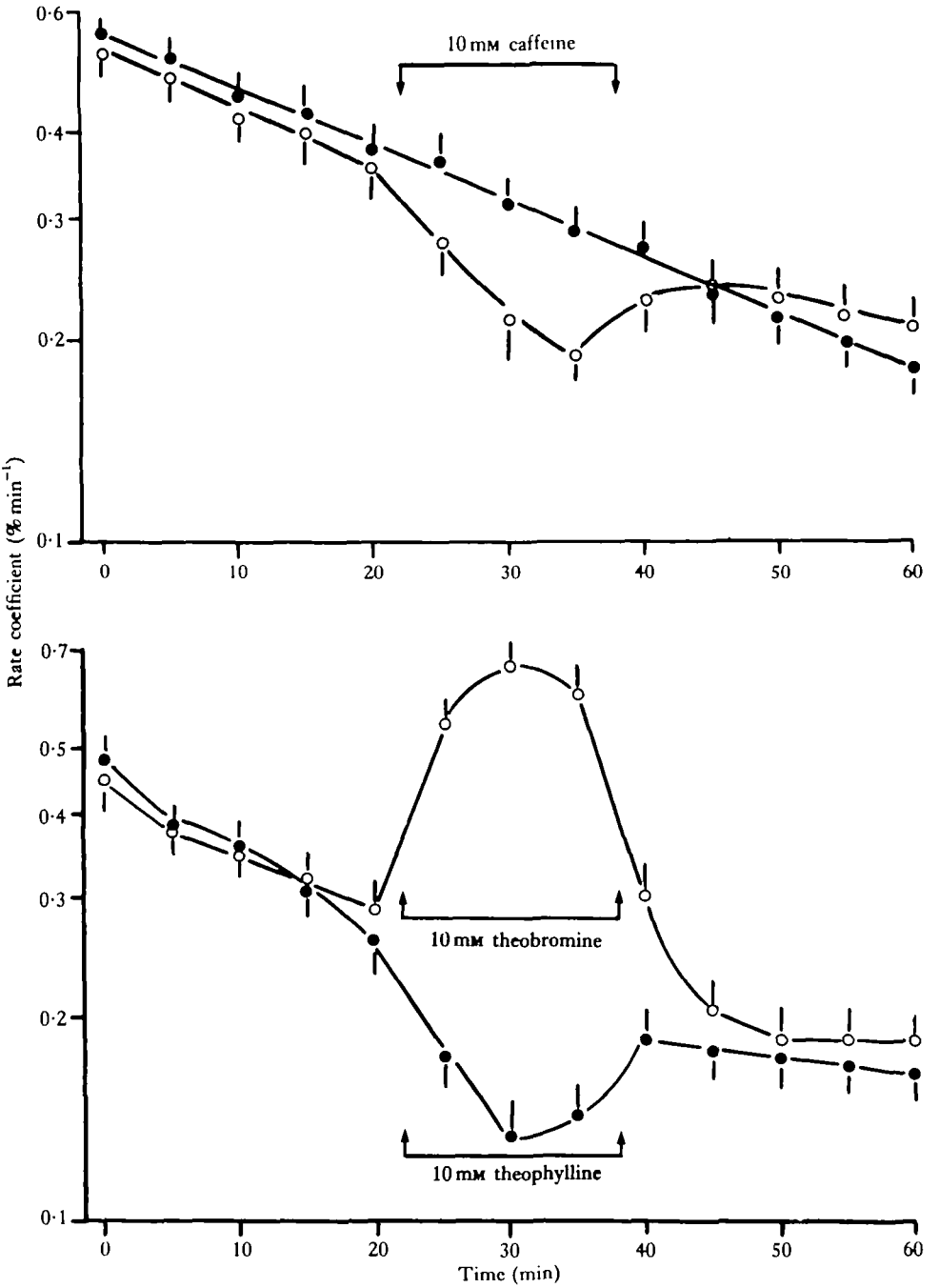

Fig. 11. The slow intracellular ^{45}Ca efflux from bladder smooth muscle strips expressed as a rate coefficient. In the upper panel, filled circles show the control slow component, and addition of caffeine (open circles) causes a small fall in calcium efflux. In the lower panel, switching the effluxing medium to one containing theophylline causes a significant fall in calcium efflux while theobromine causes a considerable stimulation of efflux. These curves represent the mean of four experiments ±S.E. of mean.

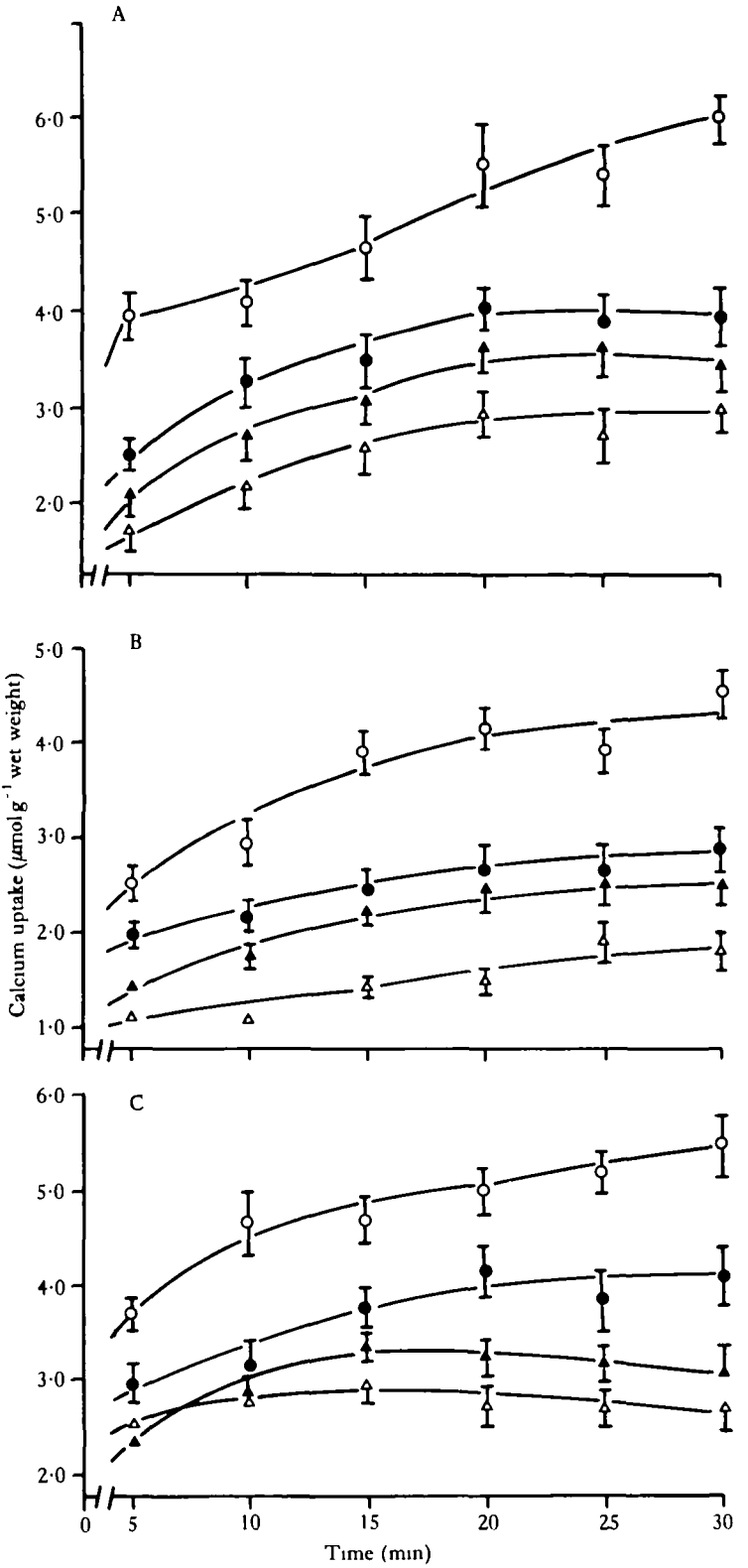
A
B
C
6·0
5·0
4·0
3·0
2·0
1·0
Calcium uptake (μmol g⁻¹ wet weight)
0
5
10
15
20
25
30
Time (min)

Fig. 12

Table 1. *The effect of methylxanthines on calcium binding by microsomes and mitochondria isolated from smooth muscle of ileum, vas deferens and bladder*

Experimental conditions	Calcium uptake (nmol mg^{-1} protein)		
	Ileum	Vas deferens	Bladder
Microsomes			
Control	$6{\cdot}35 \pm 0{\cdot}68 (N = 5)$	$6{\cdot}83 \pm 0{\cdot}56 (N = 5)$	$8{\cdot}40 \pm 0{\cdot}72 (N = 5)$
Caffeine 4 mM	$9{\cdot}20 \pm 0{\cdot}88 (N = 4)$**	$8{\cdot}13 \pm 0{\cdot}88 (N = 4)$*	$9{\cdot}10 \pm 0{\cdot}86 (N = 4)$†
Theophylline 4 mM	$7{\cdot}40 \pm 0{\cdot}60 (N = 4)$*	$7{\cdot}03 \pm 0{\cdot}76 (N = 4)$†	$9{\cdot}21 \pm 0{\cdot}75 (N = 4)$†
Theobromine 2 mM	$4{\cdot}31 \pm 0{\cdot}50 (N = 4)$**	$3{\cdot}86 \pm 0{\cdot}40 (N = 4)$**	$4{\cdot}29 \pm 0{\cdot}36 (N = 4)$**
Mitochondria			
Control	$5{\cdot}38 \pm 0{\cdot}44 (N = 6)$	$4{\cdot}34 \pm 0{\cdot}44 (N = 6)$	$7{\cdot}57 \pm 0{\cdot}60 (N = 6)$
Caffeine 4 mM	$6{\cdot}06 \pm 0{\cdot}80 (N = 5)$†	$4{\cdot}40 \pm 0{\cdot}38 (N = 5)$†	$7{\cdot}45 \pm 0{\cdot}90 (N = 5)$†
Theophylline 4 mM	$5{\cdot}06 \pm 0{\cdot}63 (N = 5)$†	$4{\cdot}56 \pm 0{\cdot}50 (N = 5)$†	$7{\cdot}26 \pm 0{\cdot}66 (N = 5)$†
Theobromine 2 mM	$2{\cdot}58 \pm 0{\cdot}36 (N = 5)$**	$2{\cdot}14 \pm 0{\cdot}28 (N = 5)$**	$3{\cdot}92 \pm 0{\cdot}42 (N = 5)$**

† Not significant.
* $P < 0{\cdot}05$.
** $P < 0{\cdot}01$.

DISCUSSION

In rhythmically active visceral muscles such as the rat ileum the tension developed takes two forms, a basic resting tonus upon which is superimposed cyclic transient tension fluctuations responsible for the minute rhythmicity. At the cellular level, the basic tonus reflects a particular steady state cellular free calcium level, while the rhythmic contractions result from a cyclical influx of additional extracellular calcium which transiently increases myoplasmic free calcium.

Caffeine and theophylline reduce resting tonus while caffeine at high concentrations and theophylline at most concentrations also abolish spontaneous rhythmic contractions in the ileum. This is correlated with the abolition by caffeine of spike discharges in this preparation (Syson & Huddart, 1976). Caffeine and theophylline may thus inhibit the cyclical calcium influx phase of the excitation-contraction coupling sequence upon which such contractions depend.

In a number of visceral muscles there is evidence that the phasic and tonic components of K-induced contractures depend upon different sources of activator calcium (Imai & Takeda, 1967; Urakawa & Holland, 1964; Shimo & Holland, 1966; Syson & Huddart, 1973). The phasic response accompanies a massive increase in spike discharge (Burnstock & Holman, 1963; Chapman & Holman, 1968; Syson & Huddart, 1976) and can be readily blocked by lanthanum and calcium-free media (Huddart & Saad, 1977, 1978; Barratt & Huddart, 1979). The phasic response may thus result from the influx of extracellular calcium which accompanies the burst of spikes during initial depolarization. The tonic response reflects an increased myoplasmic free calcium level which is maintained during protracted depolarization. Studies of

Fig. 12. The effect of methylxanthines on ^{45}Ca influx into (A) ileal smooth muscle strips, (B) vas deferens and (C) bladder smooth muscle strips. Solid circles represent control influx conditions, open circles represent uptake in 2 mM theobromine saline, closed triangles represent uptake in 4 mM caffeine saline and open triangles represent uptake in 4 mM theophylline saline. Each point is the mean of five determinations ±S.E. of mean.

calcium-induced release of calcium (Cheng, 1976; Barratt & Huddart, 1979; Alohan & Huddart, 1979) suggest that the K-induced tonic contracture depends upon the stimulation of calcium release from cellular sources which is induced by the initial calcium influx (also see Saida, 1982). The results reported here for vas deferens and bladder muscle showed that neither caffeine nor theophylline affected the phasic contracture response to any significant extent but the tonic response was markedly inhibited by both agents in both muscles. Caffeine and theophylline may thus not have much effect on calcium influx but they may reduce intracellular activator calcium upon which the tonic response depends.

This hypothesis was investigated by first examining the effect of methylxanthines upon the slow phase of calcium efflux, which represents calcium movement from the intracellular compartment and can be expected to reveal intracellular actions of pharmacologically active agents (Isaacson & Sandow, 1967*a*,*b*; Deth & Van Breemen, 1977). The efflux rate coefficient was reduced by caffeine and theophylline, which is consistent with their actions on tension development and indicates that these methylxanthines have caused a fall in cellular calcium level.

Calcium influx into these muscles was then examined coupled with a study of calcium binding by subcellular fractions known from other studies to be involved in cellular calcium regulation (see Saad & Huddart, 1981). Theophylline caused a small decrease in ^{45}Ca influx into all three muscles while the effect of caffeine was even smaller. These effects (only a 10–15 % inhibition of influx) are of little significance since large reductions in extracellular calcium are needed to affect tension in smooth muscle (Syson & Huddart, 1973; Huddart & Hunt, 1975).

Caffeine had little effect on mitochondrial calcium uptake in any of the muscles but it did enhance calcium uptake by microsomes from ileum and vas deferens. Similarly theophylline was without much effect on mitochondrial calcium uptake, although it did enhance calcium uptake by microsomes from all three muscles.

The actions of theobromine on tension were the reverse of those of caffeine and theophylline. Theobromine increased tension in the ileum and with vas deferens and bladder muscle both the phasic and tonic components of the K response were enhanced. The ^{45}Ca efflux studies showed that theobromine increased slow compartment efflux in all three muscles, indicating an increase in myoplasmic calcium. Theobromine also enhanced ^{45}Ca influx into all three muscles by between 40 and 70 %, which is consistent with the tension studies and the efflux studies, and additionally theobromine strongly inhibited calcium binding by both microsomes and mitochondria from all three muscles.

All three methylxanthines appear directly to affect the calcium level in the fibres of these muscles. Caffeine and theophylline, by slightly reducing influx and by stimulating microsomal calcium binding, reduce cellular calcium level. On the other hand theobromine stimulates calcium influx and inhibits microsomal and mitochondrial calcium binding. These actions must play the major role in increasing cellular calcium levels in all the muscles, as detected in the theobromine efflux experiments.

At first sight it seems strange that closely related methylxanthines should have such differing actions on calcium levels in these smooth muscles. The dimethylxanthines theophylline and theobromine are, in fact, natural degradation products of the trimethylxanthine caffeine (see Williams, Lowitt, Polson & Szentivanyi, 1978). Their

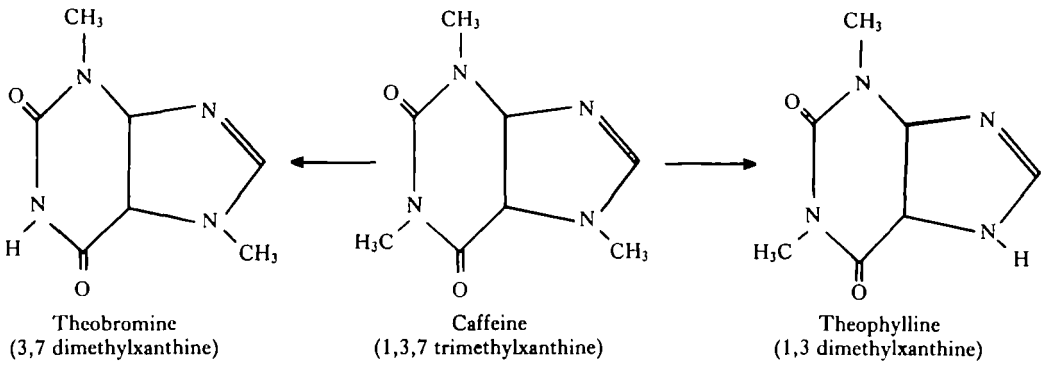

Fig. 13. The structural interrelationship of caffeine, theophylline and theobromine.

structures are shown in Fig. 13. The differences in cellular action relating to calcium influx and microsomal and mitochondrial binding by caffeine and theophylline on the one hand and theobromine on the other may have a structural explanation. This may result from differing actions of these methylxanthines on membrane bound receptors operating calcium channels in the cell membranes and in microsomal and mitochondrial membranes. Attempts have been made previously to deduce a structural basis for methylxanthine actions on muscle. Bianchi (1968), Johnson & Inesi (1969) and Chapman & Miller (1974) have examined methylxanthine interactions with the sarcolemma of skeletal muscle and with the sarcoplasmic reticulum of cardiac and skeletal muscle. The effects of caffeine on the calcium permeability of the sarcolemma and sarcoplasmic reticulum and its competition with ATP in reticular calcium transport suggest a structural basis which can be envisaged as an agonist/receptor interaction. This could have implications for the modulation of secondary messengers.

Caffeine and theophylline have similar actions on both influx and microsomal calcium binding, so if this involves an agonist/receptor mechanism then the 7-substituted methyl group cannot be relevant to this action. In their study of methylxanthine relaxation of tracheal muscle, Williams *et al.* (1978) noticed that 1 methylxanthine and 3 methylxanthine were each less effective than theophylline (1,3 dimethylxanthine). Structural interactions of di- or trimethylxanthines with a receptor may require methyl substitution in both the 1 and 3 positions for maximal agonist effect. Theobromine possesses no 1-substituted methyl group and this could be the basis for the different action of theobromine compared with theophylline and caffeine on calcium influx and subcellular binding activity were a receptor operated channel to be involved in calcium modulation. It is interesting that in a quite different tissue, rat epididymal fat cells, Beavo *et al.* (1970) noticed that theobromine was considerably less effective than theophylline in the stimulation of lipolysis which is correlated with extent of inhibition of cyclic AMP phosphodiesterase. In this tissue differences in methylxanthine actions may also reflect structural differences in the reaction with intracellular receptors associated with phosphodiesterase activity.

Additionally, or alternatively, there could be another explanation for the differing actions of these methylxanthines revolving around secondary messengers such as

cyclic AMP and/or cyclic GMP. Caffeine and theophylline are known to inhibit cyclic nucleotide phosphodiesterase. Although 3 methylxanthine also inhibits this enzyme system (Williams *et al.* 1978) responsible for the conversion of cyclic AMP and cyclic GMP to inactive 5'AMP and 5'GMP, we have no information about theobromine action in this respect. It is known that cyclic AMP and cyclic GMP are responsible for phosphorylating, and therefore activating, a number of protein kinases responsible for modulating membrane permeability and altering ion movements within the cell (Posternack, 1974; Friedman, 1976; Weiss & Hait, 1977). However, the relationship between the contractile state of smooth muscle and cyclic nucleotide level is often contradictory. Although contraction is thought to result from either a fall in cyclic AMP and/or an increase in cyclic GMP (for review see Berridge, 1975), in many smooth muscles there is no correlation between cyclic AMP level and contractile state (Diamond & Hartle, 1974; Reinhardt, Rogenbach, Brodde & Schumann, 1977). Furthermore, dibutyryl cyclic AMP enhances tension and K responses in rat ileum and vas deferens but does not affect ^{45}Ca uptake by microsomes (Saad & Huddart, 1980). These data argue against any obvious casual relationship between cyclic AMP level and smooth muscle relaxation. Any direct link between methylxanthines and relaxation or activation of these smooth muscles *via* a cyclic AMP/cyclic GMP involvement seems even more tenuous.

A more likely explanation of methylxanthine actions may revolve around interaction with adenosine receptors, which in turn does not exclude indirect actions on secondary messengers. Caffeine, theophylline, theobromine and other xanthine derivatives have been shown to antagonize adenosine receptors in fat cells and central nervous tissue (Fredholm, 1980; Daly *et al.* 1981; Fredholm & Persson, 1982). They may well have such an action in muscle cells since Chapman & Miller (1974) showed that caffeine competed with ATP for calcium transport sites in frog myocardial sarcoplasmic reticulum. This seems a more likely mode of action than the rather weak inhibitory effects of methylxanthines on cyclic nucleotide phosphodiesterase. There is now evidence that adenosine receptors may be involved in the modulation of cellular responses to some hormones and neurotransmitters (Schubert *et al.* 1979; Fredholm & Hedquist, 1980), and purine nucleotides themselves may act as transmitters or modulators in mammalian visceral muscles (for review see Burnstock, 1981). This strengthens the argument for a structure/activity relationship for methylxanthine action based on an agonist/receptor model.

We are most grateful for the excellent technical assistance of Mr H. Latham with some of the experiments reported here.

REFERENCES

ALOHAN, F. I. & HUDDART, H. (1979). Spontaneous activity of an annelid visceral muscle and related calcium movements. The effect of KCl depolarization, caffeine, acetylcholine and adrenaline. *Comp. Biochem. Physiol.* **63C**, 161–171.

ANDERSSON, R. G. G. (1973). Role of cyclic AMP and Ca^{2+} in mechanical and metabolic events in isometrically contracting vascular smooth muscle *Acta physiol. scand.* **87**, 84–95.

BARRATT, L. & HUDDART, H. (1979). Spontaneous activity and related calcium movements of fish intestinal smooth muscle. The effect of depolarization, caffeine and lanthanum. *Gen. Pharmac.* **10**, 21–30.

BEAVO, J. A., ROGERS, N. L., CROFFORD, O. B., HARDMAN, J. G., SUTHERLAND, E. W. & NEWMAN, E. V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine 3', 5'-monophosphate phosphodiesterase activity. *Mol. Pharmac.* **6**, 597–603.

BENNETT, A. (1873). An experimental inquiry into the physiological actions of thein, caffeine and guaranine, cocaine and theobromine. *Edin. Med. J.* **19**, 323–341.

BERRIDGE, M. J. (1975). The interaction of cyclic nucleotides and calcium in the control of cellular activity. *Adv. cyclic Nucleotide Res.* **6**, 1–98.

BIANCHI, C. P. (1961). The effect of caffeine on radiocalcium movement in frog sartorius. *J. gen. Physiol.* **44**, 945–958.

BIANCHI, C. P. (1968). Pharmacological actions on excitation-contraction coupling in striated muscle. *Fedn Proc. Fedn Am. Socs exp. Biol.* **27**, 126–131.

BLAUSTEIN, M. P. (1974). The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmac.* **70**, 33–82.

BLAUSTEIN, M. P. (1976). Sodium-calcium exchange and the regulation of cell calcium in muscle fibres. *Physiologist* **19**, 525–540.

BLAUSTEIN, M. P., RATZLAFF, R. W. & KENDRICK, N. K. (1978). The regulation of intracellular calcium in presynaptic nerve terminals. *Ann. N.Y. Acad. Sci.* **307**, 195–212.

BRINLEY, F. J. (1978). Calcium buffering in squid axons. *Ann. Rev. Biophys. Bioeng.* **7**, 363–392.

BURNSTOCK, G. (1981). Neurotransmitters and trophic factors in the autonomic nervous system. *J. Physiol., Lond.* **313**, 1–35.

BURNSTOCK, G. & HOLMAN, M. E. (1963). Smooth muscle: autonomic nerve transmission. *Ann. Rev. Physiol.* **25**, 61–90.

CARVALHO, A. P. (1968). Calcium binding properties of sarcoplasmic reticulum as influenced by ATP, caffeine, quinine and local anaesthetics. *J. gen. Physiol.* **52**, 622–642.

CHAPMAN, J. B. & HOLMAN, M. E. (1968). Potassium contractures in the ureter of the guinea pig. *Aust. J. exp. Biol. med. Sci.* **46**, 503–514.

CHAPMAN, R. A. & LEOTY, C. (1976). The time-dependent and dose-dependent effects of caffeine on the contraction of ferret heart. *J. Physiol., Lond.* **256**, 287–314.

CHAPMAN, R. A. & MILLER, D. J. (1974). The effects of caffeine on the contraction of the frog heart. *J. Physiol., Lond.* **242**, 589–613.

CHEN, S., BITTAR, E. E., TONG, E. & DANIELSON, B. G. (1972). Stimulation by caffeine of the calcium efflux in barnacle muscle fibres. *Experientia* **28**, 807–809.

CHENG, J. T. (1976). Calcium-induced release of calcium in rectal smooth muscle of mice. *Jap. J. Pharmac.* **26**, 73–78.

DALY, J. W., BRUNS, R. F. & SNYDER, S. H. (1981). Adenosine receptors in the central nervous system: relationship to the central actions of methylxanthines. *Life Sci.* **28**, 2083–2097.

DETH, R. & CASTEELS, R. (1977). A study of releasable calcium fractions in smooth muscle cells of the rabbit aorta. *J. gen. Physiol.* **69**, 401–416.

DETH, R. & VAN BREEMEN, C. (1977). Agonist-induced release of intracellular Ca^{2+} in the rabbit aorta. *J. Membr. Biol.* **30**, 363–380.

DIAMOND, J. & HARTLE, D. K. (1974). Cyclic nucleotide level during spontaneous uterine contraction. *Can. J. Physiol. Pharmac.* **52**, 763–767.

FREDHOLM, B. B. (1980). Are methylxanthine effects due to antagonism of endogenous adenosine? *Trends Pharmac. Sci.* **1**, 129–132.

FREDHOLM, B. B. & HEDQUIST, P. (1980). Modulation of neurotransmission by purine nucleotides and nucleosides. *Biochem. Pharmac.* **29**, 1635–1643.

FREDHOLM, B. B. & PERSSON, C. G. A. (1982). Xanthine derivatives as adenosine receptor antagonists. *Eur. J. Pharmac.* **81**, 673–676.

FRIEDMAN, D. L. (1976). The role of cyclic nucleotides in cell growth and differentiation. *Physiol. Rev.* **56**, 652–708.

HERZ, R. & WEBER, A. (1965). Caffeine inhibition of calcium uptake by muscle reticulum. *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 208.

HEUMANN, H. G. (1976). The subcellular localization of calcium in vertebrate smooth muscle: calcium-containing and calcium-accumulating structures in muscle cells of the mouse intestine. *Cell Tiss. Res.* **169**, 221–231.

HILL, R. B. (1980). Use of an ionophore to maintain repeated caffeine contractures in holothurian muscle. *Life Sci.* **27**, 1967–1973.

HUDDART, H. (1969). Caffeine activation of crab skeletal muscle. *Comp. Biochem. Physiol.* **29**, 1031–1038.

HUDDART, H. (1981). Calcium regulation in ileal smooth muscle – I. Sodium-calcium counterexchange. *Gen. Pharmac.* **12**, 155–160.

HUDDART, H. & ABRAM, G. R. (1969). Modification of excitation-contraction coupling in locust skeletal muscle induced by caffeine. *J. exp. Zool.* **171**, 49–58.

HUDDART, H. & HUNT, S. (1975). *Visceral muscle – It's Structure and Function*. Glasgow: Blackie & Son.

HUDDART, H. & LATHAM, H. (1981). Calcium regulation in ileal smooth muscle. II. Interaction of sodium and magnesium in calcium counterexchange. *Gen. Pharmac.* **12**, 161–168.

HUDDART, H. & SAAD, K. H. M. (1977). Quinine and lanthanum effects on contractility and calcium movements of rat ileal smooth muscle. *Gen. Pharmac.* **8**, 341–347.

HUDDART, H. & SAAD, K. H. M. (1978). The effect of sodium and magnesium and their interaction with quinine and lanthanum on spontaneous activity and related calcium movements of rat ileal smooth muscle. *J. comp. Physiol.* **126**B, 233–240.

HUDDART, H. & SYSON, A. J. (1975). The effect of caffeine on calcium efflux and calcium translocation in skeletal and visceral muscle. *J. exp. Biol.* **63**, 131–142.

HUDDART, H. & WILLIAMS, A. J. (1974). The effect of caffeine and quinine on calcium uptake by sarcoplasmic reticulum isolated from crustacean skeletal muscle in relation to the disruption of excitation-contraction coupling. *J. comp. Physiol.* **94**, 331–338.

IMAI, S. & TAKEDA, K. (1967). Actions of calcium and certain multivalent cations on potassium contracture of guinea pig's taenia coli. *J. Physiol., Lond.* **190**, 155–169.

ISAACSON, A. (1969). Caffeine-induced contractures and related calcium movements of muscle in hypertonic media. *Experientia* **25**, 1263–1265.

ISAACSON, A. & SANDOW, A. (1967*a*). Caffeine effects on radiocalcium movements in normal and denervated rat skeletal muscle. *J. Pharmac. exp. Ther.* **151**, 376–387.

ISAACSON, A. & SANDOW, A. (1967*b*). Quinine and caffeine effects on Ca^{45} movements in frog sartorius muscle. *J. gen. Physiol.* **50**, 2109–2128.

ITO, Y. & KURIYAMA, H. (1971). Caffeine and excitation-contraction coupling in gastrointestinal smooth muscle. *J. gen. Physiol.* **57**, 448–463.

JANIS, R. A. & DANIEL, E. E. (1977). Ca^{2+} transport by subcellular fractions from smooth muscle. In *The Biochemistry of Smooth Muscle*, (ed. N. L. Stephens), pp. 653–671. Baltimore: University Park Press.

JOHNSON, P. N. & INESI, G. (1969). Effect of methylxanthines and local anesthetics on fragmented sarcoplasmic reticulum. *J. Pharmac. exp. Ther.* **169**, 308–314.

KUKOVETZ, W. R. & POCH, G. (1970). Inhibition of cyclic 3′, 5′-nucleotide phosphodiesterase as a possible mode of action of papaverine and similarly acting drugs. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **267**, 189–194.

MCFARLAND, S. A. & PFAFFMAN, M. A. (1972). The effect of caffeine on excitation-contraction coupling in gastrointestinal smooth muscle. *Arch. int. Pharmacodyn.* **198**, 49–60.

NILSSON, K. B., ANDERSSON, R. G. G., MOHME-LUNDHOLM, E. & LUNDHOLM, L. (1978). Some properties of calcium-binding microsomal sub-fractions isolated from rabbit colon muscle. *Acta Pharmac. Toxicol.* **43**, 185–193.

PICKERING, J. W. (1893). Observations on the physiology of the embryonic heart. *J. Physiol., Lond.* **14**, 383–466.

POCH, G. & UMFAHRER, W. (1976). Differentiation of intestinal smooth muscle relaxation caused by drugs that inhibit phosphodiesterase. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **293**, 257–268.

POSTERNACK, T. (1974). Cyclic AMP and cyclic GMP. *Ann. Rev. Pharmac.* **14**, 23–34.

REINHARDT, D., ROGENBACH, W., BRODDE, O. E. & SCHUMANN, H. J. (1977). Influence of papaverine and isoprenaline on contractility and cyclic AMP level of left guinea pig atria at different rates of beat. *Nauyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **299**, 9–15.

RAEMAEKERS, L., WUYTACK, F., BATRA, S. & CASTEELS, R. (1977). A comparative study of calcium accumulation by mitochondria and microsomes isolated from smooth muscle of guinea pig taenia coli. *Pflügers Arch. ges. Physiol.* **368**, 217–223.

SAAD, K. H. M. & HUDDART, H. (1980). The effect of cyclic dibutyryl adenosine 3′, 5′-monophosphate on mechanical activity and calcium movement of the smooth muscle of rat ileum and vas deferens. *Gen. Pharmac.* **11**, 315–321.

SAAD, K. H. M. & HUDDART, H. (1981). Influence of noradrenaline and KCl on calcium translocation in rat vas deferens smooth muscle and its subcellular fractions. *Gen. Pharmac.* **12**, 373–380.

SAIDA, K. (1982). Intracellular calcium release in skinned smooth muscle. *J. gen. Physiol.* **80**, 191–202.

SAKAI, T. & IIZUKA, T. (1972). The effect of caffeine and rapid cooling on smooth muscle. *Jap. J. Physiol.* **22**, 135–145.

SANDOW, A. (1965). Excitation-contraction coupling in skeletal muscle. *Pharmac. Rev.* **17**, 265–320.

SCHUBERT, P., REDDINGTON, M. & KREUTZBERG, G. W. (1979). On the possible role of adenosine as a modulatory messenger in the hippocampus and other regions of the CNS. *Prog. Brain Res.* **51**, 149–165.

SHIMO, Y. & HOLLAND, W. C. (1966). Effects of potassium on membrane potential, spike discharge and tension in taenia coli. *Am. J. Physiol.* **211**, 1299–1304.

SIEGMAN, M. H. & GORDON, A. R. (1972). Potentiation of contraction: effects of calcium and caffeine on active state. *Am. J. Physiol.* **222**, 1587–1592.

SYSON, A. J. & HUDDART, H. (1973). Contracture tension in rat vas deferens and ileal smooth muscle and its modification by external calcium and the tonicity of the medium. *Comp. Biochem. Physiol.* **45**A, 345–362.

SYSON, A. J. & HUDDART, H. (1976). The effect of caffeine on excitation-contraction coupling in skeletal and smooth muscle. *J. exp. Biol.* **64**, 789–798.

THEOBALD, T. C., SYSON, A. J. & BURRIN, D. H. (1978). The effect of caffeine and quinine on calcium efflux and cyclic AMP levels in bovine ileal smooth muscle. *Comp. Biochem. Physiol.* **61**C, 395–400.

UCHIDA, M. (1976). Active extrusion of calcium ions by smooth muscle microsomes. *Biochem. biophys. Res. Commun.* **73**, 127–132.

URAKAWA, N. & HOLLAND, W. C. (1964). ^{45}Ca uptake and tissue calcium in K-induced phasic and tonic contraction in taenia coli. *Am. J. Physiol.* **207**, 873–876.

WEBER, A. & HERZ, R. (1968). The relationship between caffeine contracture of intact muscle and the effect of caffeine on the reticulum. *J. gen. Physiol.* **52**, 750–759.

WEISS, B. & HAIT, W. N. (1977). Selective cyclic nucleotide phosphodiesterase inhibitors as potential therapeutic agents. *Ann. Rev. Pharmac.* **17**, 441–478.

WILLIAMS, J. F., LOWITT, S., POLSON, J. B. & SZENTIVANYI, A. (1978). Pharmacological and biochemical activities of some monomethylxanthine and methyluric acid derivatives of theophylline and caffeine. *Biochem. Pharmac.* **27**, 1545–1550.

WIXTED, W. M. & ZIMSKIND, P. D. (1977). The effect of caffeine on ureteral motility. *Invest. Urol.* **15**, 137–139.