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Pharmacological, Radioligand Binding, and Electrophysiological Characteristics of FPL 64176, a Novel Nondihydropyridine Ca^{2+} Channel Activator, in Cardiac and Vascular Preparations

WEI ZHENG, DAVID RAMPE, and DAVID J. TRIGGLE

Department of Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York 14260 (W.Z., D.J.T.), and Marion Merrell Dow Research Institute, Cincinnati, Ohio 45215 (D.R.)

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SUMMARY

The pharmacological, radioligand binding, and electrophysiological properties of FPL 64176, a new nondihydropyridine Ca^{2+} channel activator, were studied in rat tail artery, cardiac membranes, and A7r5 smooth muscle cells. FPL 64176 induced a contractile response, with an EC_{50} value of 2.11×10^{-7} M. The maximum tension response to FPL 64176 was approximately 2-fold higher than that to (*S*)-Bay K 8644. FPL 64176 showed no significant inhibitory activity at concentrations up to 10^{-5} M. The Ca^{2+} channel antagonists nifedipine, verapamil and diltiazem noncompetitively antagonized and completely relaxed the responses induced by FPL 64176. IC_{50} values of these three drugs were 5.22×10^{-9}, 1.31×10^{-7}, and 1.95×10^{-7} M, respectively, for relaxing submaximum contractile responses to FPL 64176 (5×10^{-7} M). The washout time for FPL 64176 was about 40 min, which was much longer than that for (*S*)-Bay K 8644 (within 1 min). FPL 64176 weakly inhibited (+)-[^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 binding in rat cardiac membranes, with IC_{50} values of 1.04×10^{-5} M and 7.03×10^{-6} M for inhibition of (+)-[^{3}H]PN 200-110 and [^{3}H]TA-3090 binding, respectively, and with 23% inhibition of [^{3}H]D888 binding at a FPL 64176 concentration of 1×10^{-5} M. Dissociation kinetics of the three radioligands were allosterically accelerated by FPL 64176. Electrophysiological studies on the A7r5 smooth muscle cell line directly confirmed a large (approximately 14-fold) stimulatory effect on L-type Ca^{2+} current amplitude. The results suggest that FPL 64176 is a new type of Ca^{2+} channel activator with higher efficacy and a mechanism and site of action that are distinct from those for (*S*)-Bay K 8644.

Voltage-gated Ca^{2+} channels have been subclassified into L, N, and T types (reviewed in Refs. 1 and 2). L-type Ca^{2+} channels, which dominate in the cardiovascular system, are sensitive to nifedipine (1,4-dihydropyridine), verapamil (phenylalkylamine), and diltiazem (benzothiazepine), representing the three structural classes of calcium channel antagonist ligands (3). Potent activators of Ca^{2+} channels have been found in the enantiomers of 1,4-dihydropyridines such as (−)-(*S*)-Bay K 8644 (4), 202-791 (5), CGP 28 392 (6), and RS 30026 (7). These activators are believed to occupy the same binding site as the 1,4-dihydropyridine antagonists on the α_1 subunit of L-type calcium channel protein [reviewed by Triggle and Rampe (8)]. However, (*S*)-Bay K 8644 produces antagonist effects at concentrations higher than 1×10^{-6} M (9, 10), and potent 1,4-dihydropyridine antagonists produce transient activator effects at low concentrations (10–13). Thus, the 1,4-dihydropyridines possess dual activities and are not pure agonists or antagonists.

Recently, a nondihydropyridine, FPL 64176, a benzoylpyrrole (Fig. 1), has been reported to have Ca^{2+} channel activator-like properties (14). FPL 64176 enhanced Ca^{2+} uptake and contracted cardiac and vascular smooth muscles but was weakly effective in displacing specific (+)-[^{3}H]PN 200-110 binding. We wished to know whether the pharmacological effects of FPL 64176 are sensitive to calcium channel antagonists and how its properties compared with those of the 1,4-dihydropyridine activator (*S*)-Bay K 8644. We report on the pharmacological, radioligand binding, and electrophysiological properties of FPL 64176, using rat tail artery, A7r5 smooth muscle cells, and cardiac membranes.

Materials and Methods

Tissue preparation and recording of mechanical response. Rat tail artery strips were prepared as described by Webb *et al.* (15) and Su *et al.* (16). Briefly, male Holtzman rats of age 15–17 weeks (250–290 g body weight) were sacrificed by decapitation, and the median tail artery was removed and placed in PSS, aerated with 95% O_2/5% CO_2 at 37°, of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; KH_2PO_4, 1.18; $NaHCO_3$, 25; dextrose, 5.5; pH

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ABBREVIATIONS: PSS, physiological salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA; ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

7.4. After gentle cleaning of connective tissue from the artery, a steel needle (size 11) was inserted and the artery was cut into strips 1 cm long, at an angle of approximately 45°. The strips were suspended in glass organ baths, containing 10 ml of aerated PSS at 37°, connected to a force-displacement transducer (Grass FT03), and isometric contractions were recorded on a Grass polygraph (model 7D). Before measurement of responses, tissues were equilibrated for 2 hr under a resting tension of 600 mg, with a solution change every 15 min. During this period, the arterial strips were stimulated three times with 80 mM KCl, to initiate a steady level of response (16). The tissue was then pretreated with phentolamine (1×10^{-6} M) to block the effect of any norepinephrine released from sympathetic nerve terminals. After an additional 30-min equilibration period, a response to 80 mM KCl was recorded as control. Data were expressed as mean ± standard error for six to eight separate observations.

To determine the response to FPL 64176, cumulative dose-response curves to activator were measured in the presence of various concentrations of KCl. The concentration of activator was increased only after the response to the previous addition had attained a maximum steady level. Responses to (*S*)-Bay K 8644 were recorded in the presence of 20 mM total K^+, in which maximum tension of the tissues was generated, as previously defined in our laboratory (16).

Two procedures were used to study the inhibitory effects of nifedipine, verapamil, and diltiazem on contractile response induced by FPL 64176. First, a control cumulative dose-response curve to FPL 64176 was recorded in tissues suspended in PSS containing 25 mM K^+. The tissues were then successively washed and equilibrated in PSS for 2 hr. A second cumulative dose-response curve to FPL 64176 was determined after the tissues were preincubated with various concentrations of nifedipine, verapamil, or diltiazem for 30 min. All responses to FPL 64176 were recorded after the addition of 20 mM K^+ to the bath solution (total of 25 mM K^+) for 10 min. Arterial strips were exposed only once to a single concentration of the antagonist. In a control experiment, the second response of FPL 64176 was measured without preincubation with antagonists, to determine any time-dependent changes in tissue response to agonist. Such changes were negligible in this preparation. The second procedure determined the relaxant effects of nifedipine, verapamil, and diltiazem. Tissues were contracted with 5×10^{-7} M FPL 64176 in the presence of 25 mM K^+, and cumulative doses of the antagonists were added to the bath solution after a stable response to FPL 64176 had been obtained.

Radioligand binding assay in rat heart membranes. Rat heart ventricle muscle was minced with scissors and homogenized in 15 volumes/g of weight of ice-cold Tris buffer (50 mM, pH 7.2 at 25°), by two bursts (at maximum speed) in a Polytron (Brinkman Instruments, Inc., Westbury, NY), followed by 10 passes of a motor-driven glass-Teflon homogenizer (TRI-R-Stirrer; TRI-R Instruments Inc., Rockville Center, NY) (setting 7; nominal clearance, 0.13–0.18 mm). The homogenate was centrifuged at 1100 × *g* for 20 min at 4°, and the supernatant was recentrifuged at 40,000 × *g* for 45 min. The resultant pellet was suspended in cold Tris buffer. Protein was determined by the method of Bradford (17), using bovine serum albumin as standard.

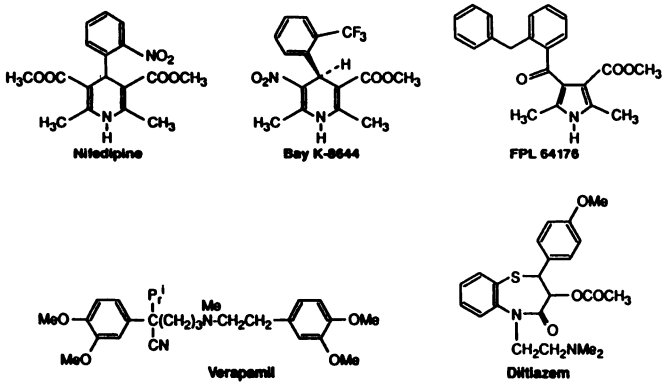

Fig. 1. Structural formulae of FPL 64176, the 1,4-dihydropyridine activator (*S*)-Bay K 8644, and the antagonists nifedipine, verapamil (phenylalkylamine), and diltiazem (benzothiazepine).

The competition binding assay of FPL 64176 with (+)-[^{3}H]PN 200-110 was carried out by the methods established previously in our laboratory (18, 19). Membrane protein (70–100 μg) was incubated with (+)-[^{3}H]PN 200-110 (5.6×10^{-11} M) and various concentrations of FPL 64176 for 2 hr, in a 5-ml incubation volume. Saturation binding of (+)-[^{3}H]PN 200-110 was performed in the presence or absence of 5 μM FPL 64176, by incubation of the membrane protein with various concentrations of (+)-[^{3}H]PN 200-110 (0.01–0.6 nM). After incubation, the samples were filtered over Whatman GF/B filters and washed twice with 5 ml of ice-cold buffer, using a cell harvester (model M-24R; Brandel Instruments, Gaithersburg, MD). Nonspecific binding was defined by addition of 10^{-7} M unlabeled PN 200-110. Binding experiments were carried out in glass tubes and in subdued light. The filters were then measured by liquid scintillation counting, at an efficiency of approximately 50%.

The saturation binding of [^{3}H]D888 and [^{3}H]TA-3090 was performed as described above, with the following modifications: [^{3}H]D888 (1–20 nM) and [^{3}H]TA-3090 (1–40 nM) were incubated with the membrane protein (40–50 μg) in a final volume of 0.5 ml of 50 mM Tris buffer (pH 7.4), containing 0.1% bovine serum albumin, for [^{3}H]D888 or 0.3 ml for [^{3}H]TA-3090, at 25° for 1 hr. GF/C filters used were pretreated with 0.5% polyethylenimine for 2 hr. Competition binding was performed using 4.2×10^{-9} M [^{3}H]D888 or 5.3×10^{-9} M [^{3}H]TA-3090 and various unlabeled drugs. Nonspecific binding was defined by 10^{-5} unlabeled verapamil or 10^{-5} unlabeled TA-3090.

Dissociation kinetics were measured by preincubating (+)-[^{3}H]PN 200-110 (0.07 nM), [^{3}H]D888 (4.2×10^{-9} M), or [^{3}H]TA-3090 (5.3×10^{-9} M) with 40–60 μg of membrane protein for 90 min at 25° for (+)-[^{3}H]PN 200-110 or 150 min at 10° for [^{3}H]D888 and [^{3}H]TA-3090. This incubation was followed by the addition of excess unlabeled (+)-PN 200-110 (10^{-7} M), verapamil (10^{-5} M), or TA-3090 (10^{-5} M), to initiate the dissociation process. The samples were filtered at defined times. All binding experiments were performed in duplicate, with four or five determinations.

Electrophysiological experiments. The A7r5 rat aortic smooth muscle cell line was used for all electrophysiological experiments. Cells were cultured in 35-mm Falcon culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Before electrophysiological recording, the cells were trypsinized and seeded on glass coverslips.

Ion currents were recorded at room temperature via the gigaseal patch-clamp technique, as described by Hamill *et al.* (20), utilizing an Axopatch-1B amplifier (Axon Instruments, Burlingame, CA). Electrodes were fashioned from TW150 glass capillary tubes (World Precision 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): CsOH, 130; aspartic acid, 80; EGTA, 15; 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, 5; $MgCl_2$, 11.5; Na_2ATP, 3; Na_3GTP, 0.1; HEPES, 10; pH 7.4. Seal formation was carried out in Tyrode's solution, after which the extracellular solution was replaced with a current recording solution of the following composition (in mM): *N*-methyl-D-glucamine, 110; aspartate, 140; $BaCl_2$, 40; HEPES, 10; tetraethylamonium hydroxide, 30; 4-aminopyridine, 5; pH 7.4; with methanesulfonic acid. Cell currents were conditioned by a four-pole low-pass filter with a cutoff frequency of 1 kHz. Currents were sampled at 2 kHz and stored, analyzed, and corrected using a laboratory computer (IBM-AT) and pCLAMP software (Axon Instruments).

Materials and data analysis. (+)-[^{3}H]PN 200-110 (71.5 Ci/mmol) and [^{3}H]D888 (84 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). [^{3}H]TA-3090 (109 Ci/mmol) was generously provided by Dr. Ray H. Zobrist (Marion Merrell Dow Inc., Kansas City, MO).

All data were processed using an IBM personal computer. Pharma-

cological data were analyzed using a standard set of Pharmacological Programs (21). Significance of difference was accepted at the 0.05 level. The binding data were analyzed with the program LIGAND (22).

Results

Pharmacological Studies

Effect of K^+ on contractile response to FPL 64176. Application of FPL 64176, at a concentration of 10^{-8} to 10^{-5} M, to rat tail arterial strips induced a small contractile response. The contractile activities of FPL 64176 increased greatly when the tissues were preincubated with additional K^+ (added to PSS) for 10 min. The maximum tension induced by FPL 64176 increased from 382 mg to 636–1078 mg in the presence of additional K^+ between 5 to 80 mM. The optimum total concentration of K^+ was 25 mM, in which FPL 64176 produced maximum tension with an EC_{50} value of $2.11 \pm 0.40 \times 10^{-7}$ M (Fig. 2).

Comparisons of the maximum tension induced by K^+, (*S*)-Bay K 8644, and FPL 64176. Dose-response curves to KCl and (*S*)-Bay K 8644 were determined in rat tail arterial stripes, yielding EC_{50} values of 42.4 mM and 6.13×10^{-9} M respectively, consistent with values reported previously (16, 23). Maximum tension responses induced by KCl, (*S*)-Bay K 8644, and FPL 64176 are compared in Fig. 3. The tension produced by (*S*)-Bay K 8644 was 79.2% of the K^+ response, whereas FPL 64176 induced a maximum tension of 242.3% of the K^+ response, which was significantly different from the maximum tension induced by (*S*)-Bay K 8644 ($p < 0.05$).

Time course of FPL 64176 action. Both (*S*)-Bay K 8644 and FPL 64176 dose-dependently increased the rat tail artery contraction in the presence of the optimum K^+ concentration in PSS. The times for both drugs to achieve the maximum responses were similar, approximately 30 min, although the magnitude of the maximum response was some 2-fold higher with FPL 64176. Washout times of the two drugs were, however, significantly different. The maximum response to (*S*)-Bay K 8644 in rat tail artery dropped rapidly after washing with PSS, and the tension returned to base-line within 1 min. The contractile responses induced by FPL 64176 decreased slowly, with complete relaxation occurring after 40 min (Fig. 4).

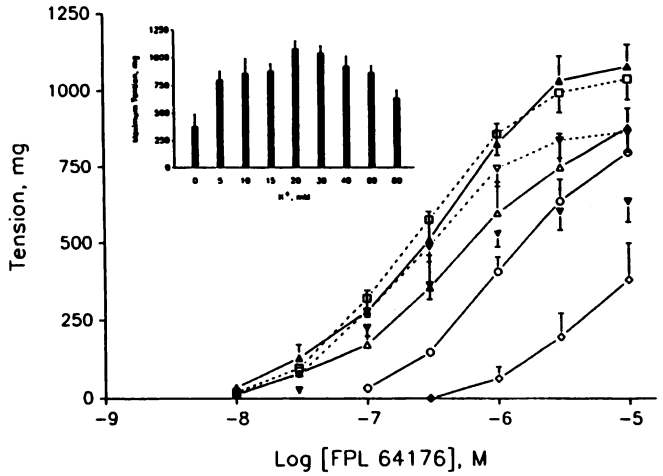

Fig. 2. Contractile responses of FPL 64176 in rat tail artery in the absence (◇) and presence of additional extracellular K^+ concentrations (○ 5 mM; △, 15 mM; ▲, 20 mM; □, 30 mM; ▽, 60 mM; ▼, 80 mM K^+). *Inset*, Maximum tension responses to 10 μM FPL 64176 in the absence or presence of various K^+ concentrations.

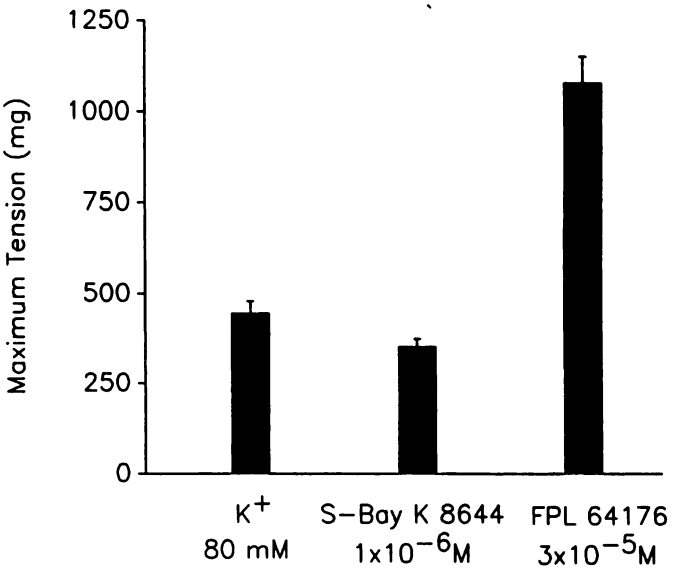

Fig. 3. Comparison of the maximum tension responses to K^+, (*S*)-Bay K 8644, and FPL 64176 in rat tail artery. (*S*)-Bay K 8644 and FPL 64176 responses were induced in the presence of 15 mM and 20 mM K^+, respectively.

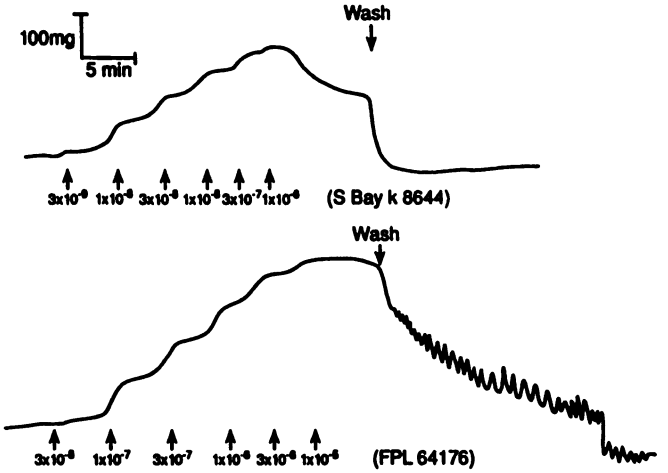

Fig. 4. Trace of tension responses of rat tail artery to accumulated doses of (*S*)-Bay K 8644 and FPL 64176 in the presence of 15 and 20 mM K^+, respectively, at the indicated conditions.

Inhibition of maximum tension response to FPL 64176 by Ca^{2+} channel antagonists. Cumulative dose-response curves to FPL 64176 were determined in the absence and presence of nifedipine, verapamil, and diltiazem, in rat tail arterial strips. These agents produced noncompetitive antagonism of the FPL 64176 response (Fig. 5). The IC_{50} values of nifedipine, verapamil, and diltiazem were 2.90×10^{-8}, 2.56×10^{-6}, and 3.14×10^{-6} M, respectively (Table 1).

Inhibition of submaximum tension response to 5×10^{-7} M FPL 64176 by Ca^{2+} channel antagonists. The relaxing effects of nifedipine, verapamil, and diltiazem were determined after a stable contractile response to 5×10^{-7} M FPL 64176 was obtained (Fig. 6). All three Ca^{2+} channel antagonists dose-dependently relaxed the contractions, with IC_{50} values of $5.22 \pm 0.62 \times 10^{-9}$, 1.31×10^{-7}, and 1.95×10^{-7} M, respectively (Table 1). Complete relaxation was reached at concentrations of 1×10^{-7} M nifedipine, 3×10^{-6} M verapamil, and 3×10^{-6} M diltiazem.

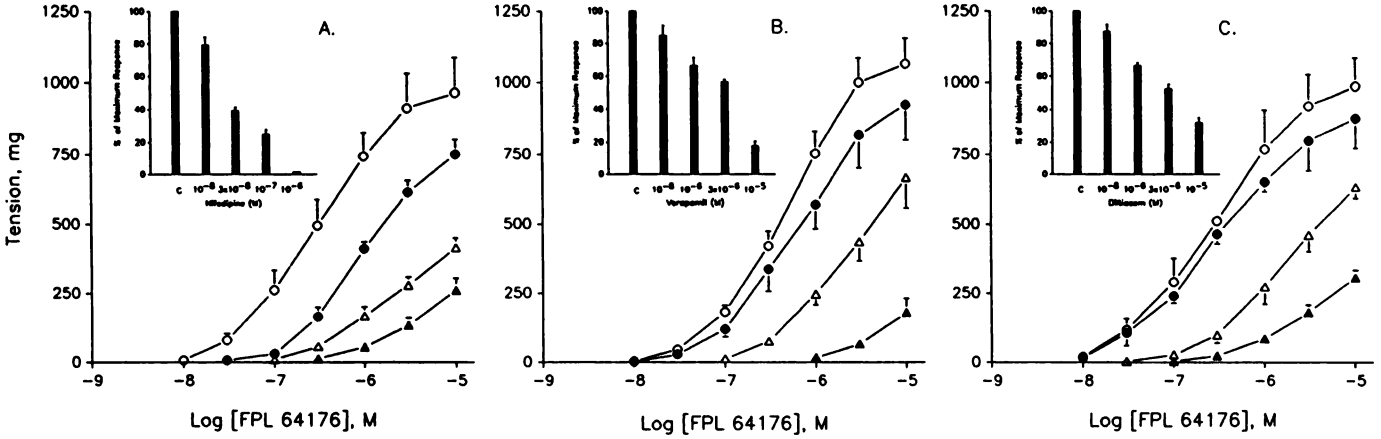

Fig. 5. Inhibition of FPL 64176 contractile responses in rat tail artery by nifedipine, verapamil, and diltiazem. A, Effects of various concentrations of nifedipine. ○, 0; ●, 1×10^{-8} M; △, 1×10^{-7} M; ▲, 1×10^{-6} M. B, Effects of various concentrations of verapamil. ○, 0; ●, 1×10^{-8} M; △, 1×10^{-6} M; ▲, 1×10^{-5} M. C, Effects of various concentrations of diltiazem. ○, 0; ●, 1×10^{-6} M; △, 1×10^{-6} M; ▲, 1×10^{-5} M. *Insets*, inhibition of maximum tension responses to FPL 64176 (1×10^{-5} M) in rat tail artery by nifedipine (A), verapamil (B), and diltiazem (C).

TABLE 1

IC_{50} values of nifedipine, verapamil, and diltiazem on FPL 64176-induced contractile responses in rat tail artery

Drug	IC_{50}		Ratio
	Relaxation of response induced by 0.5 μm FPL 64176	Inhibition of maximum tension response to FPL 64176	
	m		
Nifedipine	$5.22 \pm 0.62 \times 10^{-9}$	$2.92 \pm 0.27 \times 10^{-8}$	5.6
Verapamil	$1.31 \pm 0.23 \times 10^{-7}$	$2.56 \pm 0.21 \times 10^{-6}$	19.5
Diltiazem	$1.95 \pm 0.25 \times 10^{-7}$	$3.14 \pm 0.33 \times 10^{-6}$	16.1

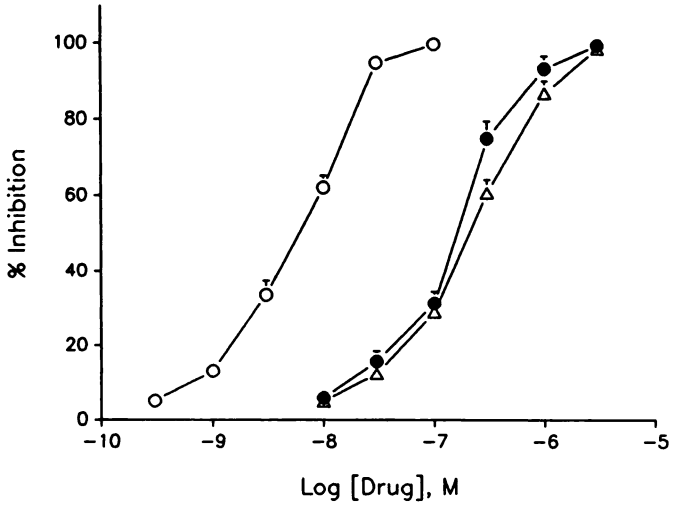

Fig. 6. Inhibition of submaximum FPL 64176 (5×10^{-7} M) responses in rat tail artery by nifedipine (○), verapamil (●), and diltiazem (△).

Binding Studies

Inhibition of (+)-[^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 binding by FPL 64176 in rat cardiac membranes. These three ligands represent three classes of Ca^{2+} channel antagonists, i.e., (+)-[^{3}H]PN 200-110 for 1,4-dihydropyridines, [^{3}H]D888 for phenylalkylamines, and [^{3}H]TA-3090 for benzothiazepines. [^{3}H]TA-3090 is a new radiolabeled diltiazem analog that has been reported to be more potent than diltiazem (24). Specific binding of (+)-[^{3}H]PN 200-110 in rat cardiac membranes was displaced by FPL 64176 at concentrations between 1×10^{-6} and 1×10^{-5} M (Fig. 7A). The maximum inhibition of (+)-[^{3}H]PN 200-110 specific binding by FPL 64176 was 50.8 ± 1.2%. Further inhibition could not be achieved, because of the limited solubility of this drug. An IC_{50} value of FPL 64176 was calculated as $1.04 \pm 0.13 \times 10^{-5}$ M; this value should be regarded as an estimate only. Nifedipine concentration-dependently inhibited (+)-[^{3}H]PN 200-110 binding, with an IC_{50} value of $3.30 \pm 0.14 \times 10^{-9}$ M.

Similarly, weak inhibition of [^{3}H]D888 and [^{3}H]TA-3090 binding by FPL 64176 was also observed (Fig. 7, B and C). Only 23 ± 0.21% inhibition of [^{3}H]D888 binding in rat cardiac membranes was obtained at an FPL 64176 concentration of 1×10^{-5} M, but verapamil showed complete inhibition of [^{3}H]D888 binding, with an IC_{50} value of $3.32 \pm 0.39 \times 10^{-8}$ M. FPL 64176 inhibited 62 ± 0.53% of [^{3}H]TA-3090 binding at a concentration of 1×10^{-5} M ($IC_{50} = 7.30 \pm 0.83 \times 10^{-6}$ M). An IC_{50} value of $5.28 \pm 0.22 \times 10^{-8}$ M was obtained for diltiazem inhibition of [^{3}H]TA-3090 binding.

Effect of FPL 64176 on the equilibrium binding of (+)-[^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 in rat cardiac membranes. Saturation analysis of equilibrium binding of these three radiolabeled ligands showed that the binding data fit a single class of high affinity binding sites (Fig. 8). Addition of FPL 64176 significantly changed the binding affinities of (+)-[^{3}H]PN 200-110 and [^{3}H]TA-3090 ($p < 0.05$), with small and nonsignificant changes in B_{max} (Table 2). Nonspecific binding was 20–40% for (+)-[^{3}H]PN 200-110, 20–50% for [^{3}H]D888, and 30–65% for [^{3}H]TA-3090.

Effect of FPL 64176 on dissociation kinetics of (+)-[^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 in rat cardiac membranes. Dissociation of these radiolabeled ligands was initiated in the presence or absence of FPL 64176, to examine further the mechanism of FPL 64176 interaction at these binding sites. The dissociation rates of (+)-[^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 were best fitted as monoexponential processes (Fig. 9). FPL 64176 significantly accelerated the dissociation process of these three radioligands. In the absence or presence of 1×10^{-5} M FPL 64176, k_{-1} values were 0.0042 ± 0.0004 and 0.0095 ± 0.0003 min^{-1} for (+)-[^{3}H]PN 200-110 ($p < 0.05$), 0.100 ± 0.006 and 0.139 ± 0.01 min^{-1} for [^{3}H]

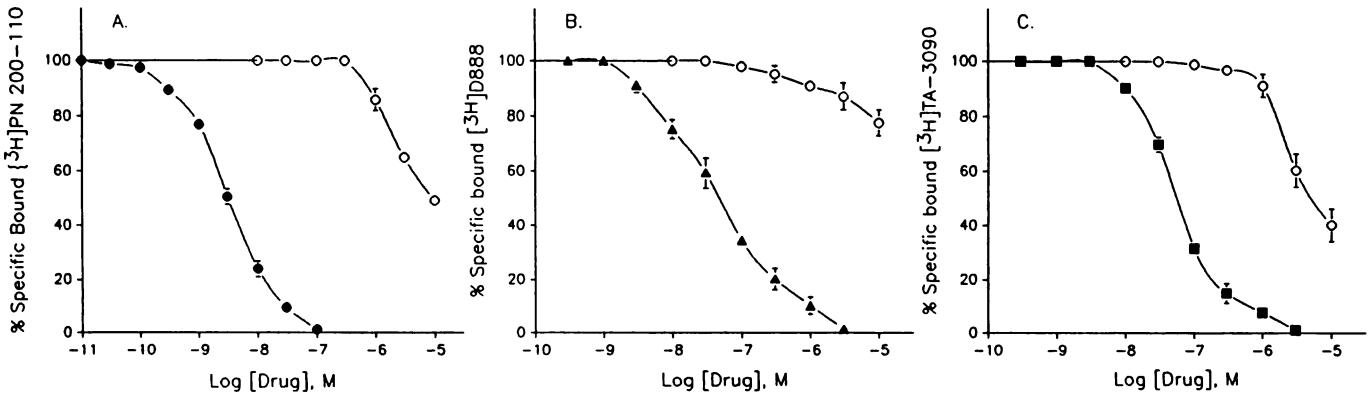

Fig. 7. Effects of FPL 64176 on the binding of (+)-[^{3}H]PN 200-110 (5.6×10^{-11} M) (A), [^{3}H]D888 (4.2×10^{-9} M) (B), and [^{3}H]TA-3090 (5.3×10^{-9} M) (C) in rat cardiac membranes. ○, FPL 64176; ●, nifedipine; ▲, verapamil; ■, diltiazem.

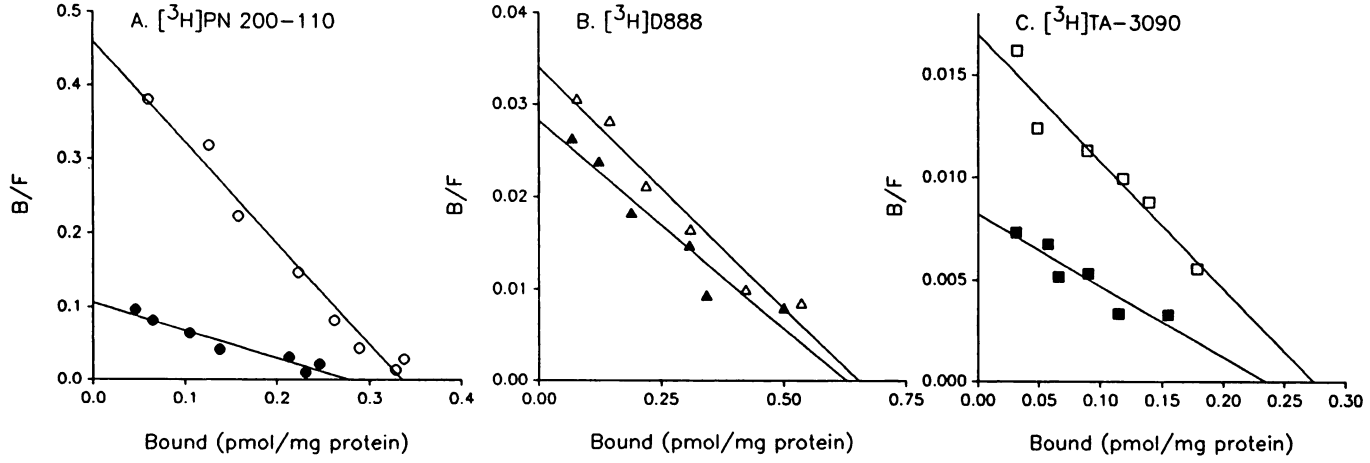

Fig. 8. Effects of FPL 64176 on saturation binding of (+)-[^{3}H]PN 200-110 (A), [^{3}H]D888 (B), and [^{3}H]TA-3090 (C) in rat cardiac membranes. A, Scatchard plots of (+)-[^{3}H]PN 200-110 binding in the absence (○) or presence of 5 μM FPL 64176 (●). B, Scatchard plots of [^{3}H]D888 binding in the absence (△) or presence of 10 μM FPL 64176 (▲). C, Scatchard plots of [^{3}H]D888 binding in the absence (□) or presence of 10 μM FPL 64176 (■).

TABLE 2

Effects of FPL 64176 on the binding of [^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 in rat heart membranes

	K_D	B_{max}	n_H
	M	fmol/mg of protein	
[^{3}H]PN 200-110			
Control	$2.65 \pm 0.16 \times 10^{-11}$	353 ± 13	1.00 ± 0.01
+ FPL 64176 (5 μM)	$1.21 \pm 1.10 \times 10^{-10}$[a]	299 ± 13[a]	0.98 ± 0.02
[^{3}H]D888			
Control	$6.07 \pm 1.04 \times 10^{-9}$	685 ± 56	0.98 ± 0.01
+ FPL 64176 (10 μM)	$7.90 \pm 0.51 \times 10^{-9}$	613 ± 28	0.98 ± 0.01
[^{3}H]TA-3090			
Control	$7.33 \pm 0.65 \times 10^{-9}$	268 ± 11	0.97 ± 0.01
+ FPL 64176 (10 μM)	$1.69 \pm 0.36 \times 10^{-8}$[a]	214 ± 32	0.98 ± 0.01

[a] Significance of difference from individual control data, $p < 0.05$.

D888 ($p < 0.05$), and 0.030 ± 0.004 and 0.048 ± 0.006 min^{-1} for [^{3}H]TA-3090 ($p < 0.05$), respectively.

Electrophysiological Studies

We used whole-cell patch-clamp electrophysiology to examine the effects of FPL 64176 directly on Ca^{2+} channels in A7r5 cells. This cell line has previously proven useful for studying drug interactions at the L-type Ca^{2+} channel (25). Fig. 10 shows a summary of the effects of 10^{-6} M FPL 64176 on L-type Ca^{2+} currents carried by 40 mM external Ba^{2+} (I_{Ba}). FPL 64176 caused a dramatic increase in I_{Ba}, which was reversible upon washing of the cell with drug-free external solution. In four cells tested, I_{Ba} was increased an average of 1398 ± 353% 1 min after addition of 10^{-6} M FPL 64176. FPL 64176 also caused a prolongation of tail current duration and a slowing of the activation kinetics of I_{Ba} during the pulse.

Fig. 11 shows the effects of FPL 64176 on the current-voltage (I-V) relationship of the L-type Ca^{2+} channel in A7r5 cells. Current records under control conditions and 1 min after the addition of 10^{-6} M FPL 64176 are shown in Fig. 11, A and B, respectively. The corresponding I-V relationships are plotted in Fig. 11C. After addition of 10^{-6} M FPL 64176, the amplitude of I_{Ba} was increased at all test potentials, although this enhancement was more pronounced at negative test potentials. Additionally, the threshold and peak of the I-V relationship were shifted by approximately 10 mV in the hyperpolarizing direction after FPL 64176 treatment.

Discussion

Our results showed that FPL 64176 dose-dependently induces a contractile response in rat tail artery, with characteristics apparently similar to those reported for (*S*)-Bay K 8644 and (+)-PN-202 791 (16, 23). These responses to FPL 64176

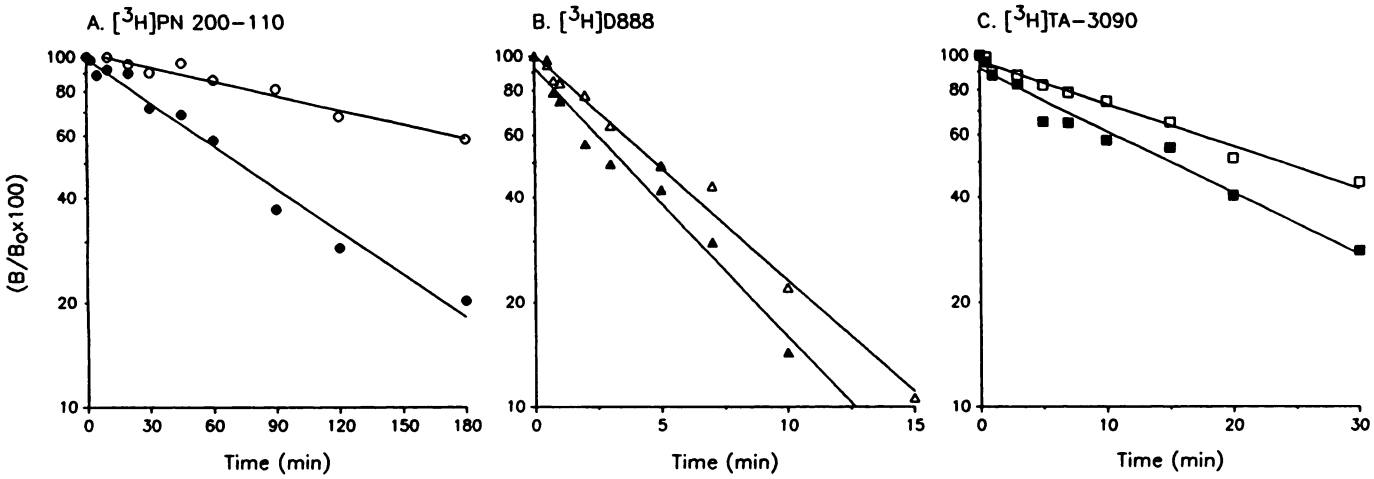

Fig. 9. Effects of FPL 64176 on dissociation kinetics of (+)-[^{3}H]PN 200-110 (A), [^{3}H]D888 (B), and [^{3}H]TA-3090 (C). A, Dissociation of (+)-[^{3}H]PN 200-110 was initiated by addition of 0.1 μM (+)-PN 200-110, with 10 μM FPL 64176 (●) or without FPL 64176 (○). B, Dissociation of [^{3}H]D888 was initiated by addition of 10 μM verapamil, with 10 μM FPL 64176 (▲) or without FPL 64176 (△). C, Dissociation of [^{3}H]TA-3090 was initiated by addition of 10 μM TA-3090, with 10 μM FPL 64176 (■) or without FPL 64176 (□).

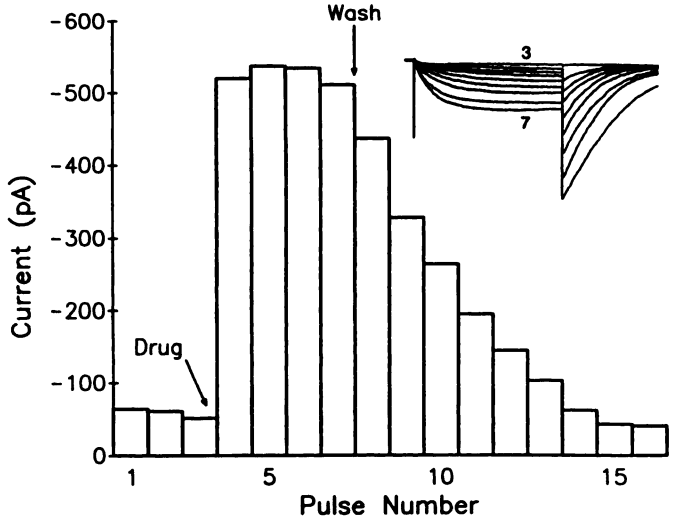

Fig. 10. Effects of FPL 64176 (10^{-6} M) on L-type Ca^{2+} channel currents in A7r5 cells. Currents were induced by 325-msec clamp pulses to +10 mV, from a holding potential of −40 mV, every 15 sec. Currents were recorded in 40 mM extracellular Ba^{2+} and were measured at the end of each pulse. Drug addition and washout are indicated. *Inset*, current traces from pulses 3 and 7, as well as during progressive washout of drug (pulses 8–14), are shown.

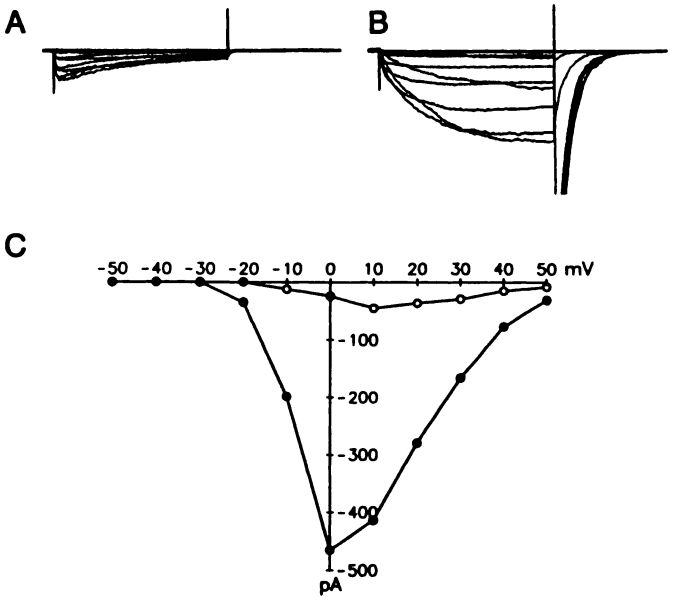

Fig. 11. Effects of FPL 64176 on the L-type Ca^{2+} channel I-V relationship in A7r5 cells. Currents were induced by 325-msec clamp pulses to various test potentials every 10 sec. A, Control current traces. B, Current traces in the same cell 1 min after the addition of 1 μM FPL 64176. C, Calcium channel I-V relationship for the experiments illustrated in A (○) and B (●). Currents were sampled at the end of the pulse to generate the I-V relationship.

are completely antagonized by nifedipine, verapamil, and diltiazem, consistent with a process of activation of voltage-dependent Ca^{2+} channels. However, important differences exist between the actions of (*S*)-Bay K 8644 and FPL 64176.

The contractile responses to FPL 64176 are noncompetitively antagonized by nifedipine, verapamil, and diltiazem, suggesting an absence of direct interaction at the three primary ligand binding sites of the Ca^{2+} channel. In contrast, contractile responses to (*S*)-Bay K 8644 are competitively antagonized by nifedipine and noncompetitively inhibited by verapamil and diltiazem (16). The abilities of nifedipine, verapamil, and diltiazem to inhibit maximum and submaximum tension responses to FPL 64176 are different. The ratio of activities is smallest for nifedipine and much larger for verapamil and diltiazem (Table 1). This may represent different voltage- and frequency-dependent interactions known to exist between the three antagonist classes (26–28).

(*S*)-Bay K 8644 and (+)-PN-202 791 produce biphasic responses in cardiovascular tissues, with antagonism being exerted at higher concentrations (9, 10, 23). In contrast, FPL 64176 does not show significant antagonist effects at concentrations up to 10^{-5} M. Other data also suggest that Bay K 8644 and FPL 64176 act by different mechanisms. The maximum tension generated by FPL 64176 is some 2-fold greater than that produced by (*S*)-Bay K 8644. This higher tension induced by FPL 64176 may be due to its nonexistent or weak antagonist activity, relative to (*S*)-Bay K 8644. Obviously, the efficacy of FPL 64176 is higher than that of (*S*)-Bay K 8644, although the

potency is weaker than that of (*S*)-Bay K 8644. Additionally, the responses to (*S*)-Bay K 8644 in rat tail artery are dependent on the presence of elevated K^+ (16), whereas FPL 64176 induces responses without additional K^+, although the tension increased further in the presence of higher K^+ concentrations. Finally, the prolonged washout period for FPL 64176 and the spontaneous activity observed during this period are also in contrast to the action of (*S*)-Bay K 8644 and are consistent with a site and a mechanism of action of FPL 64176 that are distinct from those of (*S*)-Bay K 8644.

(+)-[^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 (24), representing three major classes of Ca^{2+} channel antagonists (1,4-dihydropyridines, phenylalkylamines, and benzothiazepines, respectively), are thought to occupy different binding sites on the α_1 subunit of the Ca^{2+} channel protein (2). Recently, locations of the binding sites for the first two classes of ligands on the α_1 subunit have been indicated (29, 30). Our binding data show that FPL 64176 is a very weak inhibitor of [^{3}H]PN 200-110 binding, in agreement with the previous report by McKechine *et al.* (14). The IC_{50} value for FPL 64176 inhibition of [^{3}H]PN 200-110 binding is some 45-fold higher than the EC_{50} value for stimulation of contractile response. FPL 64176 is a similarly weak inhibitor of [^{3}H]D888 and [^{3}H]TA-3090 binding. In contrast, (*S*)-Bay K 8644 competitively inhibits radiolabeled 1,4-dihydropyridine antagonist binding with an affinity similar to its pharmacological activity (16, 31). These data collectively suggest that FPL 64176 interacts at a discrete site. This is confirmed by FPL 64176 acceleration of the dissociation process of (+)-[^{3}H]PN 200-110, [^{3}H]D888, and [^{3}H]TA-3090 from their separate binding sites.

Finally, electrophysiological studies in the A7r5 smooth muscle cell line directly confirm the stimulatory activity of FPL 64176 on L-type Ca^{2+} channels. Indeed, currents are increased approximately 14-fold after the addition of 10^{-6} M FPL 64176. Undoubtedly, this dramatic increase in Ca^{2+} channel current underlies the great efficacy with which FPL 64176 contracts vascular smooth muscle. However, in contrast to our pharmacological studies, we found that the effects of FPL 64176 on Ca^{2+} channel currents are rapidly reversible. At present we have no explanation for this discrepancy, but it may involve some partitioning of FPL 64176 in whole tissue that is not observed on the single-cell level.

Like Bay K 8644, FPL 64176 shifts the threshold and the peak of L channel I-V relationship in the hyperpolarizing direction and prolongs the decay of inward tail currents (11). Unlike Bay K 8644, however, FPL 64176 slows current activation and fails to promote inactivation of L channel current during step depolarizations (11, 32, 33). Thus, FPL 64176 has a mechanism of action on L-type Ca^{2+} channels that is, at least in some respects, unique from that of Bay K 8644. Future studies on the single-channel level should prove valuable in determining further the action of FPL 64176.

In conclusion, our pharmacological, radioligand binding, and electrophysiological data indicate that FPL 64176 is a new type of Ca^{2+} channel activator, with a mechanism and site of action obviously different from those of the 1,4-dihydropyridine class of Ca^{2+} channel activators. An important property of FPL 64176 is that its efficacy is greater than that of (*S*)-Bay K 8644, without significant inhibitory effects at high concentrations. Thus, this new benzoylpyrrole-type Ca^{2+} activator is a very useful tool to study further the structure-function relationship and the gating mechanism of voltage-dependent Ca^{2+} channels.

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Send reprint requests to: David J. Triggle, Ph.D., 126 Cooke Hall, State University of New York at Buffalo, Buffalo, NY 14260.