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Characterization of the Effects of a New Ca^{2+} Channel Activator, FPL 64176, in GH_3 Cells

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SUMMARY

We examined the effects of the benzoylpyrrole-type Ca^{2+} channel activator FPL 64176 on voltage-dependent L-type Ca^{2+} channels in rat anterior pituitary (GH_3) cells. FPL 64176 increased K^+-dependent Ca^{2+} influx into GH_3 cells with an EC_{50} value of 1.2×10^{-7} M but had no effect on the binding of [^{3}H]PN200–110 to GH_3 cell membranes at concentrations up to 10^{-6} M. Whole-cell patch-clamp electrophysiology revealed that FPL 64176 (1 μM) increased L-type Ca^{2+} channel current amplitude and shifted the current-voltage relationship in the hyperpolarizing direction. Furthermore, Ca^{2+} channel current activation and deactivation were prolonged. Single-channel analysis showed that FPL 64176 increased both the probability of channel opening and the mean channel open time. Interestingly, the effect of FPL 64176 on channel open time was highly voltage dependent, with much longer openings being observed at more hyperpolarized potentials. We conclude that FPL 64176 represents a new class of L-type Ca^{2+} channel activator with a novel site and mechanism of action.

The influx of Ca^{2+} ions into excitable cells is important for a variety of physiological events, including neurotransmitter release and muscle contraction. An important means through which Ca^{2+} gains entry into these cells is via voltage-dependent Ca^{2+} channels. Although many classes and subclasses of Ca^{2+} channels are likely to be discovered, current information supports the existence of four categories of Ca^{2+} channels, termed T, L, N, and P (1–3). Of these, the L-type channel dominates in the cardiovascular system and many secretory cells. This channel is also the site of action of drugs such as the dihydropyridines, the phenylalkylamines, and the benzothiazepines (for review, see Ref. 4). These Ca^{2+} channel antagonists, in addition to their clinical importance, have been indispensable as tools for examining the structure and function of L-type channels.

It is clear that a number of chemically distinct compounds can act as antagonists of L-type Ca^{2+} channels (5). By comparison, few ligands act primarily as L-channel activators. Indeed, the only synthetic ligands classified as L-channel activators are confined to certain *S*-enantiomers of the 1,4-dihydropyridines, such as (*S*)-Bay K 8644 (4). However, in a recent report a new benzoylpyrrole molecule, FPL 64176, was shown to have pharmacological activity consistent with L-channel activation (6). Thus, this compound increased $^{45}Ca^{2+}$ influx into GH_3 cells and caused a positive inotropic effect in guinea pig aorta and atria. Subsequently, the pharmacological, biochemical, and electrophysiological effects of this compound on smooth and cardiac muscle have been detailed (7, 8). The present study was undertaken to examine further the molecular mechanisms of L-channel activation by FPL 64176 in rat anterior pituitary (GH_3) cells.

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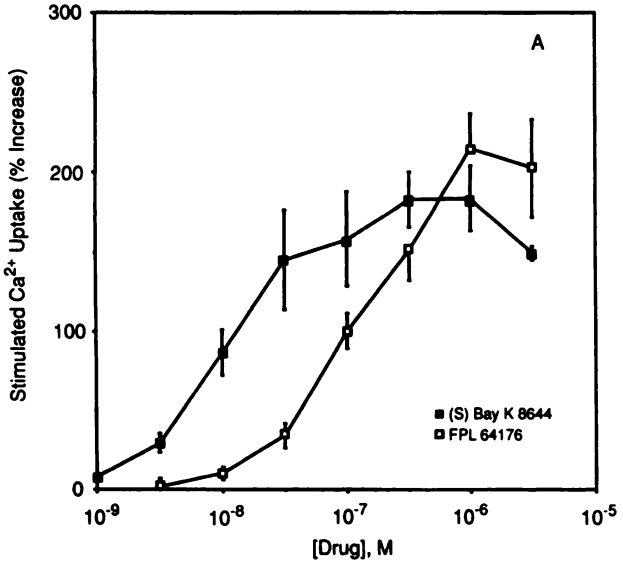
Materials and Methods

Cell culture. GH_3 cells used for $^{45}Ca^{2+}$ uptake experiments were grown to confluency (4–6 days) in 35-mm Falcon culture dishes, in F-10 nutrient mixture (GIBCO Laboratories, Grand Island, NY) supplemented with 15% horse serum and 2.5% fetal bovine serum, in an atmosphere of 95% air/5% CO_2. Those cells used for [^{3}H]PN200–110 binding were grown in 100-mm Falcon culture dishes. Cells used for electrophysiological measurements were grown in a similar fashion for 2–6 days, on glass coverslips.

Ca^{2+} uptake and [^{3}H]PN200–110 binding. For Ca^{2+} influx experiments GH_3 cells were preincubated with various concentrations of drugs for 15 min. After this time the culture medium was aspirated off and replaced by 1 ml of a solution containing (in mM) 100 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 20 HEPES, and 25 glucose, pH 7.4, or the same solution containing 42 mM KCl in equimolar substitution for NaCl. Both solutions also contained appropriate amounts of drug and 1 μCi of $^{45}CaCl_2$. Uptake was allowed to proceed at room temperature for 2 min, after which time it was terminated by two 1.5-ml washes with an ice-cold lanthanum solution containing (in mM) 110 NaCl, 5.4

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid.



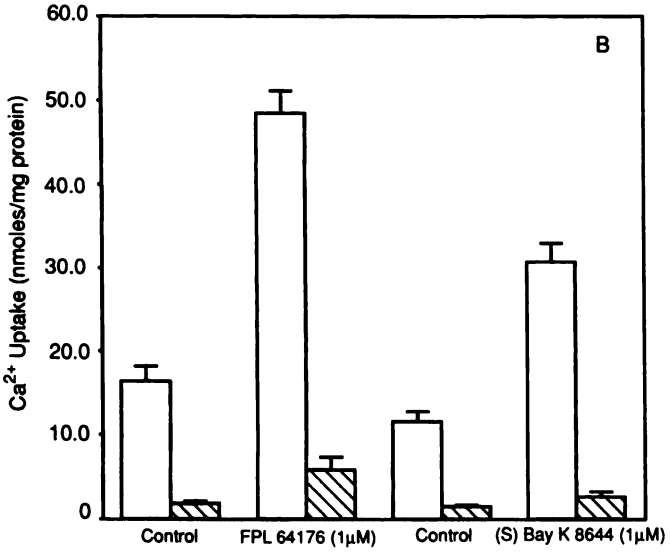


Fig. 1. Effects of (*S*)-Bay K 8644 and FPL 64176 on Ca^{2+} uptake in GH_3 cells. A, Dose-response curves for (*S*)-Bay K 8644 (EC_{50} = 9.90 ± 0.17 × 10^{-9} M) and FPL 64176 (EC_{50} = 1.20 ± 0.20 × 10^{-7} M) effects on stimulated Ca^{2+} uptake (Ca^{2+} uptake in 42 mM K^+ solution minus Ca^{2+} uptake in 5.4 mM K^+ solution). *Error bars*, standard errors (three or four experiments). B, Effects of 1 μM FPL 64176 and 1 μM (*S*)-Bay K 8644 on Ca^{2+} uptake. Drug data are shown next to paired control values. □, Uptake in 42 mM K^+ solution; ▨, uptake in 5.4 mM K^+ solution. *Error bars*, standard errors (three or four experiments).

KCl, 1.0 $LaCl_3$, 20 HEPES, and 25 glucose, pH 7.4. Cells were then digested with 0.5% NaOH and ^{45}Ca uptake was quantitated using liquid scintillation counting.

[^{3}H]PN200–110 binding to GH_3 cell membranes was carried out as described previously (9). Briefly, cells were washed off the culture dishes and allowed to swell on ice in 5 mM Tris (pH 7.4) for 30 min. Cells were homogenized and centrifuged at 1000 × *g* for 15 min. The supernatant was collected and recentrifuged at 42,000 × *g* for 30 min. The resultant pellet was resuspended in 50 mM Tris (pH 7.4) and used for all binding studies. [^{3}H]PN200–110 binding reactions were carried out in 1 ml of 50 mM Tris for 75 min. Nonspecific binding was defined using 5 × 10^{-7} M nifedipine. Incubation was terminated by filtration through Whatman GF/B filter strips, using a Brandel (Gaithersburg, MD) cell harvester. Filters were washed with 3 × 5 ml of ice-cold 50 mM Tris buffer and radioactivity was quantitated using liquid scintillation counting. For all experiments protein concentrations were determined by the method of Lowry *et al.* (10).

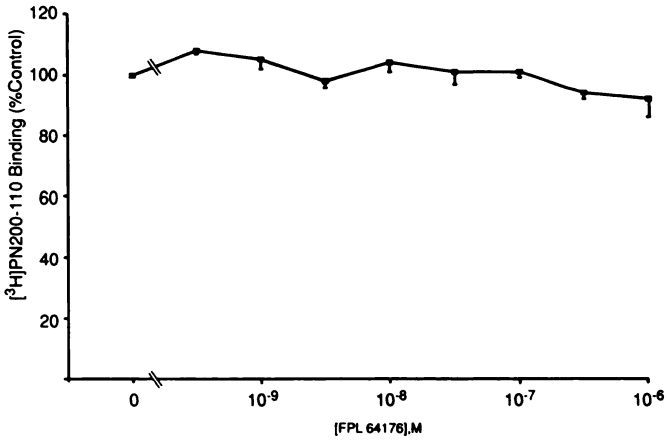


Fig. 2. Effects of FPL 64176 on [^{3}H]PN200–110 binding in GH_3 cell membranes. Binding was carried out as described in Materials and Methods, using 50 pM [^{3}H]PN200–110. *Error bars*, standard errors (three experiments).

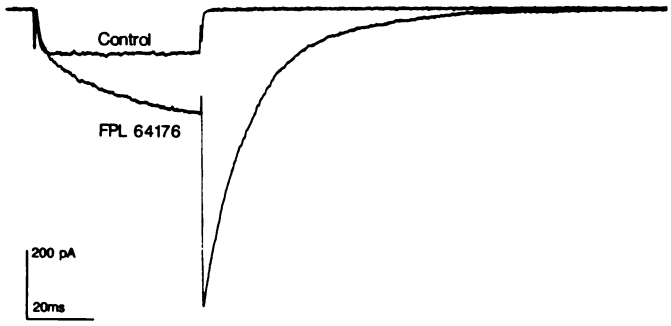


Fig. 3. Effects of FPL 64176 on whole-cell Ca^{2+} channel currents in GH_3 cells. Currents were induced by a 50-msec clamp pulse to 0 mV from a holding potential of −60 mV. Control current and current 1 min after the addition of 1 μM FPL 64176 are shown. Note slowing of current activation and prolongation of tail current duration after FPL 64176 treatment.

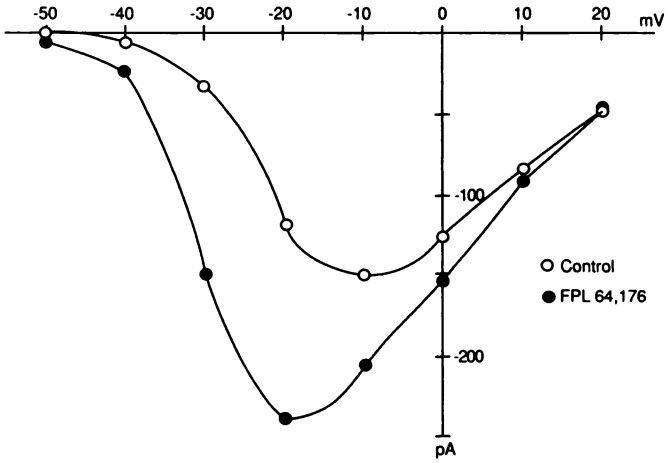


Fig. 4. Effects of FPL 64176 on Ca^{2+} channel current-voltage relationship in GH_3 cells. Currents were induced by 100-msec clamp pulses from a holding potential of −60 mV every 20 sec. Currents were recorded under control conditions (○) or 1 min after the addition of 1 μM FPL 64176 (●).

Whole-cell electrophysiology. Ion currents were recorded at room temperature via the gigaseal patch-clamp technique (11), using an Axopatch-1D amplifier (Axon Instruments, Burlingame, CA). Electrodes were fashioned from TW150 glass capillary tubes (World Pre-

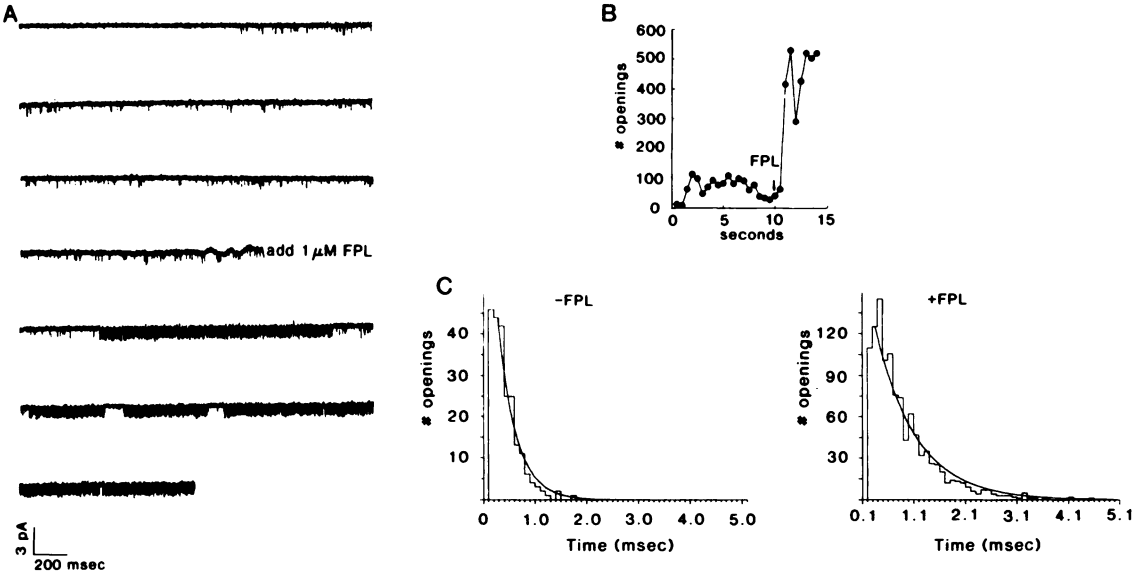


Fig. 5. Effect of FPL 64176 on single-channel activity in a cell-attached patch. A, Continuous recording at a holding potential of 0 mV illustrates the increase in activity when 1 μM FPL 64176 is added to the bath. B, The number of openings per 0.5-sec interval is plotted against time. C, The mean open time distributions in the absence and presence of FPL 64176 are fit with single exponentials with time constants of 0.34 and 0.78 msec, respectively. Data were filtered at 1 kHz.

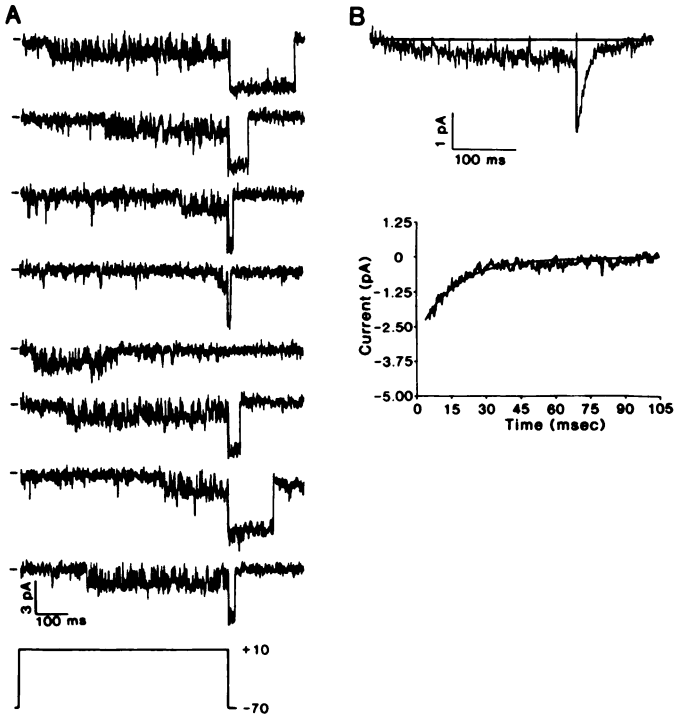


Fig. 6. Effect of FPL 64176 on single-channel activity of a cell-attached patch in response to the step depolarization protocol illustrated in the figure. A, Selected records demonstrate the prolonged tail current in the presence of 1 μM FPL 64176. B, Individual records from the cell are summed and averaged and the tail current is fit to a single-exponential decay.

cision Instruments, New Haven, CT) and had resistances of 2–4 MΩ when filled with internal solution. Pipettes used for intracellular recordings were filled with the following solution (in mM) 130 CsOH, 80 aspartic acid, 15 EGTA, 5 BAPTA, 11.5 $MgCl_2$, 3 Na_2ATP, 0.1 Na_3GTP, and 10 HEPES, pH 7.4 with CsOH. Seals were made in Tyrode's solution. After seal formation, Tyrode's solution was replaced with a Ca^{2+} channel recording solution containing (in mM) 110 *N*-methyl-D-glucamine, 140 aspartate, 10 $Ba(OH)_2$, 10 HEPES, 30 tetraethylammonium hydroxide, and 5 4-aminopyridine, pH 7.4 with methylsulfonic acid. Cell currents were conditioned by a four-pole low-pass filter with a cutoff frequency of 1 KHz. Data were stored, analyzed, and corrected using a Compaq 386 computer and pCLAMP software (Axon Instruments).

Single-channel recording. Activity was recorded from cell-attached patches. The pipette solution contained (in mM) 100 $BaCl_2$ and 10 HEPES, with pH adjusted to 7.2 with BaOH. The bathing solution contained (in mM) 140 potassium aspartate, 10 EGTA, 1 $MgCl_2$, and 10 HEPES, pH adjusted to 7.4 with KOH. All experiments were performed at room temperature.

Patch pipettes of 3–10-MΩ resistance were prepared from 8161 or 7040 glass (Garner Glass). The ground electrode was a silver-silver chloride pellet connected to the bath via a KCl/Ag-AgCl agar bridge. Data were recorded using a List EP7 patch-clamp amplifier. The single-channel data were analog filtered at a cutoff frequency of 5–10 kHz using a four-pole Bessel filter (Wavetek) and were digitized on-line at 10–20 kHz. A four-pole zero-phase digital filter was used during the analysis for additional filtering. Data were analyzed using a threshold, half-amplitude method previously described (12) or using FETCHAN and PSTAT (Axon Instruments). All current traces are illustrated with inward current being displayed in the downward direction.

Chemicals. [^{3}H]PN200–110 (85.9 Ci/mmol) and $^{45}CaCl_2$ (20.4 Ci/g) were obtained from New England Nuclear (Boston, MA). (*S*)-Bay K 8644 was obtained from Miles Laboratory (New Haven, CT). FPL 64176 was synthesized by Dr. John P. Paolini (Marion Merrell Dow, Cincinnati, OH). All other materials were obtained from commercial sources.

Results

Fig. 1A shows the effects of (*S*)-Bay K 8644 and FPL 64176 on Ca^{2+} uptake in GH_3 cells. This Ca^{2+} uptake has been shown previously to be antagonized by the 1,4-dihydropyridines, as well as other L-type Ca^{2+} channel blockers (13). Stimulated Ca^{2+} uptake (uptake in 42 mM K^+ solution minus uptake in 5.4 mM K^+ solution) was enhanced in a dose-dependent manner by both compounds, with (*S*)-Bay K 8644 being more potent (EC_{50} = 9.9 ± 0.17 × 10^{-9} M) than FPL 64176 (EC_{50} = 1.20 ± 0.02 × 10^{-7} M). Although FPL 64176 was less potent than (*S*)-

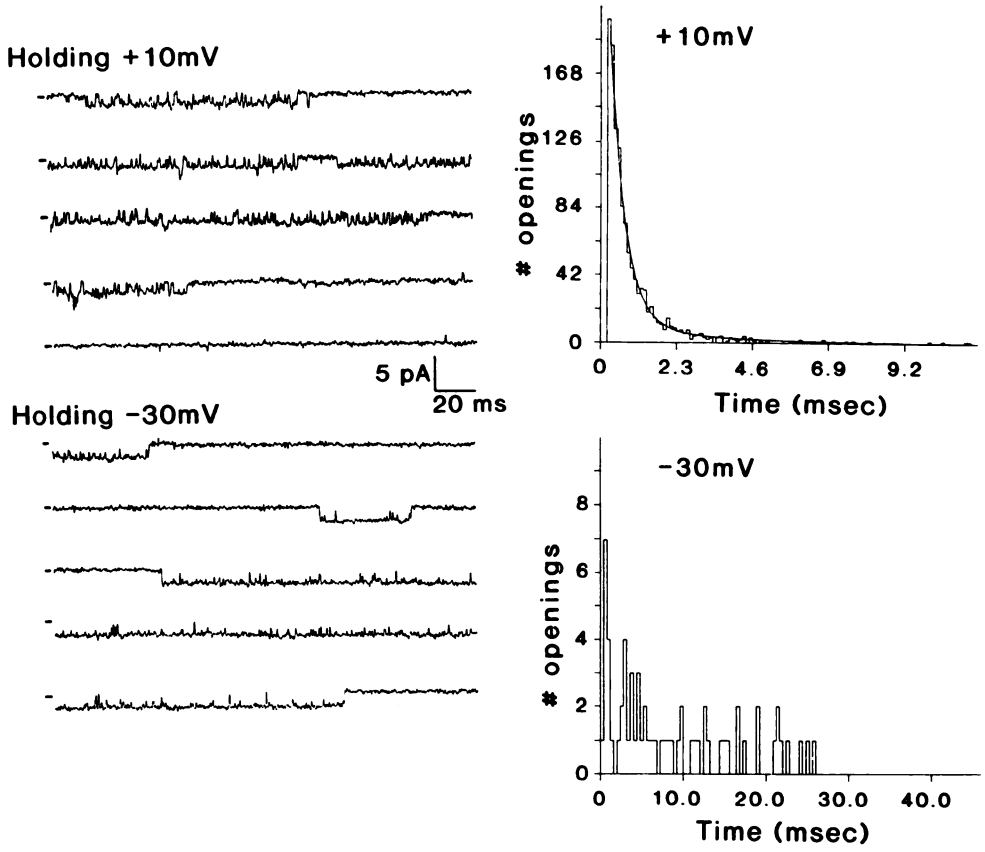


Fig. 7. Effect of voltage on mean open time in the presence of 1 μM FPL 64176. Selected records of activity in a cell-attached patch at a holding potential of +10 mV and −30 mV demonstrate the reduction in mean open time that occurs with depolarization. The mean open time distribution at +10 mV is best fit with a double-exponential fit with time constants of 0.44 and 2.40 msec. The distribution at −30 mV was not fit because the prolonged openings meant that few individual events were recorded. The mean ± standard deviation for all openings at −30 mV was 8.6 ± 7.5 msec.

Bay K 8644, it was somewhat more efficacious. This was especially true in resting (5.4 mM) K^+ solutions (Fig. 1B). FPL 64176 at 1 μM increased Ca^{2+} influx under resting conditions by 214%, compared with 80% for 1 μM (*S*)-Bay K 8644.

Fig. 2 shows the effects of FPL 64176 on [^{3}H]PN200–110 binding in GH_3 cell membranes. FPL 64176 had no significant effect on [^{3}H]PN200–110 binding at concentrations as high as 1 μM. These results are in agreement with those previously reported in cardiac membranes (6, 7).

We next turned to whole-cell patch-clamp electrophysiology to examine directly the effects of FPL 64176 on L-type Ca^{2+} channel currents in GH_3 cells. Using a holding potential of −60 mV we were able to examine L-type Ca^{2+} channels without contamination from T-type channels, which are also known to exist in this preparation (14, 15). Fig. 3 shows a typical response of L-channel current to 1 μM FPL 64176. In seven cells tested, 1 μM FPL 64176 increased peak L-channel current by 98 ± 9%. In addition to an increase in current amplitude, FPL 64176 also slowed current activation. Under control conditions current activation could only be fit to a single exponential with a time constant (τ) of 1.16 ± 0.10 msec (n = 7). After addition of 1 μM FPL 64176, activation could be fit with two exponentials, a fast component similar to control (τ = 1.44 ± 0.25 msec) and a much slower component (τ = 14.79 ± 1.97 msec). Furthermore, tail current duration was greatly prolonged in the presence of FPL 64176 (Fig. 3). When fit to a single exponential between 80% and 20% of their peak values, tail current time constant values averaged 17.71 ± 1.64 msec (n = 7) in the presence of 1 μM FPL 64176, compared with approximately 250 μsec in control.

Fig. 4 shows the effects of 1 μM FPL 64176 on the Ca^{2+} channel current-voltage (I-V) relationship in GH_3 cells. With the exception of the highest test potential examined (+20 mV), FPL 64176 increased L-channel current throughout the I-V relationship. This stimulation was more pronounced at negative test potentials. Additionally, FPL 64176 shifted both the threshold and the peak of the I-V relationship by approximately 10 mV in the hyperpolarizing direction.

The mechanism of the FPL 64176-induced increase in whole-cell current was examined at the single-channel level using barium as the ion-carrying species. An example of channel activity recorded from a cell-attached patch before and after the addition of 1 μM FPL 64176 to the bath solution is shown in Fig. 5. The diary of open events illustrates the large increase in the probability of opening. The comparison of the open times at 0 mV in the presence (0.78 msec) and absence of drug (0.34 msec) illustrates that FPL 64176 also prolongs open time.

The effect of FPL 64176 on the tail currents in the whole-cell recordings was confirmed at the single-channel level, as demonstrated in Fig. 6. Activity from a cell-attached patch in the presence of 1 μM FPL 64176 was recorded in response to a step depolarization. Prolonged channel openings were present as tail currents when the voltage was returned to the holding potential of −70 mV. The single-channel records were summed (Fig. 6B), and the tail current was fit to a single exponential with a time constant of 16 msec (Fig. 6B), similar to the value obtained for the whole-cell tail current. Furthermore, as indicated by the records in Fig. 6A and the summed current in Fig. 6B, channel activity was most pronounced after a delay upon depolarization.

The prolonged open time in the tail currents suggested that the effect of FPL 64176 on mean open time was voltage de-

pendent. Therefore, mean open time was examined as a function of the voltage. As the membrane potential was depolarized the mean open time was reduced (Fig. 7). The mean open time at −30 mV was 8.6 msec. At +10 mV the open time distribution was fit by a double exponential; the major component had a time constant of 0.44 msec and a smaller component was fit with a τ of 2.4 msec.

Discussion

The present studies detail some of the effects of the benzoylpyrrole FPL 64176 on L-type voltage-dependent Ca^{2+} channels in GH_3 pituitary cells. As described previously (6), both (*S*)-Bay K 8644 and FPL 64176 increased Ca^{2+} uptake into GH_3 cells in a dose-dependent manner. We found that FPL 64176 was about 10-fold less potent, in terms of concentration, than (*S*)-Bay K 8644. However, the efficacy of FPL 64176 for increasing Ca^{2+} uptake was somewhat greater than that of (*S*)-Bay K 8644, especially in low-K^+ solutions. Furthermore, FPL 64176 had no effect on 1,4-dihydropyridine binding in these cells. This is in agreement with our previous findings, which showed only a weak allosteric interaction between FPL 64176 and several Ca^{2+} channel antagonist binding sites in cardiac membranes (7). Thus, it is reasonable to assume that FPL 64176 defines a new category of L-type Ca^{2+} channel agonist.

We used whole-cell patch-clamp recordings to confirm directly a stimulatory effect of FPL 64176 on L-type Ca^{2+} channels. In addition to an increase in current amplitude, FPL 64176 caused a dramatic increase in tail current duration and a hyperpolarizing shift in the L-channel I-V relationship. These effects are qualitatively similar to those observed for Bay K 8644 in other reports (16, 17). However, upon depolarization FPL 64716 also produced a very prolonged component of activation, which has not been reported for Bay K 8644 in this preparation (16, 18).

The present study is the first to examine the mechanisms of action of FPL 64176 at the single-channel level. We found that FPL 64176 dramatically increased the probability of opening of L-type Ca^{2+} channels at depolarized potentials. This was accompanied by a moderate prolongation of mean channel open time. Again, these effects are similar to those seen for Bay K 8644 (19). However, when single-channel currents were summed and averaged they demonstrated slow activation similar to that seen for whole-cell currents. It is clear from the traces presented in Fig. 6A that the greatest channel activity generally occurred with some delay upon depolarization. This behavior no doubt underlies, at least in part, the slow activation kinetics observed after FPL 64176 treatment and suggests that Ca^{2+} channels may need to open before the full effects of FPL 64176 can be realized.

The dramatic increase in tail current duration observed at both the whole-cell and single-channel levels suggested to us that the effects of FPL 64176 were in some way voltage dependent. Examination of single-channel currents at various test potentials showed that this was indeed the case. At a holding potential of −30 mV mean channel open time was nearly 10 msec. Upon further depolarization (+10 mV) these long openings were interrupted by brief high frequency closures. This type of voltage-dependent behavior has not been observed with 1,4-dihydropyridine Ca^{2+} channel activators like Bay K 8644 (19). Furthermore, we feel that the prolonged channel openings observed at hyperpolarized potentials contribute not only to the prolongation of tail current duration but probably also to the enhanced $^{45}Ca^{2+}$ influx observed in resting K^+ solutions.

In summary, we have investigated some of the effects of the new L-type Ca^{2+} channel activator molecule FPL 64176 in GH_3 cells. FPL 64176 stimulates L-type Ca^{2+} channels in GH_3 cells at a site and with a mechanism of action distinct from those of other activators such as Bay K 8644. The mechanism of action of FPL 64176 involves an increase in the probability of channel opening as well as an increase in channel open time, with the latter effect being highly voltage dependent. Drug interactions at the benzoylpyrrole binding site promise to shed new light on the mechanisms of Ca^{2+} channel gating.

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