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Ca^{2+}-DEPENDENT BLOCK AND POTENTIATION OF L-TYPE CALCIUM CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. The caged calcium compound nitr-5 has been used to investigate the response of the L-type calcium current (I_{Ca}) of guinea-pig ventricular cells to a rapid increase in the free intracellular calcium concentration ($[Ca^{2+}]_i$).

2. When 2 mM nitr-5 or 3 mM DM-nitrophen was loaded into cells via a patch pipette and photolysed during the decay phase of I_{Ca}, a partial block of the current developed within 75 ms. The block was reduced by increasing the pre-flash $[Ca^{2+}]_i$ and enhanced by adding high concentrations of Ca^{2+} chelators to the pipette-filling solution.

3. The photolysis-induced block was not suppressed in the presence of isoprenaline, suggesting a direct action of Ca^{2+} on the channels rather than a mechanism involving channel phosphorylation.

4. The most prominent effect of nitr-5 photolysis was a slow potentiation of I_{Ca}. When I_{Ca} was activated at frequencies between 0·05 and 0·7 Hz with various levels of pre-flash $[Ca^{2+}]_i$, peak I_{Ca} was approximately doubled in amplitude following photolysis.

5. At a stimulation frequency of 0·05 Hz, when nitr-5 was the only chelator present in the pipette, the time course of the potentiation was fitted by a single exponential with a time constant (τ_P) of 2·7 min. When 1 mM $CaCl_2$ was added to the pipette-filling solution, the time course of the potentiation was slowed (τ_P = 6 min), although its amplitude was unchanged. With 12 mM BAPTA (a calcium chelator) added instead of $CaCl_2$, the response was accelerated (τ_P = 1·7 min).

6. Equimolar substitution of extracellular Ca^{2+} with Ba^{2+} significantly suppressed the flash-induced potentiation. The time course of the potentiation of the barium current, I_{Ba} (τ_P = 1·9 min) was similar to that of I_{Ca} with BAPTA in the pipette. Potentiation of I_{Ba} was largely blocked in Ca^{2+}-depleted cells when $CaCl_2$ was omitted from the pipette.

7. When I_{Ca} was activated at frequencies of $\geqslant$ 0·1 Hz, with 1 mM $CaCl_2$ added to the nitr-5 (2 mM) in the pipette, the onset of the flash-induced potentiation was best fitted by two exponentials; one was similar to the single component seen at 0·05 Hz and the other was approximately one order of magnitude faster. The contribution of the faster component was positively correlated to the stimulation frequency.

8. The flash-induced potentiation of I_{Ca} was suppressed in the presence of a

supramaximal concentration of the β-adrenergic agonist isoprenaline. Stimulation of I_{Ca} by isoprenaline was significantly reduced after augmentation of the current by photolysis of nitr-5.

9. The protein kinase inhibitors H-7 and Rp-cAMP-S had no significant effect on the photolysis-induced potentiation, but the non-hydrolysable ATP analogue AMP-PNP caused a greater than twofold increase in the potentiation when compared with ATP. The magnitude of the potentiation was dependent on the concentration of ATP added to the pipette solution.

10. We conclude that the response of I_{Ca} to a rapid increase in $[Ca^{2+}]_i$ in guinea-pig ventricular cells is complex. The immediate block of the current is probably caused by a direct interaction between the photoreleased Ca^{2+} and the Ca^{2+} channel. The Ca^{2+} dependence of the photoinduced potentiation is explained in terms of a model with two binding sites for Ca^{2+}; one site is close to the channel mouth and triggers the response, while the other is inhibitory. The potentiation is not mediated by Ca^{2+}-dependent phosphorylation, but it does involve a nucleotide.

INTRODUCTION

The role of Ca^{2+} in the excitation–contraction coupling of heart muscle has been the subject of extensive investigation. Ca^{2+} entry during I_{Ca} provides the trigger for Ca^{2+}-activated Ca^{2+} release from the sarcoplasmic reticulum (SR) (Fabiato & Fabiato, 1975; Valdeolmillos, O'Neill, Smith & Eisner, 1989), which in different mammalian species provides varying proportions of the Ca^{2+} that binds to the myofilaments to initiate contraction (reviewed in Bers, 1991). Since I_{Ca} has a crucial role in excitation–contraction coupling, the regulation of this current has been the focus of many studies. It is modulated by Ca^{2+} in a complex way, with increased $[Ca^{2+}]_i$ causing both inhibition and potentiation of I_{Ca}. The inactivation of I_{Ca} is dependent both on voltage and Ca^{2+} influx through the channel (Eckert & Chad, 1984; Lee, Marban & Tsien, 1985), with greater Ca^{2+} influx producing more pronounced inactivation. The dependence of I_{Ca} inactivation on $[Ca^{2+}]_i$ in cardiac muscle was first demonstrated by intracellular injection of $CaCl_2$ or EGTA into Purkinje fibres (Isenberg, 1977). It was later demonstrated in isolated guinea-pig heart cells by intracellular dialysis with various Ca^{2+} chelators via a patch pipette (Lee *et al.* 1985; Bechem & Pott, 1985). Studies on single Ca^{2+} channels in chick ventricular myocytes (Mazzanti & DeFelice, 1990) and guinea-pig ventricular cells (Yue, Backx & Imredy, 1990) have recently demonstrated that individual Ca^{2+} channels are inactivated by the Ca^{2+} which passes through them, observations which confirm previous studies of macroscopic Ca^{2+} channel currents.

Increased intracellular Ca^{2+} has also been shown to potentiate I_{Ca} in heart. Digitalis has been shown to stimulate the slow inward current in ferret ventricular muscle (Marban & Tsien, 1982) and this effect was linked to an elevation of $[Ca^{2+}]_i$. Repetitive activation of I_{Ca} in guinea-pig ventricular cells can cause potentiation of the peak amplitude of I_{Ca} and slow its inactivation (Lee, 1987; Fedida, Noble & Spindler, 1988*a*, *b*; Zygmunt & Maylie, 1990). These effects have been shown to depend on Ca^{2+} entry (but see Lee, 1987) and it was suggested that they involve phosphorylation of the Ca^{2+} channels (Fedida *et al.* 1988*b*). More recent studies using the same preparation have provided further evidence that Ca^{2+}-induced potentiation

involves the binding of Ca^{2+} to a site near to the cytoplasmic face of the channel and phosphorylation of the channel (Zygmunt & Maylie, 1990). It has been postulated that such Ca^{2+}-dependent potentiation of I_{Ca} may underlie the positive force staircase seen in many cardiac preparations (Zygmunt & Maylie, 1990).

Direct demonstrations of Ca^{2+}-dependent modulation of I_{Ca} have come from studies using flash photolysis of caged Ca^{2+} molecules (Adams, Kao, Grynkiewicz, Minta & Tsien, 1988; Kaplan & Ellis-Davies, 1988; Gurney, 1991) introduced into cells via a patch pipette. In guinea-pig ventricular myocytes, a rapid increase in $[Ca^{2+}]_i$ can cause both inactivation and potentiation of I_{Ca} (Gurney, Charnet, Pye & Nargeot, 1989; Hadley & Lederer, 1991). Hadley & Lederer (1991) have studied the mechanisms underlying the inactivation produced by photorelease of Ca^{2+} from DM-nitrophen. They found that the Ca^{2+}-induced inactivation could be blocked by the β-adrenergic agonist, isoprenaline, and concluded that Ca^{2+} acts by stimulating Ca^{2+}-dependent phosphatases, which dephosphorylate and inactivate the Ca^{2+} channels. Inhibition of I_{Ca} is also produced by photolysis of nitr-5 (Gurney *et al.* 1989; Hadley & Lederer, 1991). One of the aims of this study was to determine the role of dephosphorylation in the response when nitr-5 is employed as the Ca^{2+} donor, and to examine how the photoinduced inhibition is influenced by varying the $[Ca^{2+}]_i$. In contrast to the inhibitory effect of Ca^{2+} on I_{Ca}, virtually nothing is known about the Ca^{2+}-induced augmentation of I_{Ca} in guinea-pig ventricular cells. In an earlier study describing this effect (Gurney *et al.* 1989), we concentrated mainly on frog atrial cells, where augmentation appeared to be the only response to photolysis of caged Ca^{2+}. We have now examined the Ca^{2+}-induced augmentation of I_{Ca} in guinea-pig ventricular cells in more detail. In particular, we have investigated the Ca^{2+} dependence of this response and the possible role of phosphorylation, which was suggested to mediate the Ca^{2+}-induced augmentation of I_{Ca} in frog (Gurney *et al.* 1989; Charnet, Richard, Gurney, Ouadid, Tiaho & Nargeot, 1991). Some of this work has appeared in abstract form (Bates & Gurney, 1992*a*, *b*).

METHODS

Cell dissociation

Experiments were performed on ventricular myocytes isolated from male guinea-pigs approximately 250–500 g in weight. Cells were isolated using a method similar to that of Harding, O'Gara, Jones, Brown, Vescovo & Poole-Wilson (1990). Animals were killed by cervical dislocation, the hearts quickly removed and perfused for 5 min at 36 °C with a nominally Ca^{2+}-free solution containing (mM): NaCl, 120; KCl, 5·4; $MgSO_4$, 5; pyruvate, 5; glucose, 20; taurine, 20; Hepes, 10 (pH 6·96). The perfusate was then replaced with one containing 4 U ml^{-1} protease (Sigma type XXIV). After 2 min the perfusate was switched to a solution containing collagenase (Worthington Class 2, approximately 0·3 mg ml^{-1}) and hyaluronidase (Sigma, 0·6 mg ml^{-1}). Perfusion was continued for between 5 and 10 min, after which the heart was removed from the cannula and chopped finely in warmed 'KB' solution (see Belles, Malécot, Hescheler & Trautwein, 1988) containing (mM): KOH, 85; KCl, 30; KH_2PO_4, 30; $MgSO_4$, 3; glutamic acid, 50; taurine, 20; EGTA, 0·5; Hepes, 10; glucose, 10 (pH 7·4). The tissue was strained through four layers of gauze, centrifuged briefly and resuspended in 'KB' solution. The cells were then spun again and resuspended in either the recording solution, containing (mM): NaCl, 120; KCl, 2·5; $MgCl_2$, 2·5; NaH_2PO_4, 0·5; KH_2PO_4, 0·5; $NaHCO_3$, 15; $CaCl_2$, 1; glucose, 10; Hepes, 5 (pH 7·4), or 'KB' solution until use.

Electrophysiology

For electrophysiological experiments, cells were transferred to a recording chamber of approximately 0·5 ml volume and continuously superfused with recording solution. In some

experiments 1 mM Ba^{2+} was substituted for Ca^{2+}. All experiments were performed at room temperature ($\approx$ 22–24 °C). Cells were usually voltage clamped at −70 mV using the whole-cell configuration of the patch-clamp technique. Pipettes had resistances of between 2 and 4 MΩ and were coated close to their tips with black Sylgard (Sylgard 170 silicone elastomer, Dow Corning) to reduce electrode capacitance and protect the bulk of the pipette solution from light flashes. Voltage was controlled with a Biologic RK 300 patch-clamp amplifier. After making a high resistance seal, the cell membrane under the tip of the pipette was disrupted by suction. Series resistance compensation was used routinely; currents were not leak subtracted. Pipette solution usually contained (mM): CsCl, 130; $MgCl_2$, 1; Hepes, 15; nitr-5, 2; ATP-Na_2, 2 (pH 7·2). In some experiments, either 1 mM $CaCl_2$, 12 mM BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) or 25 mM EGTA was added. DM-nitrophen (usually 3 mM) with a variable amount of added $CaCl_2$, replaced nitr-5 in some experiments. I_{Ca} was activated by a 300 ms depolarization to 0 mV. This step was preceded by a 100 ms prepulse to −40 mV to eliminate T-type Ca^{2+} current and Na^+ current. Contaminating outward K^+ currents were blocked by the Cs^+-containing pipette solution. Unless otherwise noted, the stimulation frequency was 0·05 Hz. At least 10 min was allowed for dialysis of cells before flash photolysis, and results were discarded if I_{Ca} did not stabilize during this period. The stimulus protocol was generated and the flashlamp triggered, with pCLAMP data acquisition and analysis software (Version 5.5.1, Axon Instruments, Burlingame, CA, USA). Data was collected with a Compaq Deskpro 286 microcomputer for later analysis with pCLAMP. Graphs were generated and curves fitted to the mean data with the Sigmaplot Scientific Graph System (Jandel Scientific, USA). Mean data in tables and figures are presented ± the standard error of the mean, with the number of observations shown in parentheses.

Flash photolysis

Ca^{2+} release was initiated by < 1 ms flashes of ultraviolet light, delivered from a xenon short-arc flashlamp (Dr G. Rapp, Optoelektronic, Hamburg, Germany) and was usually triggered during the decay phase of I_{Ca}. Light was directed and focused onto the cells with a 5 mm diameter liquid light guide equipped with a 19 mm fused silica focusing lens (Oriel Scientific Ltd, Leatherhead, Surrey). Wavelengths below 320 nm were excluded with a glass coverslip (Chance Propper, no. 0) so that the broad spectrum output of the lamp ranged from 320 to 900 nm. Although photolysis of solution in the pipettes was minimized by coating them with black Sylgard, some of the pipette tip as well as the cell would have been irradiated. For most experiments, the energy discharged across the lamp was 150 J, with the light energy focused on the cells measured as $1{\cdot}6 \times 10^4$ J m^{-2} using a disc calorimeter (AG Electro-optics, Tarporley, Cheshire). At this intensity, a single flash photolysed around 25% of the Ca^{2+}-bound nitr-5 molecules, as measured from changes in the absorbance of droplets of nitr-5 solution after a flash (Gurney, Tsien & Lester, 1987). The 40-fold loss in Ca^{2+} affinity of the photolysed molecules resulted in a net Ca^{2+} release. The $[Ca^{2+}]_i$ before and after photolysis was calculated, assuming that the added Ca^{2+} buffers were homogeneously distributed in the cell and were the dominant buffers. A program following the same approach as Fabiato (1988) was used to calculate $[Ca^{2+}]$, with K_D (dissociation constant) values of 145 nM and 6·3 μM for nitr-5 before and after photolysis, respectively. The binding of Mg^{2+} to nitr-5 (Adams *et al.* 1988) and Ca^{2+} and Mg^{2+} binding to ATP (Fabiato, 1988) were also included in the equations. In this way, the $[Ca^{2+}]_i$ before photolysis was estimated as 177 nM when pipettes contained 2 mM nitr-5 and 1 mM $CaCl_2$, with a single flash increasing the $[Ca^{2+}]_i$ by around 85 nM. With or without BAPTA present and with no added $CaCl_2$, 2 mM nitr-5 would be expected to buffer the pre-flash bulk $[Ca^{2+}]_i$ in the low nanomolar range with negligible release of Ca^{2+} upon photolysis.

Sources of reagents

Nitr-5, BAPTA, ryanodine and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine, di-HCl (H-7) were obtained from Calbiochem-Novabiochem (Nottingham) and isoprenaline and adenylylimidodiphosphate (AMP-PNP) from Sigma (Poole, Dorset). (Rp)-cyclic adenosine-3′,5′-mono phosphothioate (Rp-cAMP-S) was from Biologic Life Science Institute, Bremen, FRG. DM-nitrophen was a gift from Dr J. Kaplan.

RESULTS

The immediate effect of photolysis on L-type calcium current

The immediate effect of photolysis of intracellular nitr-5 was a rapid block of I_{Ca}, which was complete within 75 ms. This is shown in Fig. 1, where records of I_{Ca} obtained before and at photolysis are superimposed. Currents were recorded during 300 ms steps to 0 mV from a holding potential of −70 mV, after a 100 ms prepulse to −40 mV. the position of a flash can be identified by a brief electrical artifact, produced by the discharge of the flashlamp. In Fig. 1, the early parts of the transients

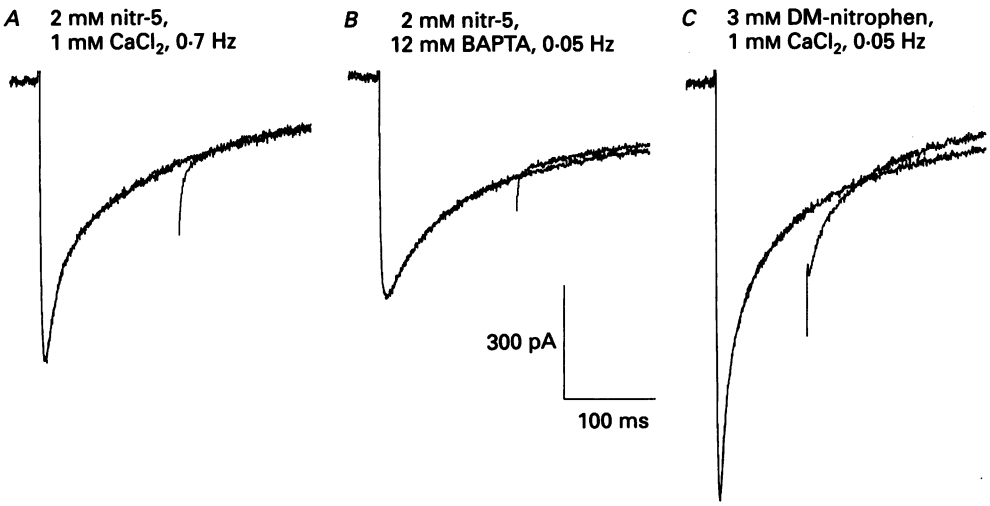

Fig. 1. Immediate effect of photolysis of intracellular nitr-5 on I_{Ca}. Cells were depolarized to 0 mV for 300 ms from a holding potential of −70 mV, following a 100 ms pre-pulse to −40 mV. After stabilization of the current and equilibration with nitr-5 (about 10 min), a flash was presented during the decay phase of I_{Ca}. The current during which the flash was presented is shown superimposed on the preceding current. The position of the flash is marked by a brief electrical artifact caused by the discharge of the flashlamp. *A*, pipette solution included 2 mM nitr-5 with 1 mM added $CaCl_2$; I_{Ca} was activated at 0·7 Hz. *B*, pipette contained 2 mM nitr-5 with 12 mM BAPTA and no added $CaCl_2$; I_{Ca} was activated at 0·05 Hz. *C*, pipette contained 3 mM DM-nitrophen with 1 mM added $CaCl_2$; I_{Ca} was activated at 0·05 Hz.

have been blanked for clarity. When the pre-flash $[Ca^{2+}]_i$ was varied by altering either the total Ca^{2+} or Ca^{2+} buffers in the pipette, or the interstimulus interval, the magnitude of the block was seen to vary. Figure 1*A* shows the effect of a light flash presented 150 ms after the onset of the test pulse, when 1 mM $CaCl_2$ was included in the pipette with nitr-5 (2 mM) and the stimulation frequency was 0·7 Hz. In this cell there was no measurable change in I_{Ca}; the mean block measured 100 ms after the flash (as the difference, in pA, between the pre- and post-flash currents), expressed as a percentage of the peak amplitude of I_{Ca} was $0{\cdot}7 \pm 0{\cdot}4\%$ ($n = 6$). With pipette contents identical to the experiments of Fig. 1*A*, but at a stimulation frequency of 0·05 Hz, the mean block was $1{\cdot}7 \pm 0{\cdot}3\%$ ($n = 25$), and when $CaCl_2$ was omitted from the pipette it was further increased to $2{\cdot}7 \pm 1{\cdot}1\%$ ($n = 6$). Figure 1*B* shows the effect of a flash presented at the same time (150 ms), when $[Ca^{2+}]_i$ was buffered to a lower

level, by including 12 mM BAPTA in the pipette with nitr-5 in the absence of added $CaCl_2$; the stimulation frequency was 0·05 Hz. A clear decrease in the inward current was apparent immediately after the flash, the mean block being 3·7 ± 0·4 % ($n = 4$). Thus, there was a significant ($P < 0·05$ by ANOVA (analysis of variance) comparing the four groups) trend showing increasing photoinduced block of I_{Ca} as the pre-flash

TABLE 1. Properties of the Ca^{2+}-induced potentiation with various levels of $[Ca^{2+}]_i$: the peak amplitudes of I_{Ca} are listed along with the measured maximum potentiation, the time constants (τ_P) and amplitudes (a_P) of the exponential fits to the rising phase of the mean potentiation for I_{Ca} and I_{Ba}

	Pipette contents (mM)					
Permeant ion	$CaCl_2$	BAPTA	I_{Ca} peak (pA)	τ_P (min)	a_P (%)	Maximum potentiation (%)
Ca^{2+}	1	—	567 ± 76 (12)	6·0	95 (12)	86 ± 12 (10)
Ca^{2+}	—	—	491 ± 118 (6)	2·7	87 (3)	85 ± 10 (3)
Ca^{2+}	—	12	642 ± 153 (5)	1·7	93 (3)	87 ± 15 (3)
Ba^{2+}	1	—	426 ± 75 (4)	1·9	37 (4)	45 ± 13 (4)

$[Ca^{2+}]_i$ was reduced. This was associated with a slowing of I_{Ca} decay, the half-time ($t_{\frac{1}{2}}$) ranging from 30 ± 4 ms ($n = 8$) with nitr-5 and $CaCl_2$ present at 0·7 Hz, to 42 ± 5 ms ($n = 6$) with internal BAPTA at 0·05 Hz ($P < 0·05$). The apparent sensitivity of the flash-induced block to $[Ca^{2+}]_i$ did not reflect differences in the peak I_{Ca} amplitude, since this did not vary significantly between the conditions (see Table 1). However, the magnitude of the block was dependent on the timing of the flash during the decay phase of I_{Ca}. In three cells stimulated at 0·05 Hz, with 1 mM added $CaCl_2$, a flash was applied 100 ms after I_{Ca} was triggered. The mean block was 3·3 ± 0·7 %, which is significantly different from the block when the flash was applied 50 ms later ($P < 0·05$). In two cells where the flash was presented 25 ms after I_{Ca} was triggered, the block was 7·3 and 5·1 %. The degree of block also varied with the amount of Ca^{2+} released from nitr-5, which was altered by varying the intensity of the flash (not shown). In agreement with Hadley & Lederer (1991), brighter flashes produced a greater degree of block. When pipettes contained nitr-5 (2 mM) with 1 mM $CaCl_2$, replacing the extracellular Ca^{2+} with Ba^{2+} resulted in a slowing of I_{Ca} decay ($t_{\frac{1}{2}} >$ 100 ms). In these conditions, a flash presented 150 ms after triggering I_{Ca} resulted in a rapid block of 1·4 ± 0·3 % ($n = 4$), which was similar to that observed with extracellular Ca^{2+}.

The currents shown in Fig. 1*C* were obtained when the caged Ca^{2+} compound DM-nitrophen (3 mM) was included in the pipette instead of nitr-5, with 1 mM added $CaCl_2$; the frequency of stimulation was 0·05 Hz. The immediate effect of the flash was to induce a rapidly activating inward current, with a peak distinct from the flash artifact. I_{Ca} became outward with respect to the pre-flash current approximately 75 ms after the flash, suggesting that a fast block of I_{Ca} similar to that seen after nitr-5 photolysis, was superimposed upon a transient inward current, which took more than 75 ms to decay. Photolysis of DM-nitrophen has been shown to activate a Na^+–Ca^{2+} exchange current in guinea-pig ventricular cells (Niggli & Lederer, 1990), which appears qualitatively similar to the inward current here. It seems likely that the rapid block was partly masked by a Na^+–Ca^{2+} exchange current activated by the

jump in $[Ca^{2+}]_i$, which would have been larger than in the nitr-5 experiments. In contrast to nitr-5, there was no increase in the amplitude of subsequent post-flash currents (see later); instead the current following each flash was slightly decreased. Qualitatively similar results were seen in four cells in the same conditions, and in a

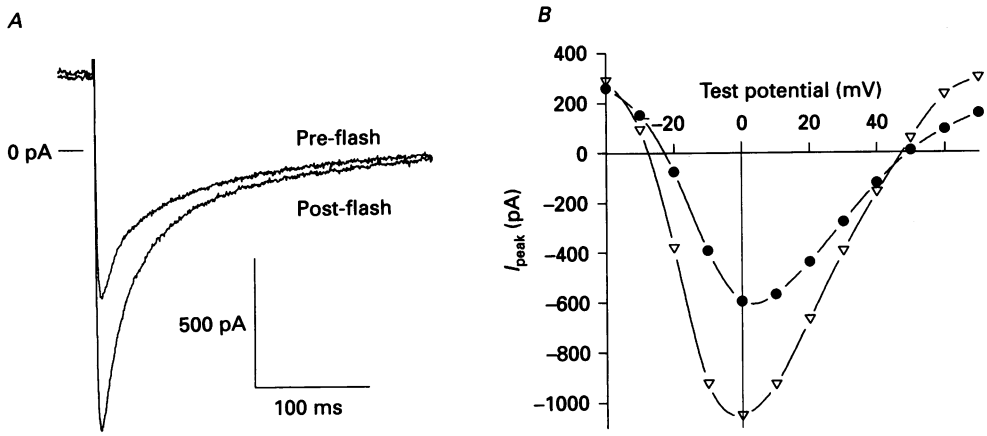

Fig. 2. Effect of photolysis of intracellular nitr-5 on peak I_{Ca}. The data shown are from one cell, recorded with a pipette containing 2 mM nitr-5 and 1 mM added $CaCl_2$. The stimulation frequency was 0·05 Hz. *A*, pre-flash I_{Ca} superimposed on maximally potentiated I_{Ca}. *B*, peak I_{Ca} plotted against test potential, before the flash (●) and at the peak of the potentiation (▽).

further four cells with added $CaCl_2$ increased to 2·7 mM. A fast block of I_{Ca} was also produced when pipettes contained 500 μM DM-nitrophen and only 12·5 μM added $CaCl_2$ ($n = 2$). However, when DM-nitrophen was reduced to 200 μM in an attempt to reduce Ca^{2+} buffering before photolysis and reduce the size of the Ca^{2+} jump, photolysis had no reproducible effect on I_{Ca}. In most experiments, 1 mM $MgCl_2$ was present in the pipettes; however, when it was omitted similar results were obtained.

I_{Ca} is potentiated by an increase in $[Ca^{2+}]_i$

The most prominent post-flash effect was a long-term potentiation of I_{Ca}, as previously described in frog atrial and guinea-pig ventricular cells (Gurney *et al.* 1989). Figure 2 shows results from one cell, in which the holding potential was −70 mV, the stimulation frequency was 0·05 Hz and there was 1 mM added $CaCl_2$ in the pipette solution. In this cell, I_{Ca} amplitude reached a maximum about 10 min after the flash, after which the current began to decline. Figure 2*A* shows a potentiated I_{Ca}, superimposed on the pre-flash current, recorded during voltage steps to 0 mV. Peak I_{Ca} was measured during steps to various potentials (after a 100 ms pre-pulse to −40 mV), both before the flash and when the potentiation had reached a maximum. Figure 2*B* shows peak I_{Ca} amplitude plotted against the test voltage at these times. I_{Ca} was enhanced at all potentials; the threshold for activation of the current and the apparent reversal potential did not appear to be altered after the

flash, but the voltage for peak activation was shifted slightly in the hyperpolarizing direction. In contrast to the effect on fast block of I_{Ca}, the timing of the flash during the decay of the current had no effect on the subsequent facilitation. Potentiation was also observed when photolysis was triggered 10 ms before the prepulse to −40 mV, although we have not yet examined responses to flashes triggered before I_{Ca} in detail. As found previously (Hadley & Lederer, 1991), the amplitude of the potentiation increased with increasing flash intensity, as more Ca^{2+} would have been released.

Light alone does not affect I_{Ca}

To exclude the possibility that flash-induced changes in I_{Ca} were due to direct effects of the high intensity light flashes, cells were flashed when pipette solution contained no nitr-5 or $CaCl_2$, but 1 mM EGTA or 15 mM BAPTA. No measurable effects on I_{Ca} were observed. It therefore seems likely that both the immediate block of I_{Ca} and the subsequent enhancement resulted directly or indirectly from a rapid increase in $[Ca^{2+}]_i$.

The effect of lowering $[Ca^{2+}]_i$ *on the photolysis-induced potentiation of* I_{Ca}

The time course of the potentiation was highly reproducible from cell to cell during the first several minutes after the flash. However, with time the response became more variable, I_{Ca} beginning to decline after varying periods of potentiation. This variability might represent differential dialysis of individual cells with the pipette solution and/or the onset of some process causing an inhibition or 'run-down' of I_{Ca}. Because of the variability, in each condition we only considered the period during which peak I_{Ca} was increasing in all the cells, when investigating the time course of the potentiation.

If the potentiation of I_{Ca} after photolysis was due to photoreleased Ca^{2+}, then under conditions where nitr-5 is not bound to Ca^{2+}, photolysis should produce no potentiation. Merely omitting $CaCl_2$ from the pipette did not block the response (Fig. 3*A*), presumably because Ca^{2+} entering the cell during I_{Ca} and Ca^{2+} from intracellular stores was available to the nitr-5. The Ca^{2+} chelator BAPTA was therefore included in pipettes at a concentration of 12 mM, with nitr-5, in an attempt to block the potentiation by further lowering the bulk $[Ca^{2+}]_i$ and by preventing intracellular diffusion of Ca^{2+} that had entered through the Ca^{2+} channels. As shown in Fig. 3, which illustrates the results of these alterations of $[Ca^{2+}]_i$, potentiation was not prevented although its time course was altered. With 1 mM $CaCl_2$, 0 $CaCl_2$ or 12 mM BAPTA added to the pipette solution, potentiation of I_{Ca} was well fitted by a single exponential (Fig. 3*A*). The time constant (τ_P) and the maximum potentiation (a_P) given by the fit to eqn (1) (see Fig. 3), are summarized in Table 1 for each condition, along with the pre-flash peak I_{Ca} (expressed as the mean peak I_{Ca} over the 2 min prior to the flash for 0·05 Hz stimulation and over 1 min before the flash for currents elicited at 0·7 Hz) and the measured maximum increases in I_{Ca} amplitude for those cells where a clear maximum was reached. There is good agreement between the derived and measured amplitudes. In contrast to what might be expected, the speed of the potentiation was reduced significantly when 1 mM $CaCl_2$ was added to the 2 mM nitr-5 in the pipette, and increased when 12 mM BAPTA was added instead,

with little change in the maximum potentiation in either case. A similar result was obtained when 25 mM EGTA replaced BAPTA in the pipette solution. Thus, it appears that a reduced bulk $[Ca^{2+}]_i$ results in accelerated potentiation of I_{Ca}.

The effect of removing extracellular calcium on the potentiation of I_{Ca}

At low levels of $[Ca^{2+}]_i$, I_{Ca} would be the major source of Ca^{2+} available to intracellular Ca^{2+} chelators but, even at higher resting $[Ca^{2+}]_i$ I_{Ca} may provide a

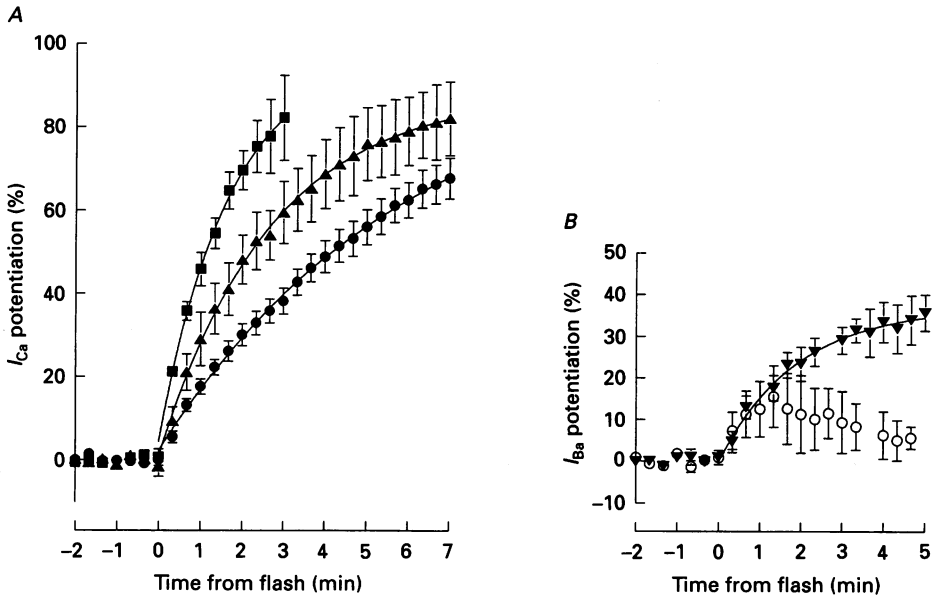

Fig. 3. Photolysis-induced potentiation of peak I_{Ca} and I_{Ba} activated at 0·05 Hz, with various levels of $[Ca^{2+}]_i$. current potentiation, expressed as a percentage of the mean I_{Ca} or I_{Ba} recorded during the 2 min prior to the flash, is plotted against the time after presentation of a flash. The fitted curves are described by:

$$P = a_P\left[1-\exp\left(\frac{-t}{\tau_P}\right)\right]+z, \quad (1)$$

where P is the potentiation at time t, a_P and τ_P are the amplitude and the time constant of the fitted curve, and z is the zero intercept. Values of a_P and τ_P are listed in Table 2. The data were fitted from the first post-flash current. *A*, potentiation of I_{Ca} when pipettes contained 2 mM nitr-5 with 1 mM added $CaCl_2$ (●), no added $CaCl_2$ (▲) or 12 mM added BAPTA (■). *B*, potentiation of I_{Ba} when pipettes contained 2 mM nitr-5 with 1 mM added $CaCl_2$ (▼), or in Ca^{2+}-depleted cells with no added $CaCl_2$ (○).

significant fraction of the Ca^{2+} bound by nitr-5. To determine the contribution of Ca^{2+} carried into the cell via I_{Ca} to the post-flash potentiation, external Ca^{2+} was replaced by equimolar Ba^{2+} prior to recording. The effect of photolysis on I_{Ba} amplitude, when the pipette contained nitr-5 with 1 mM $CaCl_2$, is shown in Fig. 3*B*, with the results summarized in Table 1. In the presence of extracellular Ba^{2+}, the mean post-flash potentiation of the current was reduced from 86 to 45%, but the time course of the potentiation was similar to that seen for I_{Ca} when 12 mM BAPTA

replaced $CaCl_2$ in the pipette. Thus, Ca^{2+} entering the cell during I_{Ca} contributed significantly to the flash-induced potentiation.

Suppression of potentiation of I_{Ba} in calcium-depleted cells

Since neither depletion of $[Ca^{2+}]_i$ nor removal of $[Ca^{2+}]_o$ by themselves completely blocked the flash-induced potentiation, we depleted Ca^{2+} on both sides of the membrane in an attempt to abolish the response. In order to prevent Ca^{2+} accumulation by the SR or other intracellular organelles, cells were transferred directly from the nominally Ca^{2+}-free medium, in which they were stored for 2–12 h after isolation, to Ba^{2+}-containing recording solution. Ca^{2+} channel currents were recorded from these cells, using pipettes containing nitr-5 but no added $CaCl_2$. The effect of a flash is shown in Fig. 3*B* along with the response of I_{Ba} when the pipette solution did contain added $CaCl_2$. The potentiation of I_{Ba} was largely blocked, although the size of the residual response was highly variable. The mean response initially followed a similar time course to the potentiation of I_{Ba} in cells that were not Ca^{2+}-depleted, but it was not maintained. The current reached a maximum within 1·5 min after the flash and returned to the pre-flash level over the next 3 min.

Dependence of potentiation on the rate of activation of I_{Ca}

The frequency at which I_{Ca} is activated affects the amplitude and time course of the current (Fedida *et al.* 1988*a*; Zygmunt & Maylie, 1990) and may also influence the $[Ca^{2+}]$ in various intracellular compartments. Figure 4 shows the mean flash-induced potentiation of I_{Ca} at frequencies of 0·7 and 0·05 Hz, when pipettes contained nitr-5 with 1 mM added $CaCl_2$. The maximum potentiation was similar for both frequencies (Table 2). However, during the first minute after photolysis, I_{Ca} was more rapidly potentiated at the higher frequency. The time course of the mean potentiation at 0·7 Hz was best fitted by two exponentials, as described by eqn (2) (Fig. 4), with time constants of 0·3 and 4·5 min, which contributed 20 and 80 % respectively to the total potentiation (Table 2). Flash-induced potentiation was also measured with stimulation frequencies of 0·1 and 0·2 Hz. In both cases, the time course of the mean potentiation could be well fitted by two exponentials with the same time constants as those measured at 0·7 Hz. To ease comparison of the relative contributions of the two components in each condition, the time constants were fixed during the fitting procedure at $\tau_{P1} = 0{\cdot}3$ min and $\tau_{P2} = 4{\cdot}5$ min, with the amplitudes a_{P1} and a_{P2} allowed to vary to give the best fit to eqn (2). The relative contribution of the fast component was calculated from the amplitude values giving the best fit. As shown in Table 2, the relative contribution of each component to the total potentiation varied with the stimulation frequency, although the maximum potentiation was constant. As the frequency was decreased, the contribution of the fast component became smaller and, at 0·05 Hz, the lowest frequency tested, the potentiation was adequately fitted by a single exponential as already described.

Effect of depletion of sarcoplasmic reticulum storage sites

To test whether or not the potentiation was mediated by Ca^{2+}-induced Ca^{2+} release from the SR, triggered by the small quantity of photoreleased Ca^{2+}, SR Ca^{2+} stores were depleted before photolysis of nitr-5, by inclusion of 1 μM ryanodine in the

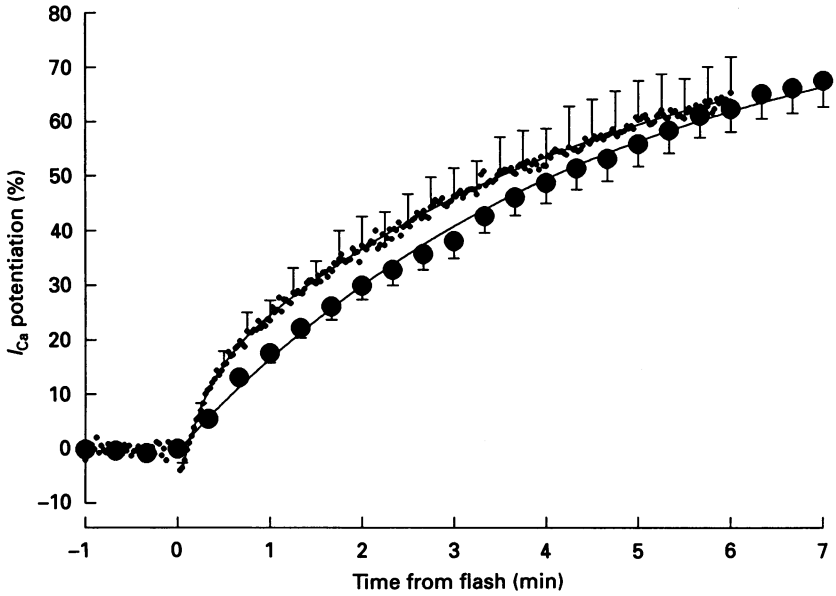

Fig. 4. Photolysis-induced potentiation of I_{Ca} when currents were activated at 0·05 (●) and 0·7 Hz (•). Peak I_{Ca} potentiation, expressed as a percentage of the pre-flash peak I_{Ca}, plotted against time after presentation of a flash. The curves are fitted to two exponentials, described by:

$$P = a_{P1}\left[1-\exp\left(\frac{-t}{\tau_{P1}}\right)\right]+a_{P2}\left[1-\exp\left(\frac{-t}{\tau_{P2}}\right)\right]+z, \quad (2)$$

where P and t are defined as for eqn (1), τ_{P1} and τ_{P2} are the time constants of the two components of potentiation, with a_{P1} and a_{P2} their respective amplitudes. The data were fitted from the first post-flash current for 0·05 Hz and the third post-flash current for 0·7 Hz. The best fit to the data at 0·7 Hz was found with $a_{P1} = 18\%$, $\tau_{P1} = 0{\cdot}3$ min, $a_{P2} = 73\%$, $\tau_{P2} = 4{\cdot}5$ min. The data at 0·05 Hz is fitted with $a_{P1} = 0$, $a_{P2} = 95\%$ and $\tau_{P2} = 4{\cdot}5$ min.

TABLE 2. Frequency dependence of the Ca^{2+}-induced potentiation: the maximum potentiation and the relative contribution of the fast component to the total response are listed

Stimulation frequency (Hz)	$\frac{a_{P1}\times 100}{a_{P1}+a_{P2}}$	Maximum potentiation (%)
0·05	0	86±12 (10)
0·1	9	60±7 (3)
0·2	17	87±16 (4)
0·7	20	80±7 (4)

pipette solution. At a stimulation frequency of 0·05 Hz, with 1 mM $CaCl_2$ in the pipette, the measured flash-induced potentiation of I_{Ca} (78±15%, $n = 4$) was of a similar magnitude to that seen in cells with a functional SR. The mean data were best fitted by a single exponential with $\tau_P = 3{\cdot}9$ min. At a stimulation frequency of 0·7 Hz, the mean data from three cells were best fitted by two exponentials with $\tau_{P1} = 0{\cdot}4$ min and $\tau_{P2} = 3{\cdot}1$ min, the faster component comprising 37% of the total

potentiation. Ryanodine therefore slightly accelerated the slow component of the flash-induced potentiation at both stimulation frequencies, with a slight increase in the contribution of the fast component at high frequencies. It is not clear if these effects are significant, but it is clear that ryanodine did not block either component of potentiation.

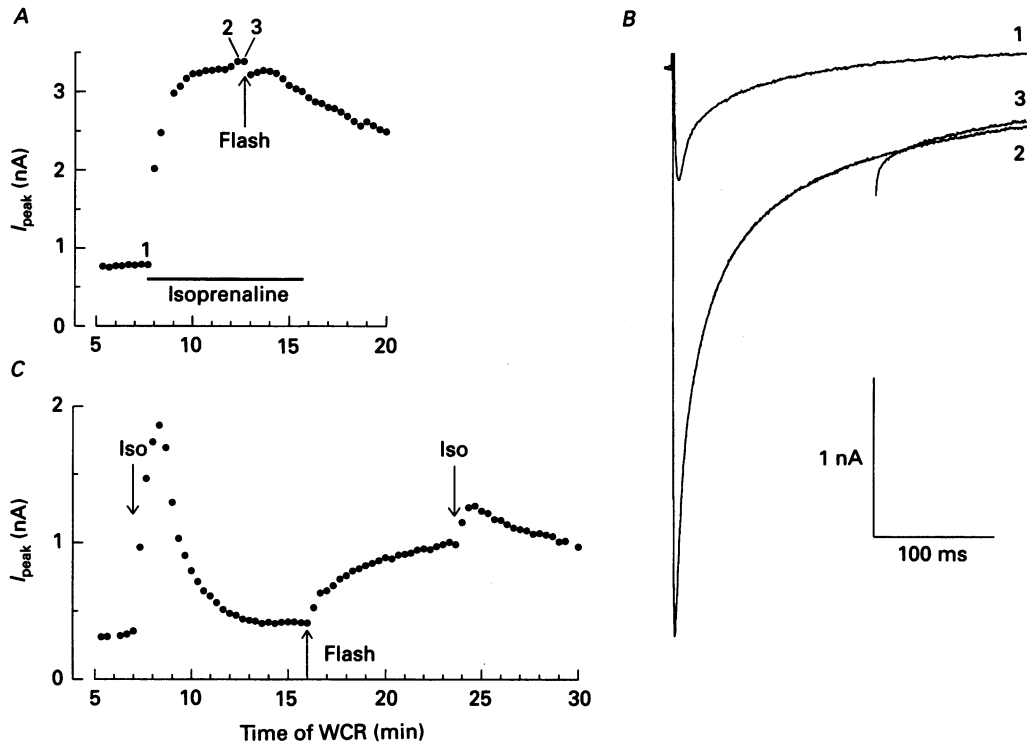

Fig. 5. Interactions between isoprenaline and nitr-5 photolysis. *A*, peak I_{Ca} plotted against the time from the onset of whole-cell recording (WCR). Application of 10 μM isoprenaline is indicated by the bar, and the flash by the arrow. *B*, currents recorded at times indicated in Fig. 5*A* from the same cell, before application of isoprenaline (trace 1), and during maximal isoprenaline-induced potentiation (traces 2 and 3). During the current recording shown in trace 3, a flash was presented. *C*, peak I_{Ca} plotted as in *A*. The flash is indicated by the upright arrow and 300 ms applications of 10 μM isoprenaline (Iso) by inverted arrows.

Combined effects of β-adrenergic stimulation and nitr-5 photolysis

It has been suggested that Ca^{2+}-induced potentiation of I_{Ca} in frog atrial cells occurs through phosphorylation of the Ca^{2+} channels (Gurney *et al.* 1989; Charnet *et al.* 1991). To test whether this is the case in guinea-pig ventricular cells, Ca^{2+}-bound nitr-5 (1 mM added $CaCl_2$) was photolysed either before or after the β-adrenergic agonist isoprenaline (10 μM) was applied by pressure ejection from a pipette placed approximately 300 μm from the cell. Figure 5 illustrates interactions between the effects of isoprenaline and nitr-5 photolysis. When a flash was given during continuous application of this supramaximal concentration of isoprenaline

(Fig. 5*A* and *B*), the immediate effect was a rapid block of I_{Ca} (Fig. 5*B*) which did not differ significantly from that observed in the absence of isoprenaline (3·0 ± 1·9 %, $n = 4$). This suggests that the block was not due to an alteration in the phosphorylation state of the L-type Ca^{2+} channels. However, the potentiating effect of the flash *was* blocked (Fig. 5*A*), with similar results obtained from all four cells

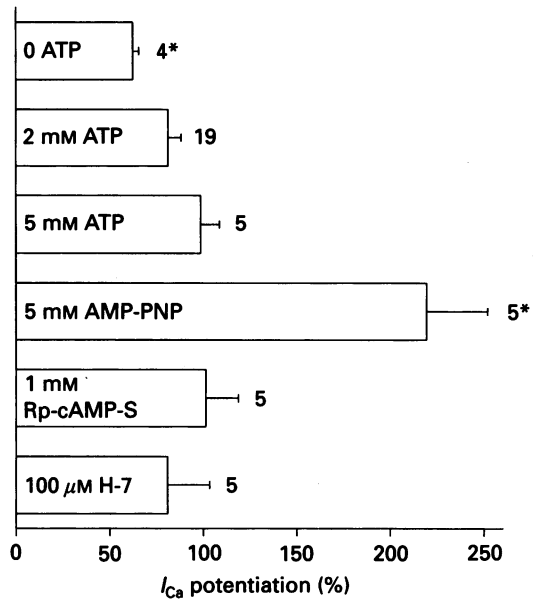

Fig. 6. Ca^{2+}-dependent potentiation of I_{Ca} with different concentrations of intracellular ATP and when phosphorylation was suppressed. The concentrations of ATP and drugs shown in the bars indicate the concentrations included in the pipette-filling solution. For the experiments with Rp-cAMP-S and H-7, 2 mM ATP was included in the pipette solution. The number of cells in each treatment is shown to the right of the bars. Asterisks indicate a significant difference at the 1 % probability level from the potentiation with 5 mM ATP.

tested. Figure 5*C* illustrates the effect of isoprenaline after flash-induced I_{Ca} potentiation. First, isoprenaline (10 μM) was applied briefly (300 ms), then after complete recovery of I_{Ca} a flash was given, followed 8 min later by a second, identical application of isoprenaline. The second isoprenaline-induced increase in the current was greatly attenuated. In fact, the initial response to isoprenaline was larger than the combined effects of nitr-5 photolysis and isoprenaline, suggesting differences in the mechanisms underlying the two responses. Similar results were obtained from three cells. In several other cells, repeated applications of isoprenaline induced reproducible responses in the absence of nitr-5 photolysis.

Phosphorylation does not mediate the photolysis-induced potentiation of I_{Ca}

In order to test whether or not cAMP-dependent phosphorylation was involved in the Ca^{2+}-dependent potentiation of I_{Ca}, we included Rp-cAMP-S (1 mM), a competitive inhibitor of protein kinase A (Botelho, Rothermel, Coombs & Jasorff,

1988), in the pipette solution containing nitr-5 and 1 mM added $CaCl_2$. At that concentration, Rp-cAMP-S has been shown to block the stimulation of I_{Ca} by isoprenaline in frog cardiac cells by 98% (Hartzell, Méry, Fischmeister & Szabo, 1991). The results of these experiments are summarized in Fig. 6. The presence of Rp-cAMP-S did not significantly alter the magnitude of the flash-induced potentiation of I_{Ca}, suggesting that cAMP-dependent phosphorylation was not necessary for the potentiation to occur. The broad-spectrum protein kinase inhibitor H-7 (Hidaka, Inagaki, Kawamoto & Sasaki, 1984) was used to test whether phosphorylation by a different protein kinase was involved in the flash-induced potentiation. At 100 μM, H-7 in the pipette solution also had no significant effect on the post-photolysis potentiation of I_{Ca} (see Fig. 6), although it largely suppressed the response to isoprenaline (not shown).

In order to determine whether an H-7-resistant kinase might be involved in the Ca^{2+}-dependent stimulation of I_{Ca}, we first examined the effect of varying the ATP concentration in the pipette solution, which also contained 2 mM nitr-5 and 1 mM $CaCl_2$. As can be seen in Fig. 6, photorelease of Ca^{2+} produced a larger potentiation of I_{Ca} when ATP was present then when it was omitted from the pipette. This suggests that the Ca^{2+}-induced potentiation is ATP dependent, although since simply omitting ATP from the pipette solution would not deplete ATP, we cannot be sure if there is an essential requirement for the nucleotide. We next attempted to suppress all types of phosphorylation by replacing ATP in the pipette-filling solution with the non-hydrolysable analogue AMP-PNP. The concentration used was 5 mM, which blocked approximately 80% of the response to a supramaximal dose of isoprenaline (data not shown). The pipette solution also contained 1 mM $CaCl_2$. In the presence of AMP-PNP, the flash-induced potentiation of 220% was significantly larger than that observed in the presence of ATP (Fig. 6). This implies that protein phosphorylation does not mediate the potentiation of I_{Ca} by a rapid rise in $[Ca^{2+}]_i$. The response does, however, appear to depend on the presence of nucleotides, AMP-PNP being more effective than ATP.

DISCUSSION

As found previously, a sudden increase in $[Ca^{2+}]_i$, produced by photolysis of nitr-5, triggered a rapid inhibition of I_{Ca} followed by a slowly developing augmentation of the current. Both responses were altered by varying the $[Ca^{2+}]_i$ before photolysis or the amplitude of the Ca^{2+} step, confirming that both responses were initiated by Ca^{2+}. However, the $[Ca^{2+}]_i$ was not fully controlled by nitr-5. Replacing the extracellular Ca^{2+} with Ba^{2+} reduced the potentiation response to photolysis by 40%, implying that Ca^{2+} entering the cell from the outside was bound by nitr-5 before photolysis and contributed to the response. This helps to explain why the photoinduced modulation of I_{Ca} was not prevented by 12 mM BAPTA. Although at this concentration, BAPTA should buffer Ca^{2+} well in the bulk cytoplasm, it may be less effective close to Ca^{2+} channels during Ca^{2+} entry (Stern, 1992). Thus we propose that with extracellular Ca^{2+} and with BAPTA in the pipette, photolysis of nitr-5 selectively raised the $[Ca^{2+}]$ at the inner membrane surface, close to the Ca^{2+} channel

pore. In contrast, with Ba^{2+} as the permeant ion, photolysis of nitr-5 would presumably raise $[Ca^{2+}]_i$ uniformly throughout the cell. In this condition the $[Ca^{2+}]$ jump near the channels would be closest to that predicted from the calibration experiments (85 nM). With extracellular Ca^{2+} and with $CaCl_2$ added to the pipette (no BAPTA), $[Ca^{2+}]_i$ would also rise throughout the cell, but larger increases may occur next to the membrane. Such a non-uniform distribution of photoreleased Ca^{2+} has been observed with nitr-5 in neurones (Marrion, Zucker, Marsh & Adams, 1991). Non-uniform release could also result from Ca^{2+}-induced Ca^{2+} release from the SR, which is well developed in guinea-pig ventricular cells, but not in frog where adding BAPTA to the pipette *could* block the photoinduced potentiation of I_{Ca} (Gurney *et al.* 1989). Also, Ca^{2+} release from the SR mediates the contractile response of guinea-pig ventricle to nitr-5 photolysis (Valdeolmillos *et al.* 1989; Näbauer & Morad, 1990; Niggli & Lederer, 1990). However, the flash-induced potentiation was little affected by ryanodine at concentrations used previously to prevent Ca^{2+}-induced Ca^{2+} release in this preparation (e.g. Zygmunt & Maylie, 1990). Ryanodine did not block the potentiation even at a stimulation frequency of 0·7 Hz, when SR stores should be well filled (Bers, 1991).

As noted previously (Gurney *et al.* 1987), the cell may provide additional sources of Ca^{2+}, because small, but significant responses to nitr-5 photolysis were observed even when Ba^{2+} carried the current through the Ca^{2+} channels and $CaCl_2$ was omitted from the pipette. Responses were sometimes observed even in cells depleted of Ca^{2+} by pre-incubation in a Ca^{2+}-free medium. These were unlikely to be an artifact of photolysis, because flashes had no effect in the absence of photolabile chelators and full blockade of the response was observed in some Ca^{2+}-depleted cells. We considered the possibility that in the absence of added $CaCl_2$, nitr-5 might bind and release Mg^{2+} upon photolysis, because Mg^{2+} appears to potentiate I_{Ca} when Ca^{2+} buffering is high (O'Rourke, Backx & Marban, 1992). However, since nitr-5 has a low affinity for Mg^{2+} (K_D = 8 mM) and shows no loss of affinity upon photolysis (Adams *et al.* 1988), it is more likely that the observed responses resulted from Ca^{2+} release, and that the cells contain Ca^{2+} stores that are not easily depleted but are accessible to nitr-5.

Photolysis of nitr-5 results in a biphasic modulation of I_{Ca}, whereas photorelease of Ca^{2+} from DM-nitrophen primarily causes inhibition (Hadley & Lederer, 1991). The explanation for their qualitatively different effects presumably lies in their very different Ca^{2+} and Mg^{2+} binding properties. Several observations confirm that larger $[Ca^{2+}]_i$ transients were produced with DM-nitrophen. For instance, flashes often contracted cells dialysed with DM-nitrophen, whereas nitr-5-loaded cells usually remained relaxed. Furthermore, photolysis of DM-nitrophen, but not nitr-5, was frequently associated with a transient inward current, with the properties expected of the Na^+–Ca^{2+} exchange current (Niggli & Lederer, 1990). The chelators also differed in that nitr-5 (2 mM), but not DM-nitrophen (3 mM), suppressed the depolarization-induced contractions. This is despite the 30-fold higher Ca^{2+} affinity of DM-nitrophen (Kaplan & Ellis-Davies, 1988). Näbauer & Morad (1990) explained this property of DM-nitrophen by hindered diffusion into the cell. However, since the DM-nitrophen and nitr-5 molecules are of comparable size, a more likely explanation is that Ca^{2+} binding by DM-nitrophen is slow compared to nitr-5, allowing Ca^{2+} entry

through the Ca^{2+} channels to trigger Ca^{2+} release from the SR and contraction. This is further suggested by the rapid decay of I_{Ca} observed in DM-nitrophen-loaded cells. Although Näbauer & Morad rejected this hypothesis because EDTA, from which DM-nitrophen is derived, did suppress contraction, it is not unreasonable to suppose that the dimethoxynitrophenyl moiety in DM-nitrophen (Kaplan & Ellis-Davies, 1988) might slow its binding kinetics.

While variance in the magnitudes and time courses of the $[Ca^{2+}]_i$ transients induced by photolysis may explain the distinct responses with nitr-5 and DM-nitrophen (Hadley & Lederer, 1991), it is clear from the present study that differences in the pre-flash $[Ca^{2+}]_i$ could be just as important. With nitr-5, the inhibitory response was enhanced by lowering the pre-flash $[Ca^{2+}]$; the higher Ca^{2+} affinity of DM-nitrophen means that it would buffer $[Ca^{2+}]_i$ to lower levels than nitr-5 before photolysis. Intracellular Mg^{2+} or MgATP could also play a role; Mg^{2+} was omitted from the intracellular solutions used by Hadley & Lederer (1991) to study Ca^{2+}-induced inactivation with DM-nitrophen. However, we found that following DM-nitrophen photolysis, inhibition was the dominant effect whether or not Mg^{2+} was present.

Ca^{2+}-induced inactivation of I_{Ca}

The rapid block of I_{Ca} produced by photolysis of caged Ca^{2+} molecules increases with the magnitude of the Ca^{2+} jump (see also Hadley & Lederer, 1991), but varies inversely with the $[Ca^{2+}]_i$ present before photolysis. When $[Ca^{2+}]_i$ was raised by including $CaCl_2$ in the pipette and increasing the stimulation frequency, I_{Ca} decayed more rapidly and post-photolysis block was minimal. This suggests that Ca^{2+}-dependent inactivation was already substantial at the time of the flash. Conversely, when $[Ca^{2+}]_i$ was lowered with BAPTA, I_{Ca} decay was slowed and flashes caused significantly more block, consistent with more non-inactivated channels being available to respond to the Ca^{2+} jump. That flashes produced a similar degree of block when Ba^{2+} or Ca^{2+} carried the current is not at variance with this. Ca^{2+}-induced inactivation contributes little to the decay of I_{Ba}, but as discussed above, replacing the extracellular Ca^{2+} with Ba^{2+} probably reduced the amount of Ca^{2+} photoreleased next to the membrane. Alternatively, by modifying the channels in some other way before the flash (e.g. through phosphorylation), Ca^{2+} may make the channels less susceptible to a further increase in $[Ca^{2+}]_i$. This is consistent with the pronounced block resulting from DM-nitrophen photolysis, despite the relatively rapid decay of I_{Ca} in DM-nitrophen-loaded cells.

Whether Ca^{2+} was photoreleased from nitr-5 or DM-nitrophen, block of I_{Ca} was fully developed within 75 ms. This block has been proposed to reflect Ca^{2+}-dependent dephosphorylation of the Ca^{2+} channels, the rapidity of the response being explained by a Ca^{2+}-dependent phosphatase located near to the channel mouth (Hadley & Lederer, 1991). An alternative mechanism put forward by the same authors involves a direct action of Ca^{2+} on the channels, the effect being regulated by their phosphorylation state. The evidence for the involvement of channel phosphorylation is that the β-adrenergic agonist, isoprenaline, blocked the response. Contrary to this, the block caused by photolysis of nitr-5 was unaffected by a supramaximal concentration of isoprenaline, implying that even maximally phosphorylated channels are susceptible to Ca^{2+}-dependent inactivation. The discrepancy between

the results of our study and that of Hadley and Lederer, probably reflects the different experimental approaches taken. In the present study, flashes were presented during I_{Ca} decay, both in the absence and presence of isoprenaline and without excess Ca^{2+} loading of the cells. Hadley and Lederer removed external Na^+ to prevent Ca^{2+} extrusion via the Na^+–Ca^{2+} exchanger, resulting in a $[Ca^{2+}]_i$ jump that was large enough to irreversibly contract the cell, and was therefore much larger than in the present study. DM-nitrophen was photolysed 20 ms before activating I_{Ca}; in the presence of isoprenaline, the effect of photolysis was measured 30 s after the flash, although I_{Ca} was shown to have recovered within 30 s under control conditions (Na^+ present). Since isoprenaline did not prevent the Ca^{2+}-induced block of I_{Ca} with nitr-5, we favour a model in which Ca^{2+} interacts directly with the Ca^{2+} channel rather than involving intracellular messengers. The magnitude of the block caused by photolysis was larger when flashes were presented near the peak of I_{Ca}, as opposed to later during the decay phase of the current. The simplest mechanism that can account for this is one in which Ca^{2+} binds to a site on the open channel to block permeation.

The Ca^{2+}-induced potentiation

Photolysis of intracellular nitr-5 initiates a slow potentiation of I_{Ca} which superficially resembles that seen in frog atrial cells (Gurney *et al.* 1989), although there are important differences. In frog, the response was fully reversible and reproducible in individual cells, whereas in guinea-pig, I_{Ca} did not recover from a maximal potentiation within the time course of an experiment, and a second flash invariably led to inactivation of I_{Ca} without further potentiation. The results presented represent the responses to the first flash exposure.

The amplitude of the potentiation varied with the flash intensity and hence the size of the $[Ca^{2+}]_i$ jump (see also Hadley & Lederer, 1991). Thus the fact that varying the pre-flash $[Ca^{2+}]_i$ had no effect on the amplitude of the potentiation implies that, in each condition, a similar amount of Ca^{2+} was released at the receptor site responsible for the potentiation. This requires that the fraction of nitr-5 complexed with Ca^{2+} before photolysis was comparable in each case. Since Ca^{2+} entry into the cell strongly influenced the response, the proportion of nitr-5 complexed with Ca^{2+} close to the membrane may be largely determined by Ca^{2+} entry. Assuming binding kinetics similar to the related molecule fura-2, binding between Ca^{2+} and nitr-5 or BAPTA should be close to equilibrium in 0·5 μs (Adler, Augustine, Duffy & Charlton, 1991). Thus Ca^{2+} entering during I_{Ca} would have equilibrated with nitr-5 before the flash was presented. Both nitr-5 and BAPTA become saturated above a free Ca^{2+} concentration of 1 μM, when a flash could release several micromolar Ca^{2+}. Close to open Ca^{2+} channels, $[Ca^{2+}]_i$ may reach hundreds of micromolar or more (Fogelson & Zucker, 1985; Simon & Llinás, 1985; Smith & Augustine, 1988), even in the presence of high intracellular concentrations of BAPTA (Stern, 1992). Such high $[Ca^{2+}]_i$ may also be reached at neurotransmitter release sites, 60 nm from the channels (Adler *et al.* 1991). To determine at what distance from the inner mouth of a Ca^{2+} channel nitr-5 would become saturated by Ca^{2+} entering through the channel, we applied the model of Stern (1992), which describes Ca^{2+} buffering in the vicinity of a channel pore, to the conditions of our experiments. The intracellular diffusion of nitr-5 and BAPTA and the kinetics of Ca^{2+} binding were assumed to have values similar to

those for fura-2 in mammalian cells. That is, the diffusion coefficient was set at 5×10^{-10} m^2 s^{-1} (Pusch & Neher, 1988) and the forward rate constant for Ca^{2+} binding was assumed to be 5×10^8 M^{-1} s^{-1} (Jackson, Timmerman, Bagshaw & Ashley, 1987). A Ca^{2+} channel current of 0·04 pA, as predicted from the data of Hess, Lansman & Tsien (1986) for 1 mM extracellular Ca^{2+}, was also assumed. These values predict that 2 mM nitr-5 would be saturated 12 nm from the channel pore, even with 12 mM BAPTA present. This is only a rough estimate, since the calculation relies on untested assumptions. Nevertheless, it strongly suggests that the Ca^{2+} binding site that triggers I_{Ca} potentiation may be very close to the Ca^{2+} channel.

The onset of the potentiation was slowed if $[Ca^{2+}]_i$ was increased and accelerated if $[Ca^{2+}]_i$ was lowered. BAPTA and EGTA accelerated the response by lowering the pre-flash $[Ca^{2+}]_i$, rather than by increasing the Ca^{2+}-buffering capacity of the cell, because simply omitting Ca^{2+} from the pipette had a similar, although less pronounced effect. This result also precludes an effect due to light sensitivity of the Ca–BAPTA complex, which has been suggested previously (Noel & Capiod, 1991) but was not apparent in the present study. The mechanism by which a reduced $[Ca^{2+}]_i$ might accelerate the potentiation is not clear. Since low $[Ca^{2+}]_i$ also enhanced the blocking effect of photolysis, it may modify the channels to make them more susceptible to the Ca^{2+} jump. The faster response may also be a direct result of the reduced Ca^{2+}-dependent inactivation present before the flash. The simplest model that can account for the acceleration, both in extracellular Ba^{2+} and with reduced $[Ca^{2+}]_i$, includes two Ca^{2+} binding sites. One site located close to the channel would trigger the potentiation when activated by Ca^{2+}. The second site would be inhibitory, such that when occupied by Ca^{2+}, it would slow the response to a subsequent increase in $[Ca^{2+}]_i$.

Frequency dependence of the potentiation

Most experiments employed low frequency stimulation (0·05 Hz). When flashes were presented during higher frequency stimulation, I_{Ca} potentiation was accelerated and became biphasic. The first component developed about ten times faster than the potentiation at 0·05 Hz and became more pronounced as the frequency was increased, comprising $\sim 20\%$ of the response at the highest frequency tested (0·7 Hz). Greater Ca^{2+} influx at higher frequencies cannot explain the accelerated potentiation, because elevating the pre-flash $[Ca^{2+}]_i$ was shown to slow the potentiation. It could, however, reflect a use-dependent or voltage-dependent effect of Ca^{2+}, although more experiments are required to determine which. The faster component developed over a time course similar to the Ca^{2+}-induced I_{Ca} potentiation in frog (Gurney *et al.* 1989). It is therefore possible that the faster component corresponds to the response in frog, and that the slow potentiation is a different process. Unfortunately, we could not ascertain if, as in the frog, the fast component was transient, because it superimposed on the slow response. Whatever the mechanism, the frequency dependence is of interest in relation to the stimulation-dependent facilitation of I_{Ca} that is characteristic of guinea-pig ventricle (Fedida *et al.* 1988*a*, *b*; Zygmunt & Maylie, 1990). The flash-induced potentiation during low frequency stimulation develops too slowly to account for it, but it could reflect the accelerated response to Ca^{2+} at higher frequencies.

Mechanism of Ca^{2+}-induced I_{Ca} potentiation

The flash-induced potentiation was sensitive to the intracellular ATP concentration and was suppressed in the presence of a supramaximal concentration of isoprenaline. Similar observations in frog atrial cells were suggested to reflect an involvement of Ca^{2+}-dependent phosphorylation (Gurney *et al.* 1989). However, new observations argue against phosphorylation as a mediator of the response. Although the response to isoprenaline was suppressed after I_{Ca} had been potentiated by nitr-5 photolysis, the combined effects of isoprenaline and Ca^{2+} were smaller than the effect of isoprenaline alone, implying differences in their modes of action. The response to Ca^{2+} does not involve cAMP-dependent phosphorylation, because the increase in I_{Ca} was not blocked by either Rp-cAMP-S, a competitive inhibitor of protein kinase A (Botelho *et al.* 1988), or H-7, a broad-spectrum kinase inhibitor (Hidaka *et al.* 1984), at concentrations that suppressed the response to isoprenaline. Experiments with AMP-PNP, which prevents transfer of the γ-phosphate of the nucleotide required for phosphorylation (Yount, Babcock, Ballantyne & Ojala, 1971), also rule against this mechanism. Rather than blocking the flash-induced potentiation, intracellular AMP-PNP enhanced the response. This suggests that rather than acting through phosphorylation, the Ca–nucleotide complex directly modulates I_{Ca} through a phosphorylation-independent mechanism. The Mg–nucleotide complex was also reported recently to directly modulate I_{Ca} in guinea-pig ventricular cells (O'Rourke *et al.* 1992). This was shown using photolytic release of Mg^{2+} from caged Mg^{2+} in a similar way to the present experiments. Perhaps the Ca^{2+} and Mg^{2+} complexes act at the same site, possibly through an allosteric interaction as suggested by O'Rourke *et al.* (1992). It should be possible to determine this by comparing the ability of different nucleotides to support the photoinduced potentiation of I_{Ca} in the presence of caged Mg^{2+} or caged Ca^{2+}.

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REFERENCES

ADAMS, S. R., KAO, J. P. Y., GRYNKIEWICZ, G., MINTA, A. & TSIEN, R. Y. (1988). Biologically useful chelators that release Ca^{2+} upon illumination. *Journal of the American Chemical Society* **110**, 3212–3220.

ADLER, E. M., AUGUSTINE, G. J., DUFFY, S. N. & CHARLTON, M. P. (1991). Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *Journal of Neuroscience* **11**, 1496–1507.

BATES, S. E. & GURNEY, A. M. (1992*a*). Modulation of L-type calcium current in mammalian ventricular myocytes by photolysis of caged calcium. *Advances in Experimental and Medical Biology* **311**, 385–386.

BATES, S. E. & GURNEY, A. M. (1992*b*). Modulation of L-type calcium current in guinea-pig ventricular myocytes by photolysis of caged calcium. *Journal of Physiology* **446**, 553*P*.

BECHEM, M. & POTT, L. (1985). Removal of Ca current inactivation in dialysed guinea-pig atrial cardioballs by Ca chelators. *Pflügers Archiv* **404**, 10–20.

BELLES, B., MALÉCOT, C. O., HESCHELER, J. & TRAUTWEIN, W. (1988). 'Run-down' of the Ca current during long whole-cell recordings in guinea-pig heart cells: role of phosphorylation and intracellular calcium. *Pflügers Archiv* **411**, 353–360.

BERS, D. M. (1991). *Excitation–Contraction Coupling and Cardiac Contractile Force.* Kluwer Academic Publishers, The Netherlands.

BOTELHO, L. H., ROTHERMEL, J. D., COOMBS, R. V. & JASORFF, B. (1988). cAMP analog antagonists of cAMP action. *Methods in Enzymology* **159**, 159–172.

CHARNET, P., RICHARD, S., GURNEY, A. M., OUADID, H., TIAHO, F. & NARGEOT, J. (1991). Modulation of Ca currents in isolated frog atrial cells studied with photosensitive probes. Regulation by cAMP and Ca^{2+}: a common pathway? *Journal of Molecular and Cellular Cardiology* **23**, 343–356.

ECKERT, R. & CHAD, J. (1984). Inactivation of Ca channels. *Progress in Biophysics and Molecular Biology* **44**, 215–267.

FABIATO, A. (1988). Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods in Enzymology* **157**, 378–417.

FABIATO, A. & FABIATO, F. (1975). Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *Journal of Physiology* **249**, 469–495.

FEDIDA, D., NOBLE, D. & SPINDLER, A. J. (1988*a*). Use-dependent reduction and facilitation of Ca^{2+} current in guinea-pig myocytes. *Journal of Physiology* **405**, 439–460.

FEDIDA, D., NOBLE, D. & SPINDLER, A. J. (1988*b*). Mechanism of the use dependence of Ca^{2+} current in guinea-pig myocytes. *Journal of Physiology* **405**, 461–475.

FOGELSON, A. L. & ZUCKER, R. S. (1985). Presynaptic calcium diffusion from various arrays of single channels. Implications for transmitter release and synaptic facilitation. *Biophysical Journal* **48**, 1003–1017.

GURNEY, A. M. (1991). Photolabile calcium buffers to selectively activate calcium-dependent processes. In *Cellular Neurobiology: A Practical Approach*, ed. CHAD, J. & WHEAL, H., pp. 153–177. IRL Press at Oxford University Press, Oxford.

GURNEY, A. M., CHARNET, P., PYE, J. M. & NARGEOT, J. (1989). Augmentation of cardiac calcium current by flash-photolysis of intracellular caged-Ca^{2+} molecules. *Nature* **341**, 65–68.

GURNEY, A. M., TSIEN, R. W. & LESTER, H. A. (1987). Activation of a potassium current by rapid photochemically generated steps of intracellular calcium in rat sympathetic neurones. *Proceedings of the National Academy of Sciences of the USA* **84**, 3496–3500.

HADLEY, R. W. & LEDERER, W. J. (1991). Ca^{2+} and voltage inactivate Ca^{2+} channels in guinea-pig ventricular myocytes through independent mechanisms. *Journal of Physiology* **444**, 257–268.

HARDING, S. E., O'GARA, P., JONES, S. M., BROWN, L. A., VESCOVO, G. & POOLE-WILSON, P. A. (1990). Species dependence of contraction velocity in single isolated cardiac myocytes. *Cardioscience* **1**, 49–54.

HARTZELL, H. C., MÉRY, P.-F., FISCHMEISTER, R. & SZABO, G. (1991). Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylation. *Nature* **351**, 573–576.

HESS, P., LANSMAN, J. B. & TSIEN, R. W. (1986). Calcium channel selectivity for divalent and monovalent cations: voltage and concentration dependence of single channel current in ventricular heart cells. *Journal of General Physiology* **88**, 293–319.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**, 5036–5041.

ISENBERG, G. (1977). The slow inward current component under the influence of modified $[Ca^{2+}]_i$. *Pflügers Archiv* **371**, 61–69.

JACKSON, A. P., TIMMERMAN, M. P., BAGSHAW, C. R. & ASHLEY, C. C. (1987). The kinetics of calcium binding to fura-2 and indo-1. *FEBS Letters* **216**, 35–39.

KAPLAN, J. H. & ELLIS-DAVIES, G. C. R. (1988). Photolabile chelators for the rapid photorelease of divalent cations. *Proceedings of the National Academy of Sciences of the USA* **85**, 6571–6575.

LEE, K. S. (1987). Potentiation of the calcium-channel currents of internally perfused mammalian heart cells by repetitive depolarization. *Proceedings of the National Academy of Sciences of the USA* **84**, 3941–3945.

LEE, K. S., MARBAN, E. & TSIEN, R. W. (1985). Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *Journal of Physiology* **364**, 395–411.

MARBAN, E. & TSIEN, R. W. (1982). Enhancement of calcium current during digitalis inotropy in mammalian heart: positive feed-back regulation by intracellular calcium? *Journal of Physiology* **329**, 589–614.

MARRION, N. V., ZUCKER, R. S., MARSH, S. J. & ADAMS, P. R. (1991). Modulation of M-current by intracellular Ca^{2+}. *Neuron* **6**, 533–545.

MAZZANTI, M. & DEFELICE, L. J. (1990). Ca channel gating during cardiac action potentials. *Biophysical Journal* **58**, 1059–1065.

NÄBAUER, M. & MORAD, M. (1990). Ca^{2+}-induced Ca^{2+} release as examined by photolysis of caged Ca^{2+} in single ventricular myocytes. *American Journal of Physiology* **258**, C189–193.

NIGGLI, E. & LEDERER, W. J. (1990). Voltage-independent calcium release in heart muscle. *Science* **250**, 565–568.

NOEL, J. & CAPIOD, T. (1991). Photolytic release of cAMP activates Ca^{2+}-dependent K^+ permeability in guinea-pig liver cells. *Pflügers Archiv* **417**, 546–548.

O'ROURKE, B., BACKX, P. H. & MARBAN, E. (1992). Phosphorylation-independent modulation of L-type calcium channels by magnesium–nucleotide complexes. *Science* **257**, 245–248.

PUSCH, M. & NEHER, E. (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Archiv* **411**, 204–211.

SIMON, S. M. & LLINÁS, R. R. (1985). Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophysical Journal* **48**, 485–498.

SMITH, S. J. & AUGUSTINE, G. J. (1988). Calcium ions, active zones and synaptic transmitter release. *Trends in Neurosciences* **11**, 458–464.

STERN, M. D. (1992). Buffering of calcium in the vicinity of a channel pore. *Cell Calcium* **13**, 183–192.

VALDEOLMILLOS, M., O'NEILL, S. C., SMITH, G. L. & EISNER, D. A. (1989). Calcium-induced calcium release activates contraction in intact cardiac cells. *Pflügers Archiv* **413**, 676–678.

YOUNT, R. G., BABCOCK, D., BALLANTYNE, W. & OJALA, D. (1971). Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P–N–P linkage. *Biochemistry* **10**, 2484–2489.

YUE, D. T., BACKX, P. H. & IMREDY, J. P. (1990). Calcium-sensitive inactivation in the gating of single calcium channels. *Science* **250**, 1735–1738.

ZYGMUNT, A. C. & MAYLIE, J. (1990). Stimulation-dependent facilitation of the high threshold calcium current in guinea-pig ventricular myocytes. *Journal of Physiology* **428**, 653–671.