Sarcoplasmic reticulum Ca^{2+} content, L-type Ca^{2+} current and the Ca^{2+} transient in rat myocytes during β-adrenergic stimulation

M. Hussain and C. H. Orchard *

Department of Physiology, University of Leeds, Leeds LS2 9NQ, UK

1. The effect of β-adrenergic stimulation on the relationship between the intracellular Ca^{2+} transient and the amplitude of the L-type Ca^{2+} current (I_{Ca}) has been investigated in ventricular myocytes isolated from rat hearts. Intracellular [Ca^{2+}] was monitored using fura-2 during field stimulation and while membrane potential was controlled using voltage clamp techniques.

2. The increase in the amplitude, and the rate of decline, of the Ca^{2+} transient produced by isoprenaline (1·0 μmol l^{-1}) was not significantly different in myocytes generating action potentials and in those voltage clamped with pulses of constant duration and amplitude.

3. Under control conditions, the current–voltage (I–V) relationship for I_{Ca} was bell shaped. The amplitude of the Ca^{2+} transient also showed a bell-shaped voltage dependence. In the presence of isoprenaline, the amplitude of both I_{Ca} and the Ca^{2+} transient was greater at all test potentials and the I–V relationship maintained its bell-shaped voltage dependence. However, the size of the Ca^{2+} transient was no longer graded with changes in the amplitude of I_{Ca}: a small I_{Ca} could now elicit a maximal Ca^{2+} transient.

4. Rapid application of caffeine (10 mmol l^{-1}) was used to elicit Ca^{2+} release from the sarcoplasmic reticulum (SR). Isoprenaline increased the integral of the subsequent rise in cytoplasmic [Ca^{2+}] to 175 ± 13% of control.

5. Abbreviation of conditioning pulse duration in the presence of isoprenaline was used to reduce the amplitude of the Ca^{2+} transient to control levels. Under these conditions, the amplitude of the Ca^{2+} transient was again graded with the amplitude of I_{Ca} in the same way as under control conditions.

6. Nifedipine (2 μmol l^{-1}) was also used to decrease Ca^{2+} transient amplitude in the presence of isoprenaline. In the presence of isoprenaline and nifedipine, the amplitude of the Ca^{2+} transient again showed a bell-shaped voltage dependence.

7. The SR Ca^{2+}-ATPase inhibitor thapsigargin (2·5 μmol l^{-1}) reduced the effect of isoprenaline on the amplitude of the Ca^{2+} transient. In the presence of thapsigargin, the size of the Ca^{2+} transient increased as I_{Ca} increased in response to isoprenaline.

8. These data suggest that the increase in the amplitude of the Ca^{2+} transient produced by β-adrenergic stimulation in cardiac muscle is due to an increase in the gain of the SR Ca^{2+} release process, due principally to an increase in the Ca^{2+} content of the SR.

β-Adrenergic stimulation increases the force of contraction and the rate of relaxation of cardiac muscle. These changes appear to be due to an increase in the amplitude, and abbreviation, of the Ca^{2+} transient that initiates contraction. The precise mechanisms responsible for these changes in the Ca^{2+} transient are unclear, although it is known that β-agonists stimulate adenylate cyclase, thus increasing cAMP within the cell and activating cAMP-dependent protein kinase (PKA). There are a number of subsequent changes in the excitation–contraction coupling pathway which could bring about the β-adrenergic-induced changes in the Ca^{2+} transient.

(i) β-Adrenergic stimulation alters action potential duration, although the effect is variable; action potential duration has been reported to increase, stay the same, or decrease (Tsien,

* To whom correspondence should be addressed.

1977). An increase in action potential duration might be expected to increase the strength of contraction and prolong the twitch (Morad & Goldman, 1973), and a decrease in action potential duration to decrease the strength of contraction and abbreviate its time course. Thus a change in action potential duration cannot account for both the increase in the Ca^{2+} transient *and* its abbreviation, but could enhance one of these changes, while offsetting the other.

(ii) One of the components of the change in action potential duration observed during β stimulation is an increase in the L-type Ca^{2+} current (I_{Ca}) brought about by PKA-induced phosphorylation of this channel (Reuter & Scholz, 1977). This Ca^{2+} helps to load the sarcoplasmic reticulum (SR; Fabiato, 1985; and see below) and is an important trigger for the release of Ca^{2+} from the SR (Beuckelmann & Wier, 1988) via Ca^{2+}-induced Ca^{2+} release (CICR; Fabiato, 1985). Since CICR is graded with the size of the trigger (Fabiato, 1985; Beuckelmann & Wier, 1988), Callewaert, Cleeman & Morad (1988) suggested that the increase in the size of the Ca^{2+} transient observed during β-adrenergic stimulation was due to the increase in I_{Ca} triggering the release of more Ca^{2+} from the SR. However, in that study Cs^+ was present in the electrode solution, and Cs^+ alters the amplitude of the Ca^{2+} transient (Han, Schiefer & Isenberg, 1994), the voltage dependence of contraction (Levi, Mitcheson & Hancox, 1996) and the response to isoprenaline (Hussain & Orchard, 1996) of cardiac myocytes, so that the role of this mechanism in the absence of Cs^+ is unclear.

(iii) β stimulation increases the phosphorylation of the regulatory protein phospholamban (Lindemann, Jones, Hathaway, Henry & Watanabe, 1983). This causes phospholamban to dissociate from the SR Ca^{2+}-ATPase, thus increasing the rate of Ca^{2+} uptake into the SR (Tada, Kirchberger, Repke & Katz, 1974). This enhanced Ca^{2+} uptake, and the increase in I_{Ca} (above), would be expected to increase the Ca^{2+} content of the SR and hence to increase the size of the Ca^{2+} transient observed during β-adrenergic stimulation. The observation that mice which do not express phospholamban show little change in the time course of contraction in response to such stimulation (Luo *et al.* 1994) supports the idea of an important role for phospholamban and the SR in the observed response. However, attempts to demonstrate an increase in SR Ca^{2+} content during β-adrenergic stimulation have produced equivocal results (Frampton, 1991; Steele & Miller, 1992; Patel, Coronado & Moss, 1995), thus leaving unresolved whether SR Ca^{2+} content does increase, and hence whether it has a role, in the response to β stimulation.

(iv) The activity of the isolated SR Ca^{2+} release channel (the ryanodine receptor) is altered by phosphorylation by either PKA or Ca^{2+}–calmodulin-dependent protein kinase (CaM kinase II; Hain, Onoue, Mayrleitner, Fleischer & Schindler, 1995), although the response to endogenous CaM kinase II is different from the response to exogenous CaM kinase II (Hain *et al.* 1995). The response to such phosphorylation, and its role, *in vivo* is unclear, but it has been suggested that such phosphorylation underlies the increased Ca^{2+} release from the SR observed during β stimulation (Patel *et al.* 1995), by increasing the sensitivity of the release channel to the Ca^{2+} trigger.

In the present study, we have investigated whether the increase in Ca^{2+} release from the SR, which appears to underlie much of the inotropic response to β-adrenergic stimulation, is due to changes in action potential duration (point (i)), the increase in I_{Ca} triggering more Ca^{2+} release from the SR (point (ii)), or whether a given I_{Ca} triggers the release of more Ca^{2+} from the SR (points (iii) and (iv)). The data show that the increased Ca^{2+} transient amplitude produced by β-adrenergic stimulation in cardiac muscle is due to an increase in the gain of the SR Ca^{2+} release process which appears to be due, principally, to an increase in the Ca^{2+} content of the SR.

Preliminary data from this study have been presented previously to The Physiological Society (Hussain & Orchard, 1995).

METHODS

Cell isolation

The methods used for cell isolation have been described previously (Frampton, Orchard & Boyett, 1991). In brief, male Wistar rats were killed by stunning followed by cervical dislocation. The heart was removed and Langendorff-perfused for 2–3 min with physiological salt solution containing 0·75 mmol l^{-1} Ca^{2+} (see below for composition) followed by perfusion with Ca^{2+}-free salt solution supplemented with 1·0 mmol l^{-1} EGTA. Finally, the heart was perfused for 8–10 min with salt solution containing collagenase (1 mg ml^{-1}; Worthington Type II, Lorne Laboratories) and protease (0·1 mg ml^{-1}; Type XIV, Sigma). The ventricles were removed, opened and gently agitated in the enzyme solution supplemented with 1 % bovine serum albumin (Sigma). After 5 min the ventricles were separated from the solution by filtration, and ventricular myocytes were collected by centrifugation of the filtrate (40 *g* for 30 s), resuspended in enzyme-free isolation solution (see below for composition) and stored until use. The ventricles were returned to another aliquot of the enzyme solution and shaken for a further 5 min before re-filtration. The process was repeated 2–3 times. Only cells that showed clearly defined striations and responded to field stimulation with a rapid twitch were used in the present study. All experiments were carried out at room temperature (22–24 °C), to minimize SR Ca^{2+} release triggered by Na^+–Ca^{2+} exchange (Vornanen, Shepherd & Isenberg, 1994).

Measurement of $[Ca^{2+}]_i$

Myocytes were loaded with the fluorescent Ca^{2+} indicator fura-2 either by incubation with the membrane-permeant acetoxymethyl ester (AM) form of fura-2 (5 μmol l^{-1} for 10 min at room temperature) for experiments on intact cells and when the perforated patch clamp technique was used, or by inclusion of the membrane-impermeant K_5fura-2 (Molecular Probes Inc.) in the pipette solution during whole-cell voltage clamp. In the initial experiments the concentration of fura-2 in the pipette was 240 μmol l^{-1}; this was subsequently reduced to 120 and then to 60 μmol l^{-1}. Similar results were obtained at each dye concentration.

Myocytes were allowed to settle onto the coverslip which formed the base of the experimental chamber, and were superfused with Tyrode solution (see below for composition). The chamber was mounted on the stage of an inverted microscope (Nikon Diaphot) and fura-2 fluorescence was elicited by alternate (every 2 ms) illumination with 340 and 380 nm light, obtained using a rotating filter wheel (Cairn Research Ltd) in front of a Xenon excitation lamp (Frampton *et al.* 1991). The fluorescence emitted at 510 nm was monitored using a photomultiplier tube (Frampton *et al.* 1991). The ratio of the fluorescence emitted at 510 nm during excitation at 340 nm to that emitted during excitation at 380 nm was obtained using an analog divide circuit and was used as a measure of $[Ca^{2+}]_i$. Data were discarded from any cell that showed spontaneous increases in $[Ca^{2+}]_i$ between stimulated contractions either under control conditions or in the presence of isoprenaline.

The problems of using fura-2 to monitor $[Ca^{2+}]_i$ have been discussed previously (e.g. Frampton *et al.* 1991). However, one problem which should be addressed here is compartmentation of the dye when the AM form is loaded, since such compartmentation will not occur when the free acid is loaded via the patch electrode. We have previously presented evidence that compartmentation of fura-2 is very small using our loading conditions (Frampton *et al.* 1991). In the present study we did not see any differences between AM-loaded cells and cells loaded with the free acid which could be ascribed to compartmentation. In addition, if whole-cell patch electrodes were used on AM-loaded cells, fluorescence decreased rapidly to undetectable levels following breakthrough of the patch, presumably as the dye diffused out of the cell into the patch electrode. This also suggests that there is little compartmentation of fura-2 when the dye is introduced as the AM ester.

The amplitude of the Ca^{2+} transient was measured as the difference between end diastolic $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ at the peak of the rapidly rising phase of the Ca^{2+} transient (see Isenberg & Han, 1994, who used this index as a measure of SR Ca^{2+} release). In voltage clamped cells, when no rapidly rising phase was apparent (e.g. at negative test potentials), then $[Ca^{2+}]_i$ was measured at the *time* at which peak $[Ca^{2+}]_i$ occurred in the $[Ca^{2+}]_i$ transient under the same condition (control or isoprenaline) at the nearest test potential that did show a rapidly rising phase.

Recording membrane currents

Conventional whole-cell recordings were obtained using a patch clamp amplifier (model 8800; Dagan Corporation). This amplifier was controlled, and command pulses generated, by an A/D CED interface (model 1401; Cambridge Electronic Design) and a microcomputer (model PC-433; Elonex plc). Micropipettes made from borosilicate glass (type GC200F; Clark Electromedical Instruments) were filled with a potassium glutamate-based solution (see below), to give a resistance of 0·5–1·0 MΩ, which increased only slightly (by < 0·5 MΩ) after fire polishing.

Perforated patch voltage clamp recordings were obtained using amphotericin B (250 or 500 μg ml^{-1}) as the pore-forming agent, which gave a series resistance (before compensation) of ~6 MΩ. In this configuration, membrane currents and the Ca^{2+} transient reached a steady state within 5 to 15 min of seal formation and remained stable for 1–3 h, without the run-down attributed to washout of intracellular constituents. Voltage clamp pulses were used to depolarize the membrane potential from −40 mV, to inactivate the Na^+ current, to a test potential between −30 and +70 mV; the difference between peak inward current elicited on depolarization and the current remaining at the end of the voltage clamp pulse was used as a measure of the amplitude of I_{Ca}. Adequate voltage control was ascertained by the time to peak of I_{Ca}, which was less than 15 ms in all of the experiments described below, by ensuring that I_{Ca} was maximal between 0 and +10 mV, and that the position of the current–voltage (I–V) relationship was stable. Data from cells which did not meet these criteria were discarded; for example, if the application of isoprenaline produced a significant shift of the I–V relationship along the voltage axis, or significantly skewed the I–V relationship to more negative potentials, this was taken as evidence of inadequate voltage control and data from that cell were discarded. This is considered further in the Discussion.

Data acquisition and analysis

Command pulses, membrane currents, fura-2 fluorescence elicited by excitation by 340 and 380 nm light, and the fluorescence ratio were displayed on a 6-channel chart recorder (Model RS3600; Gould Electronics Ltd) and were digitized (Model D-890; Neuro Data Instrument Corporation) and recorded on videotape for later off-line analysis. The signals were also digitized at 2·5 kHz and recorded directly to the hard disk of the computer via the CED interface. Digitized fluorescence transients were used to measure the amplitude (PA), time to peak (TTP) and the half-time of decline ($t_{½}$) of the transient. Digitized current records were also used to measure the amplitude of I_{Ca}, as defined above.

Data are presented as the means ± S.E.M. of n preparations. Statistical significance was determined using either Student's t test or a paired t test as appropriate. $P < 0·05$ was taken as statistically significant. The linear fits shown in the figures were obtained using least-squares linear regression, the curved fits (e.g. the bell shape of the transient–voltage (V) relationship) were fitted by eye.

Solutions and drugs

The physiological salt solution used for isolation and storage of myocytes contained (mmol l^{-1}): NaCl, 130; KCl, 5·4; $MgCl_2.6H_2O$, 1·1; NaH_2PO_4, 0·4; creatine, 10·0; taurine, 20·0; glucose, 10·0; and Hepes, 10·0; titrated to pH 7·3 with NaOH. This was supplemented with EGTA or Ca^{2+} for the different stages of the isolation procedure, as described above.

The Tyrode solution used during the experiments contained (mmol l^{-1}): NaCl, 93·0; $NaHCO_3$, 20·0; Na_2HPO_4, 1·0; $MgSO_4.7H_2O$, 1·0; KCl, 5·0; glucose, 10·0; sodium acetate, 20·0; and insulin, 5 units l^{-1}; equilibrated with 5% CO_2–95% O_2 (pH 7·35). The cells were superfused at 4·5 ml min^{-1} (bath volume, 0·5 ml); all measurements reported in this paper were obtained when the cell had reached a steady state, as judged by the amplitude and time course of the Ca^{2+} transient and membrane currents, under each condition.

The normal pipette solution for both conventional and perforated whole-cell recordings contained (mmol l^{-1}): potassium glutamate, 120·0; KCl, 20·0; NaCl, 10·0; MgATP, 4·0; and Hepes, 10·0; titrated to pH 7·3 with KOH. The Cs^+-containing pipette solution used in some experiments contained (mmol l^{-1}): caesium glutamate, 120·0; KCl, 20·0; NaCl, 10·0; MgATP, 4·0; and Hepes, 10·0; titrated to pH 7·3 with KOH.

Isoprenaline was prepared daily from ampoules containing 2·0 mmol l^{-1} isoprenaline sulphate and used within 3–4 h of preparation. Nifedipine was prepared daily from a 10·0 mmol l^{-1} stock in DMSO. Caffeine was dissolved directly in Tyrode solution just before use to give a final concentration of 10 mmol l^{-1}, and was applied rapidly to the cell by pressure ejection from a patch pipette positioned close to the cell using a micromanipulator. Both the amplitude and the integral of the rise of $[Ca^{2+}]_i$ induced by caffeine were monitored. The integral was less dependent than amplitude on the rate of application of caffeine to the cell; changes in the integral

were therefore used to monitor changes in the amount of Ca^{2+} released from the SR by caffeine (see Harrison, Frampton, McCall, Boyett & Orchard, 1992). Both the amplitude and the integral will be decreased by mechanisms which enhance Ca^{2+} removal from the cell (e.g. isoprenaline) so that this technique will underestimate the isoprenaline-induced changes in SR Ca^{2+} content. Thus the increase in the integral that we observed in the presence of isoprenaline (see Results) is probably an underestimate of the isoprenaline-induced increase in SR Ca^{2+} content.

RESULTS

The effect of controlling membrane potential on the response to isoprenaline

Figure 1 shows the well-recognized effects of isoprenaline (1·0 μmol l^{-1}) on the Ca^{2+} transient, in a representative field-stimulated ventricular myocyte: Fig. 1*A* shows that isoprenaline increased the amplitude of the transient (by 95 ± 21%, $n = 8$, $P < 0{\cdot}001$; Fig. 1*C*); Fig. 1*B* shows that isoprenaline also accelerated the decline of the transient, decreasing $t_{½}$ from 285 ± 16 to 165 ± 12 ms ($P < 0{\cdot}001$; Fig. 1*C*) with no significant effect on TTP (Fig. 1*C*).

To investigate whether changes in action potential duration might modulate the response of the Ca^{2+} transient to isoprenaline (see Introduction), the response to isoprenaline was investigated in another group of myocytes in which the period of depolarization was maintained constant using the perforated patch clamp technique (see Methods), which avoids dialysis of the cell which might alter the response to isoprenaline (see below). Membrane potential was held at −40 mV and stepped to 0 mV for 300 ms every 2 s. Under these conditions, isoprenaline significantly increased the amplitude of I_{Ca} (by 165 ± 16%, $P < 0{\cdot}02$, $n = 9$; Fig. 2*A* and *B*, upper traces) and the Ca^{2+} transient (by 67 ± 12%, $P < 0{\cdot}02$; Fig. 2*A*, *B* (lower traces) and *C*). This was accompanied by an abbreviation of the Ca^{2+} transient similar to that observed in the cells generating action potentials (Fig. 2*B*): isoprenaline decreased $t_{½}$ to 199 ± 12 ms ($P < 0{\cdot}001$; Fig. 2*C*) with no significant change in TTP (Fig. 2*C*). Neither the increase in the amplitude of the Ca^{2+} transient, nor $t_{½}$ in the presence of isoprenaline, was significantly different from that observed in field-stimulated cells.

These data suggest that: (i) changes in the action potential (and resting potential) cannot be the sole determinants of the changes in the Ca^{2+} transient observed during β-adrenergic stimulation. Thus other mechanisms must be involved in these changes. (ii) The use of the perforated patch voltage clamp technique in subsequent experiments is

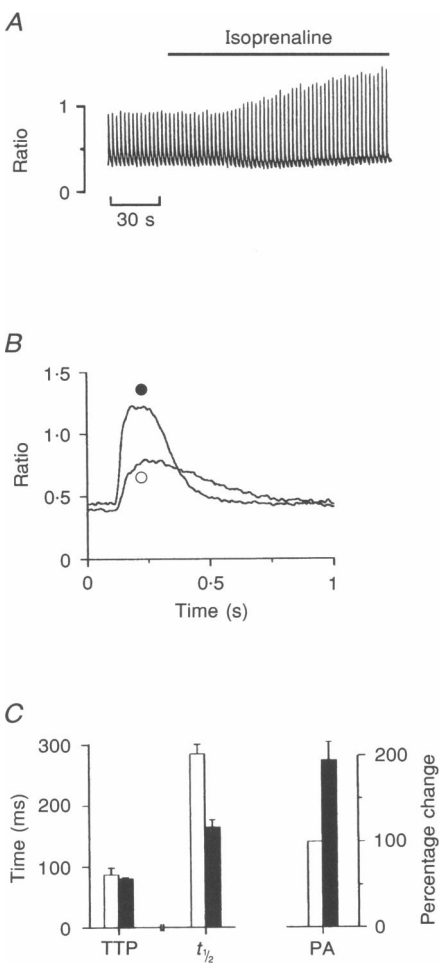

Figure 1. Effect of isoprenaline on Ca^{2+} transients in field-stimulated rat ventricular myocytes

A, slow time base chart recording of Ca^{2+} transients (fura-2 fluorescence ratio; $F_{340/380}$) in a representative myocyte. Isoprenaline (1 μmol l^{-1}) was applied during the period indicated by the horizontal bar. *B*, fast time base averaged (30 sweeps) Ca^{2+} transients under control conditions (○) and in the presence of 1 μmol l^{-1} isoprenaline (●). *C*, time to peak (TTP), time to 50% decline ($t_{½}$) and increase in the peak amplitude (PA) of the Ca^{2+} transients in the absence (□) and presence (■) of isoprenaline. Values are means ± S.E.M. ($n = 8$).

unlikely to have altered the response of the Ca^{2+} transient to isoprenaline. (iii) The isoprenaline-induced changes in the Ca^{2+} transient are secondary to changes in I_{Ca} and/or SR function (see Introduction). The following experiments were designed to investigate these possibilities.

The effect of isoprenaline on the relationship between I_{Ca} and the amplitude of the Ca^{2+} transient

Although isoprenaline increased both I_{Ca} and the Ca^{2+} transient (Fig. 2), it is not clear whether the Ca^{2+} transient is larger because the increased I_{Ca} triggers more Ca^{2+} release from the SR (in which case the increase in the size of the Ca^{2+} transient would be expected to be graded with, and depend on, the increase in I_{Ca}) or because the SR releases more Ca^{2+} for a given I_{Ca}. To distinguish between these possibilities, we have investigated the effect of isoprenaline on the relationship between the amplitude of I_{Ca} and the Ca^{2+} transient.

In an initial series of experiments, Cs^+ was present in the pipette (whole-cell configuration) so that I_{Ca} and the Ca^{2+} transient could be monitored in the absence of contaminating K^+ currents. The protocol used for these experiments is shown in the top traces in Fig. 3*A* and *B*: membrane potential was held at −40 mV and stepped to 0 mV for 300 ms every 2 s for a train of either five or ten conditioning pulses. The membrane potential was then stepped to a variable test potential between −30 and +60 mV for 300 ms before the next series of conditioning pulses. The lower traces in Fig. 3*A* and *B* show the effect of isoprenaline on membrane current and the Ca^{2+} transient, monitored using this protocol. The voltage dependence of I_{Ca} and the Ca^{2+} transient was obtained during the intercalated test pulses under control conditions (Fig. 3*A*) and in the presence of isoprenaline (Fig. 3*B*). Under control conditions the size of the Ca^{2+} transient was graded with I_{Ca}.

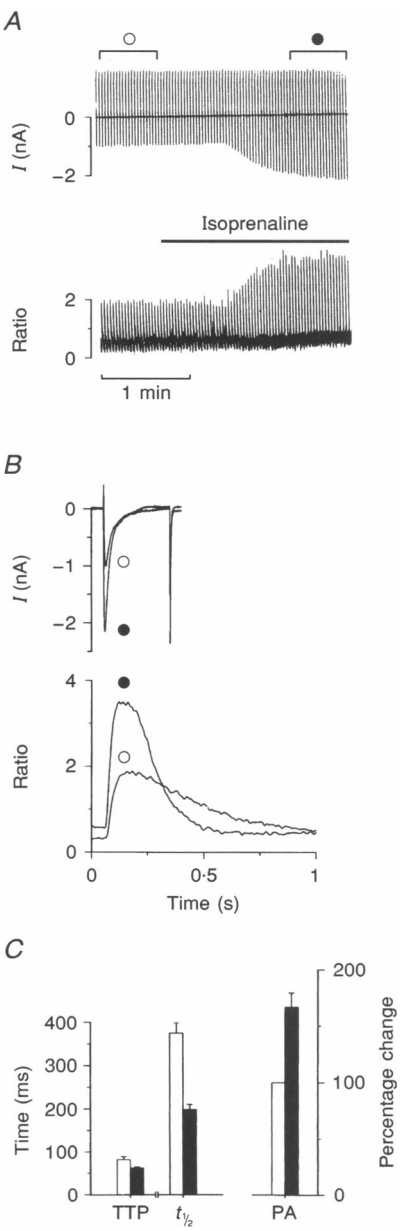

Figure 2. Effect of isoprenaline on Ca^{2+} transients and I_{Ca} monitored in rat ventricular myocytes using perforated patch clamp

A, slow time base chart recording of membrane current (top; the upward deflections represent capacity transients, the negative-going capacity transients have been removed using a sample and hold circuit so that the peak of the downward deflections represents peak inward current during the voltage clamp pulses) and Ca^{2+} transients (bottom; fura-2 fluorescence ratio) in a representative cell. Membrane potential was held at −40 mV and stepped to 0 mV for 300 ms every 2 s to elicit I_{Ca} and the Ca^{2+} transient. Isoprenaline (1 μmol l^{-1}) was applied during the period indicated by the horizontal bar. *B*, fast time base average records (30 sweeps) of membrane current (including both positive (truncated) and negative-going capacity transients) and Ca^{2+} transients under control conditions (○) and in the presence of 1 μmol l^{-1} isoprenaline (●). *C*, TTP, $t_{½}$ and the increase in PA of the Ca^{2+} transients in the absence (□) and presence (■) of isoprenaline. Values are means ± S.E.M. ($n = 9$).

Both I_{Ca} and the amplitude of the Ca^{2+} transient increased in response to isoprenaline, by 267 ± 37·5 and 87·3 ± 26%, respectively, at a test potential of 0 mV ($n = 4$) and the amplitude of the Ca^{2+} transient was still graded with I_{Ca} (Fig. 3*B*) so that the relationship between the amplitude of the Ca^{2+} transient and I_{Ca} was not significantly different in the absence and presence of isoprenaline (not shown).

When this protocol was repeated with K^+, rather than Cs^+, in the pipette (whole-cell configuration), isoprenaline still increased the amplitude of I_{Ca}, by 289 ± 143% ($n = 4$); this was not significantly different from the increase observed in the presence of Cs^+. However, the amplitude of the Ca^{2+} transient increased by only 14 ± 6%, which was significantly less than that observed in intact cells (Fig. 1), or when perforated patch clamp was used (Fig. 2), or in the presence of Cs^+ (Fig. 3). Under these conditions, isoprenaline also abolished the gradation of the Ca^{2+} transient with I_{Ca} that was observed under control conditions, although isoprenaline still decreased $t_{½}$ (from 341 ± 54 to 189 ± 24 ms; $P = 0·03$).

Since the response to isoprenaline is different in the presence of Cs^+ from that in the presence of the physiological cation K^+, it appears that Cs^+ alters the response of the cardiac cell to isoprenaline. This may be due to the previously reported effects of Cs^+ on the SR (Levi *et al.* 1996) and hence the Ca^{2+} transient (Han *et al.* 1994). Similarly, since the increase in the amplitude of the Ca^{2+} transient in response to isoprenaline was different during whole-cell clamp in the presence of K^+ from that observed in the perforated patch configuration and in intact cells, it seemed possible that cell dialysis altered the response to isoprenaline. Thus subsequent experiments were carried out using the perforated patch clamp technique and in the absence of Cs^+.

Since Cs^+ could not be used to inhibit K^+ currents, an alternative method was used to ensure that isoprenaline-induced changes in K^+ currents were not confounding the measurement of I_{Ca}: the effect of isoprenaline was investigated on the current remaining after inhibition of I_{Ca} with 100 μmol l^{-1} Cd^{2+}. The protocol for these experiments was the same as that used for the measurement of I_{Ca} (above) using the perforated patch clamp technique. Figure 4*A* shows membrane currents obtained in response to the intercalated test pulses to potentials between −30

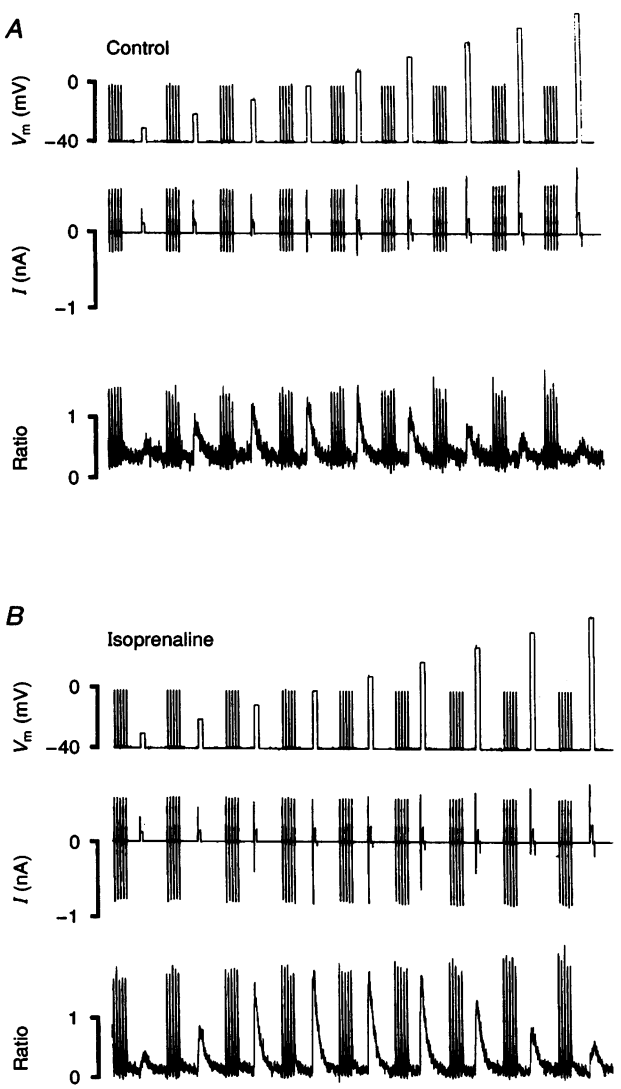

Figure 3. Effect of isoprenaline on the *I–V* relationship for I_{Ca} and the transient–*V* relationship during whole-cell clamp in the presence of Cs^+

A and *B*, chart records of membrane potential (V_m; top), membrane currents (middle) and the Ca^{2+} transient monitored using fura-2 (bottom) in the absence (*A*) and presence (*B*) of isoprenaline (1 μmol l^{-1}). Membrane potential was held at −40 mV and stepped to 0 mV for 5 conditioning pulses before it was stepped to each test potential (see text for further details). The chart was speeded up for each test pulse to enable the membrane current and the Ca^{2+} transient observed in response to each test potential to be seen more easily.

and +60 mV following inhibition of I_{Ca} with Cd^{2+}. Figure 4*B* shows membrane currents from the same cell, using the same protocol, following application of 1 μmol l^{-1} isoprenaline, showing little change in the response to isoprenaline. Figure 4*C* shows mean (± S.E.M., $n = 3$) *I–V* relationships in the absence and presence of isoprenaline, and the difference between these two *I–V* relationships, showing that isoprenaline had no significant effect on this current. Similar data were obtained using the Ca^{2+} channel blocker D600 (methoxyverapamil; not shown). These data show that, using a protocol identical to that used to monitor I_{Ca}, isoprenaline appears to have no significant effect on the currents remaining after inhibition of I_{Ca}, making it unlikely that the observed changes in I_{Ca} are contaminated by isoprenaline-induced changes in other currents. This is supported by the observation (above) that the increase in I_{Ca} produced by isoprenaline in the presence of K^+ was not significantly different from that observed in the presence of Cs^+.

Figure 5 shows the effect of isoprenaline on membrane current and the Ca^{2+} transient, monitored during the protocol described above, using the perforated patch clamp technique in the absence of Cs^+. The voltage dependence of I_{Ca} and the Ca^{2+} transient was obtained during the intercalated test pulses under control conditions (Fig. 5*A*) and in the presence of isoprenaline (Fig. 5*B*). Under control conditions, the size of the Ca^{2+} transient was graded with the size of I_{Ca}, but in the presence of isoprenaline this gradation was lost, so that the amplitude of the Ca^{2+} transient was the same regardless of the amplitude of I_{Ca}. Figure 6 shows fast time base recordings of membrane current (top traces) and the fura-2 fluorescence ratio (bottom traces) at different test potentials in the absence (Fig. 6*A*) and presence (Fig. 6*B*) of isoprenaline, which are representative of data from seven cells. These traces show that, as well as increasing Ca^{2+} transient amplitude, isoprenaline also significantly increased the rate of rise of $[Ca^{2+}]_i$, as might be expected for an increase in SR Ca^{2+} release (Isenberg & Han, 1994). This accelerated rise of the Ca^{2+} transient was maintained at all test potentials except the most positive and the most negative, suggesting that the voltage dependence of both Ca^{2+} transient amplitude and rate of rise are altered by isoprenaline. Ca^{2+} transient amplitude and I_{Ca}, measured as described in the Methods, are shown graphically in Fig. 7 for a representative cell. Under control conditions (○), both the *I–V* relationship (Fig. 7*B*) and the transient–*V* relationship (Fig. 7*A*) were bell shaped: both increased as the test potential was increased up to about 0 mV, and then decreased at more positive potentials. In the presence of isoprenaline (●) both I_{Ca} and the amplitude of the Ca^{2+} transient were greater at any given potential than under control conditions (see also Figs 5 and 6). However, the amplitude of the Ca^{2+} transient was no longer graded with changes in potential (and I_{Ca}); a submaximal I_{Ca} could now trigger a maximal Ca^{2+} transient. This is shown graphically in Fig. 7*C*, which shows the amplitude of the Ca^{2+} transients plotted against the size of

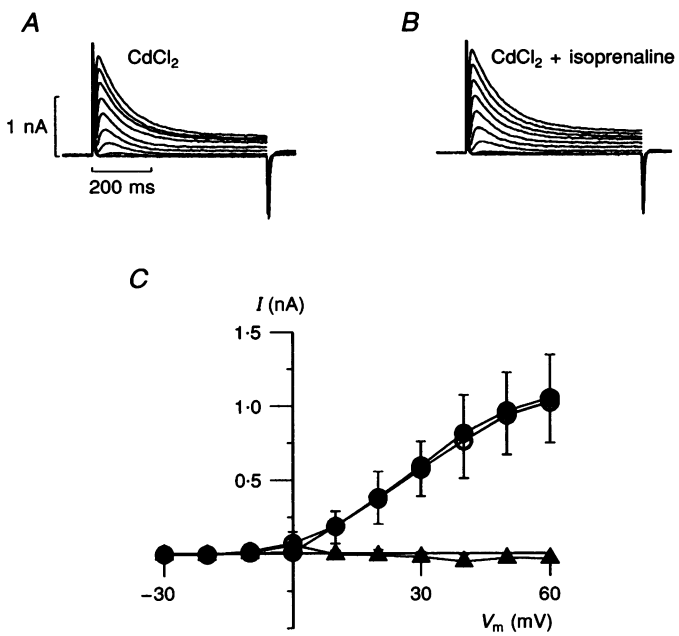

Figure 4. Effect of isoprenaline on the currents remaining after inhibition of I_{Ca} with Cd^{2+}

A, currents obtained at test potentials between −30 and +60 mV (see text for details of protocol used) in the absence of isoprenaline. *B*, currents obtained at test potentials as in *A*, but in the presence of isoprenaline (1 μmol l^{-1}). *C*, *I–V* relationships for these currents in the absence (○) and presence (●) of isoprenaline, and the difference current (i.e. the change in current induced by isoprenaline; ▲). Values are means ± S.E.M. ($n = 3$).

the associated I_{Ca}. Under control conditions (○), the amplitude of the Ca^{2+} transient increased as I_{Ca} increased; the relationship was linear with a slope of $0{\cdot}78 \pm 0{\cdot}23$ ($n = 7$). If the isoprenaline-induced increase in the size of the Ca^{2+} transient is due to an increase in I_{Ca} triggering greater Ca^{2+} release from the SR, it might be expected that the data obtained in the presence of isoprenaline would also fall on this line, or an extension of it. However, Fig. 7*C* shows that the points obtained in the presence of isoprenaline (●) form a different relationship from that observed in the control: the size of the Ca^{2+} transient no longer appeared to be graded with changes in I_{Ca}, so that even a small I_{Ca} could produce a Ca^{2+} transient which was larger than that observed in the control, and the slope of the relationship was significantly ($P < 0{\cdot}01$) less than in the absence of isoprenaline. Furthermore, increases in I_{Ca} produced little further increase in the amplitude of the Ca^{2+} transient. This lack of increase in the amplitude of the Ca^{2+} transient is unlikely to be due to saturation of fura-2 with Ca^{2+}, since during the increase in $[Ca^{2+}]_i$ that accompanies

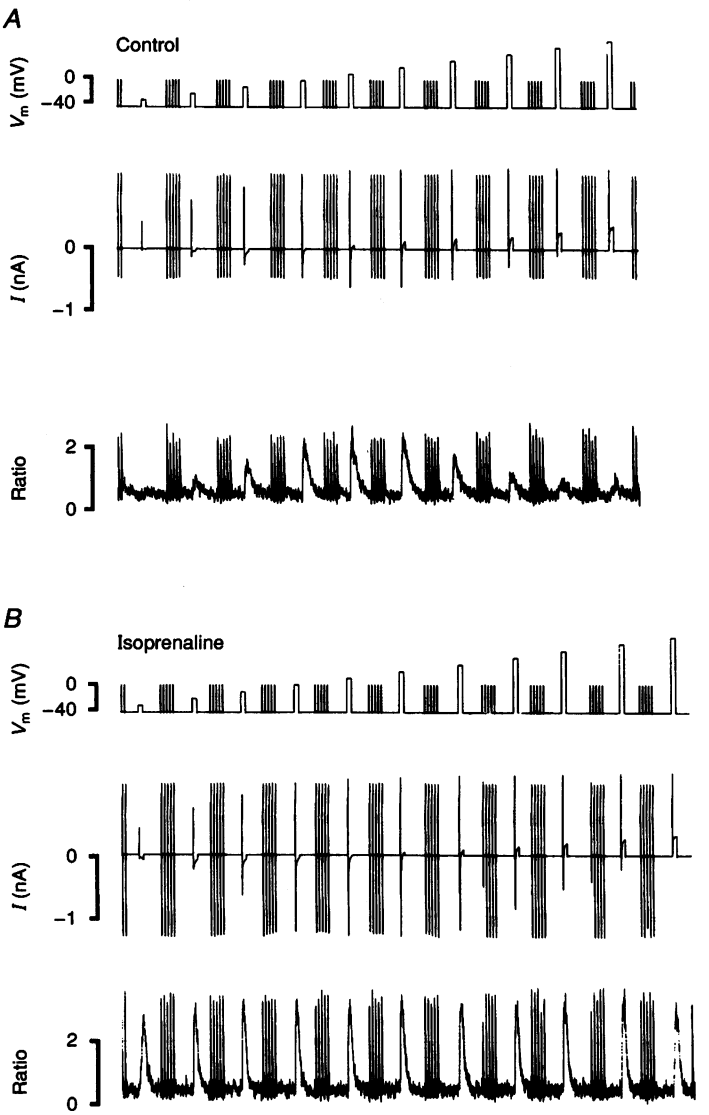

Figure 5. The effect of isoprenaline on the *I–V* relationship for I_{Ca} and the transient–*V* relationship during perforated patch clamp

A and *B*, chart records of membrane potential (top), membrane currents (middle; the large upward deflections represent capacity transients, the negative-going capacity transients have been removed using a sample and hold circuit so that the peak of the downward deflections represents peak inward current during the voltage clamp pulses) and the Ca^{2+} transient monitored using fura-2 (bottom) in the absence (*A*) and presence (*B*) of isoprenaline (1 μmol l^{-1}). Membrane potential was held at −40 mV and stepped to 0 mV for 5 conditioning pulses before being stepped to each test potential (see text for further details). The chart was speeded up for each test pulse to enable the membrane current and the Ca^{2+} transient observed in response to each test potential to be seen more easily.

cell death, the fluorescence ratio increases to 2–3 times the level observed in isoprenaline (not shown).

These data suggest, therefore, that an increase in I_{Ca} is not necessary to trigger greater Ca^{2+} release from the SR during β-adrenergic stimulation, since in the presence of isoprenaline a maximal Ca^{2+} release could be triggered by a small I_{Ca}. Instead, the SR releases more Ca^{2+} in response to a given Ca^{2+} trigger. To test whether such increased release could be due to an increase in the SR Ca^{2+} content (see Introduction), we have investigated firstly, whether we could detect such an increase in SR Ca^{2+} content, and secondly, whether interventions designed to decrease the Ca^{2+} content of the SR could reverse the effects of isoprenaline on the relationship between I_{Ca} and Ca^{2+} transient amplitude.

The effect of isoprenaline on SR Ca^{2+} content assessed using caffeine

Caffeine has been widely used to release Ca^{2+} from the SR in order to assess SR Ca^{2+} content. Figure 8 shows the effect of isoprenaline on caffeine-induced Ca^{2+} release from the SR. Figure 8*A* shows a slow time base record of fura-2 fluorescence in a representative myocyte during field stimulation and then, 20 s after cessation of stimulation, during application of caffeine. Caffeine caused a rapid rise of $[Ca^{2+}]_i$ as Ca^{2+} was released from the SR; subsequent applications of caffeine had little effect, suggesting that the SR was depleted of Ca^{2+} by the first application. Following removal of caffeine and the start of stimulation, the size of the Ca^{2+} transient slowly increased, presumably as the SR

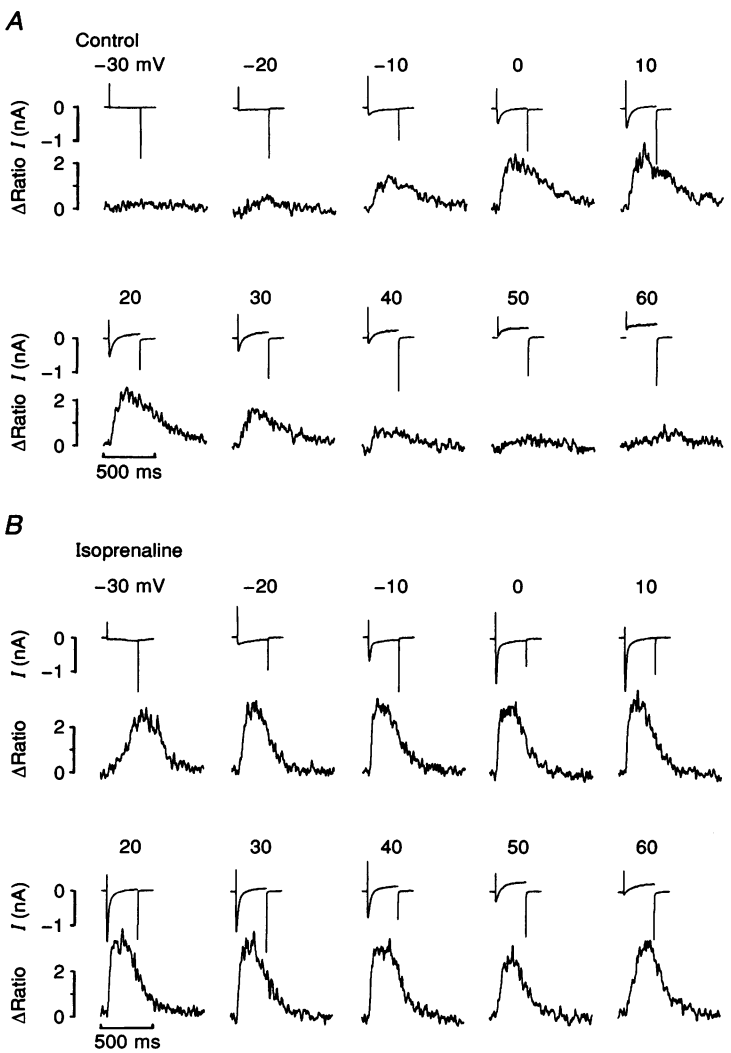

Figure 6. Fast time base records of membrane current (top traces) and fura-2 fluorescence ratio (lower traces) at the potentials indicated in the absence (*A*) and presence (*B*) of isoprenaline (1 μmol l^{-1})

The records were obtained during the intercalated test pulses in an experiment of the type shown in Fig. 5. ΔRatio, change in fura-2 fluorescence ratio. The apparent variation in the amplitude of the capacity transients is due to digitization of the very fast transients; inspection of the original chart records shows that the capacity transients were of uniform amplitude (e.g. Fig. 5).

refilled with Ca^{2+}. Figure 8*B* shows that, in the presence of isoprenaline, not only was the size of the Ca^{2+} transient increased, but that the amplitude of the caffeine-induced Ca^{2+} release was also increased, suggesting that isoprenaline increases SR Ca^{2+} content. When stimulation was recommenced following washout of caffeine, but in the continued presence of isoprenaline, the size of the Ca^{2+} transient increased more rapidly than in the control (Fig. 8*A*) suggesting that the SR refills with Ca^{2+} more rapidly in the presence of isoprenaline. Figure 8*C* shows the increase in the caffeine-induced release of Ca^{2+} more clearly; superimposed records of the caffeine-induced increase in $[Ca^{2+}]_i$ obtained in the absence and presence of isoprenaline, show that isoprenaline increased both the amplitude and integral of the rise of $[Ca^{2+}]_i$ (see Methods). In four cells, isoprenaline increased the integral of the rise of $[Ca^{2+}]_i$ produced by caffeine to 175 ± 13% of control ($P < 0{\cdot}01$). It appears, therefore, that isoprenaline increases SR Ca^{2+} content. If this increase underlies the change in the relationship between I_{Ca} and the Ca^{2+} transient observed in isoprenaline, it might be predicted that manoeuvres that reverse this increase in SR Ca^{2+} content would also reverse the isoprenaline-induced change in this relationship. The following experiments were designed to test this hypothesis.

The effect of decreasing SR Ca^{2+} content on the response to isoprenaline

Reduction of voltage clamp pulse duration from 100 to 20 ms has previously been shown to decrease the SR Ca^{2+} content in rat ventricular myocytes (Janczewski, Spurgeon, Stern & Lakatta, 1995), presumably by curtailing I_{Ca}, thus preventing the Ca^{2+}entry that occurs during the latter part of I_{Ca}, which is thought to load the SR with Ca^{2+} (Fabiato, 1985). We therefore investigated the effect of reducing voltage clamp pulse duration on the response to isoprenaline. Under control conditions (as described above), both peak inward current and the Ca^{2+} transient showed bell-shaped voltage relationships (Fig. 9*Aa* and *B*, ○). *I–V* and transient–*V* relationships were then obtained in the presence of isoprenaline, with similar results to those described above, i.e. gradation of the Ca^{2+} transient amplitude with I_{Ca} was lost (Fig. 9*B*, ●). The duration of the conditioning pulses was then reduced from 300 to 10 ms, still in the presence of isoprenaline. This resulted in a decrease in the size of the Ca^{2+} transient to a level similar to control. After 2 min, 300 ms test pulses to different potentials were intercalated between every five conditioning pulses to obtain *I–V* and transient–*V* relationships (Fig. 9*Ab* and *B*, ▼). Under these conditions, the Ca^{2+} transient was again graded with peak inward current, and

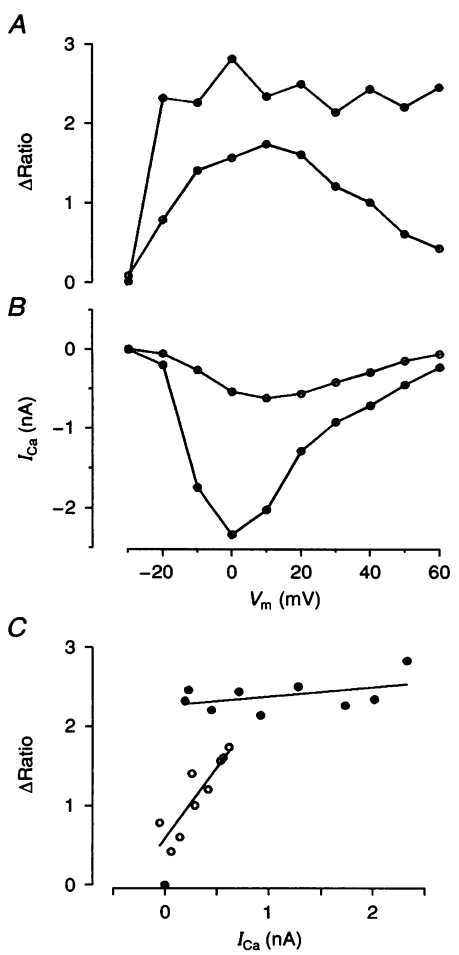

Figure 7. The effect of isoprenaline on the *I–V* relationship for I_{Ca}, and on the Ca^{2+} transient–*V* relationship during perforated patch clamp

A, amplitude of the Ca^{2+} transient (top; measured as described in the text) at different test potentials (obtained using the protocol shown in Fig. 5) in the absence (○) and presence (●) of isoprenaline in a representative myocyte in which membrane potential was controlled using perforated patch voltage clamp. *B*, *I–V* relationship for I_{Ca} in the same cell shown in *A*. *C*, amplitude of each Ca^{2+} transient plotted against the amplitude of the associated I_{Ca}.

both I_{Ca} and the Ca^{2+} transient showed bell-shaped voltage dependence in the presence of isoprenaline, despite the fact that the trigger for Ca^{2+} release (I_{Ca}) during the test pulses remained the same as before, because of the continued presence of isoprenaline and the 300 ms test pulses used.

Thus abbreviation of the conditioning pulses in the presence of isoprenaline reduced the amplitude of the Ca^{2+} transient and returned the voltage dependence of the Ca^{2+} transient to that observed under control conditions. These data are consistent with the idea that the altered characteristics of Ca^{2+} release in the presence of isoprenaline are due to an increase in the Ca^{2+} load of the SR.

The Ca^{2+} channel antagonist nifedipine (2 μmol l^{-1}) was also used to decrease trans-sarcolemmal Ca^{2+} influx, and hence the Ca^{2+} content of the SR, in the presence of isoprenaline. This concentration of nifedipine was chosen to produce only partial (~80–90%) inhibition of I_{Ca} so that Ca^{2+} transients could still be elicited by I_{Ca}. Figure 10*A* shows that, under control conditions, nifedipine-induced inhibition of peak inward current was accompanied by a decrease in the amplitude of the corresponding Ca^{2+} transients, and that the time courses were similar. Similar results were obtained in four cells. This is consistent with the observation that under control conditions the amplitude of the Ca^{2+} transient is linearly related to the amplitude of I_{Ca} (e.g. Figs 6 and 7). In the presence of isoprenaline, however, the amplitude of the Ca^{2+} transients declined more slowly, despite a decline in peak inward current which had a similar time course to that observed in the absence of isoprenaline (Fig. 10*B*); peak inward current had almost reached its minimum value before the amplitude of the Ca^{2+} transients started to decline significantly. Similar results were obtained in three cells. This dissociation between the size of peak inward current and the amplitude of the Ca^{2+} transient is consistent with the observation (above) that, in the presence of isoprenaline, a small I_{Ca} can trigger a large Ca^{2+} transient; the subsequent decline in the Ca^{2+} transient could be due to a decrease in the SR Ca^{2+} content as a result of the decrease in I_{Ca} (Fabiato, 1985). Figure 10*C* shows the voltage dependence of the Ca^{2+} transient, obtained using the voltage protocol shown, in a representative ventricular myocyte under control conditions, and in the presence of isoprenaline, and isoprenaline plus nifedipine. It is clear

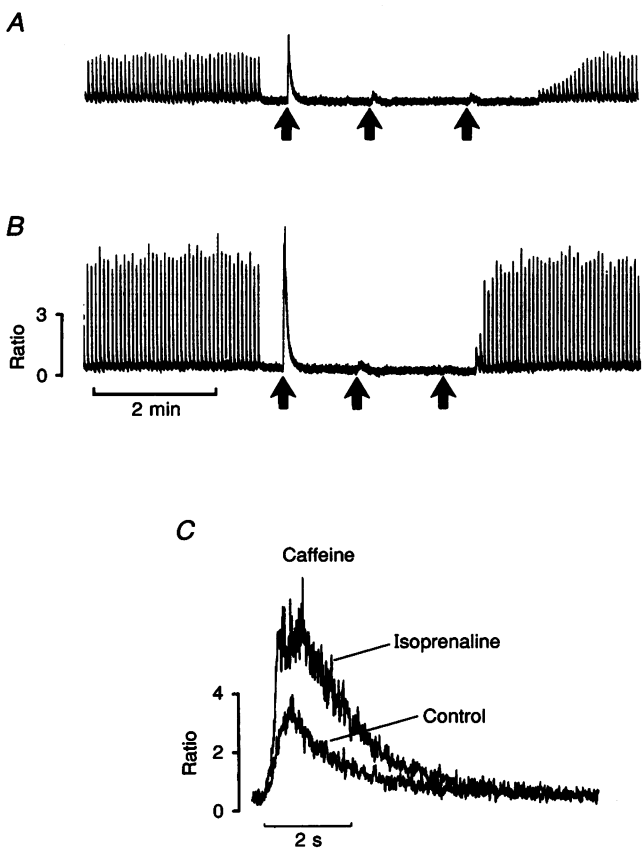

Figure 8. SR Ca^{2+} content assessed using rapid application of caffeine

A, chart record showing fura-2 fluorescence from a representative myocyte. The cell was initially field stimulated. Stimulation was then stopped before repeated rapid application of caffeine (10 mmol l^{-1}; arrows) from a pipette positioned close to the cell. Stimulation was restarted after washout of the caffeine. *B*, as for *A*, but in the presence of isoprenaline (1 μmol l^{-1}). *C*, superimposed fast time base records of the first caffeine-induced increases in $[Ca^{2+}]_i$ from *A* (Control) and *B* (Isoprenaline).

that the isoprenaline-induced alterations in the voltage dependence of the Ca^{2+} transient were reversed by nifedipine.

Effect of the SR inhibitor thapsigargin on the relationship between I_{Ca} and the Ca^{2+} transient

To investigate the role of the SR in the changes described above, the effects of isoprenaline were studied after inhibition of the SR Ca^{2+}-ATPase with thapsigargin; all measurements in the presence of thapsigargin were made after the cell had reached a steady state in the presence of the drug (> 10 min exposure to thapsigargin). Figure 11*B* shows that thapsigargin (2·5 μmol l^{-1}) resulted in a marked decrease in the amplitude of the Ca^{2+} transient and a prolongation of its time course, as expected if SR Ca^{2+} uptake, and hence release, was inhibited. There was also a small decrease in the amplitude of I_{Ca} (Fig. 11*A*), consistent with an inhibitory effect of thapsigargin on the L-type Ca^{2+} channel (Nelson, Li, Bangalore, Benson, Kass & Hinkle, 1994), and a slowed inactivation of I_{Ca}, possibly due to reduced Ca^{2+}-dependent inactivation secondary to a reduction of $[Ca^{2+}]_i$ (Boyett, Kirby & Orchard, 1988).

Figure 11*A* and *B* also shows that exposure to isoprenaline, in the presence of thapsigargin, resulted in an increase in the amplitude of I_{Ca}, and a small increase in the amplitude of the Ca^{2+} transient. However, the amplitude of the Ca^{2+} transient, albeit small, was still graded with the size of I_{Ca} in the presence of isoprenaline. This is apparent in Fig. 11*C*, which shows the amplitude of the Ca^{2+} transient obtained at different test potentials plotted against the size of the accompanying I_{Ca}. Under control conditions, the amplitude of the Ca^{2+} transient showed a steep dependence on I_{Ca}, as shown previously (e.g. Fig. 7). In the presence of thapsigargin, the relationship was flatter and lower, suggesting that most of the increase in the size of the Ca^{2+} transient observed under control conditions as I_{Ca} increases is due to increased Ca^{2+} release from the SR, rather than due directly to Ca^{2+} entry via I_{Ca} or Na^+–Ca^{2+} exchange (see

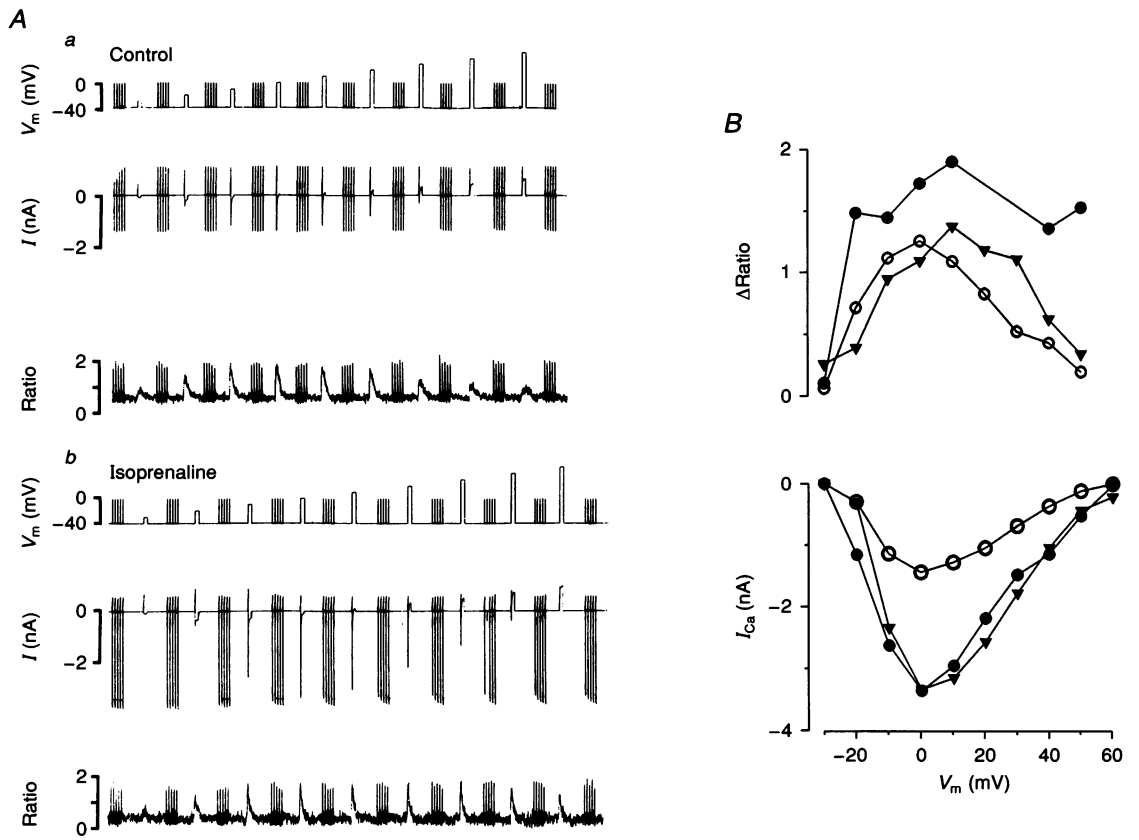

Figure 9. Effect of abbreviating the conditioning pulses on the voltage dependence of I_{Ca} and Ca^{2+} transient in the presence of isoprenaline

Aa and *b*, membrane potential (top), membrane currents (middle; the upward deflections represent capacity transients, the negative-going capacity transients have been removed using a sample and hold circuit so that the peak of the downward deflections represents peak inward current during the voltage clamp pulses) and the corresponding Ca^{2+} transients (bottom; fura-2 fluorescence ratio) under control conditions (*a*) and in the presence of isoprenaline (1 μmol l^{-1}) after the duration of the conditioning pulses had been reduced to 10 ms (*b*). All traces have been speeded up during the test pulses. *B*, *I–V* and transient–*V* relationships under control conditions (○), in 1 μmol l^{-1} isoprenaline with 300 ms conditioning pulses (●) and in 1·0 μmol l^{-1} isoprenaline with 10 ms conditioning pulses (▼).

Discussion). The addition of isoprenaline in the presence of thapsigargin resulted in an increase in the amplitude of I_{Ca} but only a small change in the Ca^{2+} transient. More importantly, the relationship between the amplitude of the Ca^{2+} transient and I_{Ca} was similar in both the absence and presence of isoprenaline. These data suggest that the isoprenaline-induced increase in the Ca^{2+} transient, when a larger Ca^{2+} transient was observed for a given Ca^{2+} current (e.g. Fig. 7), is due to increased Ca^{2+} release from the SR. The contribution of the SR to the amplitude of the Ca^{2+} transient can be envisaged as the vertical difference between the line on the transient–I_{Ca} plot obtained in the presence of thapsigargin, and the data obtained in its absence.

DISCUSSION

The present study was designed to investigate whether the increase in the amplitude of the Ca^{2+} transient that occurs during β-adrenergic stimulation was due to: (i) a change in action potential duration; (ii) an increased trigger for Ca^{2+} release; or (iii) an increase in the response of the SR to the Ca^{2+} trigger.

The possible role of a change in action potential duration

The present data show that the increase in the amplitude of the Ca^{2+} transient that occurred during β stimulation was not significantly altered when the period of depolarization was kept constant (Figs 1 and 2), suggesting that changes in action potential configuration are not the only determinant of the changes in the Ca^{2+} transient observed during β-adrenergic stimulation. However, inhibition of the SR Ca^{2+} release channel by ryanodine (Shah, Than, White, Bennett & Orchard, 1994) or the SR Ca^{2+}-ATPase by thapsigargin (Fig. 11) inhibited the increase in the amplitude of the Ca^{2+} transient during β stimulation; this suggests that the increase in the size of the transient is due to increased Ca^{2+} release from the SR during such stimulation. The subsequent Discussion focuses on the possible causes of this increased release.

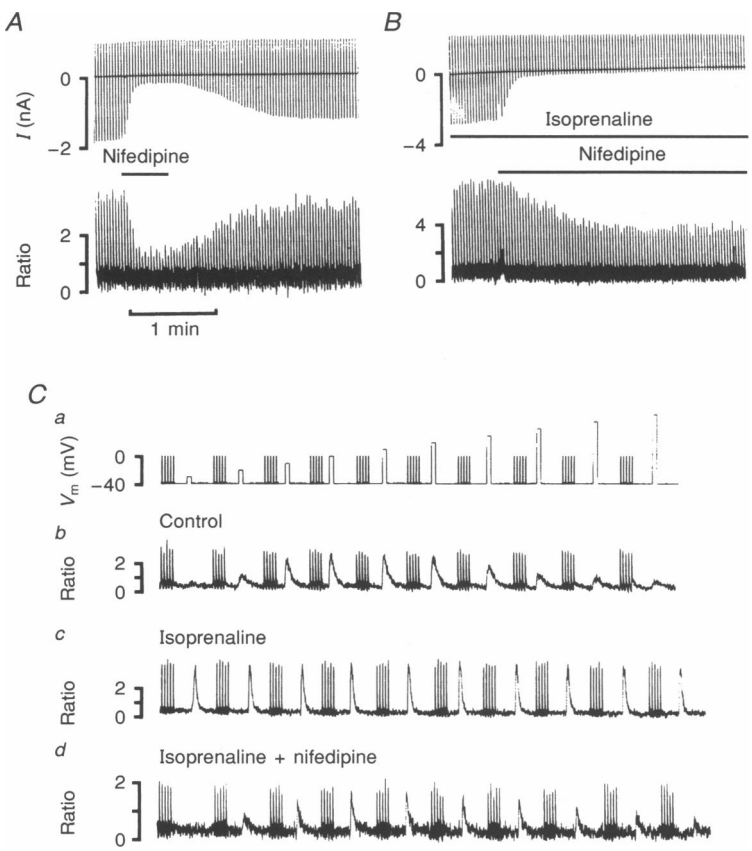

Figure 10. Effect of nifedipine (2 μmol l^{-1}) on I_{Ca} and the Ca^{2+} transient in the presence of isoprenaline (1 μmol l^{-1})

A and *B*, original chart records showing the effect of nifedipine on membrane current (top; the upward deflections represent capacity transients, the negative-going capacity transients have been removed using a sample and hold circuit so that the peak of the downward deflections represents peak inward current during the voltage clamp pulses) and Ca^{2+} transients (bottom; fura-2 fluorescence ratio) in the absence (*A*) and presence (*B*) of isoprenaline. *C*, Ca^{2+} transients obtained in control (*b*), and in the presence of isoprenaline (*c*) and isoprenaline + nifedipine (*d*), using the voltage protocol shown in *a*; all traces have been speeded up during the test pulses, which were intercalated after every 5 conditioning pulses.

The possible role of an increased trigger for SR Ca^{2+} release during β stimulation

One possible cause of the increased Ca^{2+} release from the SR is that an increase in I_{Ca} triggers the release of more Ca^{2+} from the SR. Under control conditions, the size of the Ca^{2+} transient was graded with the amplitude of I_{Ca}, which implies that an increase in I_{Ca} normally triggers the release of more Ca^{2+} (Beuckelmann & Wier, 1988). During β stimulation, however, the size of the Ca^{2+} transient was no longer graded with I_{Ca}. Even a small (i.e. similar to control) I_{Ca} could now trigger a maximal Ca^{2+} transient, so that an increase in the Ca^{2+} trigger does not appear to be necessary to account for the increased transient. This is in contrast to the conclusions of Callewaert *et al.* (1988) who suggested that the increased Ca^{2+} transient amplitude was a consequence of the increased trigger. However, in the study of Callewaert *et al.* (1988), Cs^+ was present in the patch electrode used for voltage clamp. The present study shows that in the presence of Cs^+ the Ca^{2+} transient was graded with I_{Ca} in the presence of isoprenaline, whereas in the presence of K^+, the Ca^{2+} transient was not graded with I_{Ca}. Since Cs^+ alters the response to isoprenaline, possibly by altering SR Ca^{2+} release (Levi *et al.* 1996), and hence the Ca^{2+} transient (Han *et al.* 1994), the present data probably represent more closely the normal physiological response to β-adrenergic stimulation.

In the present study, the amplitude of the rapidly rising phase of the Ca^{2+} transient was used to assess changes in the Ca^{2+} transient (Isenberg & Han, 1994; see Methods). Previous studies have used the amplitude of the rapidly rising phase of the transient, as in the present study (Isenberg & Han, 1994), the rate of rise of the transient (Isenberg & Han, 1984), peak $[Ca^{2+}]_i$ during the transient (Cannell, Berlin & Lederer, 1987; Callewaert *et al.* 1988) and $[Ca^{2+}]_i$ measured at a fixed time after the start of the transient (Callewaert *et al.* 1988). In the present study, the rate of rise of the Ca^{2+} transient increased at all potentials in the presence of isoprenaline (Fig. 6), and this accelerated rate of rise was maintained at most test potentials, consistent with an increase in SR Ca^{2+} release (Isenberg & Han, 1994), and a decreased voltage dependence of release, in the presence of isoprenaline. Previous work has shown that the relationship between $[Ca^{2+}]_i$ and I_{Ca} is flatter if peak $[Ca^{2+}]_i$ is monitored than if $[Ca^{2+}]_i$ is monitored at 25 ms (Cannell *et al.* 1987; Callewaert *et al.* 1988). The measure used in the present study will tend to enhance the 'bell shape' of the Ca^{2+} transient–V relationship under control conditions (Fig. 6), since at the most negative and

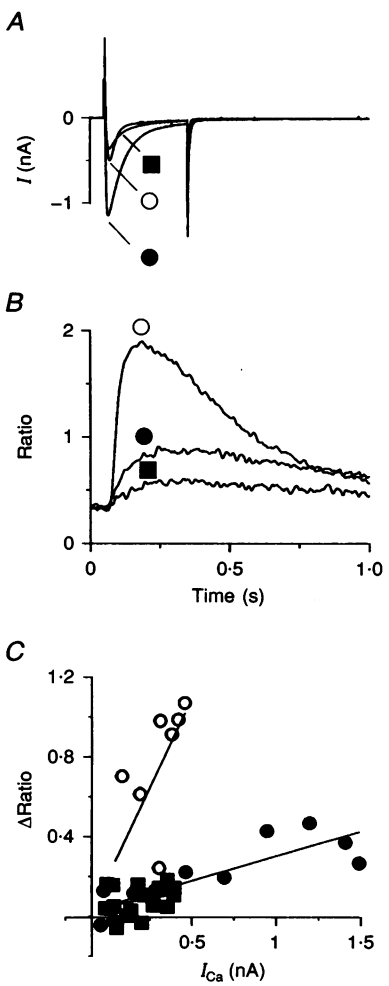

Figure 11. Effect of isoprenaline on I_{Ca} and the Ca^{2+} transient in the presence of thapsigargin

Fast time base averaged (30 sweeps) records of I_{Ca} (*A*) and Ca^{2+} transients (fura-2 fluorescence ratio; *B*) recorded under control conditions (○), in the presence of thapsigargin (2·5 μmol l^{-1}; ■) and in the presence of thapsigargin + isoprenaline (1 μmol l^{-1}) (●). *C*, the amplitude of Ca^{2+} transients obtained during test pulses to different potentials under the three different conditions, plotted against their associated I_{Ca}.

positive test potentials, $[Ca^{2+}]_i$ was lower at the time used than at the end of the pulse. However, in the presence of isoprenaline there would be little difference between the two measures, since the highest $[Ca^{2+}]_i$ is that at the peak of the fast upstroke of the transient. Thus the measure used will tend to emphasize the change from the original 'bell shape' to the flatter relationship observed. However, it is clear from Fig. 6 that, even if peak $[Ca^{2+}]_i$ were used, isoprenaline still markedly decreased the voltage dependence of the $[Ca^{2+}]_i$ transient, although it is clear that the precise relationship between $[Ca^{2+}]_i$ and I_{Ca} will depend on the measure used, and on the time course of the Ca^{2+} transient which will depend, in turn, on many factors including SR Ca^{2+} release and the relative contributions of trans-sarcolemmal and SR Ca^{2+} flux.

However, although the isoprenaline-evoked increase in I_{Ca} does not appear to be required to *trigger* more Ca^{2+} release from the SR, the data shown in Figs 9 and 10 suggest that I_{Ca} is necessary to provide Ca^{2+} to increase the load of the SR, since inhibition of I_{Ca} by nifedipine, or the use of brief depolarizing voltage clamp pulses to shorten the late component of I_{Ca} (which is thought to load the SR with Ca^{2+}; Fabiato, 1985), in the presence of isoprenaline, resulted in a decrease in the amplitude of the Ca^{2+} transient, presumably by decreasing the Ca^{2+} load of the SR (see below).

A second possibility is that Ca^{2+} entry via the Na^+–Ca^{2+} exchanger, which may also trigger Ca^{2+} release from the SR (Levi, Spitzer, Kohmoto & Bridge, 1994) may increase during β stimulation. Conditions in the present study were chosen to minimize Ca^{2+} release from the SR triggered by the exchange mechanism (Vornanen *et al.* 1994). In agreement with previous studies (Callewaert *et al.* 1988; Isenberg & Han, 1994), it appears unlikely that the exchanger contributes to the rapidly rising Ca^{2+} transient under control conditions, since no transients were observed at positive test potentials (e.g. Fig. 5*A*) when I_{Ca} was small and Ca^{2+} influx via the exchanger would be expected to be potentiated. It is also unlikely that the exchanger was responsible for much Ca^{2+} influx in the presence of isoprenaline; if this had occurred, the linear dependence of the size of the Ca^{2+} transient on I_{Ca} in the presence of thapsigargin (Fig. 11*C*) would not be expected. Rather, when I_{Ca} was small, there would be one population of points, obtained at negative test potentials, showing small Ca^{2+} transients, and another population, obtained at positive test potentials, which would enhance Ca^{2+} influx via the exchanger, in which the small I_{Ca} would be associated with a larger Ca^{2+} transient. Since this was not observed, it suggests that Ca^{2+} influx via the exchanger was negligible. Furthermore, Main & Cannell (1995) have reported that isoprenaline produced no obvious increase in the Na^+–Ca^{2+} exchange current in voltage clamped ventricular myocytes. It appears unlikely, therefore, that increased Ca^{2+} influx via the exchanger acts as an increased trigger for Ca^{2+} release during β stimulation. It is worth noting, however, that the current experiments were performed at room temperature, and that the activity of the Na^+–Ca^{2+} exchange mechanism increases with temperature (Vornanen *et al.* 1994). Thus, at 37 °C, Ca^{2+} influx via the exchanger may be sufficient to trigger SR Ca^{2+} release, so that contraction amplitude does not decline markedly, at positive potentials (Levi *et al.* 1994, 1996). In this case, it might be expected that at 37 °C the loss of voltage dependence observed in the present study would be most apparent at negative potentials, where contraction is still graded with voltage at 37 °C (Levi *et al.* 1996).

A potential problem which should be considered is whether a loss of voltage control in the presence of isoprenaline could account for the present data. This seems unlikely, however, because: (i) data from experiments in which the *I–V* curve became skewed in the presence of isoprenaline (an indication of loss of voltage control) were not used (see Methods); (ii) a similar loss of voltage dependence of the Ca^{2+} transient in the presence of isoprenaline was seen when the whole-cell configuration was used, even with the low-resistance electrodes used; (iii) in the presence of Cs^+, the voltage dependence of the Ca^{2+} transients was restored despite the continued enhancement of the Ca^{2+} current; and (iv) abbreviation of the duration of the conditioning pulses restored the voltage dependence of the Ca^{2+} transients, despite the continued enhancement of the Ca^{2+} current during the test pulses. These observations are unexpected if the loss of voltage dependence of the Ca^{2+} transient was due to loss of voltage control.

In summary, therefore, it appears that the increased Ca^{2+} release in the presence of isoprenaline is due to an increase in the amount of Ca^{2+} released by the SR for a given trigger, rather than the increase in I_{Ca} triggering the release of more Ca^{2+} from the SR.

Possible mechanisms for the increased SR Ca^{2+} release in response to a given I_{Ca}

The gain of the Ca^{2+} release mechanism (i.e. the amount of Ca^{2+} released from the SR for a given I_{Ca}) is determined by: (i) single-channel L-type Ca^{2+} currents; (ii) the sensitivity of the SR release channel to activation by Ca^{2+}; and (iii) the SR Ca^{2+} content (duBell, Lederer & Rogers, 1996). The possible role of each of these will be discussed.

(i) Current models of SR Ca^{2+} release suggest that the probability of local Ca^{2+} release from the SR is increased by *local* Ca^{2+} influx via I_{Ca} (Stern, 1992; Isenberg & Han, 1994; Wier, Egan, López-López & Balke, 1994; Cannell, Cheng & Lederer, 1995). However, because the mean open time of the SR release channel(s) is greater than that of the L-type Ca^{2+} channel, once Ca^{2+} release has been triggered, it is then normally determined by the intrinsic gating of the SR Ca^{2+} release pathway (Stern, 1992; Cannell *et al.* 1995). In this model, changes in the probability of such local release occurring bring about gradation of the whole-cell Ca^{2+} transient. The probability of such local release depends on the square of the single-channel Ca^{2+} current (Santana,

Cheng, Gomez, Cannell & Lederer, 1996). Since an increase in single-channel current increases the probability of local Ca^{2+} release, the size of the whole-cell Ca^{2+} transient for a given whole-cell I_{Ca} is greater when the single-channel currents are large than when they are small (Wier *et al.* 1994). Thus an increase in single-channel current could explain the increase in gain observed in the present study, except that isoprenaline does not appear to increase single-channel currents (Flockerzi, Oeken, Hofmann, Pelzer, Cavalie & Trautwein, 1986; Ono & Fozzard, 1992), making this explanation unlikely. Alternatively, isoprenaline could increase gain by increasing the open probability of the L-type Ca^{2+} channel, either by increasing the number of channel openings per unit time, and/or by increasing the open time of the L-type Ca^{2+} channel (Yue, Herzig & Marban, 1990). Such an increase in open probability, and the subsequent increase in Ca^{2+} influx, might be expected to increase the open probability of the SR Ca^{2+} release channel, which might be expected to have a similar effect to increasing single-channel current (i.e. to increase gain), which could explain the present data. However, gradation of Ca^{2+} transient amplitude occurred in the presence of isoprenaline when Cs^+ was present (Fig. 3) and when the conditioning pulses were abbreviated (Fig. 9), even though in both cases I_{Ca} during the test pulses was the same in the presence of isoprenaline as in the control. Since the loss of gradation was absent in the continued presence of isoprenaline-induced changes in the current, this suggests that isoprenaline-induced changes in L-type channel gating do not underlie the loss of gradation of the Ca^{2+} transient, unless a more complex interaction of SR Ca^{2+} content and Ca^{2+} trigger is postulated.

(ii) Phosphorylation of the cardiac SR Ca^{2+} release channel by purified PKA or CaM kinase II has been reported to relieve Mg^{2+} block of the channel, and phosphatases (and hence presumably dephosphorylation) have been reported to decrease the gain of the SR Ca^{2+} release process (without changing SR Ca^{2+} content or I_{Ca}; duBell *et al.* 1996). Thus it is possible that phosphorylation of the release channel could sensitize the Ca^{2+} release process so that a small trigger could cause a large Ca^{2+} release during β stimulation (Patel *et al.* 1995; duBell *et al.* 1996). However, in the present study gradation of Ca^{2+} release could be observed in the presence of isoprenaline, when such phosphorylation would still be present (Figs 3, 9 and 10). Thus although it remains possible that this mechanism modulates the sensitivity of the Ca^{2+} release mechanism, it appears unlikely that such phosphorylation underlies the isoprenaline-induced increase in gain that leads to loss of gradation of the Ca^{2+} transient. In addition, phosphorylation of the cardiac SR Ca^{2+} release channel by endogenous CaM kinase II appears to inhibit channel activity (Hain *et al.* 1995; Lokuta, Rogers, Lederer & Valdivia, 1995), which would decrease the gain of the Ca^{2+} release mechanism.

(iii) During β stimulation, enhanced Ca^{2+} uptake by the SR, and the increase in I_{Ca} (see above and Introduction), would be expected to increase the Ca^{2+} content of the SR. Other interventions that increase the SR Ca^{2+} content have been reported to increase the amount of Ca^{2+} released by the SR for a given trigger (Han *et al.* 1994; Bassani, Yuan & Bers, 1995; Janczewski *et al.* 1995), both because there is more Ca^{2+} available for release and because, at high Ca^{2+} loads, a greater fraction of the Ca^{2+} content is released (Bassani *et al.* 1995; Janczewski *et al.* 1995).

The hypothesis that an increase in SR Ca^{2+} content is involved in the response to isoprenaline is supported by the observation that rapid application of caffeine showed that there was an increase in SR Ca^{2+} content in the presence of isoprenaline (Fig. 8), and that manoeuvres that decrease the Ca^{2+} content of the SR could reverse the effects of isoprenaline on both the amplitude and voltage dependence of the Ca^{2+} transient (Figs 9 and 10). These data are consistent with the idea that an increase in SR Ca^{2+} content underlies the observed effects of isoprenaline on the gain of the Ca^{2+} release mechanism (above) and hence the amplitude of the Ca^{2+} transient. Thus we suggest that the response to isoprenaline is due to increased Ca^{2+} flux through the ryanodine receptor for a given L-type Ca^{2+} current, as a consequence of the increase in SR Ca^{2+} content.

More difficult to reconcile with this hypothesis is the observation that during conventional whole-cell recordings isoprenaline had little effect on the amplitude of the Ca^{2+} transient, which probably reflects the Ca^{2+} load of the SR, but still increased the 'gain' of the Ca^{2+} release process (i.e. a small I_{Ca} could still trigger maximal Ca^{2+} release). It has, however, previously been shown that the Ca^{2+} content of the SR needs to be increased very little (~4%) to increase markedly the fraction of its Ca^{2+} content that is released (Bassani *et al.* 1995), which could explain this apparent discrepancy. The reasons for the smaller response of Ca^{2+} transient amplitude to isoprenaline during whole-cell voltage clamp in the present study are unclear, although a similar small response has been reported before (Sham, Jones & Morad, 1991). It seems unlikely that the altered response in the present study is due to the fura-2 introduced through the patch pipette, since the same response was observed across a fourfold change in fura-2 concentration. It seems possible, however, that the altered response was due to a change in SR function, since when Cs^+ was present, the amplitude of the Ca^{2+} transient increased. One possibility is that in the whole-cell configuration the amplitude of the Ca^{2+} transient was close to maximal, which limited the possible increase in amplitude, but that the Ca^{2+} transient was decreased by Cs^+ (Han *et al.* 1994), thus allowing $[Ca^{2+}]_i$ scope to increase. The reasons for this are unclear, but it seems likely to be due to dialysis of the cell. However, since I_{Ca} and the rate of decline of the Ca^{2+} transient were still increased by isoprenaline it appears that at least some phosphorylation pathways were intact during dialysis.

In conclusion, the increase in the amplitude of the Ca^{2+} transient during β-adrenergic stimulation does not appear to depend on changes in action potential duration, but is due

to increased Ca^{2+} release from the SR. This increased release does not require an increased trigger for Ca^{2+} release, but is due to an increase in the gain of the SR Ca^{2+} release process, which can be reversed (by manoeuvres designed to decrease the Ca^{2+} content of the SR) in the continued presence of the isoprenaline-induced increase in I_{Ca} and phosphorylation of the ryanodine receptor (above), which is unexpected if these changes underlie the observed increase in gain. The simplest explanation of these data, therefore, is that the observed changes depend principally on the observed increase in the Ca^{2+} content of the SR, which will in turn depend on the rate of Ca^{2+} uptake by the SR and the size of I_{Ca}.

Mechanisms underlying the abbreviation of the Ca^{2+} transient during β-adrenergic stimulation

The present data show that the abbreviation of the Ca^{2+} transient that occurs during β-adrenergic stimulation is not altered when the period of depolarization is kept constant, in agreement with the work of Morad & Rolett (1972); this argues against a significant role for changes in action potential duration in this abbreviation. The abbreviation of the Ca^{2+} transient is, however, abolished by the SR Ca^{2+} release channel inhibitor ryanodine (Shah *et al.* 1994) and the SR Ca^{2+}-ATPase inhibitor thapsigargin (this study), suggesting an important role for the SR in the abbreviation. Isoprenaline had no effect on the time course of the Ca^{2+} transient or contraction in the presence of an antibody against phospholamban (Sham *et al.* 1991) or in mice which do not express phospholamban (Luo *et al.* 1994). It appears, therefore, that phospholamban-mediated acceleration of Ca^{2+} uptake by the SR (see Introduction) underlies the faster decline of the Ca^{2+} transient (Figs 1 and 2), and hence abbreviated contraction, that occurs during β stimulation. Such accelerated Ca^{2+} uptake could account for the more rapid recovery of the twitch in the presence of isoprenaline, following depletion of the SR Ca^{2+} content using caffeine (see Results and Fig. 8), as the SR refills with Ca^{2+} more rapidly. It would also be expected to increase the fraction of the available cytoplasmic Ca^{2+} sequestered by the SR which, in conjunction with the increase in I_{Ca}, could explain the increase in the Ca^{2+} content of the SR which appears to underlie the response of Ca^{2+} transient amplitude to β-adrenergic stimulation (above).

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Author's email address

C. H. Orchard: C.H.Orchard@Leeds.ac.uk

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