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The nature of the positive inotropic response of the isolated frog heart to theophylline and to iminazole

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Summary

1. The mechanism of the positive inotropic responses to theophylline and to iminazole has been examined in the frog heart.

2. Both theophylline and iminazole caused positive inotropic effects which declined to control amplitude with time despite continued exposure to the drugs. The duration of the response to iminazole was always longer than that to theophylline.

3. On washout of theophylline and iminazole the amplitude of heart beat slowly decreased to a value below that in the control period.

4. The theophylline response was not prevented by phentolamine or by propranolol given separately or in combination.

5. Theophylline potentiated the staircase and prolonged the post-stimulation potentiation phenomena when its own inotropic activity had subsided but iminazole reduced the staircase effect at this time.

6. Theophylline, iminazole and $3 \times [Ca^{2+}]_o$ all increased the influx of ^{45}Ca into isolated ventricles. Theophylline increased but iminazole and $3 \times [Ca^{2+}]_o$ slightly reduced ^{45}Ca efflux.

7. Total cell calcium changes were only detected in ventricles exposed for 15 min to $3 \times [Ca^{2+}]_o$ or theophylline (5×10^{-3}M). After 60 min exposure to theophylline the total cell calcium was not significantly different from controls.

8. It is concluded that the positive inotropic responses to theophylline and iminazole can be interpreted in terms of the increased calcium influx which they produce and that interpretation of effects in terms of their action on phosphodiesterase should be treated with reservation.

Introduction

Theophylline inhibits phosphodiesterase whilst iminazole activates the enzyme in broken cell preparations (Butcher & Sutherland 1962). Because of their effect on this enzyme which is responsible for the destruction of cyclic adenosine 3′,5′-monophosphate (3′,5′-AMP) they have been used to provide indirect evidence for the involvement of the nucleotide in the positive inotropic response to catecholamines (Rall & West, 1963 ; Kukovetz & Pöch, 1967).

Earlier work with theophylline has shown that it may be a relatively unspecific agent in intact tissues. Levy & Wilkenfeld (1968) using the rat uterus found that

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theophylline not only potentiated the inhibitory response to noradrenaline but also that to nitroglycerine. In addition, pretreatment of uteri with nitroglycerine also potentiated the inhibitory response of the uterus to noradrenaline. Bowman & Hall (1970) using rabbit intestine found that the actions of theophylline and especially iminazole were not specific and could be due to a depressant action on the cell membrane.

The purpose of this study was to investigate the specificity and mechanism of action of theophylline and iminazole in frog cardiac muscle.

Methods

Experiments were conducted at room temperature (18–24° C) throughout the year using hearts dissected from pithed frogs (*Rana temporaria*) of various weights and of either sex. All frogs were obtained from Haigs of Newdigate and kept in a cold room at 5–10° C for at least one week before they were used in experiments.

The perfusion fluid was the same in all experiments except those employing iminazole or high calcium ion concentrations when the NaH_2PO_4 was omitted to prevent precipitation of calcium.

The Ringer was similar to that described by Nayler (1963) for toad hearts and had the following composition (mM): NaCl, 115·0; KCl, 3·2; $NaHCO_3$, 20·6; $MgSO_4$, 1·2; NaH_2PO_4, 3·0; $CaCl_2$, 1·5; and glucose, 16·5. It was made up with glass distilled water and continuously bubbled with 95% O_2, 5% CO_2.

Perfused frog heart preparations

After pithing the frog and cutting through the pectoral girdle, the posterior vena cava was cannulated *in situ* and the two anterior venae cavae were ligated. The heart was then removed from the frog and perfused with Ringer at 2–3 ml/minute. Perfusion pressure was kept constant at 2·5 cm H_2O. The heart beats were recorded on a kymograph with an isotonic lever exerting a tension of 1 g and giving 14× magnification.

A 15–30 min equilibration period was observed before the start of all experiments. Solutions of theophylline, iminazole and calcium were made up in Ringer and stored in a Mariott bottle at the same head of pressure as the control bottle containing Ringer and exchanged for the control perfusion when required. Other drugs were dissolved in Ringer and were injected into the rubber tubing situated close to the heart so that minimal dilution of the drug took place before it entered the heart. Injection volumes were kept constant at 0·1 ml to minimize the injection artifact and a 5–10 min interval was left between successive injections.

Stimulated preparations

Ventricles only were removed from pithed frogs and strips about 10 mm × 3 mm were cut from them. One end of the strip was then tied to a hook electrode and the other end connected to a frontal writing lever which exerted a tension of 0·5 g and magnified the contractions 12 times. To prevent stretching of the preparation after it had contracted (which was found occasionally to cause rebound contractions) a stop was placed on the lever so that at 0·5 g tension the lever just rested on the stop.

Strips were stimulated with a Palmer stimulator at rates of 0·1 or 1·0 Hz and a pulse strength of 10 V. The presence or absence of the classical staircase (Bowditch, 1871) was demonstrated by increasing the frequency of stimulation from 0·1 to 1·0 Hz. The presence or absence of poststimulation potentiation (Langendorff, 1885) was demonstrated by increasing the rate of stimulation from 0·1 to 1·0 Hz for 1 min followed by a 10–15 s rest before resuming stimulation at the control rate of 0·1 Hz.

^{45}Ca-influx studies

The method used was similar to that described by Nayler (1963).

Ventricles only were removed from pithed frogs and halved longitudinally. Half the ventricle then served as the test tissue and the other half as the control. The half ventricles were transferred to a beaker of Ringer gassed with 95% O_2, 5% CO_2 and left to equilibrate for 30 min at room temperature. Any ventricles that were beating spontaneously at this time were discarded.

After the equilibration period each half ventricle was exposed to ^{45}Ca-Ringer (0·4 μCi/ml) or to ^{45}Ca-Ringer plus test drug for 15 or 60 minutes. The ventricles were then removed, lightly blotted and transferred to 1 litre of Ringer for a 45 min washout period. The tissues were then firmly blotted and weighed in lidded crucibles.

The ventricles were ashed at 400° C for 12–18 h in a muffel furnace (Hotspot Model, Gallenkamp). The amount of ^{45}Ca present in the ash was estimated by taking up the ash in 0·05 ml conc. HCl (Aristar grade, BDH) contained in 0·4 ml distilled water. Duplicate 0·2 ml samples of the resultant solution were plated onto 200 μl pre-weighed planchettes. The planchettes were then thoroughly dried, reweighed and counted in a gas flow detector.

This gave an estimate of the residual ^{45}Ca (R^{45}Ca) in the tissue; that is the ^{45}Ca remaining after a 45 min washout period in Ringer. Results were calculated as (counts/min)/g wet weight of tissue ((cpm)/g).

^{45}Ca-efflux studies

Hearts were dissected as described for ^{45}Ca influx studies and the half ventricles were equilibrated for 30 min in oxygenated Ringer. They were then transferred to ^{45}Ca-Ringer (0·4 μCi/ml) for 15 minutes. Paired half ventricles were then washed in Ringer for 1 min to remove most extracellular ^{45}Ca and subsequently washed in successive 5 ml aliquots of Ringer or Ringer plus test drug for periods of 2, 5, 15, 30, 45, 60 and 120 minutes.

Total cell calcium measurements

Ventricles were prepared as described previously and equilibrated for 30 min in oxygenated Ringer. They were then transferred to the test solutions for 15, 60 or 120 minutes. After incubation in the test solution, the ventricles were washed in Ca-free Ringer for 1 min to remove extracellular calcium ions. After blotting between filter papers, 2–3 ventricles were transferred to a crucible and weighed by difference. The tissues were then ashed as described previously.

After ashing, the crucibles were reweighed and the residue taken up in 0·2 ml conc. HCl (Aristar grade, BDH) and made up to 2·0 ml with deionized water.

The calcium ion content of the solution was estimated as described by Daniel, Massingham & Nasmyth (1970) and results expressed as mmol/kg wet weight of tissue.

Materials : Theophylline hydrate and iminazole were obtained from British Drug Houses, (±)-propranolol hydrochloride (Inderal I.C.I.) and phentolamine mesylate (Rogitine Ciba) were used.

Results

Perfused hearts

Theophylline (5×10^{-3}M) perfused through the frog heart produced a positive inotropic effect which reached a maximum in 5–10 minutes. Continued perfusion with theophylline resulted in a slow decline in the effect until, in 30–60 min, the amplitude of the beat had returned to its original size (Figure 1a). The heart rate usually slowed during the perfusion.

Iminazole (1×10^{-2}–5×10^{-2}M) caused a marked positive inotropic effect which reached a maximum in about 5–10 min and then slowly declined (Fig. 1b), until

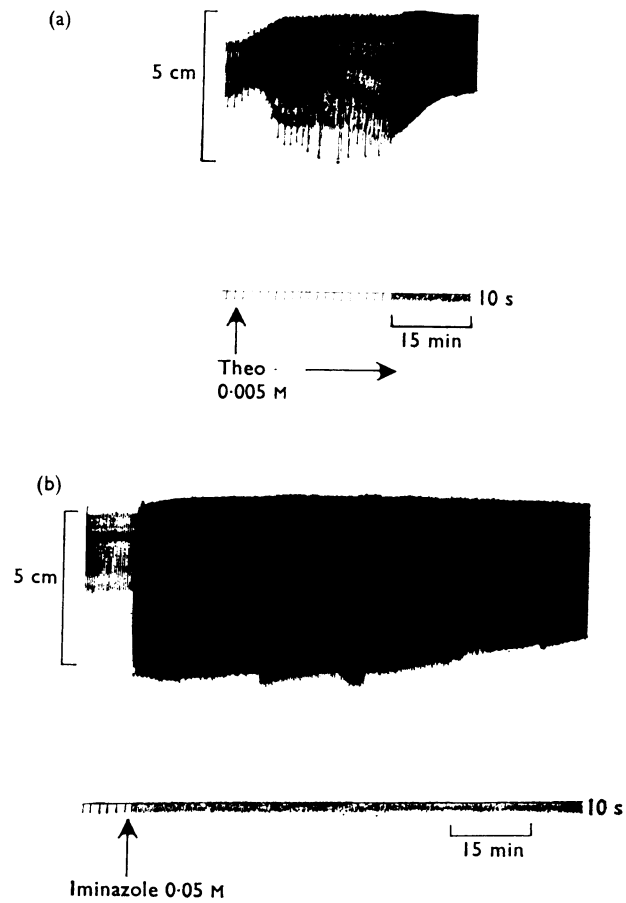

FIG. 1. The positive inotropic response of the frog heart to (a) theophylline (5×10^{-3}M) top trace, and (b) iminazole (5×10^{-2}M) lower trace.

after 60–120 min the amplitude of the beat had returned to its original size. The duration of the inotropic response to iminazole was always longer than that to theophylline.

The response to theophylline was not blocked by phentolamine ($1{\cdot}5 \times 10^{-6}$M) or by propranolol ($1{\cdot}5 \times 10^{-6}$M) given separately or in combination (Fig. 2). Theophylline (5×10^{-3}M) sometimes caused cardiac arrest in diastole before the amplitude of beat had returned to normal. This effect occurred in approximately 1 out of 7 hearts and may have been due to depression of pacemaker activity since

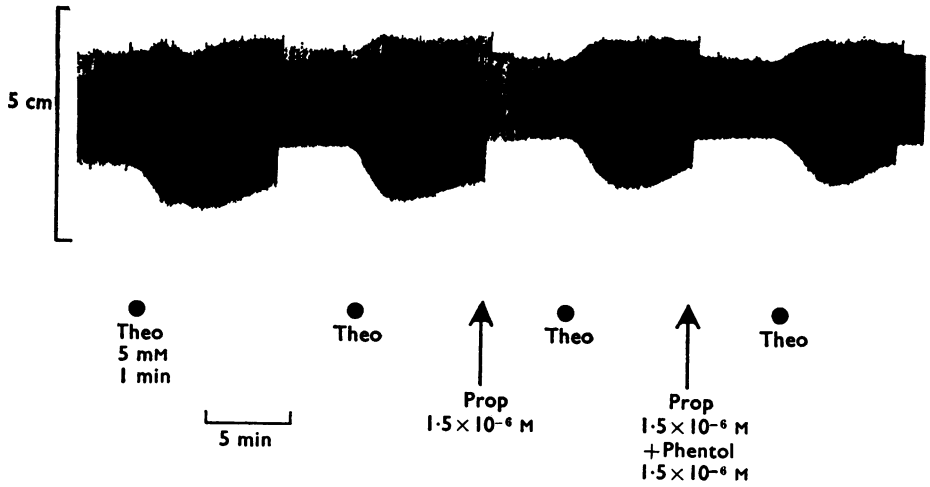

FIG. 2. The effect of propranolol (Prop.) ($1{\cdot}5 \times 10^{-6}$M) added at the first arrow and a combination of phentolamine (Phentol) ($1{\cdot}5 \times 10^{-6}$M) plus propranolol added at the second arrow on the response of the frog heart to exposure to theophylline (Theo) (5×10^{-3}M) for periods of 1 min. The kymograph was stopped for 5 min between theophylline additions.

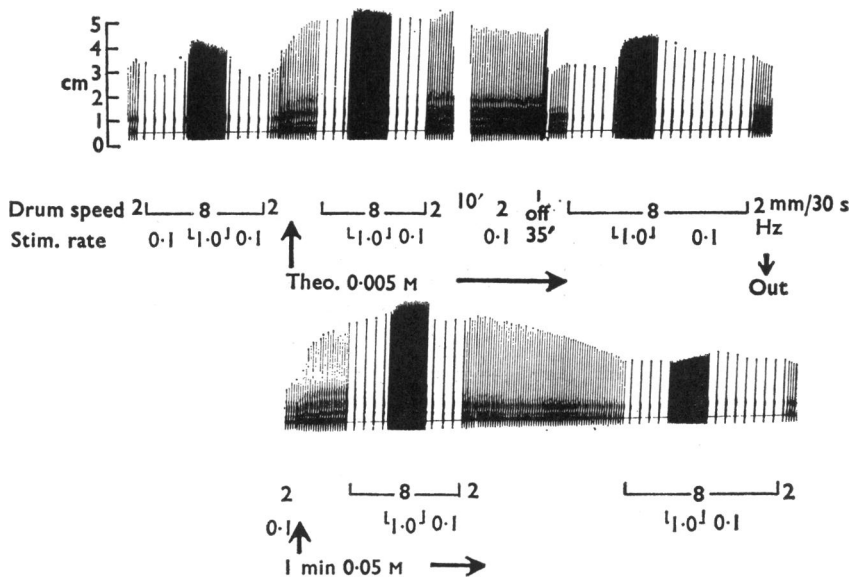

FIG. 3. Effect of theophylline (Theo) 5×10^{-3}M and iminazole (Imin) 5×10^{-2}M in a stimulated frog ventricle preparation. The trace is continuous except where shown and the drum speed was increased during the periods of increased rates of stimulation. Upper numerals= drum speed in mm/30 s. Lower numerals=stimulation rate in Hz.

hearts arrested by theophylline could be made to contract by electrical stimulation. When theophylline or iminazole was washed out the amplitude of the beat slowly decreased below its original size and then gradually returned to it.

The positive inotropic action of calcium ions (Ringer, 1883) in the frog heart was confirmed by direct injections of 50 and 100 μg of $CaCl_2 \cdot 2H_2O$ into the perfusion fluid.

Stimulated ventricle strips

When the rate of stimulating a ventricle strip was increased from 0·1 to 1·0 Hz for 1 min a staircase effect was observed. When the high rate of stimulation was stopped and a rest pause inserted before recommencing stimulation at 0·1 Hz post-stimulation potentiation could be seen with the next pulse but this quickly returned to the normal amplitude (Fig. 3).

Theophylline (5×10^{-3}M) increased the force of contraction of the ventricle strip in response to stimulation at 0·1 Hz. When the increase in force was maximal the staircase phenomenon was reduced and post-stimulation potentiation was abolished (Fig. 3). When the ventricle strip had been bathed in theophylline-Ringer for 50 min the amplitude of the contractions returned to normal. The staircase phenomenon was then larger than the control and post-stimulation

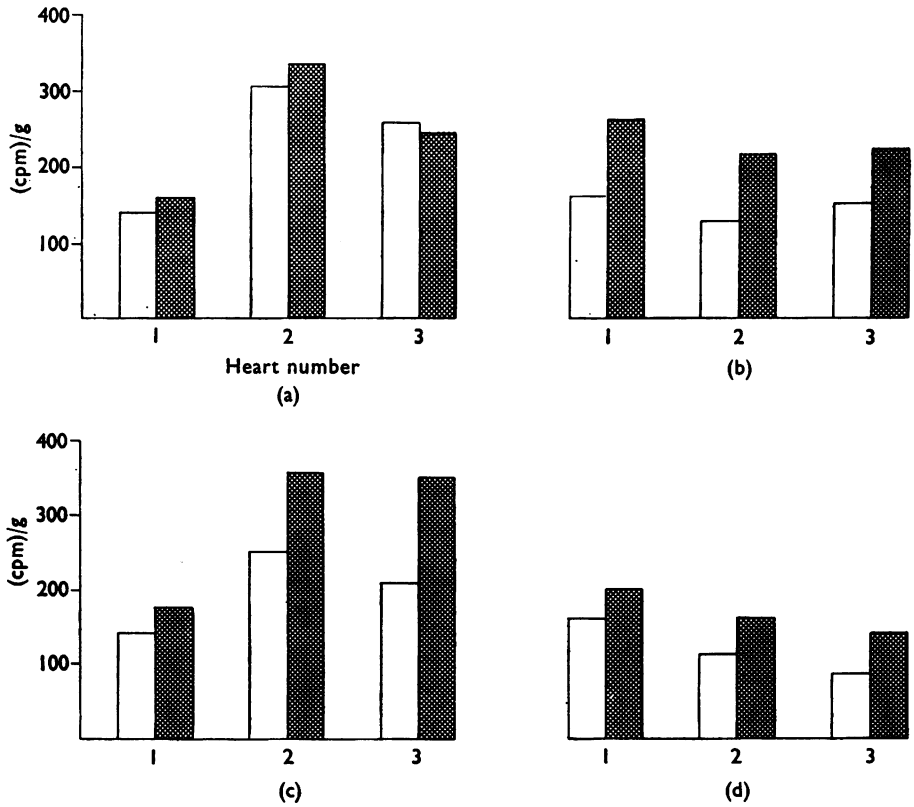

FIG. 4. ^{45}Ca-influx into frog half ventricles (a) Control heart and the effect of (b) $3 \times [Ca^{2+}]$ Ringer, (c) Theophylline 5×10^{-3}M and (d) Iminazole 1×10^{-2}M for 15 minutes. Open columns represent the control half ventricle and the cross hatched columns the test half.

potentiation returned and was longer lasting than it was in the absence of theophylline.

Iminazole (5×10^{-2}M) also increased the response of the ventricle strip to stimulation and when this effect was maximal the staircase and post-stimulation potentiation phenomena were much reduced or absent. However, when the amplitude of the contraction in the continued presence of iminazole had returned to normal the staircase phenomenon was reduced and less abrupt.

These opposing effects of iminazole and theophylline in stimulated preparations are similar to their effects on sympathomimetic amine responses in spontaneously contracting preparations (Massingham, 1969).

Calcium influx and efflux

During the calcium influx experiments it was observed that there was a large variation in the level of R^{45}Ca between ventricles but not between the halves of each ventricle (Fig. 4a). This variability between hearts has also been reported by

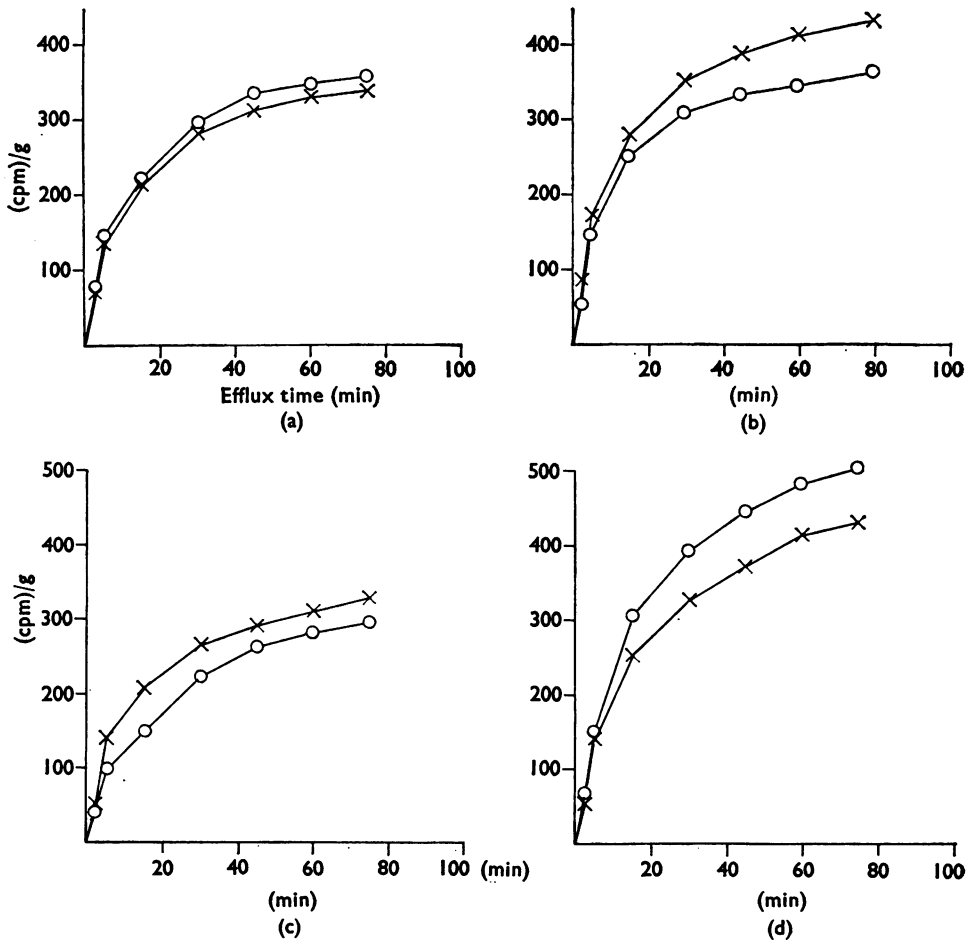

FIG. 5. (a) Control: ^{45}Ca-efflux, (b) $3 \times [Ca^{2+}]$ Ringer, (c) Iminazole 1×10^{-2}M, and (d) Theophylline 5×10^{-3}M. The curve ×——× represents the control, and ○——○ the test ^{45}Ca efflux in each case.

Nayler (1963). Because of this the results were not pooled, but each control half ventricle was compared with its test half. Differences in $R^{45}Ca$ of less than 10% were considered to be not significant because of control variability.

An increase in the Ringer concentration of calcium ions from 1·5 to 4·5 mM for 15 min increased the $R^{45}Ca$ in 3 out of 3 hearts (Fig. 4b), indicating a greater influx of ^{45}Ca into the cardiac cell in high calcium Ringer. The exposure of ventricles to theophylline (5×10^{-3}M) for 15 min significantly increased $R^{45}Ca$ in 2 out of 3 hearts (Fig. 4c). Three out of 3 test ventricles bathed in iminazole (1×10^{-2}M) for 15 min contained more $R^{45}Ca$ than their corresponding controls (Fig. 4d).

The efflux of ^{45}Ca from the heart, like ^{45}Ca influx, was found to vary when expressed as (cpm)/g of tissue. This difference was again minimized by halving the ventricle and using one half as the test and one half as the control tissue (Fig. 5a). An increase in the Ringer calcium from 1·5 to 4·5 mM caused a small reduction in efflux of ^{45}Ca in 3 out of 3 preparations (Fig. 5b). Iminazole (1×10^{-2}M) also slightly reduced efflux in 2 out of 3 preparations but slightly enhanced efflux in the other (Fig. 5c). Theophylline (5×10^{-3}M) significantly enhanced ^{45}Ca-efflux in 2 out of 2 preparations (Fig. 5d).

Total cell calcium determinations

A three-fold increase in the concentration of calcium ions in the Ringer led to an increase in total cell calcium ion concentration after a 15 min exposure period (Table 1).

Exposure of ventricles to theophylline (5×10^{-3}M) for 15 min led to a small increase in cell calcium ions. However, after 60 min exposure there was no significant difference between control and theophylline-treated ventricles (Table 1).

Iminazole (1×10^{-2}M) caused a reduction in cell calcium ion content after a 120 min exposure period but the difference was not statistically significant (Table 1).

Discussion

Nayler (1963) reported a positive inotropic effect with caffeine in toad hearts which was followed by a decline in force of contraction in the continued presence of the drug. She also observed that this decline could be hastened by replacement of caffeine–Ringer with Ringer and that the isotonic contractions which followed re-equilibration were smaller than initial contractons. Similar results have been observed in these experiments with the frog heart for both theophylline and iminazole.

The positive inotropic response to theophylline was not blocked by propranolol or by phentolamine and it was therefore not mediated entirely by catecholamine release. This result is similar to that of De Gubareff and Sleator (1965) who used caffeine in guinea-pig atrial preparations. Because theophylline inhibits phosphodiesterase and iminazole activates it (Butcher & Sutherland, 1962), the two drugs would have opposing effects on the tissue 3′,5′-AMP. It is therefore unlikely that their positive inotropism could be mediated by changes in the tissue content of the nucleotide.

Since methylxanthines have been found to release ionized calcium from intracellular storage sites this may be the process whereby the xanthines increase myo-

TABLE 1. *The effect of theophylline, iminazole and 3x[Ca^{++}] Ringer on the total cell calcium content of frog ventricles*

Drug	Exposure Time (min)	*n*	$[Ca^{++}]_i \pm$ SEM (mmol/kg)	*P*	H_2O Content (g/kg)	*P*
Control	15	10	3·26±0·07		990·8±0·72	
3x$[Ca^{++}]_o$ (4·5 mM)	15	4	4·84±0·27	<0·02	989·8±0·27	NS
Theophylline (5×10^{-3} M)	15	6	3·63±0·14	<0·001	991·8±0·72	NS
Control	60	4	3·85±0·24		997·8±0·81	
Theophylline (5×10^{-3} M)	60	4	4·03±0·13	NS	993·2±1·08	<0·01
Control	120	4	3·26±0·16		987·7±2·19	
Iminazole (1×10^{-2} M)	120	3	2·97±0·09	NS	983·2±3·2	NS

plasmic calcium ions to cause positive inotropic effects (Nayler, 1967). This hypothesis fits the present observations with theophylline and iminazole in the frog heart. Upon removal of these drugs the release of ionized calcium from cellular stores would cease, but the heart would be in calcium deficit and this may explain the reduction in force of contraction which was observed when they were washed out. However, no significant differences in total cell calcium ion concentrations were found in ventricles treated with theophylline for 60 min or iminazole for 120 min, probably because the amount of "activator" calcium (that is calcium ions which actually stimulate the contractile proteins) is small compared with the total cell calcium ion concentration (Bianchi 1968).

However, calcium ion fluxes were affected by these drugs and the results with theophylline on calcium ion influx are similar to those of Nayler (1963) who demonstrated that the enhanced influx of calcium ions which accompanies excitation in caffeine-treated toad hearts resulted in a relatively high myoplasmic calcium ion concentration and hence more forceful contractions.

These effects on cardiac calcium ions were indirectly confirmed in stimulated preparations. Both iminazole and theophylline caused a loss of, or reduction in, the staircase and post-stimulation potentiation phenomena when their inotropic activity was maximal. Similar results with caffeine have been demonstrated by Nayler (1963) in toad hearts but she did not retest for the presence of the staircase phenomenon when the positive inotropic effects had disappeared. At this time it was shown in our experiments that theophylline potentiated the staircase phenomenon but that iminazole antagonized it.

There is evidence that the staircase and post-stimulation potentiation phenomena depend on the accumulation of calcium ions inside the cell during periods of increased rates of stimulation (Niedergerke, 1956 & Nayler, 1963). The inhibition of these phenomena by theophylline and iminazole are explicable in terms of the increased influx of Ca^{++} ions which they produce as this would pre-empt the effect of the accumulation of calcium ions produced by the increased rates of stimulation. Their effects on the staircase phenomenon and on post-stimulation potentiation when their positive inotropic effect had disappeared were opposed to each other and cannot be explained in these terms because at that time neither theophylline nor iminazole had any significant effect on the total cell calcium (Table 1). After the positive inotropism has disappeared but in their continued presence iminazole inhibits responses to sympathomimetic amines and theophylline potentiates them (Massingham 1969). This parallels the effects on the staircase and post-stimulation potentiation phenomena and it seems likely that the mechanism is the same in each case. It could also be due to an effect on Ca^{2+}, since the amount of 'activator calcium' is small compared with the total cell calcium concentration, or to an increase in tissue 3′,5′-AMP in consequence of phosphodiesterase inhibition by theophylline and a decrease in the tissue content of the nucleotide caused by activation of the enzyme by iminazole. Recent evidence obtained with vascular smooth muscle indicates that methylxanthines inhibit the extraneuronal inactivation of catecholamines by inhibiting catechol-*O*-methyltransferase (Kalsner, 1971) and a similar mechanism might operate in cardiac muscle.

It is evident that all the effects of theophylline and iminazole are not explicable in terms of their action on phosphodiesterase. Bowman & Hall (1970) remarked

that the actions of theophylline and especially those of iminazole were non-specific and that interpretation of experimental results obtained with them in terms of their action on phosphodiesterase should be treated with reservation. The present experiments make it quite clear that they have at least two actions on frog heart muscle and add to the justification for this caution.

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