Modulation of single channels underlying hippocampal L-type current enhancement by agonists depends on the permeant ion

By

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Abstract

The influx of calcium (Ca\(^{2+}\)) ions through L-type channels underlies many cellular processes, ranging from initiation of gene transcription to activation of Ca\(^{2+}\)-activated potassium channels. L-type channels possess a diagnostic pharmacology, being enhanced by the dihydropyridine BAY K 8644 and benzoylpyrrole FPL 64176. It is assumed that the action of these compounds is independent of the ion conducted through the channel. In contrast to this assumption, modulation of L-type channel activity in acutely dissociated rat CA1 hippocampal neurons depended on the divalent ion identity. BAY K 8644 and FPL 64176 substantially increased single channel open time only when barium (Ba\(^{2+}\)) was the permeant ion. BAY K 8644 increased single channel conductance when either Ba\(^{2+}\) or Ca\(^{2+}\) ions were the charge carrier, an effect not observed with FPL 64176. BAY K 8644 enhanced the whole-cell L-type channel Ca\(^{2+}\)- or Ba\(^{2+}\)-carried current without a change in deactivation tail kinetics. In contrast, enhancement by FPL 64176 was associated with a dramatic slowing of deactivation kinetics only when Ba\(^{2+}\) and not Ca\(^{2+}\) was the charge carrier. Current activation was slowed by FPL 64176 with either charge carrier, an effect arising from a clustering of agonist-modified long duration openings towards the end of the voltage step. These data indicate that agonists enhanced L-type current by distinct mechanisms dependent on the permeant ion, indicating that care must be considered when used as diagnostic tools.
Introduction

L-type Ca\(^{2+}\) channels are present in hippocampal CA1 pyramidal neurons and contribute approximately 30-50\% of the total Ca\(^{2+}\) current (Elliott et al. 1995; McDonough et al. 1996). These channels arise from two separate genes CACNA1C and CACNA1D that encode the Ca\(_V\)1.2 (C class) and Ca\(_V\)1.3 (D class) subunits respectively. Channel subunits are located within the soma of hippocampal pyramidal neurons, with Ca\(_V\)1.2 subunits being clustered at the base of the major dendrites and Ca\(_V\)1.3 being more diffuse (Hell et al. 1993; Bowden et al. 2001). Both channel subtypes possess a unique pharmacology in being sensitive to dihydropyridines, although some evidence suggests that Ca\(_V\)1.3 channels may be slightly less sensitive (Xu and Lipscombe 2001). Dihydropyridine antagonists inhibit whole-cell L-type Ca\(^{2+}\) current, while agonists enhance the channel current (Hess et al. 1984). L-type current is also enhanced by the benzoarylpyrrole FPL 64176 (Rampe and Lacerda 1991). Both BAY K 8644 and FPL 64176 induce a hyperpolarizing shift in whole cell current activation and inactivation by \(~10\) mV, but only FPL 64176 slows the rates of activation of L-type current in cardiac myocytes (Fan et al. 2000). Cell-attached patch recordings of single L-type channels in cardiac myocytes (Fan et al. 2000) and recombinant Ca\(_V\)1.2 channels expressed in CHO cells (Lauven et al. 1999) have shown that enhancement of whole-cell L-type current results from increases of channel open time and probability (Lauven et al. 1999; Fan et al. 2000).
Despite the importance of L-type Ca$^{2+}$ current to hippocampal physiology, the vast majority of studies that have assessed the activity of these channels have been performed using high concentrations of Ba$^{2+}$ as charge carrier in the presence agonists. It has been assumed that the effect of these channel agonists is independent of the identity of the conducting ion. However, application of BAY K 8644 to cell-attached patches from hippocampal CA1 pyramidal neurons displaying L-type channel activity did not produce the expected long duration openings when using Ca$^{2+}$ as the permeant ion (Marrion and Tavalin 1998). In contrast, L-type channel open times were obviously lengthened by BAY K 8644 when Ba$^{2+}$ ions were the charge carrier (Fisher et al. 1990; Kavalali and Plummer 1996; Cloues et al. 1997). These data suggest that the effect of the dihydropyridine agonist depends on the identity of permeant ion. To determine whether the effects of BAY K 8644 and FPL 64176 are dependent on the charge carrier, we have used whole-cell and single channel measurements of pharmacologically isolated L-type channel currents from acutely dissociated rat hippocampal CA1 pyramidal neurons.

We have found that enhancement of L-type channel current by these compounds is from distinct mechanisms that both depend on the identity of the permeating ion. Augmentation of macroscopic L-type current by BAY K 8644 partly resulted from an increase in single channel current, which was accompanied by an increase in channel open time only when the channel was conducting Ba$^{2+}$ and not Ca$^{2+}$ ions. In contrast, enhancement of whole-cell current by FPL 64176 was not associated by a change in single channel current. Augmentation was greatest when the channel conducted Ba$^{2+}$ rather than Ca$^{2+}$ ions. This resulted in part from a dramatic recruitment of very long
duration openings, when only a modest increase in single channel open time was observed when Ca$^{2+}$ was the conducting ion. This gave rise to an obvious slowing of whole-cell current deactivation seen when the channel conducted Ba$^{2+}$ ions, compared with a slight slowing of deactivation when the current was carried by Ca$^{2+}$ ions. The use of these channel agonists as diagnostic tools to identify L-type channel current has to be reconsidered with the identity of the conducting ion.
Materials and methods

Cell Preparation

Acutely dissociated hippocampal CA1 neurons were obtained as previously described (Cloues et al. 1997). Briefly, Sprague-Dawley rats (9-14 days old) were sacrificed and hippocampi rapidly dissected and cut into 300-400 µm-thick slices. Slices were incubated at 37 °C in dissociation solution that was bubbled with O₂ of composition (mM): Na₂SO₄, 82; K₂SO₄, 30; HEPES, 10; MgCl₂, 5 (pH 7.4 with HCl), with added protease type XXIII (3 mg/ml) for 7-8 minutes. Tissue slices were then transferred to solution containing trypsin inhibitor (1 mg/ml) and bovine serum albumin (1 mg/ml) for one minute and finally rinsed in dissociation solution containing no enzyme. The CA1 region was micro-dissected and triturated onto 35 mm tissue culture plates that were coated with poly-L-lysine as needed.

Contaminating N- and P/Q-type Ca²⁺ channel currents were eliminated by pre-incubation of cells in the dissociation solution supplemented with ω-conotoxins GVIA (1 µM) and MVIIC (5 µM) for 30 minutes before recording (McCleskey et al. 1987; McDonough et al., 1996; Cloues et al. 1997; Marrion and Tavalin 1998). Cells were used for either whole-cell macroscopic current or single channel measurements for only one hour after this pre-incubation, to prevent the opening of contaminating N- and P/Q-type channels following relief of block. Hippocampal neurons possess R-type current that is resistant to block by these toxins and contributes approximately 17-35% of the macroscopic current (McDonough et al. 1996; Sochivo et al. 2003). A holding potential of –60 mV was
chosen to study both single and macroscopic L-type channel activity in toxin pre-treated neurons. This holding potential inactivates over 80% of R-type current (Sochivko et al. 2003), eliminating these channels as a source of contamination.

Whole-cell recording

Cells were superfused with a modified Krebs’ solution of composition (mM): NaCl, 125; KCl, 5.85; tetraethylammonium chloride, 22.5; MgCl₂, 1.2; HEPES(Na), 10 and D-glucose, 11 (pH 7.4 with HCl) at room temperature. The external solution was supplemented with tetrodotoxin (1 µM) to block contaminating Na⁺ currents. Whole-cell L-type Ca²⁺ current was carried by either 10 mM Ca²⁺ or Ba²⁺ using Cl⁻ salts. Whole-cell electrodes were fabricated from KG-33 (borosilicate) glass (Friedrich and Dimmock, Millville, NJ) and filled with a solution of composition (mM): CsMeSO₄, 120; tetraethylammonium chloride, 30; BAPTA, 10; MgCl₂, 5; Na₂ATP, 5 and HEPES, 10 (pH 7.2). Currents were recorded with an Axopatch 200A (Axon Instruments, Union City, CA), using electrodes of resistance 1-3 MΩ. Capacitance and series resistance compensation (>90%) was used throughout. Whole-cell currents were evoked by 50 ms voltage steps (-50 to +30 mV) from a holding potential of -60 mV, low pass filtered at 1 kHz through an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and acquired at 100 µs intervals using PULSE (HEKA, distributed by Instrutech, NY, NY). Currents were leak-subtracted with a P/4 protocol from a holding potential of -100 mV. Whole-cell macroscopic L-type channel current was analyzed using PULSEFIT (HEKA, distributed by Instrutech, NY, NY). The time-course of deactivation currents was
approximated by the fitting of a single exponential, with the fit initiated from 0.1 ms after repolarization and terminated 20 ms after the end of the activation prepulse.

Single channel recording

After incubation, cells were superfused with a solution containing (mM): K aspartate, 125; KCl, 35; MgCl$_2$, 5; HEPES(Na), 10; EGTA, 10 and CaCl$_2$, 5.64 (to give an estimated free conc. of 60 nM) (pH 7.4 with HCl). Cells in this solution had ~0 mV membrane potential. Cell-attached patch recordings were made using thick walled (1.5 mm O.D., 0.5 mm I.D.) quartz electrodes (7-10 MΩ) filled with (mM): tetraethylammonium chloride, 135; HEPES, 10 (pH 7.4). Single channel currents were carried by either Ca$^{2+}$ (10, 60 or 160 mM) or Ba$^{2+}$ (10, 30 or 110) using Cl$^-$ salts. Concentrations of divalent ion in excess of 30 mM were compensated for by equimolar substitution of tetraethylammonium chloride. The pipette solution was supplemented with ω-conotoxin GVIA (5 µM) to counteract antagonism of block by the high concentration of divalent ion used in the electrode. L-type channel activity was evoked by 200 ms voltage steps to 0 mV from a holding potential of -60 mV. All potentials are expressed as the negative of the potential imposed on the pipette, without correction for a –17 mV (for 160 mM Ca$^{2+}$) or a +16 mV (for 10 mM Ca$^{2+}$) liquid junction potential. Channel currents were recorded with an Axopatch 200A amplifier, filtered at 1 kHz with an 8-pole Bessel filter and acquired at 100 μs intervals using PULSE. Single channels were analyzed using TAC (Bruxton, dist. by Instrutech. NY, NY), which uses a cubic spline interpolation procedure. The "50% threshold" technique was used to estimate event amplitudes and durations. The threshold was adjusted for each opening, and each
transition was inspected visually before being accepted. Open duration histograms constructed from openings to level -1, were logarithmically binned and a square root transformation of the ordinate (number of events/bin) was used, with the distribution being fitted by a sum of exponential probability density functions using the maximum-likelihood method. Statistical significance of multiple components was determined using the ratio of maximum likelihoods method (Horn and Lange, 1983; Colquhoun and Sigworth, 1995). Therefore, all histograms show data fit with the minimum number of exponential distributions that were statistically required to best describe the data. Missed events were not corrected for. The rise time of the filter was \( \sim 400 \mu s \), so that any events briefer than 166 \( \mu s \) were missed because they never reached the 50% threshold after filtering. Only those events that exceeded 1 ms in duration were used for estimation of amplitudes when filtered at 1 kHz to ensure that only those channel openings where the full amplitude was achieved were included. In addition, channel opening of events carried by \( Ca^{2+} \) ions under control conditions displayed an open time that was approximately 2x the filter dead-time. Therefore, the values of open time from exponential fits may be subject to some error. Increasing the divalent concentration from 10 to 110 mM (or 160 mM in case of \( Ca^{2+} \) ions) will affect the screening of surface charges, causing a depolarizing shift in current activation. This did not affect the comparison of open times of channels conducting different concentration of divalent ion, because hippocampal L-type channel open times are insensitive to voltage (Cloues et al, 1997). Only those events that closed before the end of the voltage step were used for estimation of channel amplitude and open time. Channel open probability could not be quantified for two reasons. First, superimpositions of channel opening would not be
observed when channel open times were extremely brief. This prevented any estimate of the number of channels in the patch for correct calculation of channel open probability. Second, FPL 64176 caused a dramatic increase in channel open time, which caused openings to exceed the pulse duration. This enhanced behaviour could not be quantified and resulted in an underestimate of channel open probability, making comparison meaningless.

(±) BAY K 8644 and FPL 64176 were dissolved in ethanol to a stock concentration of 10 mM. Either compound was added to the superfusing external solution to give a final concentration of 1 µM. For whole-cell recordings, compounds were added after current amplitude had stabilized and current-voltage relationships had been established. In some experiments, whole-cell macroscopic currents were recorded in alternating Ca$^{2+}$- and Ba$^{2+}$-containing external solution before addition of the agonists. In single channel studies, either compound was added after formation of the cell-attached patch. Additional cell-attached patches were recorded from the same culture dish, in the continued presence of compound. Data were pooled from patches acutely treated with agonists and those subjected to the continued presence of the agonist. The results indicated that neither compound produced long duration openings when Ca$^{2+}$ ions were used as the charge carrier. The charge carrier was alternated (between Ca$^{2+}$ and Ba$^{2+}$ ions) in successive patches to confirm that the compound was active. All control data were obtained on experimental equipment that had not been in contact with either compound, because neither BAY K 8644 nor FPL 64176 could be completely removed by washing. Values are given as mean ± S.E.M.
All compounds and reagents were from Sigma (Poole, Dorset, UK), except (±) BAY K 8644 and HEPES (Na and free acid) were purchased from Calbiochem (San Diego, CA). ω-conotoxins GVIA and MVIIC were obtained from BACHEM California, Torrance, CA).
Results

Enhancement of macroscopic L-type currents by BAY K 8644 and FPL 64176.

Whole-cell recording from acutely dissociated rat CA1 hippocampal neurons pre-incubated in ω-conotoxins MVIIIC and GVIA revealed the L-type channel current (see Materials and Methods). Macroscopic currents showed a modest decay (~ 20-25%) during the voltage step when Ca\(^{2+}\) ions were the permeant ion (Figure 1A and C). In contrast, the whole-cell L-type current was sustained throughout a step depolarization from a holding potential of –60 mV to 0 mV when Ba\(^{2+}\) ions were the charge carrier (Figures 1B and D). The macroscopic L-type current recorded using either 10 mM Ca\(^{2+}\) or Ba\(^{2+}\) ions as the charge carrier activated from approximately –40 mV, with the peak current observed at 0 to +10 mV (Figure 1). Deactivation of L-type current was rapid regardless of the identity of the charge carrier (Figure 1 and Table 1). The decay time-course of deactivation currents was voltage-dependent, increasing e-fold in approximately 30 mV with τ increasing from 1.28 ± 0.08 ms at –60 mV to 3.37 ± 0.32 ms at –30 mV (n=11, data not shown).

Whole-cell L-type channel current was enhanced by application of BAY K 8644, regardless of whether Ca\(^{2+}\) or Ba\(^{2+}\) ions were the charge carrier (Figure 1A and B), producing an approximately 10 mV hyperpolarizing shift in current activation (Figure 1A and B). However, BAY K 8644 enhanced whole-cell current carried by Ca\(^{2+}\) ions much less (approximately 40% less) than that carried by Ba\(^{2+}\) (Figure 1A and B). BAY K 8644 did not affect the activation time course of whole-cell currents carried by either ion.
(Figure 1A and B). The effect on the macroscopic L-type channel current of FPL 64176 was markedly different from that of BAY K 8644. The enhancement of the peak current by FPL 64176 was significantly greater than that seen with BAY K 8644 (Figure 1) and was associated with an approximately 20 mV hyperpolarizing shift in current activation. In addition, FPL 64176 slowed the activation of the whole-cell current, regardless of the identity of the charge carrier (Figures 1C and D).

The time course of deactivation of macroscopic currents is determined, at least in part, by the channel open time. Both agonists have been shown to prolong L-type channel open time (e.g. Hess et al. 1984; Fan et al. 2001), suggesting that the time course of deactivation of macroscopic currents should be slowed. In contrast to this prediction, BAY K 8644 did not change the decay rate of deactivation current carried by either permeant ion (Figure 1A and B and Table 1). In accord with these predictions, FPL 64176 produced a modest slowing of the deactivation tail current time course when Ca$^{2+}$ ions were the charge carrier, but produced a dramatically slow deactivation tail current when Ba$^{2+}$ was the permeant ion (Figures 1C and D and Table 1). These effects suggested that the mechanism(s) underlying enhancement of the macroscopic current by the dihydropyridine were very different from that produced by the benzoylpyrrole. Single channel studies were carried out to determine the effects on channel gating that underlie enhancement of the macroscopic current.
Effect of L-type channel agonists on single channel properties

*Increased single channel current induced by BAY K 8644.*

BAY K 8644 or the related dihydropyridine agonist (+)-(S)-202-791 increased single L-type channel conductance in hippocampal neurons and GH3 pituitary cells when Ba\(^{2+}\) was the permeating ion (Mantegazza et al. 1995; Cloues et al. 1997). In contrast, no effect of BAY K 8644 on channel conductance was observed in dorsal root ganglion cells (Fox et al., 1987), pancreatic β cells (Smith et al., 1993) or cardiac myocytes (Hess et al. 1986). Therefore, single L-type channels were resolved using 10, 30 and 110 mM Ba\(^{2+}\) and 10, 60 and 160 mM Ca\(^{2+}\) in the electrode solution to determine if this effect was common to both divalent ion charge carriers. Figure 2A shows examples of single channel currents recorded at 0 mV in the presence of 10, 30 and 110 mM Ba\(^{2+}\). The mean values of single channel current (i) at 0 mV are plotted as a function of divalent ion concentration in Figure 2D (Hess et al., 1986). Increasing the concentration of the permeant ion increased the amplitude of single channel currents recorded at 0 mV, with the amplitude displaying saturation at high divalent ion concentration. The relationship was fitted with a hyperbolic Langmuir isotherm giving an apparent dissociation constant (K\(_d\)) of 13.5 mM (Figure 2D). Single channel open times and probability were obviously enhanced (see below) in the presence of BAY K 8644 (Figure 2B). In agreement with a previous report (Cloues et al. 1997), single channel amplitude was increased in the presence of the dihydropyridine. Amplitudes were well described by a Langmuir isotherm, giving a K\(_d\) of 18.6 mM (Figure 2D).
The dihydropyridine also increased single channel current carried by Ca$^{2+}$ ions. Figure 3A shows examples of elementary currents recorded at 0 mV in the presence of 10, 60 and 160 mM Ca$^{2+}$. Under control conditions, channel amplitude increased with Ca$^{2+}$ concentration yielding an apparent K$_d$ of 4.2 mM (Figure 3D). The amplitude of single channel currents at 0 mV was enhanced at all Ca$^{2+}$ concentrations by BAY K 8644 (Figure 3B), while channel open time was not obviously affected (see below). Plotting the amplitudes as a function of divalent concentration gave a distribution that was described by a Langmuir isotherm with a K$_d$ of 8.9 mM (Figure 3D).

**Effect of FPL 64176 on single channel current amplitude.**

BAY K 8644 increased single L-type channel amplitude, which questions whether this effect was conserved across distinct classes of L-type channel agonist. Figure 4A shows examples of single channel currents recorded at 0 mV in the presence of 10, 30 and 110 mM Ba$^{2+}$. Measured amplitudes were plotted as a function of divalent ion concentration and fitted with a saturating Langmuir isotherm that gave a K$_d$ of 13.5 mM (Figure 4C). In the presence of FPL 64176, the open time of single channel currents carried by Ba$^{2+}$ ions was obviously prolonged (Figure 4B, see below). The amplitudes of channel currents carried were similar to those recorded under control conditions, giving an apparent K$_d$ of 15.4 mM (Figure 4C). The amplitude of single channel currents recorded with 10, 60 and 160 mM Ca$^{2+}$ ions as the charge carrier were also not affected by the presence of FPL 64176, with K$_d$’s in the absence and presence of the benzoylpyrrole
being 4.2 and 4.7 mM respectively (inset of Figure 4C). Therefore, FPL 64176 did not affect channel amplitude, in contrast to the effect of BAY K 8644.

Effect of BAY K 8644 and FPL 64176 on single channel open times.

Effect of L-type channel agonists on channel open times with Ba\(^{2+}\) as the permeant ion.

The open times of L-type channels in the absence of channel agonists are extremely brief (Cloues et al. 1997; Marrion and Tavalin 1998; Guia et al. 2001; Aoyama et al. 2003). Representative openings with 10 and 110 mM Ba\(^{2+}\) as the charge carrier are shown in Figures 5 (control) and 6 (control) respectively. These openings were best described by the sum of two exponentials of time constants (τ) 0.4 and 2.8 ms (Figures 5 and 6 (control)). Channel open times were obviously prolonged in the presence of BAY K 8644 (Figure 5, BAY K 8644; Figure 6, BAY K 8644). Openings were best described by the sum of three exponentials of 1.0, 3.1 and 13.8 ms when 10 mM Ba\(^{2+}\) was the permeant ion and 0.6, 2.2 and 6.5 ms when 110 mM Ba\(^{2+}\) ions were the charge carrier. Therefore, the dihydropyridine evoked an additional open state with an open time constant at 0 mV of 6.5-14 ms (Figures 5 and 6).

In the presence of BAY K 8644, openings tended to be grouped in bursts with relatively few single long duration openings. This observation was in contrast to L-type channel openings in the presence of FPL 64176, which tended to be of very long duration with very short duration closures within the burst (Figures 5, FPL 64176, Figure 6, FPL 64176). The open time histogram of events carried by 10 mM Ba\(^{2+}\) ions was best
described by the sum of two exponentials of time constants 0.5 and 6.8 ms (Figure 5, FPL 64176). In contrast, the sum of three exponentials of time constants 0.5, 3.8 and 19.6 ms were necessary to adequately describe channel openings in 110 mM Ba$^{2+}$ (Figure 6, FPL 64176).

**Effect of L-type channel agonists on channel open times with Ca$^{2+}$ as the permeant ion.**

In contrast to expectations, BAY K 8644 and FPL 64176 had only a modest effect on L-type single channel open times carried by Ca$^{2+}$ ions (Figures 7 and 8). This was observed when either 10 mM (Figure 7) or 160 mM (Figure 8) Ca$^{2+}$ was the permeant ion. The amplitude of single channel currents recorded with 160 mM Ca$^{2+}$ at 0 mV was identical to those recorded previously that were sensitive to the dihydropyridine antagonist nimodipine (Marrion and Tavalin 1998). In addition, recordings using Ba$^{2+}$ or Ca$^{2+}$ as the charge carrier were alternated to confirm that each channel agonist was functional. As presented above, each channel agonist evoked long open time activity when Ba$^{2+}$ was the charge carrier. Therefore, the lack of a substantial effect of agonist on channels conducting Ca$^{2+}$ ions is a property of the agonist and not merely a problem of keeping the compound active. Single L-type channels conducting Ba$^{2+}$ ions displayed variable open times under control conditions, with some patches that displayed a monoexponential open duration distribution ($\tau \sim 0.4$ ms) while others exhibited a bi-exponential distribution that included additional set of openings with longer durations ($\tau \sim 2.3$ ms) (see Figures 7 and 8 and Cloues et al. 1997). Single channel open time was extremely brief when channels conducted Ca$^{2+}$ ions under control conditions, being best described by a single exponential of time constant 0.34 – 0.54 ms (Figures 7 and 8, control). It appeared that
channels conducting Ca\textsuperscript{2+} ions did not display the slower open time constant (\(\tau \sim 2.5\) ms) observed when Ba\textsuperscript{2+} was the permeating ion (Figures 5 and 6, control). Addition of BAY K 8644 promoted a minority of openings to display the slower open time component (\(\tau \sim 2.5\) ms) (Figures 7 and 8, BAY K 8644). In contrast, single channels showed no change in open state kinetics when recorded with 10 mM Ca\textsuperscript{2+} as the charge carrier and in the presence of FPL 64176 (Figure 7, FPL 64176). FPL 64176 may have produced a small effect on channels recorded with 160 mM Ca\textsuperscript{2+}, evoking the slower open time component seen in the presence of BAY K 8644 (\(\tau \sim 2.3\) ms, Figure 8, FPL 64176). Therefore, neither agonist affected channel open time beyond that already observed by channels conducting Ba\textsuperscript{2+} ions under control conditions.

Slowing of current activation by FPL 64176.

A slowing of current activation evoked by FPL 64176 has been observed in cardiac myocytes, an effect that was independent of the identity of the charge carrier (Fan et al. 2000). In many single channel sweeps, FPL 64176-modified long duration openings appeared clustered at the end of the voltage step, with channels remaining open after repolarization (Figures 4B, 5, 9A and 9C). In most sweeps, long duration openings were preceded by short duration openings (Figures 4B, 5 and 9A). This is illustrated in Figure 9A, where a membrane patch containing a single channel is shown in the presence of FPL 64176. Step depolarization to 0 mV from a holding potential of –60 mV evoked channel opening. Long duration openings characteristic of being modified by the benzoylpyrrole were observed after a marked delay and subsequent to short duration openings (marked *)
Open time analysis of short duration events that immediately preceded an opening in excess of 10 ms duration were best described by a single exponential with time constant of 0.73 ms (Figure 9C). This open time distribution was reminiscent of channel openings in the absence of FPL 64176 (compare Figure 8C to Figures 5-8). The combination of short duration openings followed by long duration activity produced a kinetic slowing of the ensemble current (Figure 9B).
Discussion

L-type channel agonists have been used to characterize the channels or to make them easier to resolve. In the absence of these compounds, L-type channels display a very brief mean open time ($\tau \sim 0.4$ ms) (Cloues et al. 1997; Kavalali et al. 1997; Figures 2, 5 and 6), which is prolonged by either BAY K 8644 or FPL 64176 when channel currents are carried by $\text{Ba}^{2+}$ ions (Figures 3, 6 and 7). It has been assumed that the effect of channel agonists is independent of the identity of the permeant ion. However, this study has shown that the mode of action of both agonists is dependent on the divalent ion charge carrier.

L-type channel agonists enhanced the macroscopic L-type current by different effects on single channel gating. For example, BAY K 8644 enhanced the whole-cell L-type channel $\text{Ba}^{2+}$ current, partly by an increase in single channel conductance (Figure 2) and a recruitment of a longer duration open state (Figures 5 and 6). A similar BAY K 8644-induced increase in single channel conductance was observed when $\text{Ca}^{2+}$ ions were the charge carrier (Figure 3), contributing to enhancement of the macroscopic current. This increased single channel current amplitude for either charge carrier in the presence of BAY K 8644 may result from the lowering of affinity for the permeant ion during conduction. Whole-cell $\text{Ba}^{2+}$ currents were augmented to a greater extent than $\text{Ca}^{2+}$ currents by BAY K 8644, an effect that can be as least partially explained by a lack of effect of the dihydropyridine on channel open times when $\text{Ca}^{2+}$ was the permeant ion (Figures 7 and 8). Similar effects of BAY K 8644 have been reported in hair cells, where
agonist-induced long duration openings were only observed when channels conducted 
Ba$^{2+}$ and not Ca$^{2+}$ ions (Rodríguez-Contreras & Yamoah, 2003). FPL 64176 strongly 
enhanced the whole-cell L-type channel Ba$^{2+}$ current, an effect achieved in part by a 
dramatic increase in channel open time (Figures 5 and 6) that underlied the dramatic 
slowing in the time course of current deactivation (Figures 1D and 9). FPL 64176 
enhancement of whole-cell Ca$^{2+}$ current did not result from an increase of single channel 
current (Figure 4) or channel open time (Figures 7 and 8), thus suggesting that it was 
mediated primarily by an increase in the frequency of opening. Collectively, these data 
suggests that while the two classes of L-channel agonists have different effects on 
conductance both have qualitatively similar effects on open time behavior that are 
dependent on the permeant ion.

L-type channels exhibit Ca$^{2+}$-dependent inactivation, resulting in a decline in whole-cell 
current during a maintained depolarization (Eckert & Chad, 1984). Macroscopic current 
from hippocampal neurons displayed clear Ca$^{2+}$-dependent inactivation, a loss of current 
that was not observed when using Ba$^{2+}$ ions as the charge carrier (Figure 1). It is possible 
that the action of channel agonist may be state-dependent, with a greater effect being 
observed on channels not inactivated by a Ca$^{2+}$-dependent process. This is consistent 
with the larger effect of BAY K 8644 on macroscopic current carried by Ba$^{2+}$ ions 
(Figure 1). It has been reported that BAY K 8644 increases the rate of inactivation of 
Ca$^{2+}$-carried macroscopic current in both cardiac L-type (Sanguinetti et al, 1986) and 
recombinant Ca$_V$1.2 subunits expressed in *Xenopus* oocytes (Noceti et al, 1998). 
Stationarity plots of channel open probability at 0 mV showed that BAY K 8644 and FPL
64176 increased the rate of decay of channel open probability conducting Ca\textsuperscript{2+} ions, an effect that was more prominent at higher concentrations of external Ca\textsuperscript{2+} ions (data not shown; Noceti et al, 1998). This was not observed when channels conducted Ba\textsuperscript{2+} ions. This data demonstrates that Ca\textsuperscript{2+}-dependent inactivation is sensitive to open probability (Noceti et al, 1998). However, it is unclear whether this suggests channel agonists favor channels that are not inactivated or that they promote inactivation by increasing channel open probability at the start of the voltage step. The open time of channels conducting Ca\textsuperscript{2+} ions within the first 50 ms of the voltage step was longer (τ~0.69 ms) than in the remaining 150 ms (τ~0.47 ms). It is possible that hippocampal L-type channels display the change in gating resulting from Ca\textsuperscript{2+}-dependent inactivation reported in cardiac myocytes (Imredy & Yue, 1994). However, channels in the presence of BAY K 8644 showed a similar small reduction of open time during the pulse (openings <50 ms: τ’s 0.5 and 2.8 ms, openings >50 ms: τ’s~0.41 and 1.9 ms, data not shown). Therefore, BAY K 8644 did not promote a large prolongation of channel open time at the start of the pulse. This suggests that the action of BAY K 8644 is not state-dependent.

L-type channel gating has been modeled with a modal scheme that can be illustrated as follows:

\[
\begin{align*}
C_1 & \xrightarrow{\text{C_2}} O & \text{mode 1 (short open time)} \\
* & \quad \uparrow & \\
C'_1 & \xrightarrow{\text{C'_2}} O' & \text{mode 2 (long open time)}
\end{align*}
\]

The channel is proposed to reside within mode 1 during normal gating, with occasional sojourns to mode 2 (Hess et al. 1984). BAY K 8644 is proposed to increase the transition
rate into mode 2 (marked *) (Hess et al. 1984). A minor change to this scheme can satisfy the data obtained in this study. Assuming that gating is modal (Hess et al. 1984; we have no data supporting this assumption), the channel can reside in any of the following states:

\[
\begin{align*}
C_1 & \quad C_2 & \quad C_3 & \quad \text{mode 1} \\
& \quad \quad \uparrow & \quad \downarrow & \\
& \quad O_1 & \quad O_2 & \\
& \downarrow & \quad \uparrow & \\
C_4 & \quad C_5 & \quad O_3 & \quad \text{mode 2}
\end{align*}
\]

The channel resides in mode 1 during normal gating, giving rise to open times best described by a dominant exponential distribution of time constant 0.4 ms (from O₁), with a very minor additional component of time constant 2.3 ms (from O₂) (Figures 4 and 5). Sojourns into mode 2 are not permissible under control conditions. Addition of agonist increases the transition rates marked ◊, but the transition rate between C₁ and C₄ into mode 2 can only occur when Ba^{2+} binds within the channel. This would give rise to an open time distribution consisting of three components derived from mode 1 (τ’s 0.4 and 2.3 ms) and mode 2 activity (τ 10 ms). The transition rate between C₂ and C₃ that resides within mode 1 activity is approximately 10-fold slower when the channel is conducting Ca^{2+} in contrast to Ba^{2+} ions. This would provide a monoexponential open time distribution (τ 0.4 ms) during control activity (Figures 6 and 7). Addition of agonist to the channel conducting Ca^{2+} ions would increase the transition rate between C₁ and C₂ (marked ◊), producing a dominant exponential distribution of time constant 0.4 ms with
a very minor additional component of time constant 2.3 ms (Figures 6 and 7). The transition rate between C1 and C4 into mode 2 is not permitted while the channel conducts Ca\(^{2+}\) ions, with mode 2 only observed when the channel conducts Ba\(^{2+}\) ions. This scheme suggests that gating is dependent on the divalent ion, with channel states only accessible if a non-physiological charge carrier is conducted through the channel. Finally, it supports the experimental observation that agonist-modified openings are very unlikely to be seen spontaneously.

Slowing of the deactivation current by BAY K 8644 was not observed in this study with either cation as the charge carrier, despite the recruitment of long duration openings at 0 mV when using Ba\(^{2+}\) ions as the charge carrier. It is worth noting that channel openings were not observed to exceed the pulse duration, supporting the lack of a slowing of macroscopic current deactivation. BAY K 8644 slows the macroscopic deactivation tail current in cardiac myocytes, an effect thought to arise from the slow closing rate associated with mode 2 openings (Tsien et al. 1986; Bechem and Hoffmann 1993; Fan et al. 2000). Slowing of macroscopic deactivation by the related dihydropyridine agonist (+)-202-791 has been reported in a variety of neurons when using Ba\(^{2+}\) ions as the charge carrier (Plummer et al, 1989; Regan et al, 1991; Kammermeier & Jones, 1997). Contrary results have been reported from hippocampal neurons, where BAY K 8644 slowed current deactivation only when the current was carried by Ba\(^{2+}\) and not Ca\(^{2+}\) ions (Toselli and Taglietti 1992; Docherty and Brown 1986), while slowing was observed by others when using Ca\(^{2+}\) ions as the charge carrier (Ishibashi et al. 1998). In contrast, a lack of effect of BAY K 8644 on current deactivation has been reported in pancreatic β cells
(Smith et al. 1993). BAY K 8644 slowed tail currents when recombinant Ca\textsubscript{V}1.2 channels were expressed in dysgenic myotubes or in CHO cells (Wilkens et al. 2001; Aoyama et al. 2003), but not when co-expressed with the cardiac \( \beta\textsubscript{2a} \) subunit in *Xenopus* oocytes (Noceti et al. 1998). Conflicting results have also been observed for the effect of BAY K 8644 on Ca\textsubscript{V}1.3 channels. For example, BAY K 8644 slowed whole-cell deactivation tail currents from native Ca\textsubscript{V}1.3 channels in pinealocytes (Chik et al. 1997), while those in dorsal root ganglion neurons were not affected (Wyatt et al. 1997). This dichotomy is also apparent with recombinant Ca\textsubscript{V}1.3 channels, as slowing of Ca\textsubscript{V}1.3-mediated current by BAY K 8644 has been observed by some (Xu and Lipscombe 2001) but not others (Bell et al. 2001; Koschak et al. 2001). Recent evidence indicates that the related dihydropyridine agonist (+)-202-791 produces only a modest slowing of deactivation tail currents in both PC12 cells and rat superior cervical ganglion cells whose L-type current is thought to be predominantly mediated by Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 respectively (Liu et al. 2003). Thus, the deactivation rate in the presence of dihydropyridine agonists does not appear to distinguish between either class of channel.

FPL 64176 evoked an obvious slowing of the deactivation tail current carried by Ca\textsuperscript{2+} ions in cardiac myocytes (Fan et al. 2000). In the present study, FPL 64176 also dramatically slowed the whole-cell deactivation current when Ba\textsuperscript{2+} was the charge carrier (Figure 1D) but produced only a modest prolongation of deactivation time-course when Ca\textsuperscript{2+} was the conducting ion (Figure 1C). In addition, the agonist did produce a kinetic slowing of macroscopic current activation, regardless of the identity of the charge carrier (Figures 1C and D). This resulted from a delay of FPL 64176-induced long duration
channel openings, which occurred subsequent to brief openings and caused most openings to occur towards the end of the voltage step (Figures 4, 5, 6 and 9A). Similar behavior has been observed for recombinant CaV1.2 channels treated with FPL 64176 (Lauven et al. 1999). These data suggest that the binding site for FPL 64176 is primarily accessible from the open state. Similar FPL 64176-induced slowing of whole-cell activation and deactivation in Ba\textsuperscript{2+} containing solutions has also been recently observed in other neuronal cell types, which like hippocampal neurons may contain a combination of CaV1.2 and CaV1.3 (Liu et al. 2003). This suggests that the slowing of activation reflects a property of the agonist that is likely to be common to both classes of L-type channels.

The amplitude of single hippocampal L-type channels conducting either Ba\textsuperscript{2+} (110 mM) or Ca\textsuperscript{2+} (160 mM) was much smaller than that recorded in cardiac tissue. For example, single L-type channels in the absence of channel agonists conducting Ca\textsuperscript{2+} ions (160 mM) at 0 mV was approximately 191 fA in hippocampal neurons (Marrion and Tavalin 1998; Figures 2 and 7) while those conducting Ba\textsuperscript{2+} ions (110 mM) was approximately 950 fA (Figures 2 and 6). In contrast, L-type channel amplitude in saturating Ca\textsuperscript{2+} and Ba\textsuperscript{2+} ion concentrations was approximately 410 fA and 1.4 pA respectively in the absence of channel agonists, in cardiac myocytes (Guia et al. 2001; Hess et al., 1986). These differences may be partly attributed to the apparent affinity of the ion for the channel being higher in hippocampus (K\textsubscript{d} \sim 4.2 mM for Ca\textsuperscript{2+} and \sim 13.5 mM for Ba\textsuperscript{2+}) compared to cardiac tissue (K\textsubscript{d} \sim 14 mM for Ca\textsuperscript{2+} and \sim 28 mM for Ba\textsuperscript{2+}; Hess et al. 1986).
The effect of these L-type channel agonists on single channel conductance has been controversial. For example, BAY K 8644 increased single channel conductance in hippocampal neurons (Figures 2 and 3; Cloues et al. 1997), GH3 pituitary cells (Mantegazza et al. 1995), cardiac myocytes (Kokubun & Reuter, 1984; Lacerda & Brown, 1989) and smooth muscle cells (Caffrey et al, 1986). In contrast, no effect of the dihydropyridine agonist on channel conductance was observed in dorsal root ganglion cells (Fox et al, 1987), pancreatic β cells (Smith et al, 1993) or cardiac myocytes (Hess et al. 1986). In contrast, FPL 64176 increases single L-type channel conductance in cardiac myocytes (Fan et al. 2001), but not in hippocampal neurons (Figure 4). It is tempting to suggest that Cav1.3 channels dominated the hippocampal L-type current, with little arising from Cav1.2 channels, because hippocampal neurons express both Cav1.2 and Cav1.3 (Bowden et al. 2001) while cardiac myocytes exclusively express Cav1.2 subunits. Certainly, the larger conductance expected from Cav1.2 channels was not observed in this study. Therefore, it is possible that even though the Cav1.2 channel is present, it does not provide a significant component of Ca$^{2+}$ entry into hippocampal neurons. However, several splice variants of the pore forming α subunit exist for both Cav1.2 and Cav1.3 and may contribute to the conflicting behavior of L-type Ca$^{2+}$ channels in various tissues (Welling et al. 1997, Safa et al. 2001). In addition, auxiliary subunits may also contribute to the aforementioned differences hippocampal neurons and other tissues. Therefore, it remains important to systematically determine the effects of channel composition on L-channel activity and pharmacology.
Regardless of the precise molecular identity of the L-type channels recorded in the present study, it is clear that use of these compounds with non-physiological charge carriers may not be simply extrapolated to physiological conditions. Therefore the identity of permeant ion must be taken into consideration when using agonists to characterize L-type channels.

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Table 1. Effects of L-type channel agonists on macroscopic current deactivation time course.

<table>
<thead>
<tr>
<th>divalent cation</th>
<th>Control</th>
<th>(±)BAY K 8644 (1 µM)</th>
<th>FPL 64176 (1 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})</td>
<td>1.28 ± 0.08 (n=10)</td>
<td>1.18 ± 0.14 (n=8)</td>
<td>1.98 ± 0.1* (n=4)</td>
</tr>
<tr>
<td>Ba(^{2+})</td>
<td>1.32 ± 0.18 (n=10)</td>
<td>1.29 ± 0.29 (n=4)</td>
<td>6.88 ± 0.9* (n=3)</td>
</tr>
</tbody>
</table>

* denotes p<0.05 significance from values observed under control conditions, Student’s t test.

Macroscopic current was evoked by a voltage step to 0 mV from a holding potential of –60 mV (50 ms duration). The time course of current deactivation at –60 mV was approximated by the fitting of a single exponential function from 0.1 ms after the termination of the voltage step.
Figure 1. Enhancement of whole-cell Ca\textsuperscript{2+}- or Ba\textsuperscript{2+}-carried L-type currents by BAY K 8644 or FPL 64176.

Cells were whole-cell voltage-clamped at –60 mV and macroscopic L-type channel current was evoked by a step depolarization to 0 mV. Macroscopic currents were carried by either 10 mM Ca\textsuperscript{2+} (A and C) or Ba\textsuperscript{2+} ions (B and D). Shown as an inset (right) to panels A-C is the normalized control (grey) and drug-modified (black) deactivation tail currents to illustrate the effects of L-type channel agonists on the time course of current deactivation. An inset is not shown for deactivation current in the presence of FPL 64176 because the greatly slowed rate did not permit a full decay within the 50 ms repolarization segment. BAY K 8644 enhanced Ca\textsuperscript{2+} (109 ± 18 % enhancement of peak current, n=8) (A) or Ba\textsuperscript{2+} (149 ± 24 %, n=4) (B) carried L-type current without a change in deactivation tail kinetics. This enhancement was accompanied by a 10 mV hyperpolarizing shift in current activation (A and B, left insets. Plotted is normalized current against membrane voltage, where current was normalized to the amplitude at 0 mV before addition of agonist). In contrast, enhancement of Ca\textsuperscript{2+} (193 ± 64 % enhancement of peak current, n=4) (C) or Ba\textsuperscript{2+} (394 ± 32 %, n=5) (D) by FPL 64176 was associated with a dramatic slowing of deactivation kinetics when Ba\textsuperscript{2+} was the charge carrier (D). A modest significant slowing of the deactivation tail current was observed when Ca\textsuperscript{2+} was the permeant ion (C) (see Table 1). This enhancement was accompanied by an approximately 20 mV hyperpolarizing shift in current activation (C and D, right insets). % enhancement was calculated as \( \frac{\{I(\text{drug})-I(\text{control})\}}{I(\text{control})} \times 100 \).
Figure 1. Tavalin et al
Figure 2. BAY K 8644 increased single channel current carried by Ba\textsuperscript{2+} ions.

Single channel currents were recorded in the cell-attached patch configuration and evoked by a step depolarization from a holding potential of –60 mV to 0 mV. A. L-type channel openings were resolved with either 10, 30 110 mM Ba\textsuperscript{2+} in the electrode solution under control conditions. Openings were of very short duration in each concentration of Ba\textsuperscript{2+}. B. Channel openings were obtained in the presence of BAY K 8644 using 10, 30 and 110 mM Ba\textsuperscript{2+} in the electrode solution. The L-type channel agonist caused a dramatic increase in channel open time (see Figures 5 and 6). C. Amplitude versus duration plots for channel openings conducting either 10 or 110 mM Ba\textsuperscript{2+} evoked at 0 mV from a holding potential of –60 mV. Closed symbols represent openings under control conditions and open symbols are events in the presence of 1 µM BAY K 8644. Single channel open times were of extremely short duration, particularly under control conditions. The plots show that events of duration shorter than 1 ms exhibited a lower apparent amplitude. This resulted from filtering preventing these events from reaching full amplitude and indicated that only those events that exceeded 1 ms duration should be used for estimation of channel amplitudes (see Materials and Methods). In addition, the amplitude-duration plots illustrate the effect of BAY K 8644 on channel amplitude and duration. D. Plotted is the relationship between single channel current amplitude at 0 mV (i) and concentration of Ba\textsuperscript{2+} in the electrode solution. Channel amplitude was obtained by fitting of amplitude histograms to a single Gaussian distribution. Only openings that exceeded 1 ms in duration were used for amplitude estimates (see Materials and Methods). Data was fitted with the equation $i = i_{\text{max}}/(1+K_d/[X])$, where $i$ is the elementary current, [X] is the ion concentration, $i_{\text{max}}$ is the saturating value of $i$ at [X] =
infinity, and \( K_d \) is the equilibrium dissociation constant. Under control conditions the equation yielded values of \( i_{\text{max}} = 0.989 \ \text{pA} \) and \( K_d = 13.5 \ \text{mM} \) (\( n=15, 5 \) and \( 8 \) for \( 10, 30 \) and \( 110 \ \text{mM} \ \text{Ba}^{2+} \) respectively). In the presence of BAY K 8644, \( i_{\text{max}} \) was increased to \( 1.44 \ \text{pA} \) and the \( K_d \) increased to \( 18.6 \ \text{mM} \) (\( n=21, 5, 10 \) for \( 10, 30 \) and \( 110 \ \text{mM} \ \text{Ba}^{2+} \) respectively).
Figure 2. Tavalin et al
**Figure 3. BAY K 8644 increased single channel current carried by Ca\(^{2+}\) ions.**

Shown are single channel events recorded under control conditions (A) or in the presence of BAY K 8644 (B). Openings were resolved using 10, 60, and 160 mM Ca\(^{2+}\) in the electrode solution. Openings were of very short duration using all Ca\(^{2+}\) concentrations, even in the presence of the dihydropyridine (B). C. Amplitude-duration plots for events evoked at 0 mV under control conditions (filled symbols) and in the presence of 1 µM BAY K 8644 (open symbols). As seen when channels conducted Ba\(^{2+}\) ions, openings shorter than 1 ms duration tended to display a lower apparent amplitude that resulted from filtering (see Materials and Methods). In addition, plots show the effect of BAY K 8644 on channel amplitude and open time. Inset shows an amplitude histogram of events recorded in 10 mM Ca\(^{2+}\). The distribution was well described a single Gaussian function. D. Plotted is the relationship between single channel amplitude at 0 mV (i) and concentration of Ca\(^{2+}\) in the electrode solution. The smooth curves are the best fit to the equation \(i = i_{\text{max}}/(1+K_d/[X])\) (see Figure 2 legend). \(i_{\text{max}} = 0.191\) pA and \(K_d = 4.24\) mM under control conditions (n=8, 4 and 9 for 10, 60 and 160 mM Ca\(^{2+}\) respectively). In the presence of BAY K 8644, \(i_{\text{max}}\) was increased to 0.299 pA and \(K_d\) was increased to 8.87 mM (n=9, 5 and 6 for 10, 60 and 160 mM Ca\(^{2+}\) respectively).
Figure 3. Tavalin et al
Figure 4. FPL 64176 did not affect single channel current amplitude.

A. Representative single channel openings are shown evoked by a step depolarization from –60 mV to 0 mV in the presence of either 10, 30 or 110 mM Ba\(^{2+}\) in the electrode solution. Events were of very short duration under all divalent ion concentration conditions. B. Addition of FPL 64176 evoked openings of very long duration in each concentration of Ba\(^{2+}\). C. The single channel amplitudes at 0 mV (i) recorded in A. and B. are plotted as a function of divalent ion concentration. The smooth curve is the best fit to the equation \(i = i_{\text{max}}/(1+K_d/[X])\) (see Figure 2 legend), with \(i_{\text{max}} = 0.99\) pA and \(K_d = 13.54\) mM under control conditions (n=15, 5 and 8 for 10, 30 and 110 mM Ba\(^{2+}\) respectively). In the presence of FPL 64176, \(i_{\text{max}}\) and \(K_d\) were unaffected (1.05 pA and 15.3 mM respectively) (n=4, 4 and 5 for 10, 30 and 110 mM Ba\(^{2+}\) respectively). Inset shows single channel amplitude at 0 mV (i) of Ca\(^{2+}\)-carried currents as a function of Ca\(^{2+}\) concentration, with the smooth curve representing by \(i_{\text{max}} = 0.194\) pA and \(K_d = 5.16\) mM for control and FPL 64176 openings (n=8, 4 and 9 for 10, 60 and 160 mM Ca\(^{2+}\) respectively under control conditions and n=5 and 5 for 10 and 160 mM Ca\(^{2+}\) respectively in the presence of FPL 64176).
Figure 4. Tavalin et al
Figure 5. BAY K 8644 and FPL 64176 recruited long open time activity of L-type Ca\(^{2+}\) channels with Ba\(^{2+}\) (10mM) as the permeant ion.

The figure shows three panels illustrating representative single channel openings under Control, + BAY K 8644 (1 \(\mu\)M) and + FPL 64176 (1 \(\mu\)M) conditions. Channel openings were evoked by a step depolarization to 0 mV from a holding potential of –60 mV with 10 mM Ba\(^{2+}\) in the electrode solution. Single channel openings were of very short duration in control conditions. Combining openings from 15 patches (total of 4579 events) produced an open state distribution that was best fitted by the sum of two exponentials of time constants 0.41 (89\%) and 2.9 ms (11\%). Both L-type Ca\(^{2+}\) channel agonists promoted long open time activity at the expense of short duration openings. Openings in the presence of BAY K 8644 tended to be grouped into short duration bursts throughout the voltage step. These openings (3455 events from 21 patches) were best described by the sum of three exponentials of time constants 1.0 (66\%), 3.1 (25\%) and 13.8 (9\%) ms. In the presence of FPL 64176, channel openings most often comprised of long duration bursts, containing short duration closures. Combining 708 events from 4 patches gave an open duration histogram that was best fitted by the sum of two exponentials of time constants 0.53 (78\%) and 6.8 (22\%) ms.
Figure 5. Tavalin et al
Figure 6. Long duration activity evoked by L-type channel agonists with 110 mM Ba\textsuperscript{2+} as the permeant ion.

Openings evoked in the absence of L-type channel agonist were of very short duration. Combined events from 8 patches (total of 2816 events) showed that these openings were best described by the sum of two exponential components of time constants 0.46 (97%) and 2.78 (3%) ms. Addition of BAY K 8644 produced longer duration openings that tended to reside in short bursts of activity (filtered at 2 kHz). Combining 470 events from 10 patches gave an open time distribution that was best fitted by the sum of three exponentials with time constants 0.59 (59%), 2.19 (17%) and 6.51 (24%) ms. Openings in the presence of FPL 64176 were of very long duration, with channel open time limited by short duration closures. The open duration histogram of 1427 events (combined from 5 patches) was best fitted by the sum of three exponentials with time constants of 0.5 (89%), 3.81 (8%) and 19.6 (3%) ms.
Figure 6. Tavalin et al.
Figure 7. Both BAY K 8644 and FPL 64176 failed to promote very long duration openings when 10 mM Ca$^{2+}$ was the charge carrier.

Single channel openings carried by 10 mM Ca$^{2+}$ ions were of very small amplitude and duration. Sweeps displayed functionally coupled SK channels (upward channel openings) in some instances (Marrion and Tavalin, 1998). Single channel openings were best described by a single exponential distribution ($\tau \sim 0.34$ ms, 923 events from 8 patches) under control conditions. Long duration openings carried by Ca$^{2+}$ ions were not observed with either L-type channel agonist. Openings in the presence of BAY K 8644 were best described by the sum of two exponentials of time constants 0.35 (90%) and 2.43 (10%) ms. Events in the presence of FPL 64176 displayed the same open time as control openings ($\tau \sim 0.33$ ms, 1565 events from 5 patches).
Figure 7. Tavalin et al
Figure 8. Minor effect of L-type channel agonists on single channel open time carried by 160 mM Ca\(^{2+}\).

Single channel openings carried by 160 mM Ca\(^{2+}\) ions were of greater amplitude than observed with 10 mM Ca\(^{2+}\) (see Figure 3). As observed with 10 mM Ca\(^{2+}\) in the electrode solution, sweeps also displayed functionally coupled SK channels (upward channel openings) in some instances (Marrion and Tavalin, 1998). Control openings were best described by a single exponential distribution ($\tau \sim 0.54$ ms, 670 events from 9 patches). Long duration openings were not observed with either L-type channel agonist when 160 mM Ca\(^{2+}\) was used as the charge carrier, as with 10 mM Ca\(^{2+}\). Openings in the presence of BAY K 8644 were best described by the sum of two exponentials of time constants 0.46 (88%) and 2.66 (12%) ms (453 events from 6 patches). The events in the presence of FPL 64176 displayed the same longer duration open time constant as observed in BAY K 8644, with openings being best described by the sum of two exponentials of time constants 0.44 (96%) and 2.3 (4%) ms (503 events from 5 patches).
Figure 8. Tavalin et al
Figure 9. The slowing of current activation by FPL 64176 resulted from a delay of long duration openings.

A. Long duration activity carried by 110 mM Ba$^{2+}$ was evoked by membrane depolarization to 0 mV from a holding potential of –60 mV. Long duration openings were clustered at the end of the voltage step. In addition, they appeared subsequent to short open time openings (marked *). This delay to FPL 64176-induced long duration activity resulted in a kinetic slowing of current activation. B. Ensemble current derived from 33 sweeps in the presence of FPL 64176. The current showed the same waveform as observed in whole-cell recordings, where current activation was greatly slowed and a very slow deactivation current time course was evoked. C. In 4 patches that contained only a single channel (as estimated by the lack of superimposed openings in the presence of FPL 64176), the open times of those events preceding an opening longer than 10 ms duration were analyzed. These openings were best described by a single exponential function of time constant 0.73 ms.
Figure 9. Tavalin et al