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Effects of FPL 64176 on Ca transients in voltage-clamped rat venticular myocytes

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1 The L-type Ca channel agonist FPL 64176 increased the amplitude of both Ca currents and Ca transients elicited from isolated voltage clamped rat ventricular myocytes far more than it increased the rate of rise of the Ca transients. Consequently, the gain function relating the amplitude of peak Ca current to Ca transient rate of rise was greatly reduced at all potentials.

2 Furthermore, an increase in this gain function normally observed at negative potentials is abolished by FPL 64716.

3 Despite slowing the rate of decline of Ca transients, FPL 64176, at the concentration of 1 μM used throughout, had no direct effect on sarcoplasmic reticulum (SR) Ca uptake or release using isolated cardiac membranes.

4 Arguments based on results presented here and elsewhere suggest that decreased gain was not due to increased ryanodine receptor adaptation or inactivation, to decreased L-type single channel current, to decreased SR Ca content, or to decreased synchronization of release. Decreased gain instead appears to reflect a form of decrease in coupling efficiency due either to differential effects of long openings on whole cell currents as opposed to the Ca release the long openings trigger or to some compensatory mechanism activated by the increased Ca trigger or resting free $[Ca^{2+}]_i$.

5 Abolition by FPL 64176 of the increased gain normally seen at negative potentials rendered it impossible to confirm or refute the claim that a single Ca ion suffices to activate ryanodine receptors. *British Journal of Pharmacology* (2002) **135,** 1495–1504

Keywords: FPL 64176; calcium; ventricular myocytes

Abbreviations: $[Ca^{2+}]_i$, resting intracellular free Ca^{2+} concentration; CPA, cyclopiazonic acid; F, fluorescence; F_o, resting fluorescence; HEPES, N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; I_{Ca}, Ca current; NIH, National Institute of Health (U.S.A.); RR, ruthenium red; RyR, ryanodine receptor; Vt, test potential

Introduction

The effects of the Ca channel agonist FPL 64176 on cardiac L-type Ca channel currents have been extensively investigated (Fan *et al*., 2000; 2001). Ca transients have previously been recorded in the presence of FPL 64176 (Yasui *et al*., 1994; Sham *et al*., 1998; Song *et al*., 2001), but they were not systematically compared to those obtained under control conditions in the same fashion as has been done with Bay K 8644 (Adachi-Akahane *et al*., 1999).

In addition to basic characterization of drug effects, there are other reasons to utilize FPL 64176 to explore cardiac excitation–contraction coupling. FPL 64176 is an important drug whose use has revealed an inactivation or adaptation process for ryanodine receptors (Yasui *et al*., 1994; Sham *et al*., 1998) and the presence of cardiac sparklets (Wang *et al*., 2001). Aside from these issues, Ca transients obtained from voltage clamped ventricular myocytes have been reported to show a pronounced increase in gain at negative membrane potentials where the L-type Ca currents are first showing signs of activation (Wier *et al*., 1994; duBell *et al*., 1996; Adachi-Akahane *et al*., 1999; Song *et al*., 2001). This phenomenon has not been well explained, and one of the goals of the present investigation was to utilize FPL 64176 to investigate this phenomenon further.

To this end, FPL 64176 offered a convenient tool because it prolongs the tail currents associated with membrane repolarization. This affords the possibility of assessing the tail current-induced Ca transients over the same negative membrane potential region where gain has been reported to be increased. This is a region of membrane potentials where tail currents normally deactivate so quickly that associated Ca transients (Fan & Palade, 1999) cannot reasonably be compared with those obtained at more positive potentials, where the current is better maintained. Some of the results reported here have previously been reported in abstract form (Fan & Palade, 2000).

Methods

Ventricular myocytes were prepared from 200–300 g male Sprague Dawley rats as described (Fan & Palade, 1999). Ca

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currents and Ca transients were recorded simultaneously using a List EPC-7 patch clamp and fluo 3, as described (Fan & Palade, 1999), except that perforated patch recording with β-escin (Fan & Palade, 1998) was employed instead of conventional ruptured patch whole cell recording. Stimuli were applied at 3 s intervals in control experiments and at 5 s intervals in the presence of FPL 64176, to allow for complete recovery of transients to baseline. In all experiments, a low Ca, 3 mM Co solution was applied at the end of the experiment in order to isolate Co-sensitive, L-type Ca currents, which represent the traces displayed.

The composition of the external solution for the experiments reported here was (mM): NaCl 140, $CaCl_2$ 1, $MgCl_2$ 0.5, CsCl 3, glucose 5.6, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) 10, pH 7.3. The composition of the internal solution in the patch pipette was (mM): Cs aspartate 120, CsCl 20, Na_2ATP 3, $MgCl_2$ 3.5, and HEPES 5, pH 7.3, supplemented with 0.1 mM fluo 3. FPL 64176 was obtained from RBI (Natick, MA, U.S.A.) and fluo 3 from Molecular Probes (Eugene, OR, U.S.A.) or Teflabs (Austin, TX, U.S.A.). FPL 64176 was applied to the external solution at a concentration of 1 μM in all experiments.

Ca transients were recorded from cells that were pre-'stained' with 2.2 μM fluo 3 AM (1 : 200 dilution of a stock solution of 50 μg 100 μl^{-1}) for 10 – 15 min at room temperature and then extensively rinsed. To prevent rundown of Ca transients due to bleaching, a computer-operated shutter system prevented light from reaching the preparation except during measurements. Ca transients are presented in terms of F (fluorescence at peak of Ca transient) to F_o (the initial basal fluorescence at rest, with fluorescence due to minimal scatter and bleeding through both filters in the filter cube subtracted by measuring from a cell-free optical section). Unless otherwise specified, rundown due to escape of dye from cell to pipette was reduced by inclusion of 0.1 mM unesterified dye in the pipette solution at a concentration that maintained Ca transients under control conditions at a constant level of F/F_o.

Drug effects on fluo 3 fluorescence were determined in two ways. First, a Gilford Fluoro IV spectrofluorometer was used to determine fluorescence at 500 nm excitation and 534 nm emission of 0.5 μM fluo 3 in solutions of the Molecular Probes (Eugene, OR, U.S.A.) Calcium Calibration Kit 2 in the presence and absence of 1 and 10 μM FPL 64176. Second, fluo 3 AM-loaded myocytes were exposed to Tyrode to round up Ca-intolerant cells, then returned to KB storage solution (Isenberg & Klöckner, 1982) for washing and exposed to 2 μM A23187 in 0.1 μM or 0.603 μM free Ca^{2+} Molecular Probes Calibration 2 kit solutions, and fluorescence recorded in the presence and absence of 1 μM FPL 64176 using the electrophysiology-optical set-up.

Effects of FPL 64176 on Ca uptake and release by canine cardiac microsomes were performed essentially as outlined in Dettbarn & Palade (1998) using antipyrylazo III absorbance changes in an HP 8453 diode array spectrophotometer (Hewlett Packard, Palo Alto, CA, U.S.A.). Antipyrylazo III (Fluka, Milwaukee, WI, U.S.A.) was used at concentrations of (mM) 0.15 or 0.25 in KCl 50, K-phosphate 62.5, MOPS 10 MgATP 1, phosphocreatine 2.5, and 20 $\mu g\ ml^{-1}$ creatine phosphokinase, pH 7.0.

Results

FPL 64176 had relatively constant kinetic effects on Ca transients but variable effects on their amplitudes. Figure 1 demonstrates effects on a cell where Ca transients were minimally enhanced. Representative Ca currents and Ca transients acquired simultaneously under control conditions under voltage clamp are shown in the second and third lines

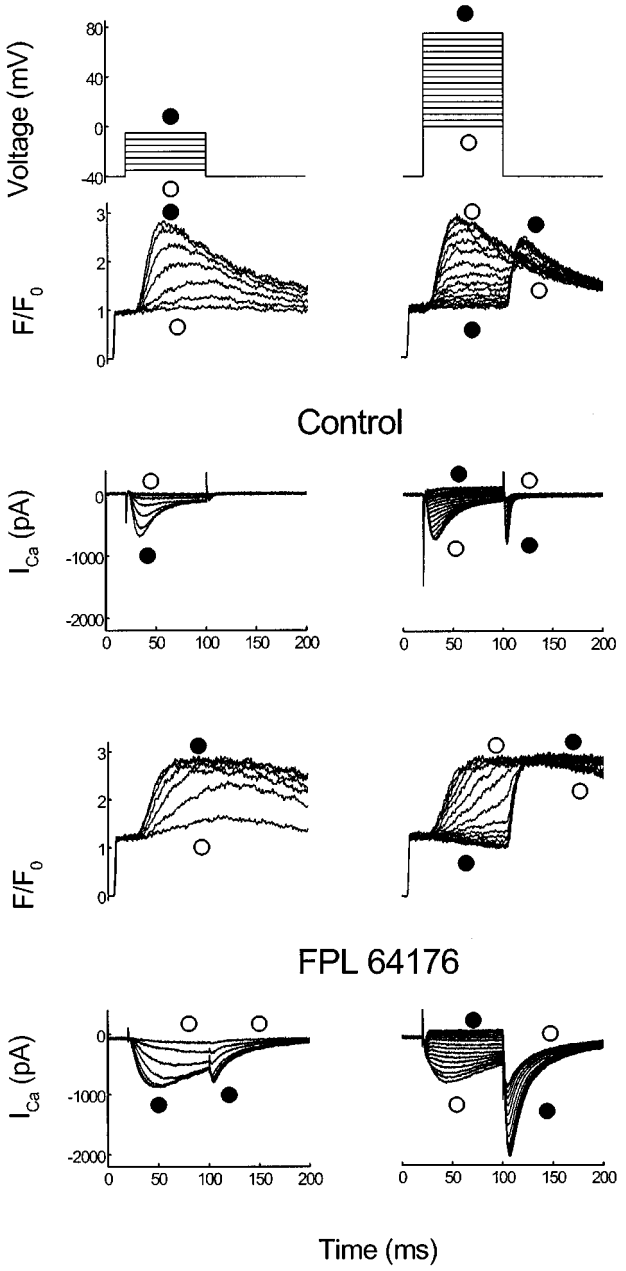

Figure 1 Kinetic effects of FPL 64176 on Ca transients elicited by Ca currents. A rat ventricular myocyte stained with fluo 3 AM was held at −40 mV and depolarized to potentials from −35 to −5 mV at left and from 0 to +80 mV at right. The voltage pulse protocol is shown in the top line. Ca transients due to fluo 3 fluorescence increase are shown in the second line, and Ca currents recorded simultaneously in the third line in the absence of FPL 64176. Subsequent measurements from the same cell after application of 1 μM FPL 64176 are shown below.

of Figure 1. At left, results are shown over the range of membrane potentials −35 to −5 mV, and at right, from 0 to +75 mV. Relatively small Ca currents at left at −30 and −25 mV result in quite sizeable Ca transients, whereas comparably small Ca currents at positive potentials at right produce significantly smaller Ca transients. At strongly positive potentials there is at best a small Ca transient during the depolarization, but subsequent repolarization causes a large Ca transient associated with the Ca tail currents.

The same myocyte was then exposed to 1 μM FPL 64176. Addition of the FPL 64176 produced significant effects on Ca currents previously described (Fan *et al*., 2000), including slowed activation, enhanced amplitude, slower inactivation, and greatly enhanced and long-lived tail currents upon repolarization. These changes are evident in the bottom two lines of Figure 1, together with several accompanying changes in the Ca transients. First, the baseline fluorescence has increased slightly, indicative of a likely rise in resting free $[Ca^{2+}]_i$. Second, the decay of both the Ca currents and the Ca transients during the depolarizing pulse has become less complete. Third, the fluorescence actually exhibits a decline at very positive potentials.

Figure 2 demonstrates the effects on Ca currents and Ca transients obtained in a representative cell which exhibited significantly increased Ca transients upon addition of FPL 64176. Potentiation of the Ca transient by >50% occurred in five of the seven cells examined with this pulse protocol. The potentiation was also accompanied by a similar increase in the F_o value obtained, suggestive that it was due to an increase in resting free $[Ca^{2+}]_i$, which in turn could have resulted in an enhanced SR Ca content that might have contributed to the potentiated Ca transient.

In Figure 3, the relationship between Ca current amplitude during the depolarization and rate of rise of the Ca transient for several cells is plotted as in Fan & Palade (1999), and appears to exhibit some hysteresis, with points at negative potentials exhibiting a faster rising Ca transient for the same peak Ca current than those obtained at positive potentials.

When equivalent data obtained in the presence of FPL 64176 are plotted in the same fashion (Figure 3), it is clear that the slope of the relationship between Ca current and the rate of rise of the Ca transient at positive potentials is reduced, but it remains linear as under control conditions. In fact, there may be slightly less of a foot representing Ca current which appears not to contribute to any increase in intracellular $[Ca^{2+}]$. At negative potentials, comparably sized Ca currents no longer elicit Ca transients with faster rates of rise. The same changes in the relationship were noted even in cells where the Ca transients were not potentiated by FPL 64176.

In Figure 4, the relationship between rate of rise of the Ca transient and the peak Ca current at that potential is plotted as a representation of 'gain'. Note that gain in all cases here is normalized to that at 0 mV in controls, where it was previously determined to be approximately 7 (Fan & Palade, 1999). Under control conditions (upper panel), gain is highest at more negative potentials, as previously reported by Wier *et al*. (1994), although this is not observed in every cell. There is a shallow fall-off of gain at positive potentials.

When data obtained in the presence of FPL 64176 are plotted (Figure 4, lower panel), it is clear that gain has been decreased at all potentials except possibly the most positive ones, where inaccuracies in I_{Ca} determinations increase scatter in the data. In addition, the increase in gain at negative potentials seen under control conditions is completely abolished. There remains a shallow decrease in gain between +20 and +70 mV, as under control conditions. Equivalent changes in this relationship were also noted whether the Ca transients had been potentiated by FPL 64176 or not.

Fan & Palade (1999) utilized a different pulse protocol in order to ensure that an equal number of channels were activated at different test pulse potentials. This protocol involved utilization of a prepulse to +80 mV, which

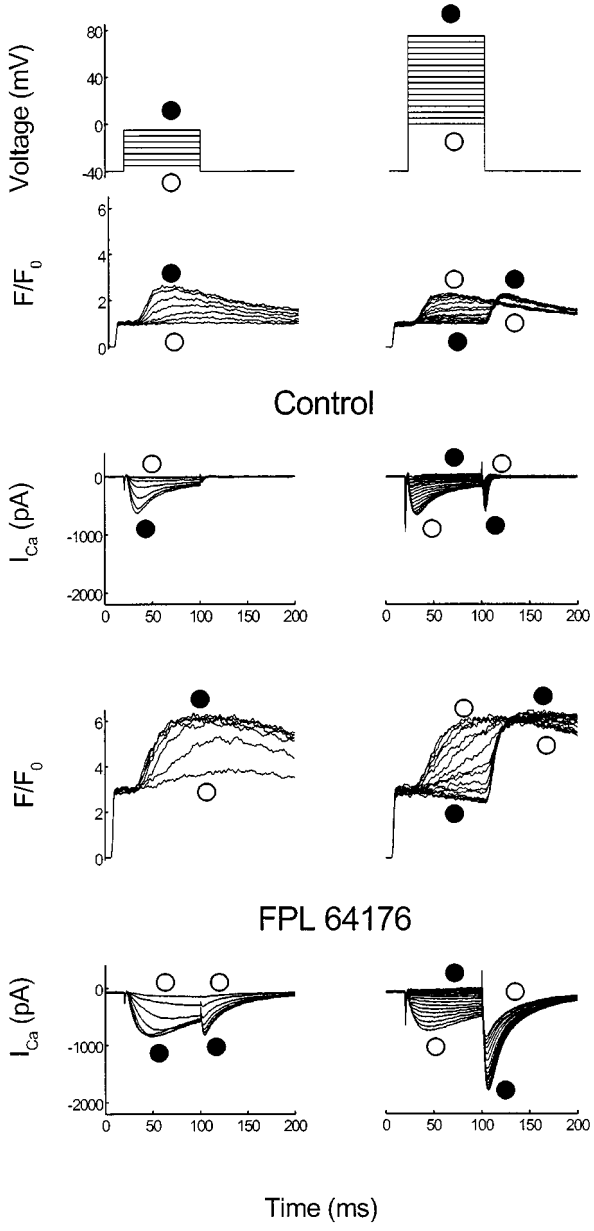

Figure 2 Kinetic effects of FPL 64176 on Ca transients elicited by Ca currents from a cell exhibiting much greater increase in its Ca transients. Experiment carried out as described for Figure 1, except that in this particular experiment, no dye was present in the pipette solution. Therefore, the increase in baseline could not have been due to greater dye concentration in the cell during exposure to 1 μM FPL 64176.

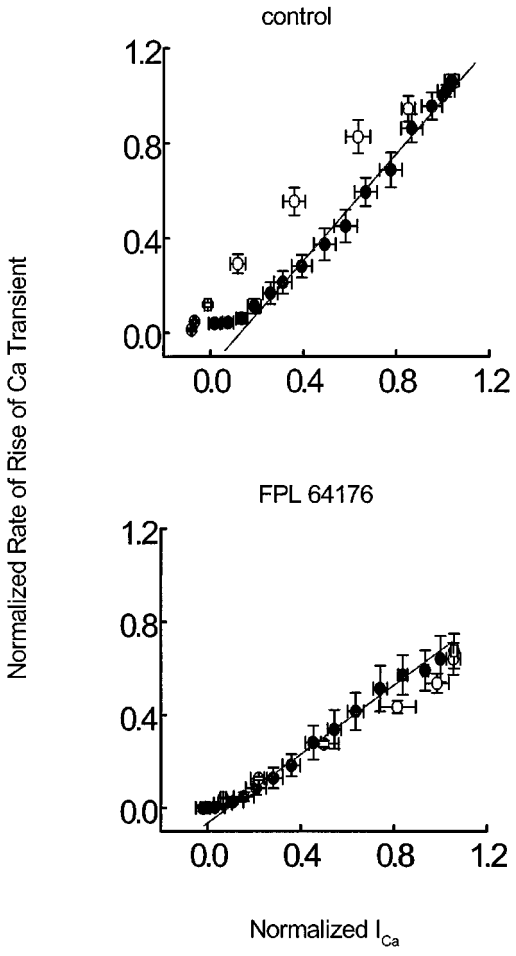

Figure 3 FPL 64176 effects on the relationship between peak Ca current and rate of rise of the Ca transient. Upper panel, the relationship between peak Ca current amplitude and rate of rise of the Ca transient is shown at negative potentials (open circles) and positive potentials (filled circles) for six cells, with results for each cell normalized to those obtained at 0 mV. Lower panel, equivalent determinations from the same six cells subsequently exposed to 1 μM FPL 64176. Error bars here and in subsequent figures represent s.e.mean.

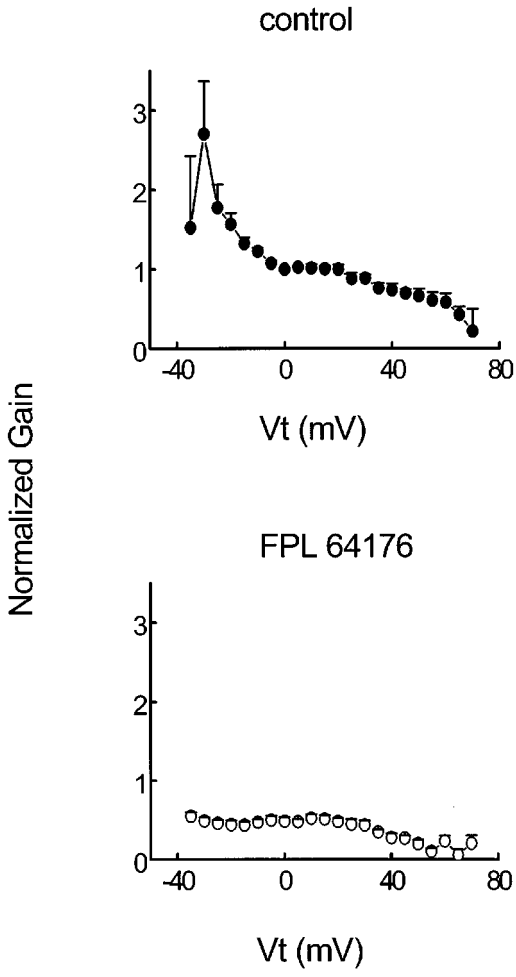

Figure 4 FPL 64176 effects on 'gain' as a function of membrane potential. Upper panel, the results from six cells are replotted so as to estimate gain as the rate of rise of the transient divided by the peak Ca current at different potentials. Lower panel, equivalent determinations from the same six cells subsequently exposed to 1 μM FPL 64176.

activated all channels but, due to the decreased driving force on Ca^{2+}, did not permit any detectable Ca entry. Ca currents and Ca transients obtained with this pulse protocol are shown in Figure 5. In this case, two sets of Ca currents were recorded, first in the presence of FPL 64176 alone, then later in the presence of 3 mM Co^{2+} in addition. The Co^{2+} blocks the L-type Ca currents (Fan & Palade, 1999) even in the presence of FPL 64176 (Fan *et al*., 2000). Subtraction of the current traces in Co from those in its absence are presented in the figure as a more accurate representation of L-type Ca currents. Since Ca transients were absent in the presence of Co, the Ca transients presented are simply those obtained in the presence of FPL 64176 alone. Ca transients and currents at negative potentials are shown at left and those at positive potentials at right. Control recordings are shown in the second and third lines, and equivalent recordings in the presence of FPL 64176 are shown in the lowest two lines.

Using this pulse protocol, it is clear that the relationship between Ca current and the rate of rise of the Ca transient is quite linear except for a small foot (Figure 6, upper left). Linearity is retained in the presence of FPL 64176, again with less evidence of a foot (lower left). Data are normalized by the control transient rate of rise value at 0 mV. The points in FPL 64176 are extended to larger tail currents because these are larger and longer maintained in the presence of the drug. They also reveal that the relationship shows signs of saturation at negative potentials. Normalized gain is plotted in Figure 6, right panels, again with data normalized to the control values for gain at 0 mV. The presence of FPL 64176 leads to a decrease in gain at negative potentials, as seen without the prepulse in FPL 64176 (Figure 4), but not as marked. As in controls, gain also falls off at potentials positive to +30 mV in the presence of FPL 64176.

The decrease in gain observed in the presence of FPL 64176 suggested that either the Ca load in the SR had been diminished in the presence of the drug or that the drug inhibited SR Ca release. To distinguish between these possibilities, two separate tests were performed. First, control and drug-treated cells were exposed to rapid application of high concentrations of caffeine (10 mM). As seen in Figure 7, the amplitude of the Ca transient elicited by caffeine

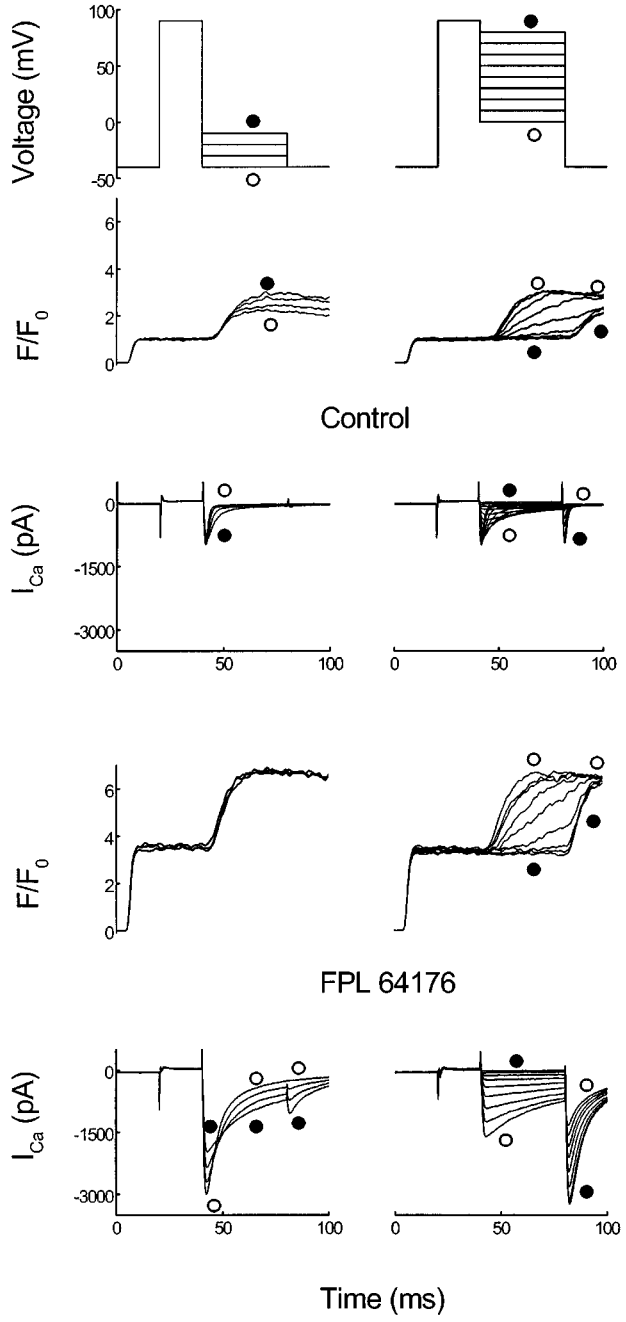

Figure 5 Effects of FPL 64176 on Ca transients elicited by Ca currents when all Ca channels are pre-activated with a prepulse. Results under control conditions are shown in second and third lines for pulses to negative (left) and positive (right) test potentials, respectively. Results from the same cell in the presence of 1 μM FPL 64176 are shown in fourth and fifth lines for pulses to negative (left) and positive (right) potentials, respectively. Results representative of eight cells under control conditions and 11 cells in the presence of FPL 64176.

application generally was not reduced in the presence of FPL 64176. The enhancements were not always correlated with one another. FPL 64176 enhanced the transients induced by electrical stimulation in six of eight cells, but it enhanced the transients elicited by caffeine in only four of the eight cells. The mean amplitude of the Ca transient elicited by a voltage clamp pulse was increased by 1 μM FPL 64176 by a factor of 1.27 ± 0.11, and the amplitude of the Ca transient elicited by squirts of 10 mM caffeine in the same cells was increased by a factor of 1.23 ± 0.20 (mean $\pm$ s.e.mean). However, the difference between FPL–caffeine and the control–caffeine data using a paired t-test was not significantly different ($P = 0.28$), and the difference between FPL-electrical stimulation and control–electrical stimulation was just barely significant ($P = 0.048$). The difference between the two values in the presence of FPL 64176 was not significantly different using an unpaired t-test ($P = 0.85$).

The lefthand panel of Figure 7 also illustrates that the declining phase of the Ca transient is slowed, even after the long-lasting Ca tail currents in the presence of FPL 64176 would have dissipated (100 ms after the end of the stimulus). Fitting the declining phase of transients from six cells before and after FPL 64176 with single exponentials indicated a decline with a time constant of 217.6 ± 74.3 ms (mean $\pm$ s.e.mean) starting 100 ms after the end of the stimulus in the presence of FPL 64176, compared with time constants of decline for the controls of 109.9 ± 26.6 ms from the peak of those transients or 101.7 ± 23.6 ms starting from the end of the stimulus. Four of the six cells showed >2x differences in taus, a fifth $>50\%$ increase, but one cell showed little difference. Paired t-tests of taus in the presence of FPL 64176 with either value indicate that the values in the presence of drug are significantly different statistically from either control value ($P = 0.034$ *vs* the first control value, $P = 0.033$ *vs* the second control value).

To examine possible direct side effects at the level of the sarcoplasmic reticulum membrane, spectrophotometric studies were undertaken on Ca uptake and release by canine cardiac microsomes. In Figure 8A (left), Ca uptake was initiated by an addition of Ca and proceeded relatively slowly at first, due to some ryanodine receptors being activated, then more rapidly as the RyRs closed. Following an addition of FPL 64176 (or vehicle), another Ca addition was made. Neither the slow nor the fast rate of Ca uptake was inhibited by 1 μM FPL 64176. In order to better observe effects on the fast rate of uptake with RyRs closed, these experiments were repeated in the presence of 10 μM ruthenium red, a RyR blocker (Figure 8A, right), and again the tabulated results from 3–4 experiments indicated no effect of FPL 64176. To assess possible very slow leaks of Ca from the vesicles, release was examined in the presence of 100 μM cyclopiazonic acid, a Ca uptake blocker. The cyclopiazonic acid induced a very slow net release of Ca, but addition of 1–10 μM FPL 64176 had no stimulatory effect on the rate of net Ca release (Figure 8B).

Discussion

The results of the present investigation indicate that FPL 64176 has a number of significant effects on the Ca transients of rat ventricular myocytes. First, it increased the baseline fluorescence. The drug did not affect the titration of 0.5 μM fluo 3 in calibration solutions *in vitro* (not shown). Nor did it affect fluo 3 fluorescence (changes $<5\%$) in normally stained myocytes treated with A23187 in Ca-free EGTA-containing media, or in media of 0.1 or 0.6 μM free Ca^{2+} containing A23187, in between the diastolic and systolic free Ca^{2+} concentrations ($n = 2$ in each solution, not shown). Thus the

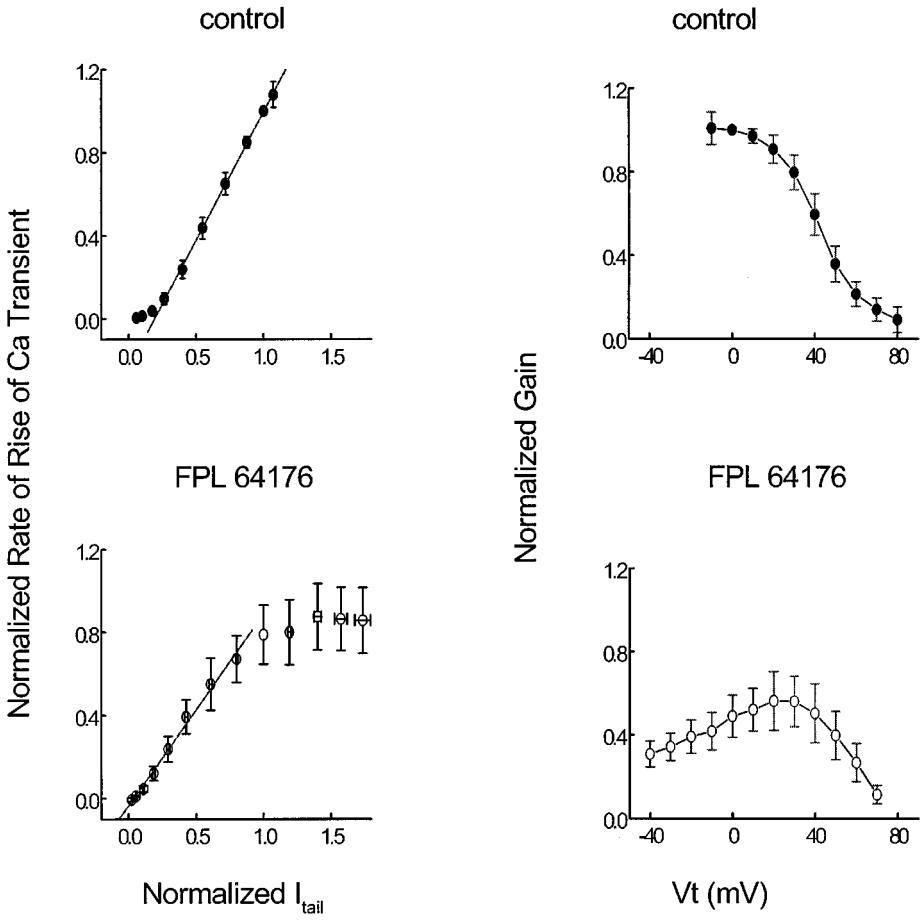

Figure 6 FPL 64176 on relationships between peak Ca current and rate of rise of Ca transient and on 'gain' as a function of membrane potential when using prepulse activation. Left panels show the relationship between peak Ca current amplitude and rate of rise of the Ca transient is shown for four cells under control conditions (above) and in the presence of FPL 64176 (below), with results for each cell normalized to those obtained at 0 mV under control conditions. At right, the results are replotted so as to estimate gain as the rate of rise of the transient divided by the peak Ca current at different potentials, again with results normalized to 0 mV under control conditions. Qualitative features of all curves were preserved with smaller s.e.mean when partially unpaired data were plotted from $n=8$ cells under control conditions and $n=11$ cells in the presence of 1 μM FPL 64176, normalized to values obtained at 0 mV, except that the decline in gain at negative potentials in the presence of FPL 64176 was not as steep.

increase in baseline fluorescence noted in experiments is consistent with an increase in resting free $[Ca^{2+}]_i$. It is surprising that the increase in some experiments was to the level of systolic Ca transients in controls, and yet the myocytes did not round up due to hypercontracture. We speculate that FPL 64176 might have some negative inotropic effect at the level of the contractile proteins, but confirmation would require further study.

An increase in resting $[Ca^{2+}]_i$ by itself is more likely to indicate altered transsarcolemmal balance than reduced SR Ca uptake, but reduced gain and results of others led us to assess direct drug effects on SR Ca uptake. Wasserstrom *et al*. (2001) recently reported activation of canine cardiac RyRs in planar lipid bilayers, but the concentrations used (20–50 μM) were much higher than used in any of the experiments reported here, and those experiments were conducted in the absence of ATP. Using canine cardiac microsomes in the more physiological presence of ATP, the experiments performed here indicate that 1 μM FPL 64176 caused no reduction in Ca uptake in the presence or absence of the RyR blocker ruthenium red (Figure 8). Thus, unless rat heart SR is considerably more sensitive to the drug than dog heart SR, direct effects of 1 μM FPL 64176 on SR Ca uptake or release appear to be minimal. Nevertheless, the declining phase of the Ca transient was significantly slowed in the presence of FPL 64176, even after allowing for decay of the prolonged Ca tail currents in the presence of drug. *In situ* it is possible that any initial increase in SR Ca content triggered some compensatory process (reduced SR Ca pumping, or increased RyR-mediated leak) which attenuated or prevented a steady-state increase in SR Ca, much as seen by Eisner *et al*. (1998; 2000) in the case of administration of low doses of caffeine. Indeed, the long-term increase in resting $[Ca^{2+}]_i$ appears to cause no statistically significant increase in the SR Ca content, as revealed by experiments involving stimulation with caffeine.

In the presence of FPL 64176, the basal fluorescence decreased at strongly positive potentials in some cells (e.g., right panels of Figures 1 and 2, observed in a majority of cells examined using this pulse protocol), affording a clue regarding the increase in resting $[Ca^{2+}]_i$. Although seen in only a minority of experiments carried out with prepulses,

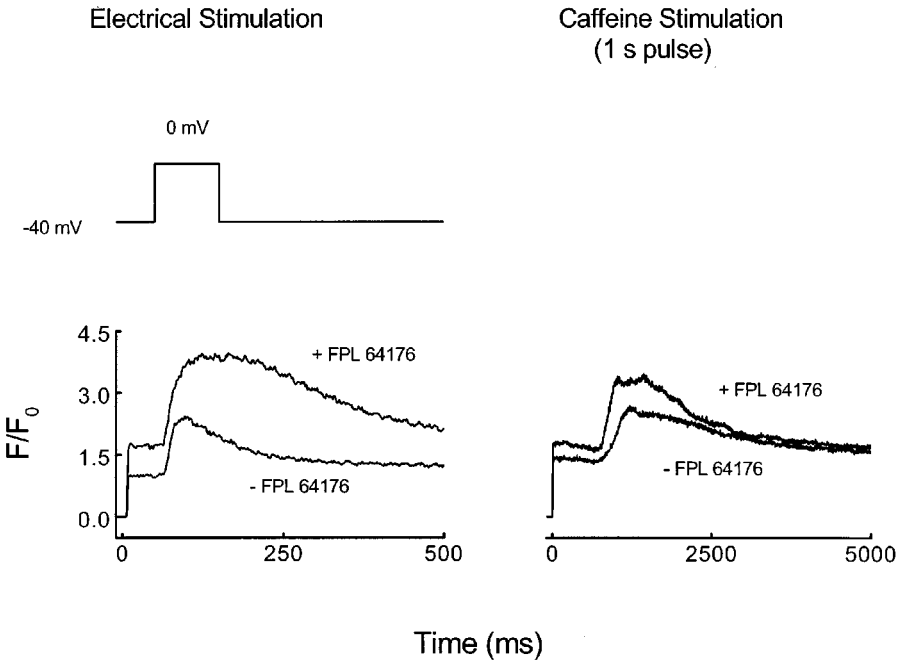

Figure 7 The decrease in gain caused by FPL 64176 is not due to a decreased SR Ca content. Left panel: Ca transients in response to depolarizations to 0 mV. Right panel: Ca transients in response to squirting of 10 mM caffeine from a wide bore pipette positioned near the myocyte. Traces before and following exposure to 1 μM FPL 64176 are superimposed. Results generally representative of those from eight cells.

this decrease might indicate that channels are active at the holding potential in the presence of drug, and that at strongly positive potentials, the driving force on Ca entry is reduced so much that net Ca entry through all activated channels is still less than that due to those few channels open at rest. Channel activity was frequently noted at rest in single-channel experiments in the presence of the drug (Fan *et al*., 2000), even with more negative holding potentials. Increases in resting $[Ca^{2+}]_i$ have also been reported with Bay K 8644 (Adachi-Akahane *et al*., 1999), even at a holding potential of -60 mV, although Bay K 8644 is reported to induce an increase in spark frequency that is not seen with FPL 64176 (Katoh *et al*., 2000). A lack of effect on spark frequency would be consistent with our failure to observe a statistically significant increase in SR Ca content and suggest that compensatory decreases in Ca uptake would be more likely to be responsible than increased leak due to RyR activity.

Several other effects of FPL 64176 on Ca transients are also likely to be due to its direct effects on the L-type Ca channels. Thus, Ca transients decay more slowly, perhaps because of the prolonged tail currents in the presence of the drug. The increase in amplitude of Ca transients without a concurrent increase in the rate of rise of the transients is puzzling. Since Yasui *et al*. (1994) observed no increase in the time constant of turn off of release by the drug, it is unlikely that diminished RyR inactivation or adaptation is responsible for either the enhanced transients or their slower decay. On the other hand, slower activation of L-type Ca current could lead to Ca transients with a slower rate of rise but a longer time-to-peak.

FPL 64176 increases both Ca currents and the Ca transients they trigger. However, it increases the Ca currents more than the Ca transient rates of rise, with a consequent decrease in the 'gain' that relates Ca release to the Ca entry that triggers it. The gain function appears in general to be reduced by FPL 64176 at all potentials (Figure 4), except possibly at very positive potentials following prepulses to $+80$ mV (Figure 6). A decrease in gain was also found by Song *et al*. (2001), who used ten times higher [FPL 64176]. They suggested a local saturation of the trigger signal was responsible, both for this effect and a similar decrease in gain observed with β-adrenergic signalling under their experimental conditions.

At first glance, decrease in gain across the entire potential range could in principle be due to enhanced RyR adaptation or inactivation, to a decreased L-type single channel current (if gain were nonlinearly dependent on current), to a saturation of the local Ca required to trigger release, to a decreased SR Ca content, to a decreased synchronization of release, or to a decrease in coupling efficiency. Adachi-Akahane *et al*. (1999) proposed increased RyR adaptation-inactivation for the decrease in gain they observed with Bay K 8644. However, since Yasui *et al*. (1994) found no faster time constant for release turn-off in the presence of FPL 64176, increased adaptation–inactivation is unlikely to be responsible for the decrease in gain seen here. Furthermore, any increased adaptation–inactivation would likely trigger a compensatory increase in SR Ca content, much as described for other SR perturbances by Eisner *et al*. (1998; 2000), and this was not observed. Prior studies have shown that the single channel current is, if anything, slightly increased by FPL 64176 (Handrock *et al*., 1998; Fan *et al*., 2001). Our measurements with caffeine suggest that the SR Ca content is not decreased. Local saturation is a likely explanation only near 0 mV (where Ca currents are largest) and at more negative potentials (where the unitary Ca currents would be

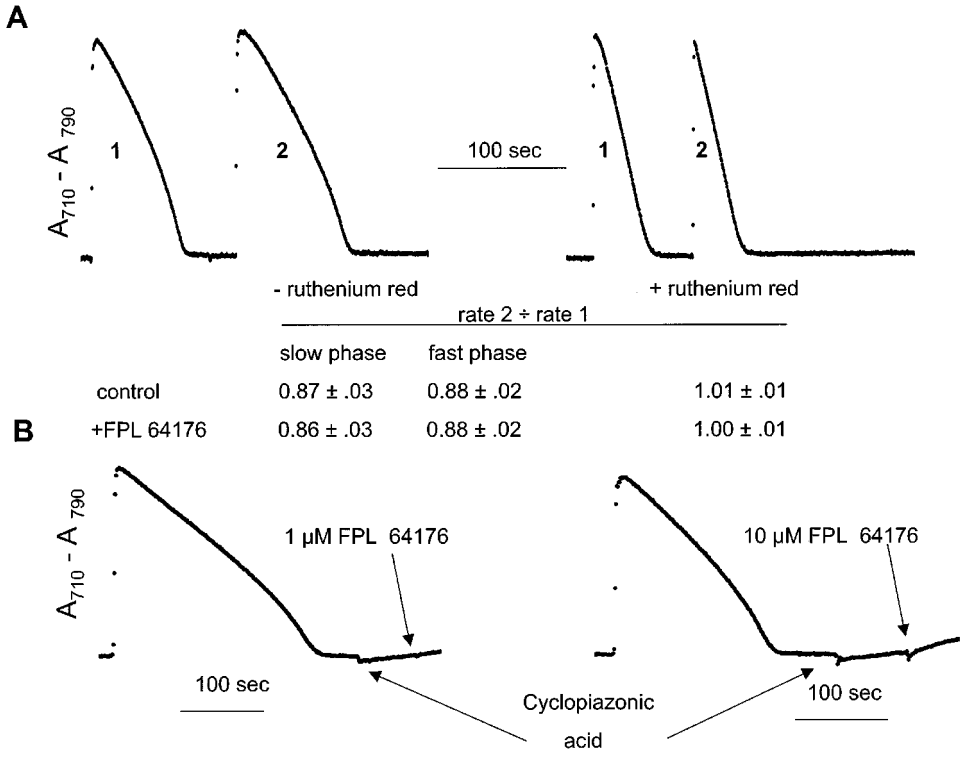

Figure 8 Ca uptake by canine cardiac microsomes is unaffected by 1 μM FPL 64176. Canine cardiac microsomes (0.5 mg protein) were administered two 50 nmol additions of $CaCl_2$ at 35°C in the presence of 62.5 mM phosphate. Ca taken up by the microsomes resulted in a slow decrease of absorbance due to reduction in the Ca-antipyrylazo III complexes outside the vesicles. Absorbance decrease became faster as trace approached baseline. (A) Following uptake of the first addition of Ca (1), 1 μM FPL 64176 was added, followed by another Ca addition (2), and results compared to a second experiment conducted in the complete absence of FPL 64176. To the right is shown an equivalent experiment in the presence of ruthenium red, a RyR blocker, throughout. Underneath is a tabulation of normalized slow and fast uptake rate results (rate 2 ÷ rate 1) conducted in the presence and absence of 10 μM ruthenium red. None of the results in the presence of FPL 64176 were significantly different from controls by means of paired *t*-tests. (B) Following uptake of an addition of Ca, 100–200 μM cyclopiazonic acid was added to block SR Ca pumps and reveal a slow leak of Ca. This leak was enhanced slightly by 1 μM FPL 64176 (left) or 10 μM FPL 64176 (right), but no more than the controls for the ethanol vehicle, which superimposed precisely over the tracings shown. Results representative of two separate experiments under each condition.

even larger). However, since gain is also decreased at positive potentials, and since gradation is maintained at both positive and negative potentials, saturation of the local Ca trigger is unlikely. Decreased synchronization of release could result in greater RyR inactivation prior to attainment of peak I_{Ca}, which is delayed in the presence of FPL 64176. This would be the opposite of the β-adrenergic effect on synchronization noted by Song *et al.* (2001). However, decreased synchronization is unlikely to account for the decrease in gain observed when prepulse-test pulse combinations were used to trigger current flow through the channels simultaneously. The final explanation, that of decreased coupling efficiency, appears most likely, but it does not necessarily indicate a direct interaction between DHPR and RyR or a direct effect of FPL 64176 on any such interaction. Rather, it might simply indicate that the long L-type channel openings caused by FPL 64176 (Fan *et al.*, 2000; 2001) sum and greatly increase whole cell Ca currents, but that these longer openings are unlikely to be significantly more effective at triggering Ca release than short openings.

Alternative explanations exist to a direct drug effect on the EC coupling gain. Increased Ca currents induced by the drug might be expected to trigger additional compensatory mechanisms than just those discussed earlier that might have attenuated increases in SR Ca load. Speculations regarding such compensation could involve increased RyR inactivation caused by either the increased trigger (Fabiato, 1985) or some kinase, phosphatase or protease activated by the increased resting $[Ca^{2+}]_i$. Alternatively, increased Na-Ca exchange might diminish the effectiveness of local Ca^{2+} at triggering release. This last possibility would suggest greater attenuation of release gain at negative potentials, as observed, where Ca extrusion *via* forward exchange would be favoured.

The abolition by FPL 64176 of the increase in gain at negative potentials normally present is especially interesting, even more so because neither Bay K 8644 nor β-adrenergic signalling exhibit the same effect (Adachi-Akahane *et al.*, 1999; Song *et al.*, 2001). As reported by Wier *et al.* (1994), the increased gain was not seen with every cell under control conditions. We have explored several possibilities to account for the normal increased gain under control conditions. It is unlikely that an action potential in tubules near threshold accounts for the high gain at negative potentials because an unclamped action potential would be even more likely in the presence of FPL 64176. Although increased gain could in principle be due to an increased SR Ca load following a rest period, we have seen, if anything, a more pronounced increase in gain at negative potentials when pulses to negative

potentials were applied only after those to positive potentials ($n=2$, not shown). Its virtual abolition by FPL 64176 might somehow be due to the slowed activation of Ca currents in the presence of drug. However, the decrease in gain in the presence of FPL 64176 was still observed at negative potentials when test pulses were preceded by prepulses which guaranteed more rapid I_{Ca} onset as well as more activated channels (Figures 5 and 6). Gain is also decreased by FPL 64176 when 'tail' Ca transients are elicited following a depolarization to strongly positive potentials. This is most obvious when comparing results at potentials $< +30$ mV in the two righthand panels of Figure 6.

Since spark amplitude is not increased similarly at negative potentials (López-López *et al*., 1995) as gain, increased gain at negative potentials could indicate that the few DHPRs which activate at negative potentials are able to activate more RyRs within a diad than can an equivalent number of additional DHPRs which open at more positive potentials. In turn, this would suggest that Ca released from one RyR does not automatically trigger all other RyRs in a diad to open regeneratively, as in the Stern (1992) cluster bomb model. Indeed, even the highest estimates of numbers of RyRs involved in sparks (Bridge *et al*., 1999; Izu *et al*., 2001) fall far short of the numbers of RyRs in diads (Franzini-Armstrong *et al*., 1999). In addition, gain could decrease at less negative potentials if a group of RyRs that could be activated by newly recruited DHPRs has already been activated by some other DHPRs. FPL 64176 does not selectively reduce the single L-type channel current at negative potentials, but its general depression of gain might be even more pronounced at negative potentials due to the longer L-type openings it induces at more negative potentials (Kunze & Rampe, 1992). While FPL 64176 might also reduce gain by selectively recruiting L-type channels not involved in triggering Ca-induced Ca release, this would reduce gain at all potentials and therefore by itself could not account for the selective decrease at negative potentials.

Use of FPL 64176 allowed exploration of possible deviations from linear behaviour in the relationship between Ca transient rate of rise and the amplitude of the peak Ca current. Linearity was still observed over the positive potential range. Rather than observing an increase in the rate of rise *vs* I_{Ca} relationship at negative potentials gain at negative potentials (as would be predicted from increased gain at those potentials), our results using prepulses to activate a similar number of Ca channels at all potentials showed clear signs of saturation in the presence of drug. However, since the drug also abolished the increase in gain observed at negative potentials when no prepulses were applied, it would be premature to attribute the decreased gain to activation of a greater number of Ca channels by use of prepulses. Accordingly, the use of FPL 64176 neither helps confirm nor refute our earlier contention that only one Ca ion is required to activate ryanodine receptor-mediated Ca release (Fan & Palade, 1999).

In conclusion, 1 μM FPL 64176 appeared to increase resting $[Ca^{2+}]_i$ with minimal direct effects on SR Ca uptake or release. More significantly, it slowed the decay of Ca transients, decreased gain associated with normal excitation–contraction coupling, perhaps due to the long L-type openings it induced, and it abolished an increase in gain at negative potentials commonly observed in its absence.

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