Calcium measurements with a new high-affinity n.m.r. indicator in the isolated perfused heart

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A new n.m.r. indicator, 1,2-bis-{2-[1-(hydroxycarbony)ethyl-(hydroxycarbonylmethyl)]amino-5-fluorophenoxy}ethane (DiMe-5FBAPTA), with a higher affinity for calcium (apparent K_d 46 nM, pH 7.2, 30 °C) than the parent 5FBAPTA chelator (K_d 537 nM, pH 7.1, 30 °C) has been used to measure the cardiac intracellular free Ca^{2+} ($[Ca^{2+}]_i$). DiMe-5FBAPTA was loaded into Langendorff-perfused ferret hearts maintained at 30 °C using the acetoxymethyl ester (AM) derivative. The intracellular concentration required to achieve an adequate signal-to-noise (S/N) ratio (> 10:1) for the n.m.r. spectra caused a similar reduction in developed pressure to that obtained using 5FBAPTA-AM. The DiMe-5FBAPTA was used to estimate $[Ca^{2+}]_i$ in diastole, through the calcium transient and at rest in the presence of the slow calcium channel blocker diltiazem. At a pacing frequency of 1.0 Hz, end-diastolic $[Ca^{2+}]_i$ was 198 ± 30 nM ($n = 9$), and reducing the pacing frequency to 0.2 Hz lowered $[Ca^{2+}]_i$ to 89 ± 13 nM ($n = 5$). Perfusion with diltiazem (100 μM) for 60 min lowered $[Ca^{2+}]_i$ to 10 ± 1 nM ($n = 4$) in unpaced hearts and to 94 ± 24 nM ($n = 4$) in hearts paced at 1.0 Hz. The $[Ca^{2+}]_i$ transient measured with DiMe-5FBAPTA was sharper and delayed compared with the transient measured previously with 5FBAPTA. Co-loading the two indicators provided evidence that the indicator with the higher K_d had a dominant effect on the end-diastolic $[Ca^{2+}]_i$. The lower values for end-diastolic $[Ca^{2+}]_i$ obtained with DiMe-5FBAPTA are consistent with fluorescent indicator measurements. These observations suggest that perturbations of $[Ca^{2+}]_i$ caused by the new indicator are less than those induced by 5FBAPTA. DiMe-5FBAPTA therefore represents a useful step in the development of ^{19}F-n.m.r. calcium indicators.

INTRODUCTION

In previous studies of the cardiac cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), we (Metcalfe et al., 1985; Kirschenlohr et al., 1988) and others (Steenbergen et al., 1987; Marban et al., 1988), have used the ^{19}F-n.m.r. indicator 5FBAPTA (Smith et al., 1983). This indicator enabled the $[Ca^{2+}]_i$ to be measured in the intact beating heart, but intracellular concentrations of 5FBAPTA above 80 μM were required because of the inherent insensitivity of the n.m.r. signal. These concentrations of indicator caused a substantial reduction in developed pressure and in the rate of recovery of the heart after contraction (Harding et al., 1993). It is presumed that these functional effects result, at least in part, from the Ca^{2+} buffering effect of cytosolic 5FBAPTA.

The functional perturbations due to the indicator are reflected in the $[Ca^{2+}]_i$ values obtained with 5FBAPTA in beating hearts. End-diastolic $[Ca^{2+}]_i$ values of 0.52 μM (Harding et al., 1993) were substantially higher than those reported using fluorescent indicators, e.g. 0.12 μM (Frampton et al., 1991) and 0.14 μM (Borzak et al., 1990) in isolated cardiac myocytes, and 0.17 μM in whole rabbit myocardium (Nakanishi et al., 1990). Other workers (Steenbergen et al., 1987) using the ^{19}F-n.m.r. with 5FBAPTA have reported $[Ca^{2+}]_i$ values averaged over the cardiac cycle in the rat heart of 0.63 μM, similar to our values from the ferret heart (Harding et al., 1989). However, one group working with ferret heart and 5FBAPTA has reported significantly lower averaged and end-diastolic $[Ca^{2+}]_i$ values of 0.35 μM (Marban et al., 1990) and 0.20 μM (Marban et al., 1988) respectively. The latter values are still significantly lower than those we have reported even when recalculated using a K_d of 537 nM for Ca^{2+} (Kirschenlohr et al., 1988).

If the end-diastolic $[Ca^{2+}]_i$ in unperturbed beating hearts is normally in the range 100–200 nM, as estimated in most studies of isolated myocytes with fluorescent indicators, the values we obtained with 5FBAPTA of approx. 0.5 μM at a pacing frequency of 1.0 Hz suggest that the indicator is elevating the end-diastolic $[Ca^{2+}]_i$ into the effective buffering range of the indicator (the apparent K_d for Ca^{2+} is 537 nM). We assumed that if we used an indicator with K_d for Ca^{2+} substantially lower than the estimated end-diastolic $[Ca^{2+}]_i$ range in unperturbed hearts, most of the indicator would be bound by Ca^{2+} in diastole and perturbation of the heart due to buffering of the Ca^{2+} transient would be greatly reduced. The use of an indicator with a lower K_d would also be appropriate for measurements in arrested hearts, as the $[Ca^{2+}]_i$ value of approx. 80 nM obtained with 5FBAPTA (Harding et al., 1993) is below the effective titration range of the indicator. We have recently described the synthesis and properties of 1,2-bis-{2-[1-(hydroxycarbony)ethyl(hydroxycarbonylmethyl)]amino-5-fluorophenoxy}ethane (DiMe-5FBAPTA) (Clarke et al., 1993), which has a K_d for Ca^{2+} of 46 nM at pH 7.2, and here we describe measurements of $[Ca^{2+}]_i$ using this indicator

Abbreviations used: BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; 5FBAPTA, 5,5'-difluoro derivative of BAPTA; 5FBAPTA-AM, tetra-acetoxymethyl ester derivative of 5FBAPTA; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DiMe-5FBAPTA, 1,2-bis-{2-[1-(hydroxycarbony)ethyl-(hydroxycarbonylmethyl)]amino-5-fluorophenoxy}ethane; DMSO, dimethyl sulphoxide; K_d, equilibrium dissociation constant; LVP, left ventricular pressure; r.f., radiofrequency; S/N ratio, signal-to-noise ratio.

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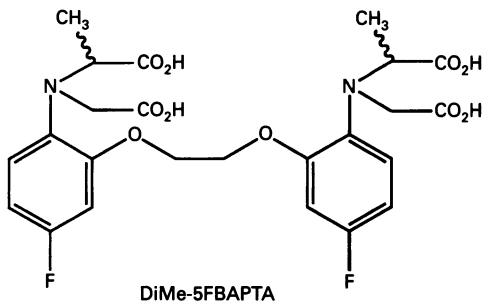
in perfused ferret hearts in diastole, through the Ca^{2+} transient and in hearts arrested by diltiazem.



DiMe-5FBAPTA

MATERIALS AND METHODS

Heart perfusion

Ferret hearts were Langendorff-perfused at a constant flow rate (4.0 ml·min^{-1}·g heart wet wt.$^{-1}$) with a modified Krebs–Henseleit bicarbonate medium (pH 7.4, 30 °C) as described previously (Harding et al., 1993). Hearts were perfused at 30 °C rather than 37 °C because washout of the indicator is much slower at the lower temperature, and pacing at low frequencies (e.g. 0.2 Hz) is stable at 30 °C but not at 37 °C. After crushing the atrio-ventricular node, hearts were paced at 1.0 Hz; this was maintained throughout indicator loading. Isovolumic left ventricular pressures (LVPs) were recorded using a balloon catheter. Maximum developed LVP was calculated as systolic LVP minus diastolic LVP. The indicators (DiMe-5FBAPTA and 5FBAPTA) were loaded as the acetoxymethyl esters (AM) as 50 mM solutions in dimethyl sulphoxide (DMSO) via the perfusion line at a rate of 250 μl/h. To obtain adequate signal-to-noise (S/N) ratios in the n.m.r. spectra, loading times were 80 min for DiMe-5FBAPTA-AM and 30 min for 5FBAPTA-AM (Kirschenlohr et al., 1988). For some experiments, hearts were rendered quiescent by perfusion with the slow calcium channel blocker diltiazem (100 μM) as previously described (Harding et al., 1993). The intracellular concentrations of DiMe-5FBAPTA were estimated by comparison of the integrated areas of the spectra with the areas of spectra of known concentrations of indicator, assuming an intracellular water fraction of 70% (Polimeni, 1974; Jacobus et al., 1982; Harding et al., 1993).

^{19}F-n.m.r. measurements

Perfused hearts were placed in a purpose-built 33 mm probehead within a Bruker AM 400 widebore spectrometer. For average $[Ca^{2+}]_i$ measurements in diltiazem-arrested hearts, ^{19}F-n.m.r. data were collected at 5 Hz, unsynchronized with the pacing pulse, in blocks of 4000 radiofrequency (r.f.) pulses over 13 min. Gated acquisition of n.m.r. spectra in the last 55 ms of the cardiac cycle allowed measurement of end-diastolic $[Ca^{2+}]_i$ (Harding et al., 1993). The cardiac $[Ca^{2+}]_i$ transient was measured in hearts paced at 1.25 Hz, with pacing stimulation coupled to the acquisition of n.m.r. data blocks in 16 periods of 50 ms within each cycle. An r.f. pulse angle of 22° was used to avoid spectral saturation (Kirschenlohr et al., 1988). $[Ca^{2+}]_i$ was estimated as previously described (Kirschenlohr et al., 1988) using a K_d for Ca^{2+} (30 °C) of 46 nM for DiMe-5FBAPTA (Clarke et al., 1993) and 537 nM for 5FBAPTA (Kirschenlohr et al., 1988). All $[Ca^{2+}]_i$ and LVP values are means ± S.E.M. unless otherwise indicated.

RESULTS AND DISCUSSION

Effect of indicator loading and washout on developed LVP and end-diastolic LVP

Hearts were loaded with DiMe-5FBAPTA-AM for 80 min to achieve adequate S/N ratios of > 10:1. A longer loading period had to be employed for DiMe-5FBAPTA-AM, compared with 30 min for 5FBAPTA-AM (Harding et al., 1993), as the hydrolysis of DiMe-5FBAPTA-AM was slower. Immediately after loading hearts with DiMe-5FBAPTA, the developed LVP was 10 ± 2% of the initial value and the end-diastolic LVP was elevated by 120 ± 30%, compared with a developed LVP of 19 ± 4% of the initial value and an increase of 230 ± 50% in end-diastolic LVP in 5FBAPTA-loaded hearts (n = 5). Unloaded hearts or hearts treated with DMSO without DiMe-5FBAPTA-AM showed no significant change in developed LVP or end-diastolic LVP over the 80 min perfusion period required for loading of DiMe-5FBAPTA. However, over 7 h of perfusion (the maximum duration of an n.m.r. experiment), unloaded hearts showed a slow fall in developed LVP to 80–90% of the initial value and a small rise in end-diastolic LVP of 30%.

As estimated from the integrated intensities of the ^{19}F-n.m.r. spectra, the intracellular concentration of DiMe-5FBAPTA immediately after loading was 150–180 μM, similar to the concentration of 5FBAPTA (Harding et al., 1993). Approximately half of the DiMe-5FBAPTA was lost over a period of 4–5 h, with no significant change in $[Ca^{2+}]_i$ (Figure 1a). This is similar to the rate of washout of 5FBAPTA (Kirschenlohr et al., 1988). Recovery of developed and end-diastolic LVPs on washout of the indicator over a 4 h period was more pronounced with DiMe-5FBAPTA than with 5FBAPTA (Figure 1b). End-diastolic LVP decreased with washout of DiMe-5FBAPTA and was only elevated by 25 ± 7% (n = 5) after 4 h compared with the initial value, whereas 5FBAPTA-loaded hearts showed no recovery (Harding et al., 1993), and in most experiments there was a small steady increase in end-diastolic LVP with time (up to 3 mmHg/h). There was minimal recovery in developed LVP (12 ± 4% of the initial value; n = 5) in 5FBAPTA-loaded hearts (Harding et al., 1993), compared with recovery of developed LVP from 10 ± 2% to 44 ± 10% (n = 5) with DiMe-5FBAPTA after 4 h.

Chemical shifts of the DiMe-5FBAPTA resonances

The ^{19}F-n.m.r. spectrum of DiMe-5FBAPTA obtained after 80 min of loading of DiMe-5FBAPTA-AM into a ferret heart is shown in Figure 2(a). The two fluorine resonances observed are 2.5 p.p.m. apart, which is substantially greater than the 1.6 p.p.m. separation of the resonances of the free and Ca^{2+}-complexed indicator in solution at pH 7.2 (Clarke et al., 1993). The intracellular pH (pH_i) calculated from the chemical shift separation of the resonances of the free and bound DiMe-5FBAPTA is anomalously high (~ 7.6). The increase in separation of the two peaks results from both an upfield shift of the resonance from the free form of the indicator and a smaller downfield shift of the resonance from the Ca^{2+}-bound form. These *in vivo* chemical shifts can be reproduced in aqueous solution by the addition of 10–20% BSA. The anomalous shift of the indicator *in vivo* may therefore be due to binding of the indicator to cytosolic protein. Binding of the indicator to BSA does not alter the affinity of the indicator for Ca^{2+}. A correction to the chemical shift separation, estimated from an independent measurement of pH_i, may therefore enable the indicator to be used for dual $[Ca^{2+}]_i$ and pH measurements.

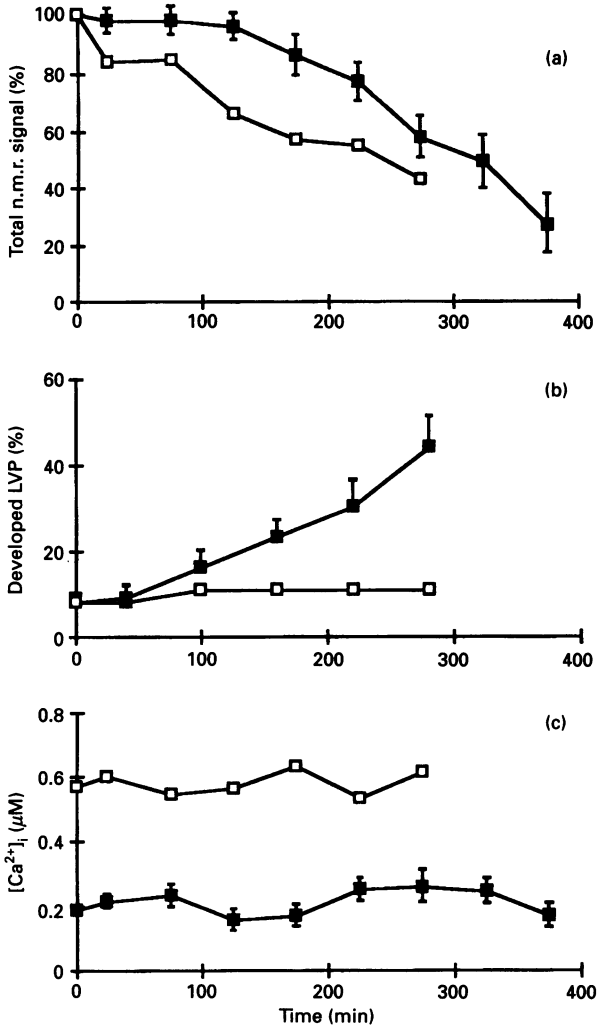


Figure 1 Indicator washout and the effect on the developed LVP and $[Ca^{2+}]_i$

Hearts were loaded with the indicators for 80 min at 30 °C and paced at 1.0 Hz (see the Materials and methods section). ■, DiMe-5FBAPTA; □, 5FBAPTA. (**a**) The total integrated ^{19}F-n.m.r. signal is shown as a percentage of the total initial signal after loading. (**b**) The developed LVP is shown as a percentage of the initial LVP before loading. (**c**) $[Ca^{2+}]_i$ values from spectra accumulated for 52 min (see the Materials and methods section) for each indicator during washout. Data for $[Ca^{2+}]_i$ and indicator washout for 5FBAPTA were replotted from Figure 1 of Kirschenlohr et al. (1988).

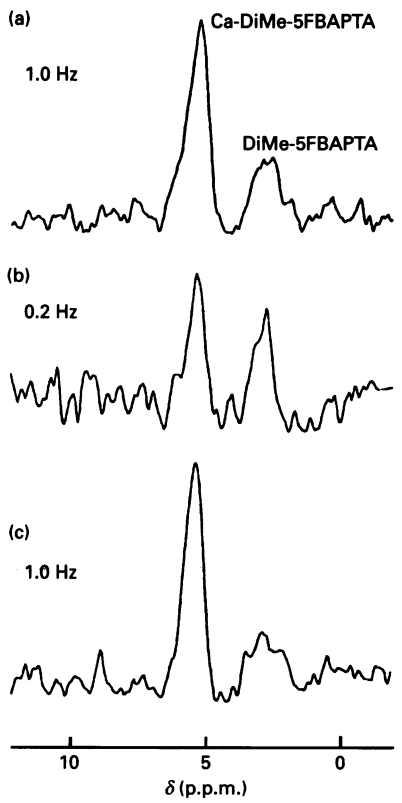


Figure 2 Effect of pacing frequency on ^{19}F-n.m.r. spectra of DiMe-5FBAPTA

The heart was loaded with DiMe-5FBAPTA for 80 min at 30 °C. End-diastolic $[Ca^{2+}]_i$ measurements were carried out over the last 55 ms of the cardiac cycle. Spectra were accumulated for 34 min (1.0 Hz) or 42 min (0.2 Hz). Ca^{2+}-free and -bound DiMe-5FBAPTA resonances are indicated and chemical shifts are referenced to free 5FBAPTA (0 p.p.m.). The heart was first paced at (**a**) 1.0 Hz, (**b**) 0.2 Hz and (**c**) 1.0 Hz repeated.

End-diastolic $[Ca^{2+}]_i$ and the effect of the pacing frequency

After loading hearts with DiMe-5FBAPTA-AM, $[Ca^{2+}]_i$ was measured in the last 55 ms of diastole (defined as end-diastolic $[Ca^{2+}]_i$). Loading of DiMe-5FBAPTA-AM for 80 min was required for an adequate S/N ratio of the free DiMe-5FBAPTA resonance, since this peak was only about 20% of the total spectral area of the indicator at a pacing frequency of 1.0 Hz. End-diastolic $[Ca^{2+}]_i$ at 1.0 Hz was 198 ± 30 nM ($n = 9$), which is less than half the corresponding end-diastolic $[Ca^{2+}]_i$ of 522 ± 54 nM ($n = 12$) measured with 5FBAPTA (Harding et al., 1993).

N.m.r. spectra obtained from a heart paced sequentially at 1.0 Hz, 0.2 Hz and then 1.0 Hz are shown in Figure 2. The 5-fold reduction in stimulation rate from 1.0 Hz to 0.2 Hz decreased end-diastolic $[Ca^{2+}]_i$ to 89 ± 13 nM ($n = 5$), which is significantly lower than the value of 299 ± 44 nM ($n = 4$) obtained with 5FBAPTA in hearts paced at 0.2 Hz (Harding et al., 1993). Reducing the pacing frequency also caused a 5-fold decrease in total developed LVP from 27 ± 2 mmHg at 1.0 Hz to 5 ± 1 mmHg at 0.2 Hz. This resulted from a decrease in systolic LVP from 61 ± 10 mmHg to 46 ± 9 mmHg and an increase in end-diastolic LVP from 33 ± 8 mmHg to 41 ± 9 mmHg. The increase in end-diastolic LVP may be due to less expulsion of venous coronary effluent associated with the weaker contraction at 0.2 Hz (Harding et al., 1993).

Measurements of $[Ca^{2+}]_i$ in diltiazem-arrested hearts

Resting $[Ca^{2+}]_i$ was measured in DiMe-5FBAPTA-loaded hearts after 60 min of perfusion with the slow calcium channel blocker diltiazem, which blocks calcium entry on depolarization (Sperelakis, 1984). Contraction was not detectable within 1 min of diltiazem perfusion which increased end-diastolic LVP from 38 ± 7 mmHg to 46 ± 6 mmHg, decreased systolic LVP from 61 ± 8 mmHg to 48 ± 7 mmHg and decreased maximum developed LVP from 21 ± 6 mmHg to < 3 mmHg.

Figures 3(a) and 3(b) show the n.m.r. spectra obtained from a heart before and after arrest with diltiazem and paced at 1.0 Hz, compared with the spectra from a heart which was not stimulated during diltiazem arrest (Figure 3c). The reversal of pacing effect on $[Ca^{2+}]_i$ during diltiazem arrest is shown in Figure 3(d). In unpaced hearts which were perfused for 60 min with diltiazem,

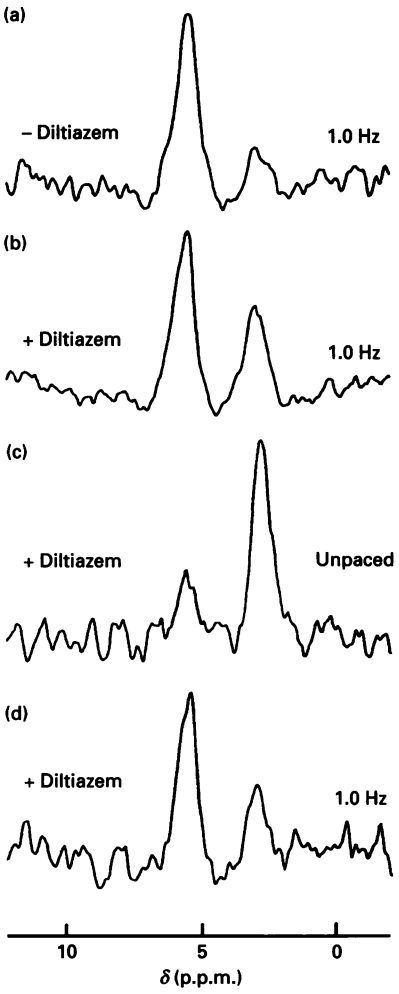


Figure 3 Effect of diltiazem on ^{19}F-n.m.r. spectra of DiMe-5FBAPTA

The heart was loaded with DiMe-5FBAPTA for 80 min at 30 °C. (**a**) Spectrum (68 min accumulation for end-diastolic $[Ca^{2+}]_i$) from a heart paced at 1.0 Hz in the absence of diltiazem. (**b**) The heart was perfused with 100 μM diltiazem; after 60 min the spectrum (52 min) was accumulated for average $[Ca^{2+}]_i$ from a heart paced at 1.0 Hz. (**c**) as in (**b**) when pacing was stopped. (**d**) Pacing restarted at 1.0 Hz.

$[Ca^{2+}]_i$ was reduced to 10 ± 1 nM ($n = 4$). Pacing of diltiazem-arrested hearts at 1.0 Hz increased the $[Ca^{2+}]_i$ to 94 ± 24 nM. By comparison, the corresponding values for $[Ca^{2+}]_i$ in 5FBAPTA-loaded hearts after perfusion for 60 min with diltiazem were 84 ± 6 nM ($n = 5$) unpaced and 157 ± 40 nM ($n = 5$) at 1.0 Hz (Harding et al., 1993). However, it should be noted that these measurements were made near the titration limit of 5FBAPTA and are therefore less accurate than the $[Ca^{2+}]_i$ values obtained with DiMe-5FBAPTA.

$[Ca^{2+}]_i$ transient

The change in $[Ca^{2+}]_i$ during the cardiac cycle was measured using gated acquisition of n.m.r. data as described in the Materials and methods section (Kirschenlohr et al., 1988). Figure 4 shows the mean $[Ca^{2+}]_i$ values obtained from four hearts in each of the 16 time intervals in the transient. The $[Ca^{2+}]_i$ rose from a diastolic value of 156 ± 8 nM to a peak of > 500 nM (10 times the K_d for Ca^{2+}) approx. 300 ms after the pacing pulse. The calcium transients measured with DiMe-5FBAPTA therefore reach maximal $[Ca^{2+}]_i$ later than the peak values measured with 5FBAPTA (150 ms) (Harding et al., 1993). After the decline of the transient, the end-diastolic $[Ca^{2+}]_i$ was 144 ± 4 nM. However, the transients measured with DiMe-5FBAPTA declined faster ($t_{\frac{1}{2}} \sim 125$ ms) after the peak $[Ca^{2+}]_i$ compared with 5FBAPTA ($t_{\frac{1}{2}} \sim 240$ ms).

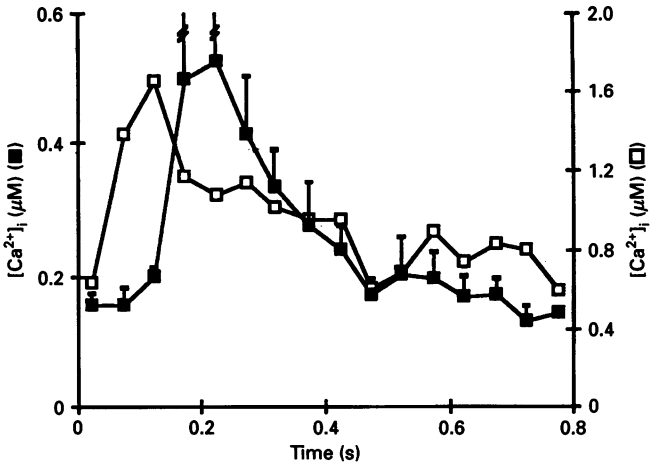


Figure 4 Cardiac calcium transients measured with DiMe-5FBAPTA (■) and 5FBAPTA (□)

The hearts were loaded with DiMe-5FBAPTA for 80 min at 30 °C. Each data point (■) corresponds to a 55 ms time interval during the cardiac cycle (pacing frequency 1.25 Hz). Values shown are the averages for four hearts. Data for 5FBAPTA (□) have been replotted from Figure 9 in Harding et al. (1993).

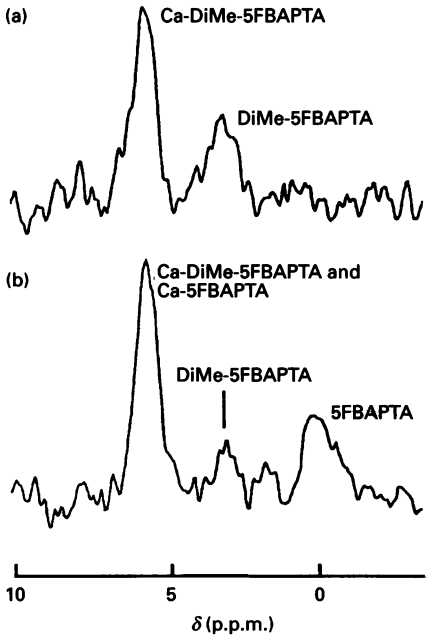


Figure 5 Co-loading of DiMe-5FBAPTA and 5FBAPTA

DiMe-5FBAPTA was loaded into a ferret heart for 80 min at 30 °C. (**a**) Spectra accumulated at end-diastole (final 55 ms of 1.0 Hz cycle) for 34 min. 5FBAPTA was then loaded into the same heart for 40 min and n.m.r. data were collected after 30 min of washout (see text). (**b**) N.m.r. spectrum of DiMe-5FBAPTA and 5FBAPTA co-loaded into heart. The spectrum shown was accumulated for 51 min at end-distole as in (**a**). The resonances for Ca^{2+}-bound and free DiMe-5FBAPTA and 5FBAPTA are indicated and referenced to free 5FBAPTA at 0 p.p.m.

Co-loading of DiMe-5FBAPTA and 5FBAPTA

Co-loading of 5FBAPTA and DiMe-5FBAPTA into the same ferret heart showed that the Ca^{2+}-bound resonances had identical chemical shifts, while the free peaks were ~ 3 p.p.m. apart (Figures 5a and 5b). The coincidence of the chemical shifts of the resonances from the Ca^{2+} complexes of the two indicators prevented simultaneous $[Ca^{2+}]_i$ measurements using the co-loaded indicators, since the relative contributions of the two indicators to the bound peak could not be determined. Furthermore, the titration ranges of DiMe-5FBAPTA and 5FBAPTA are too dissimilar to measure $[Ca^{2+}]_i$ simultaneously with each indicator with sufficient accuracy for comparison. However, the rapid decrease in the free DiMe-5FBAPTA resonance during 5FBAPTA loading into a heart pre-loaded with DiMe-5FBAPTA was consistent with a substantial increase in $[Ca^{2+}]_i$ to a concentration above the titration range of DiMe-5FBAPTA (i.e. > approx. 500 nM). Similarly, the inability to detect a peak of free DiMe-5FBAPTA when this indicator was loaded into hearts pre-loaded with 5FBAPTA was consistent with a $[Ca^{2+}]_i$ value considerably higher than the end-diastolic $[Ca^{2+}]_i$ of less than 200 nM measured with DiMe-5FBAPTA alone. In both experiments, the substantial increase in the intensity of the overlapping resonances from the Ca^{2+}-bound forms of the indicators demonstrated that both indicators were retained in the myocytes. These observations therefore suggest that 5FBAPTA, which has a higher K_d for Ca^{2+}, had a dominant influence on the end-diastolic $[Ca^{2+}]_i$.

Conclusions

Although loading of the new indicator had the same initial effect on the developed LVP as 5FBAPTA, the reversibility of this effect on washout was substantially improved using DiMe-5FBAPTA. This suggests that the new indicator causes less irreversible perturbation of heart function than 5FBAPTA over prolonged periods. Furthermore, the lower values for cardiac $[Ca^{2+}]_i$ reported using DiMe-5FBAPTA agree more closely with the values obtained with other methods (Borzak et al., 1990; Nakanishi et al., 1990; Frampton et al., 1991). These results therefore imply that when using DiMe-5FBAPTA the effects of indicator loading on developed LVP and end-diastolic $[Ca^{2+}]_i$ are uncoupled. This uncoupling presumably arises from the increased affinity of DiMe-5FBAPTA for calcium. Alternatively, the lower affinity of DiMe-5FBAPTA compared with 5FBAPTA for heavy metals might be responsible for this effect (H. L. Kirschenlohr, unpublished work). The lower K_d of the new indicator is also more appropriate for the measurement of $[Ca^{2+}]_i$ in the range of 100 nM and below, as in diltiazem-arrested hearts.

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The dominant effect of 5FBAPTA on the end-diastolic $[Ca^{2+}]_i$ in hearts co-loaded with the two indicators suggests that the end-diastolic $[Ca^{2+}]_i$ depends on the relative K_ds of the indicators, whereas the effect of the indicators on the developed LVP is not sensitive to the K_d over the range 50–500 nM. A more extensive examination of the relationship of the indicator K_d to the end-diastolic $[Ca^{2+}]_i$ will be of interest. We know from experiments with indicators of pH_i and $[Mg^{2+}]_i$ that when the K_d for Ca^{2+} is in the range 0.1–1.0 mM, there is no effect of the indicators on developed LVP or $[Ca^{2+}]_i$ (Kirschenlohr et al., 1988).

The intracellular concentrations of 5FBAPTA and DiMe-5FBAPTA required to achieve adequate S/N ratios of the spectra give rise to a similar reduction in developed LVP, presumably in response to similar calcium buffering. The main objective of further Ca^{2+} indicator development is therefore the design of more sensitive CF_3-substituted chelators, and we have recently reported progress on a prototype analogue of DiMe-5FBAPTA (Clarke et al., 1993).

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