0026-895X/92/040599-04$03.00/0

MOLECULAR PHARMACOLOGY, 41:599-602

ACCELERATED COMMUNICATION

Functional Interactions between Two Ca^{2+} Channel Activators, (*S*)-Bay K 8644 and FPL 64176, in Smooth Muscle

DAVID RAMPE and RICHARD C. DAGE
Marion Merrell Dow Research Institute, Cincinnati, Ohio 45215
Received December 11, 1991; Accepted January 23, 1992

SUMMARY

We examined the interactions of two Ca^{2+} channel activators, (*S*)-Bay K 8644 and FPL 64176, on smooth muscle L-type Ca^{2+} channels. FPL 64176 (300 nM) caused a sustained contraction of rat tail artery strips. This contractile response was inhibited by approximately 70% by (*S*)-Bay K 8644 (EC_{50} = 14 nM). (*S*)-Bay K 8644 (100 nM) increased whole-cell Ca^{2+} currents in A7r5 smooth muscle cells but effectively blocked further stimulation by 1 μM FPL 64176. When added alone, 1 μM FPL 64176 increased Ca^{2+} channel current amplitude, slowed current activation, and prolonged tail current duration. Furthermore, no inactivation of current during step depolarizations was observed in the presence of FPL 64176. After subsequent addition of (*S*)-Bay K 8644, Ca^{2+} channel current activation was accelerated and tail current duration was shortened. Additionally, pronounced inactivation of the Ca^{2+} channel current became apparent. These results are consistent with a negative allosteric interaction between the (*S*)-Bay K 8644 binding site and that of FPL 64176, in smooth muscle.

Voltage-dependent L-type calcium channels provide a major means by which Ca^{2+} gains entry into cardiovascular tissues. These channels are multimeric proteins composed of five subunits, α_1, α_2, β, γ, and δ (1). The L-type Ca^{2+} channel is the receptor site for drugs such as nifedipine, verapamil, diltiazem, and other compounds that are collectively termed the Ca^{2+} channel antagonists (2). L-type Ca^{2+} channels, as well as other voltage-dependent ion channels, can be regarded as pharmacological receptors containing discrete drug binding sites (3). However, owing to the structural complexity of the ion channels and their ability to exist in various closed, opened, and inactivated states, interactions of drugs at ion channels are sometimes more complicated than those seen for other receptor systems. Thus, functional interactions between various toxins on neuronal Na^+ channels (4, 5), as well as those seen for Ca^{2+} antagonists in smooth muscle (6, 7), are not always indicative of the allosteric interactions predicted by radioligand binding studies.

Although many drugs are known to act as antagonists at the L-type Ca^{2+} channel (8), only a few structures, confined to the dihydropyridine class of compounds, act predominantly as Ca^{2+} channel activators (2). Even these compounds, which include (*S*)-Bay K 8644, can be considered only partial agonists, due to their high affinity for the inactivated state of the Ca^{2+} channel (9). Recently, a new benzoylpyrrole compound, FPL 64176, has been synthesized and shown to have properties consistent with Ca^{2+} channel activation (10). Further studies have shown that FPL 64176 failed to interact at any of the major ligand binding sites on the L-type Ca^{2+} channel and that it possessed a pharmacological and electrophysiological profile distinct from that of (*S*)-Bay K 8644 (11, 12). The present study was undertaken to examine the functional interactions between FPL 64176 and (*S*)-Bay K 8644 in smooth muscle, using pharmacological and electrophysiological techniques.

Materials and Methods

Tissue preparation and recording of mechanical response. Rat tail artery strips were prepared essentially as described previously (13). Briefly, male Holtzman rats were sacrificed by decapitation, and the median tail artery was removed and placed in a physiological salt solution containing (in mM) NaCl, 110; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; dextrose, 11; and EDTA, 0.027. After connective tissue was cleaned from the artery, the tissue was cut into strips approximately 2 cm long. The strips were suspended in glass organ baths containing aerated physiological salt solution at 37° and were connected to a force-displacement transducer, and isometric contractions were recorded on a Grass polygraph (model 7D). Before measurement of response, tissues were equilibrated for approximately 1 hr under a resting tension of 1 g. During this time, tissues were stimulated three times with 80 mM KCl, to initiate a steady level of response. Data were expressed as mean ± standard error for six separate observations.

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

Electrophysiological experiments. Electrophysiological recordings were carried out as previously described (11). 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.* (14), using an Axopatch-1D amplifier (Axon Instruments, Burlingame, CA). Electrodes were fashioned from TW150 glass capillary tubes (World Precision Instruments, New Haven, CT) and had resistances of 1–4 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; and 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 containing (in mM) *N*-methyl-D-glucamine, 110; aspartate, 140; $BaCl_2$, 40; HEPES, 10; tetraethylammonium hydroxide, 30; and 4-aminopyridine, 5 (pH 7.4, with methanesulfonic acid). Series resistance was compensated after the seal was broken, to provide the fastest possible capacity transient without ringing. Cell currents were conditioned by a four-pole low-pass filter with a cutoff frequency of 1 KHz. Currents were sampled at 10 KHz unless otherwise stated and were stored, analyzed, and corrected using a Compaq Deskpro computer and pCLAMP software (Axon Instruments).

Results

Fig. 1A shows the effects of a submaximal dose (300 nM) of FPL 64176 on rat tail artery preincubated with 20 mM KCl (25 mM final K^+ concentration). As we reported previously (11), FPL 64176 caused a large sustained contraction under these conditions. However, when the Ca^{2+} channel activator (*S*)-Bay K 8644 (100 nM) was also added to the bath, a pronounced relaxation of the tissue was observed. Fig. 1B shows the dose-response relationship for this effect. Relaxation of the tissue occurred at all (*S*)-Bay K 8644 concentrations tested. The EC_{50} value for this effect was $1.40 \pm 0.01 \times 10^{-8}$ M, and the Hill coefficient was −0.78 ($n = 6$).

We next turned to the whole-cell patch-clamp technique, to examine the effects of (*S*)-Bay K 8644 and FPL 64176, alone and in combination, on L-type Ca^{2+} channel currents in the A7r5 smooth muscle cell line. Fig. 2A shows the effects of 100 nM (*S*)-Bay K 8644 on these currents. As reported for other smooth muscle preparations (15, 16), (*S*)-Bay K 8644 increased Ca^{2+} channel current amplitude and prolonged tail current decay (Fig. 2A, *traces 1* and *2*). However, (*S*)-Bay K 8644 effectively blocked any further stimulation of Ca^{2+} channel current by 1 μM FPL 64176 (Fig. 2A, *trace 3*). Under these conditions, the only effect of FPL 64176 we did observe was a prolongation of a small component of the Ca^{2+} channel tail current in four of five cells tested.

We next tested what effects (*S*)-Bay K 8644 would have on FPL 64176-induced Ca^{2+} currents in these A7r5 smooth muscle cells. As we have previously reported (11), FPL 64716 (1 μM) increased L-type channel current amplitude and slowed both current activation and deactivation (Fig. 2B, *traces 1* and *2*). After addition of 1 μM FPL 64176, the activation τ for the Ca^{2+} channel current was 51.0 ± 4.7 msec and the tail current τ, when measured between 80% and 20% of its peak value, was 27.8 ± 9.6 msec ($n = 5$). When 100 nM (*S*)-Bay K 8644 was subsequently added to these cells, we saw a dramatic change in the current kinetics (Fig. 2B, compare *traces 2* and *3*). Specifically, the activation τ and the tail current τ were reduced to 8.8 ± 1.4 msec and 2.6 ± 0.4 msec, respectively. These values are similar to the activation τ and tail current τ of 6.98 ± 0.34 msec and 1.95 ± 0.28 msec, respectively, recorded in the presence of 100 nM (*S*)-Bay K 8644 alone. The percentage of reduction of the activation τ at various (*S*)-Bay K 8644 concentrations is shown in Fig. 3. The EC_{50} value for this response was $4.8 \pm 0.08 \times 10^{-9}$ M, a value similar to that seen for relaxation of FPL 64176-induced contractile activity.

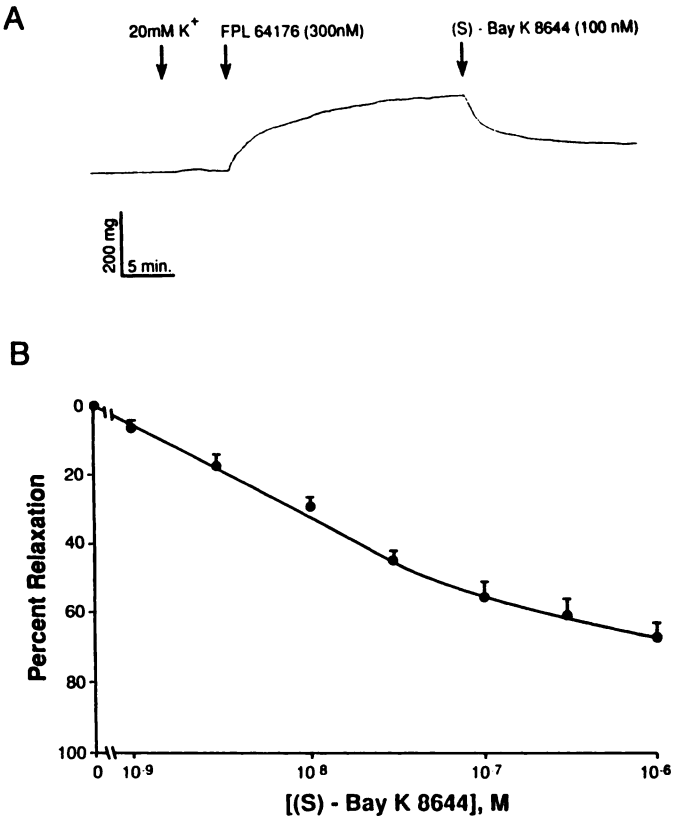

Fig. 1. Contractile responses to FPL 64176 and (*S*)-Bay K 8644 in rat tail artery strips. A, Trace of tension response to additions of FPL 64176 (300 nM) and (*S*)-Bay K 8644 (100 nM), in the presence of 20 mM K^+. B, Effects of various concentrations of (*S*)-Bay K 8644 on the contractile response induced by 300 nM FPL 64176 in the presence of 20 mM K^+. After a stable contractile response to FPL 64176, (*S*)-Bay K 8644 was added at 5-min intervals to give the indicated concentrations. The EC_{50} for the dose-response relationship was $1.40 \pm 0.01 \times 10^{-9}$ M ($n = 6$).

Fig. 4 shows the effects of 1 μM FPL 64176 on Ca^{2+} channel current in an A7r5 cell during prolonged (455-msec) depolarization. As noted previously, FPL 64176 increased Ca^{2+} channel current amplitude and prolonged both activation and tail current duration. Furthermore, we saw no obvious inactivation of Ca^{2+} channel current in the presence of FPL 64716 alone (Fig. 4, *trace 2*). However, when 100 nM (*S*)-Bay K 8644 was further added to the cell, currents displayed significant inactivation during the pulse (Fig. 4, *trace 3*). When measured at the end of the 455-msec pulse, FPL 64176-induced current amplitude was inhibited by 76 ± 4% after the addition of 100 nM (*S*)-Bay K 8644 ($n = 5$).

Discussion

The benzoylpyrrole FPL 64176 represents the first synthetic ligand, other than certain dihydropyridines, to act predomi-

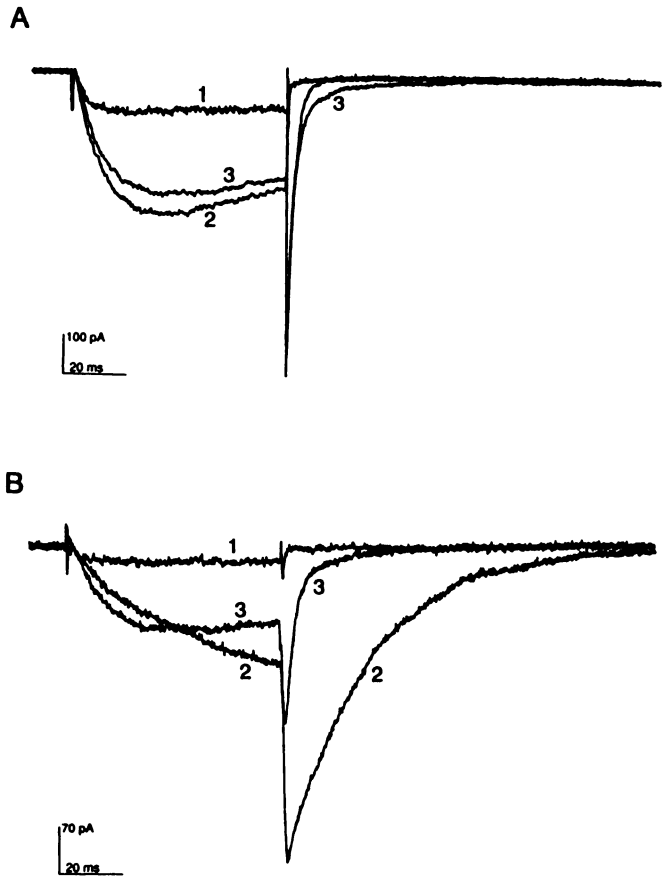

Fig. 2. Effects of FPL 64176 and (*S*)-Bay K 8644 on L-type Ca^{2+} channel currents in A7r5 smooth muscle cells. A, Currents were induced by a 70-msec clamp pulse to +10 mV, from a holding potential of −50 mV. *Traces 1* and *2*, control current and current 1 min after the addition of 100 nM (*S*)-Bay K 8644, respectively. *Trace 3*, subsequent addition of 1 μM FPL 64176 to the cell. B, The pulse protocol described for A was used to generate control current (*trace 1*) and current 1 min after the addition of 1 μM FPL 64176 (*trace 2*). Subsequent addition of 100 nM (*S*)-Bay K 8644 to the cell (*trace 3*) speeds current activation and shortens tail current duration.

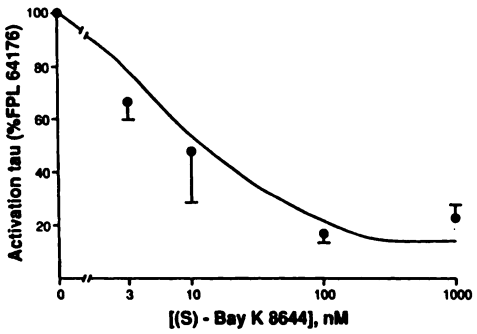

Fig. 3. Effects of (*S*)-Bay K 8644 on FPL 64176-induced Ca^{2+} channel activation kinetics. Cells were held at −50 mV and pulsed to +10 mV for 70 msec, to induce L-type Ca^{2+} channel currents. Cells were treated with 1 μM FPL 64176, followed by various concentrations of (*S*)-Bay K 8644. Ca^{2+} channel activation kinetics for both treatments were fit with a single exponential and expressed as a percentage of the FPL 64716 response. The EC_{50} value for the (*S*)-Bay K 8644 dose-response curve was $4.8 \pm 0.08 \times 10^{-9}$ M ($n = 4$ or 5).

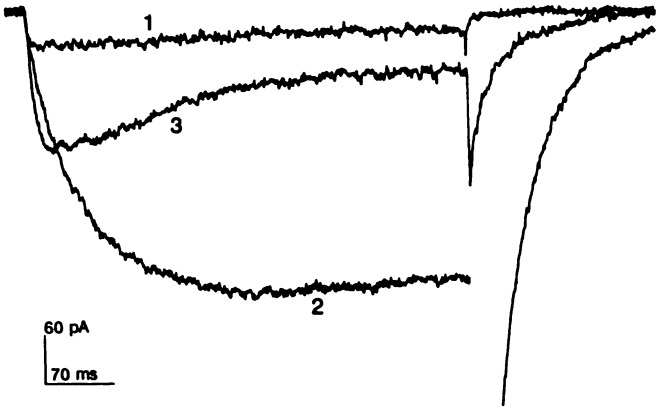

Fig. 4. Effects of FPL 64176 and (*S*)-Bay K 8644 on L-type Ca^{2+} channel currents during prolonged depolarizations. Currents were induced by a 455-msec clamp pulse to +10 mV, from a holding potential of −50 mV. Current was sampled at 1.5 KHz. *Traces 1* and *2*, control current and current in the presence of 1 μM FPL 64176, respectively. *Trace 3*, subsequent addition of 100 nM (*S*)-Bay K 8644.

nantly as an L-type Ca^{2+} channel activator. We have previously shown that, although FPL 64176 has a lower affinity for contracting smooth muscle than does (*S*)-Bay K 8644, it has a much greater efficacy (11). Due to its unique binding site and mechanism of action (11, 12), we were interested in what interactions FPL 64176 might have with a dihydropyridine activator such as (*S*)-Bay K 8644, in smooth muscle. Because these compounds are Ca^{2+} channel activators that bind to distinct sites on the channel, we expected to see additive or synergistic effects on smooth muscle contraction when the compounds were applied together. Instead, (*S*)-Bay K 8644 acted to inhibit FPL 64176-induced contractions in rat tail artery with an EC_{50} value of 14 nM. This value is similar to the EC_{50} value obtained for contraction of rat tail artery strips by (*S*)-Bay K 8644 alone (11) and suggests a high affinity interaction between (*S*)-Bay K 8644 and FPL 64176. However, inhibition of FPL 64176-induced contractions was incomplete, which is likely due to the fact that (*S*)-Bay K 8644 by itself is a positive inotropic agent in this preparation.

We examined the interactions of FPL 64176 and (*S*)-Bay K 8644 directly on L-type Ca^{2+} channels in A7r5 smooth muscle cells, using whole-cell patch-clamp electrophysiology. FPL 64176 has distinct effects on whole-cell Ca^{2+} channel currents, which include a prominent slowing of activation kinetics and a prolongation of tail current duration (11, 12). We found that cells pretreated with 100 nM (*S*)-Bay K 8644 essentially lost the ability to respond to further additions of 1 μM FPL 64176. When we treated cells with 1 μM FPL 64176 alone, we saw a pronounced prolongation of Ca^{2+} current activation kinetics and tail current duration. Further addition of 100 nM (*S*)-Bay K 8644 dramatically shortened these parameters, to the point where the currents resembled those seen in the presence of (*S*)-Bay K 8644 alone (compare Fig. 2A, *trace 2*, with Fig. 2B, *trace 3*). Because the dihydropyridines and FPL 64176 do not share the same binding site (11), we believe that the present data support the notion that (*S*)-Bay K 8644 acts as a potent allosteric inhibitor of FPL 64176 binding in intact tissue. This would allow (*S*)-Bay K 8644 to inhibit FPL 64176 responses while preserving its own stimulatory effect on smooth muscle Ca^{2+} channels. Such interactions may not be readily observed in membrane binding studies, where Ca^{2+} channels are pre-

sumed to be locked in the inactivated state and where FPL 64176 is only very weakly active ($EC_{50} \geq 10\ \mu M$) at displacing 1,4-[^{3}H]dihydropyridine binding (11).

Although commonly considered as Ca^{2+} channel activators, molecules such as (*S*)-Bay K 8644 should be regarded as partial activators, due in part to their ability to promote Ca^{2+} channel current inactivation during membrane depolarization (16–18). In the present study, FPL 64176 by itself failed to promote Ca^{2+} current inactivation during prolonged (455-msec) depolarizations. However, subsequent addition of 100 nM (*S*)-Bay K 8644 resulted in significant current inactivation in these cells. The failure of FPL 64176 to induce significant Ca^{2+} current inactivation suggests to us that it is more purely an agonist molecule than is (*S*)-Bay K 8644. These results may also explain, at least in part, the greater efficacy with which FPL 64176 contracts smooth muscle, relative to (*S*)-Bay K 8644 (11).

In summary, this report is the first to explore the interactions of two distinct Ca^{2+} channel activator ligands, FPL 64176 and (*S*)-Bay K 8644, on smooth muscle Ca^{2+} channels. We find that (*S*)-Bay K 8644 acts as a potent inhibitor of FPL 64176-induced responses. We feel that these results can be explained by a negative allosteric interaction between the 1,4-dihydropyridine agonist binding site and the benzoylpyrrole binding site in intact tissue. However, we cannot exclude other mechanisms that could also contribute to the results presented here. For example, (*S*)-Bay K 8644 could stabilize a state or substate of the Ca^{2+} channel that does not recognize FPL 64176 (18). Additionally, multiple binding sites for (*S*)-Bay K 8644 or FPL 64176 could further complicate their interactions. Future studies at the single-channel level and those using radiolabeled benzoylpyrrole ligands will be useful for exploring these possibilities.

Acknowledgments

We thank James Ficorilli and Nelson Velayo for valuable technical support and Dr. John P. Paolini for synthesis of FPL 64176.

References

1. Campbell, K. P., A. T. Leung, and A. H. Sharp. The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel. *Trends Neurosci.* **11**:425–430 (1988).
2. Janis, R. A., P. J. Silver, and D. J. Triggle. Drug action and cellular calcium regulation. *Adv. Drug Res.* **16**:311–591 (1987).
3. Triggle, D. J., D. A. Langs, and R. A. Janis. Ca^{2+} channel ligands: structure-function relationships of the 1,4-dihydropyridines. *Med. Res. Rev.* **9**:123–180 (1989).
4. Catterall, W. A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* **20**:15–43 (1980).
5. Strichartz, G., T. Rando, and G. K. Wang. An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* **10**:237–267 (1987).
6. Spedding, M. Functional interactions of calcium-antagonists in K^+-depolarized smooth muscle. *Br. J. Pharmacol.* **80**:485–488 (1983).
7. Yousif, F., and D. J. Triggle. Functional interactions between organic calcium channel antagonists in smooth muscle. *Can. J. Physiol. Pharmacol.* **63**:193–195 (1984).
8. Rampe, D., and D. J. Triggle. New ligands for L-type Ca^{2+} channels. *Trends. Pharmacol. Sci.* **11**:112–115 (1990).
9. Triggle, D. J., and D. Rampe. 1,4-Dihydropyridine activators and antagonists: structural and functional distinctions. *Trends Pharmacol. Sci.* **10**:507–511 (1989).
10. McKechnie, K., P. G. Killingback, I. Naya, S. E. O'Conner, G. W. Smith, D. G. Wattam, E. Wells, Y. M. Whitehead, and G. E. Williams. Calcium channel activator properties in a novel non-dihydropyridine, FPL 64176. *Br. J. Pharmacol.* **98**:673P (1989).
11. Zheng, W., D. Rampe, and D. J. Triggle. Pharmacological, radioligand binding, and electrophysiological characteristics of FPL 64176, a novel nondihydropyridine Ca^{2+} channel activator, in cardiac and vascular preparations. *Mol. Pharmacol.* **40**:734–741 (1991).
12. Rampe, D., and A. E. Lacerda. A new site for the activation of cardiac calcium channels defined by the non-dihydropyridine FPL 64176. *J. Pharmacol. Exp. Ther.*, **259**:982–987 (1991).
13. Webb, R. C., P. M. Vanhoutte, and D. F. Bohr. Inactivation of released norepinephrine in rat tail artery by neuronal uptake. *J. Cardiovasc. Pharmacol.* **2**:121–132 (1980).
14. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
15. Yatani, A., A. J. Seidel, and A. M. Brown. Whole-cell and single-channel calcium currents of isolated smooth muscle cells from saphenous vein. *Circ. Res.* **60**:523–533 (1987).
16. Benham, C. D., P. Hess, and R. W. Tsien. Two types of calcium channels in single smooth muscle cells from rabbit ear artery studied with whole-cell and single-channel recordings. *Circ. Res.* **61** (Suppl. I):I10–I16 (1987).
17. Kass, R. S. Voltage-dependent modulation of cardiac calcium channel current by optical isomers of Bay K 8644: implications for channel gating. *Circ. Res.* **61** (Suppl. I):I1–I5 (1987).
18. Lacerda, A. E., and A. M. Brown. Nonnodal gating of cardiac calcium channels as revealed by dihydropyridines. *J. Gen. Physiol.* **93**:1243–1273 (1989).

Send reprint requests to: David Rampe, Ph.D., Marion Merrell Dow Research Institute, 2110 E. Galbraith Road, Cincinnati, OH 45215.