# Comparison of the effects of caffeine and other methylxanthines on [Ca$^{2+}$]$_i$ in rat ventricular myocytes

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1   The effects of caffeine and other methylxanthines were investigated on intracellular calcium concentration ([Ca$^{2+}$]$_i$) and contraction in rat isolated ventricular myocytes. The use of the fluorescent indicator, Indo-1, allowed simultaneous measurement of [Ca$^{2+}$]$_i$ and the intracellular concentration of the methylxanthines.

2   Rapid application of caffeine (10 mM) produced a transient rise of [Ca$^{2+}$]$_i$ which decayed to resting levels. This was accompanied by a transient contraction which decayed to a level above baseline. The addition of theophylline also produced a transient increase of [Ca$^{2+}$]$_i$. However, following the initial transient, contraction decayed before redeveloping to a maintained level.

3   Direct measurements showed that [caffeine]$_i$ rose more quickly than did [theophylline]$_i$. The slower rise of [theophylline]$_i$ was associated with a delay in the increase of [Ca$^{2+}$]$_i$. At lower concentrations of the methylxanthines, theophylline was less effective than caffeine at initiating Ca release. The rate of entry of theobromine was similar to that of theophylline.

4   Isocaffeine did not produce a rise of [Ca$^{2+}$]$_i$. The rate of rise of [isocaffeine]$_i$ was much slower than that of either caffeine or theophylline.

5   Measurements of the oil:water partition coefficient showed that the order of relative partitioning into oil was: caffeine > theophylline > theobromine > isocaffeine. This is similar to the order of rate of entry into the cell.

6   We conclude that many of the differences in the effects of these methylxanthines can be attributed to differences in membrane permeability due to differences in oil:water partition.

**Keywords:** [Ca$^{2+}$]$_i$; cardiac muscle; contraction; caffeine; methylxanthine

## Introduction

Caffeine and other methylxanthines have a variety of effects in cardiac muscle (see O'Neill *et al.*, 1993). These include: (i) the release of Ca$^{2+}$ ions from the sarcoplasmic reticulum (SR); (ii) a shift of the relationship between [Ca$^{2+}$]$_i$ and tension such that a lower [Ca$^{2+}$]$_i$ is required to activate contraction; and finally (iii) caffeine also has actions as a phosphodiesterase inhibitor. The first two of these effects have been studied recently in isolated cardiac myocytes. The fluorescent Ca-indicator Indo-1 (Grynkiewicz *et al.*, 1985) is particularly useful in this context as it allows measurement of both [Ca$^{2+}$]$_i$ and the intracellular caffeine concentration ([caffeine]$_i$). Therefore it is possible to correlate the effects of caffeine directly with its intracellular concentration (O'Neill *et al.*, 1990; O'Neill & Eisner, 1990; Baro *et al.*, 1993).

Caffeine has been used in many studies of excitation-contraction coupling. The other methylxanthines have also been examined at both cellular (Chapman & Miller, 1974) and subcellular (Rousseau *et al.*, 1988) sites of action. One aim of this kind of study is that the use of different analogues may provide a substance which has only one of the effects of caffeine mentioned above and may therefore be of use for studying the control of contraction. In the present paper we have studied the effects of various methylxanthines on rat isolated ventricular myocytes. The results show that many of the differences between the actions of these compounds can be attributed to differences in the rate of entry into the cell and correlate with differences in the oil-water partition coefficient.

## Methods

### Ventricular myocyte experiments

The experiments were performed on single ventricular myocytes. Rats were killed by cervical dislocation. The methods for isolating cells and for measuring [Ca$^{2+}$]$_i$ and [caffeine]$_i$ from the fluorescence of Indo-1 have been published previously (O'Neill *et al.*, 1990; Eisner *et al.*, 1989). Briefly, the estimation of [caffeine]$_i$ depends on the fact that caffeine quenches the fluorescence of Indo-1 (O'Neill *et al.*, 1990). Therefore, when caffeine is applied, there are two effects on Indo-1 fluorescence: (i) The change of [Ca$^{2+}$]$_i$ will produce opposite effects at the two emission wavelengths (400 and 500 nm). (ii) Caffeine will produce a concentration-dependent (but wavelength and [Ca$^{2+}$]$_i$-independent) decrease of the fluorescence at both wavelengths. Separation of these two effects allows [Ca$^{2+}$]$_i$ and [caffeine]$_i$ to be calculated. In order to do this, the relationship between the 400:500 nm emission ratio and the fluorescence at 400 nm (F$_{400}$) is first determined in the absence of caffeine. Then in the presence of caffeine, the value of F$_{400}$ associated with a given ratio is compared with that in the absence of caffeine. This comparison gives a measure of the caffeine-induced quench from which [caffeine]$_i$ can be calculated. The method depends on the fact that the quench of Indo-1 fluorescence produced by caffeine is independent of both emission wavelength and [Ca$^{2+}$] (O'Neill *et al.*, 1990). We find that this is also the case for the quench produced by theophylline. In the case of theobromine, however, the quench is not independent of wavelength. Under these conditions it is impossible to separate [Ca$^{2+}$]$_i$- and quench-induced changes of fluorescence. In order to be able to measure the rate of entry of theobromine into the cell we have performed the experiments in a Ca-free solution after previously having discharged the SR Ca content with caffeine. Under these conditions there are no [Ca$^{2+}$]$_i$-induced changes of fluorescence and the rate of entry

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of theobromine can be estimated from the quench at any wavelength. Finally, isocaffeine fluoresces when excited at the wavelengths used to excite Indo-1. This fluorescence is greater at an emission wavelength of 400 than 500 nm. This produces a step artefact in the 400:500 nm ratio trace (e.g. Figure 5). It does not, however, appreciably perturb the estimate of the quench which develops much more slowly.

Indo-1 was loaded into the cells as the acetyoxymethyl (AM) ester. The results are presented as the ratio of the fluorescence emitted at 400 nm to that at 500 nm because of the problems of quantifying $[Ca^{2+}]_i$ when the dye is loaded in this way (Highsmith *et al.*, 1986).

The experimental solution contained (mM): NaCl 134, KCl 4, $MgCl_2$ 1, HEPES 10, glucose 11, $CaCl_2$ 1; titrated to pH 7.4 with NaOH. The solution was equilibrated with air. The various methylxanthines were added at concentrations of up to 10 mM without osmotic compensation. All experiments were carried out at 26°C.

### Determination of oil-water partition coefficients

The oil-water partition coefficients were measured from the distribution between the experimental solution (above) and either olive oil or hexadecane. In the initial experiments hexadecane was used as this is reported to be a good model for biological membranes (Stein, 1986). However we found that the hexadecane:water partition coefficient was of the order of $10^{-5}$ for isocaffeine. It was difficult to measure such small values and therefore we changed to the somewhat more polar olive oil. The concentrations of the methylxanthines in the aqueous phases were determined by measurements of the ultra-violet absorption spectrum. In the case of caffeine (which has the highest oil:water solubility ratio) 1 ml of a 0.1 mM aqueous solution was mixed with 1 ml of olive oil. The amount of caffeine in the olive oil was measured from the reduction in the concentration in the aqueous phase. For isocaffeine the oil:water partition coefficient was too low for this method to be used without having to employ impracticably large volumes of olive oil. In these cases the following indirect procedure was adopted: 2 ml of a 10 mM aqueous solution of isocaffeine was mixed with 20 ml of olive oil. The amount of isocaffeine in the oil phase was determined by removing the oil and re-extracting the isocaffeine into fresh (isocaffeine-free) aqueous phase. For theophylline and theobromine either method could be used. One concern with these determinations was that the presence of impurities in the original compounds might interfere with the estimates. For example any caffeine contamination would give an artificially high oil:water ratio. This problem was excluded by measuring the absorption spectrum of the second aqueous phase which was found to have the spectral characteristics of the compound under investigation and did not include a component with the spectral properties of caffeine. Partition coefficients were measured at 21°C.

### Nomenclature

In this paper we have tested the following compounds: caffeine (1,3,7-trimethylxanthine): isocaffeine (1,3,9-trimethylxanthine); theophylline (1,3-dimethylxanthine); theobromine (3,7-dimethylxanthine). These methylxanthines were obtained from Sigma.

### Results

Figure 1 shows a comparison of the effects of caffeine and theophylline at a concentration of 10 mM. Both agents produce a transient increase of $[Ca^{2+}]_i$ presumably due to the release of $Ca^{2+}$ ions from the SR. There are, however, differences in the contraction. In the case of caffeine the peak contraction relaxes monotonically to a plateau level. In contrast, in theophylline, the contraction relaxes to a minimum
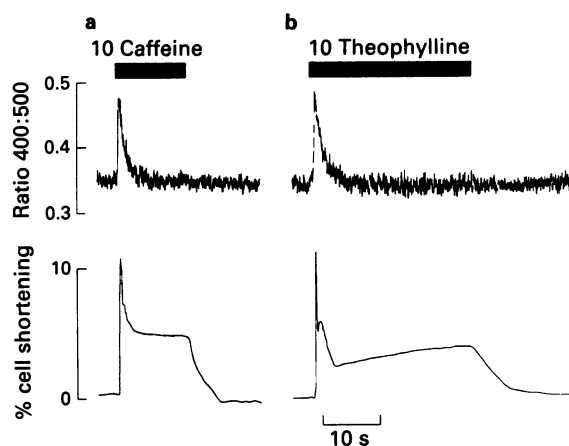


**Figure 1** Comparison of the effects of caffeine and theophylline on $[Ca^{2+}]_i$ and contraction in the same cell. In both panels traces show: top, $[Ca^{2+}]_i$ (measured from the 400:500 nm Indo-1 ratio); bottom, contraction. Caffeine (a) or theophylline (b) were added at a concentration of 10 mM as shown above the records.
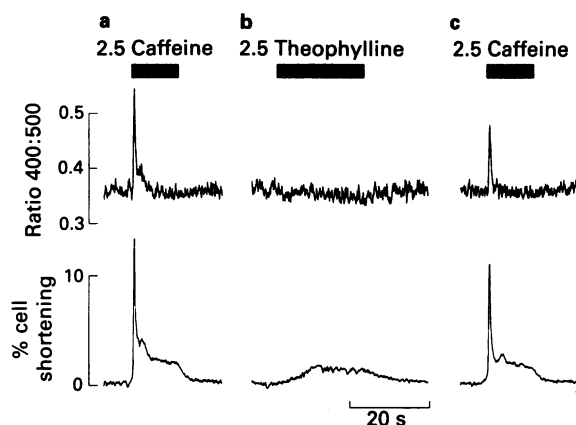


**Figure 2** Comparison of the effects of a low (2.5 mM) concentration of caffeine or theophylline on $[Ca^{2+}]_i$ and contraction in the same cell. In each panel caffeine or theophylline was added for the period shown by the solid bars. Panels show: (a) caffeine; (b) theophylline; (c) caffeine.

level before redeveloping again. The effects of lower concentrations of these agents are shown in Figure 2. The application of 2.5 mM caffeine (Figure 2a) produces a transient increase of $[Ca^{2+}]_i$ which is accompanied by a contractile response which, although mainly transient, does have a small sustained phase. In contrast when the same concentration of theophylline is applied (Figure 2b) there is no release of calcium or transient contraction. The contraction record shows only a small, slowly developing, maintained component. Finally Figure 2c shows that a subsequent exposure to caffeine produces a similar response to the initial one.

The effects of theophylline are reminiscent of those found previously for slow application of caffeine which results in a slowly developing contraction (O'Neill *et al.*, 1990). For this reason we have investigated the rate at which theophylline enters the cell. The experiment illustrated in Figure 3 shows measurements of $[Ca^{2+}]_i$ and $[methylxanthine]_i$ on the same cell during the application of 10 mM of either caffeine or theophylline. It is clear that $[caffeine]_i$ (Figure 3a) increases considerably more quickly than does $[theophylline]_i$ (Figure 3b) despite being applied at the same rate. The superimposed records (Figure 3c) show that the increase of $[Ca^{2+}]_i$ with respect to the time after addition of the methylxanthine is correspondingly delayed in theophylline compared to caffeine.
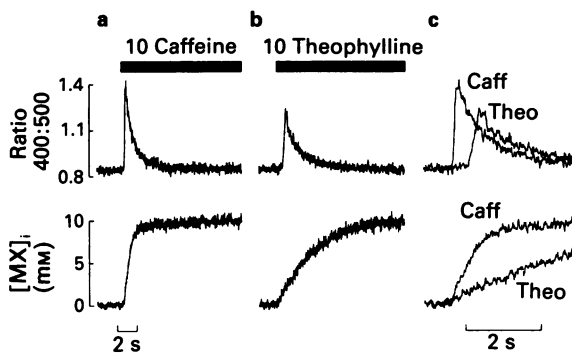
**Figure 3** Comparison of the effects of caffeine and theophylline on [Ca²⁺]ᵢ with their rate of entry into the cell. In all panels traces show: above, [Ca²⁺]ᵢ; below, methylxanthine concentration ([MX]ᵢ). Panels (a) and (b) show the effects of adding caffeine or theophylline on the same cell; (c) shows the initial part of the records of (a) and (b) superimposed on an expanded timescale.
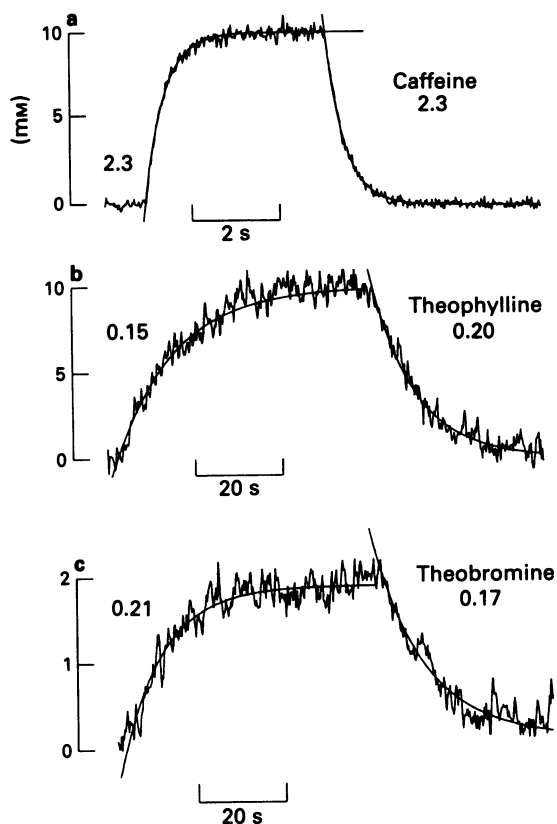


**Figure 4** A comparison of the rate of entry of caffeine, theophylline and theobromine on the same cell. In all panels the traces show the calculated concentrations of the methylxanthines: (a) 10 mM caffeine; (b) 10 mM theophylline; (c) 2 mM theobromine. The smooth curves drawn through the data are best-fit single exponentials with the rate constants (s⁻¹) indicated. Note that the timescale for (a) is different from that for (b) and (c). This experiment was performed in a Ca-free solution (see Methods).

Theobromine had a similar rate of entry into the cell to theophylline. This is illustrated in Figure 4 which shows (on the same cell) the changes of intracellular concentration for caffeine, theophylline and theobromine. In this experiment, because of the problem of dissolving higher concentrations, theobromine was used at a lower concentration (2 mM) than the other methylxanthines. This experiment was performed in a Ca-free solution. In Ca-containing solutions, theobromine produced a release of calcium from the SR as judged by a transient rise of [Ca²⁺]ᵢ of similar magnitude to that pro-

duced by theophylline. Because the quench produced by theobromine is different at the two emission wavelengths (see Methods), it is not possible to quantify the exact magnitude of the release produced by theobromine.

The experiment illustrated in Figure 5 compares the actions of caffeine with those of isocaffeine. The trace labelled 'quench' shows the percentage quenching of the fluorescence. This develops much more slowly for isocaffeine than for caffeine, indicating that isocaffeine enters the cell much more slowly than does caffeine. The Indo-1 ratio trace shows an increase when isocaffeine is added. This effect, which occurs long before isocaffeine has entered the cell is due to the fact that isocaffeine, unlike caffeine, fluoresces. The fluorescence emission of isocaffeine is greater at 400 than 500 nm and therefore increases the ratio. It should be noted that, as the increase of fluorescence is much less than the quench (particularly at 500 nm) and develops much more quickly, this effect does not interfere with the calculation of the quench. Figure 5 also shows the effects of adding caffeine at the same time as removing isocaffeine. The transient additional quench indicates that caffeine enters the cell much faster than isocaffeine leaves. This addition of caffeine produces a transient rise of [Ca²⁺]ᵢ which is much smaller than the control one, suggesting that isocaffeine has released some (but not all) of the SR calcium. This is supported by the observation (not shown) that the application of isocaffeine produces a slow decrease in the magnitude of the systolic Ca transient.

The data for the rate constants of entry of the various methylxanthines are summarized in Table 1. Caffeine has the highest rate of entry, followed by theophylline and theobromine. Isocaffeine is considerably slower than any of the others. It should be noted that the solution changing system used in the present experiments was no quicker than the rate
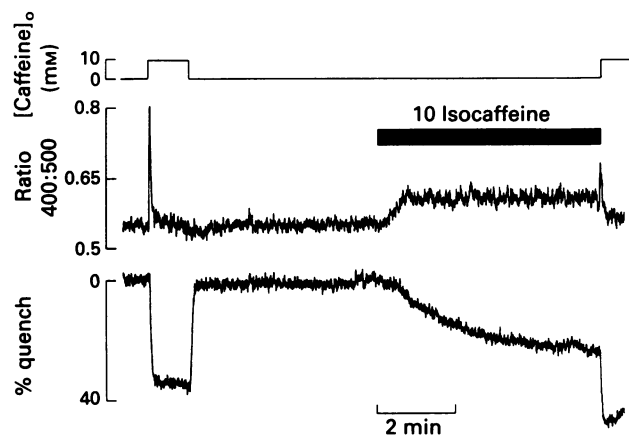


**Figure 5** A comparison of the effects of caffeine and isocaffeine. Traces show: top, [Ca²⁺]ᵢ; bottom, the % quench of the Indo-1 signal. Caffeine (10 mM) was first added and removed; then isocaffeine (10 mM) was added. Finally isocaffeine was removed while simultaneously adding caffeine (10 mM).

**Table 1** Summary of the rate constant of entry into the cell (column 2) and the oil-water partition coefficient (column 3) for the various methylxanthines

| Methylxanthine | Rate constant of entry (s⁻¹) | | Oil:water partition coefficient | |
|---|---|---|---|---|
| Caffeine | 2.3 ± 0.12 | (5) | 0.077 ± 0.007 | (4) |
| Theophylline | 0.20 ± 0.02 | (6) | 0.0216 ± 0.002 | (5) |
| Theobromine | 0.23 ± 0.03 | (4) | 0.0066 ± 0.0009 | (8) |
| Isocaffeine | 0.0045 ± 0.0009 | (4) | 0.0011 ± 0.0001 | (5) |

The data show mean ± s.e.mean (number of measurements).

constant of changes of [caffeine]$_i$. It is therefore likely that the rate constant for caffeine entry is an underestimate as higher values have been reported previously (O'Neill et al., 1990). Table 1 also shows the values for the relative oil:water partition coefficients for the various methylxanthines. It is clear that caffeine is the most oil soluble and isocaffeine the least.

## Discussion

The results presented here characterize the rate of entry of various methylxanthines into ventricular myocytes and allow these to be compared to the effects on [Ca$^{2+}$]$_i$ and contraction. We find that theophylline enters the cell more slowly than caffeine. The effects of theophylline on [Ca$^{2+}$]$_i$ and contraction are exactly those to be expected from a low rate of entry. A consistent observation was that, when the caffeine-evoked contraction had a transient component which decayed to a steady-level, that produced by theophylline would initially decline further before a secondary increase followed. As explained previously (O'Neill et al., 1990) the transient phase is due to a transient increase of [Ca$^{2+}$]$_i$. The maintained phase is due to a direct effect of the methylxanthine on the contractile machinery. Caffeine enters the cell quickly so the maintained phase develops while [Ca$^{2+}$]$_i$ is declining and the result is a smooth fall to the steady level. In contrast the lower rate of entry of theophylline means that [Ca$^{2+}$]$_i$ has declined (and contraction relaxed more fully) before the second phase begins. This lower rate of entry of theophylline compared to caffeine can explain why, although theophylline is more potent than caffeine in releasing calcium from the isolated SR (Rousseau et al., 1988), it is less effective in releasing calcium in intact cells (Figure 2). Previous work has shown that if caffeine is applied slowly the rise of [Ca$^{2+}$]$_i$ is less than if it is applied quickly (O'Neill & Eisner, 1990).

We have also found that isocaffeine enters the cell much less quickly than theophylline (or caffeine). This difference is particularly striking for two isomers. It has, however, been shown by use of u.v. and n.m.r. spectroscopy that isocaffeine is much more polarizable than is caffeine (Yanuka & Bergmann, 1986). This may account for its relative lack of solubility in the membrane. As indicated in Table 1 there is a good correlation between the rates of entry and the oil:water partition coefficient. This is not unexpected. For a given membrane the permeability to substances which simply move through the lipid phase depends on the molecular size and the partition coefficient between the membrane and the aqueous phases. It is clear that, the higher the oil:water partition coefficient, the faster the rate of entry. It should, however, be noted that the relative differences in the rate of entry are considerably greater than those of the partition coefficient. Furthermore, although theobromine and theophylline enter the cell at similar rates, their partition coefficients are different. There are two explanations for the lack of complete correlation between the partition coefficients and the rate of entry. (i) The various methylxanthines may differ in properties other than partition coefficient. Previous work has shown that permeability across a lipid membrane can be accounted for by the combination of partition coefficient and molecular size (Stein, 1986). It is therefore possible that the molecular size of the methylxanthines, when in the membrane, contributes. (ii) Another possibility is that the lipid phase of the membrane behaves in a different way as regards the relative solubilities of the methylxanthines from olive-oil. Despite these quantitative discrepancies, the major conclusion is that the rates of entry and therefore some of the effects of the methylxanthines correlate with the oil:water partition coefficients.

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