Activation of cardiac ryanodine receptors by the calcium channel agonist FPL-64176

J. ANDREW WASSERSTROM,1 LESLIE A. WASSERSTROM,1 ANDREW J. LOKUTA,2 JAMES E. KELLY,1 SIREEN T. REDDY,1 AND ANDREW J. FRANK1
1Division of Cardiology, Departments of Medicine, Molecular Pharmacology and Medicinal Chemistry and the Feinberg Cardiovascular Research Institute, Northwestern University Medical School, Chicago, Illinois 60611; and 2Department of Physiology, University of Wisconsin Medical College, Madison, Wisconsin 53706

Received 4 September 2001; accepted in final form 1 April 2002


We investigated the possibility that the Ca2+-channel agonist FPL-64176 (FPL) might also activate the cardiac sarcoplasmic reticulum (SR) Ca2+-release channel ryanodine receptor (RyR). The effects of FPL were tested on single channel activity of purified and crude vesicular RyR (RyR2) isolated from human and dog hearts using the planar lipid bilayer technique. FPL (100–200 μM) increased single channel open probability (P0) when added to the cytoplasmic side of the channel (P0 = 0.070 ± 0.021 in control RyR2; 0.378 ± 0.086 in 150 μM FPL, n = 9, P < 0.01) by prolonging open times and decreasing closed times without changing current magnitude. FPL had no effect on P0 when added to the trans (luminal) side of the bilayer (P0 = 0.079 ± 0.036 in control and 0.103 ± 0.066 in FPL, n = 4, no significant difference). The bell-shaped [Ca2+] dependence of [3H]ryanodine binding and of P0 was altered by FPL, suggesting that the mechanism by which FPL increases channel activity is by an increase in Ca2+-induced activation at low [Ca2+] (without a change in threshold) and suppression of Ca2+-induced inactivation at high [Ca2+]. However, the fact that inactivation was restored at elevated [Ca2+] suggests a competitive interaction between Ca2+ and FPL on inactivation. FPL had no effect on RyR skeletal channels (RyR1), where P0 was 0.039 ± 0.005 in control versus 0.030 ± 0.006 in 150 μM FPL (no significant difference). These results suggest that, in addition to its ability to activate the L-type Ca2+ channels, FPL activates cardiac RyR2 primarily by reducing the Ca2+ sensitivity of inactivation.

There are several classes of pharmacological agents known to activate L-type (voltage-gated) Ca2+ channels: dihydropyridines (DHP) such as BAY K 8644 (BAYK) and the benzoylpyrrole derivative FPL-64176 (FPL). The mechanism by which BAYK alters channel gating is thought to involve a change in mode from normal gating to one with a high probability of opening (6). A similar mechanism is likely to account for the actions of FPL, although its precise interactions with the Ca2+ channel are less well understood than for BAYK. For example, it is known that FPL, like BAYK, prolongs action potential duration and causes a positive inotropic effect in guinea pig papillary muscle, both of which are likely to result from increased L-type Ca2+ channel current (I_{Ca,L}) magnitude (14). Both agents induce a hyperpolarizing shift in activation and inactivation by ~10 mV (3). However, FPL slows the rates of I_{Ca,L} activation and inactivation, whereas BAYK increases current magnitude while accelerating the rate of decay (3, 14). These effects of FPL on contraction and I_{Ca,L} are presumably a result of its ability to prolong single channel open time during depolarization and immediately after repolarization, with little effect on closed times and latency to first openings (3).

Because of the differences in drug actions on I_{Ca,L}, there has been additional exploration of whether or not these agonists also share binding sites in the Ca2+ channel itself. FPL did not affect [3H]PN 200–110 binding; thus DHPs and FPL are thought to bind to different sites on the channel protein (16, 23). However, electrophysiological evidence showed that single-channel open probability (P0) for a BAYK-FPL mixture exhibited a lower P0 than either alone (11). This result was interpreted to suggest that although these agents bind to different sites on the Ca2+ channel, a functional or physical interaction does in fact exist between them.

Another interesting difference between the effects of the two I_{Ca,L} agonists is that BAYK directly increases frequency of Ca2+ sparks whereas FPL does not (10). These results were interpreted to suggest that one of the effects of BAYK binding to the Ca2+ channel may be to alter Ca2+ release from the sarcoplasmic reticulum (SR). This action might occur via a connection between the sarcolemmal Ca2+ channel and the re-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
lease channel in the SR membrane, an effect not shared by FPL (10).

We considered the possibility that, in addition to their effects on $I_{Ca,L}$, these agents might also have direct effects on cardiac ryanodine receptors (RyR2) that could influence their ability to regulate contraction. We (18) recently reported that, in addition to an effect on $I_{Ca,L}$, BAYK and its analogs also activate the purified cardiac SR calcium release channel incorporated in artificial lipid bilayers. Interestingly, the mechanism by which the channel is activated may involve a suppression of Ca$^{2+}$-induced inactivation of RyR2 that occurs at high concentrations of Ca$^{2+}$. However, this effect was only observed in purified channels and was not observed using reconstituted channels from crude microsomal vesicles. This was the first report of a direct action of BAYK on any other Ca$^{2+}$ channel, and was not observed using reconstituted channels in puriﬁed cardiac SR calcium release channel incorporated in artiﬁcial lipid bilayers. Interestingly, the mechanism by which the channel is activated may involve a suppression of Ca$^{2+}$-induced inactivation of RyR2 that occurs at high concentrations of Ca$^{2+}$. However, this effect was only observed in purified channels and was not observed using reconstituted channels from crude microsomal vesicles. This was the first report of a direct action of BAYK on any other Ca$^{2+}$ channel aside from that located in the surface membrane of many cell types and might contribute to the cellular actions of DHP agonists.

The purpose of this study was to extend these observations to the second class of Ca$^{2+}$ channel agonists represented by FPL. In particular, we wanted to determine whether FPL also activates RyR2. If so, does it share a common mechanism of action with the DHP agonists? One implication of a shared mechanism would be the existence of a common or overlapping DHP binding site on the RyR2 that acts as a putative calcium channel agonist receptor. Finally, we compared the effects of FPL on dog and human RyR2 to determine whether or not channel modification occurs in human channels in addition to animal models.

METHODS

Single channel studies. Dog hearts were obtained from animals anesthetized with pentobarbital sodium (35 mg/kg iv) before removal of the heart. Cat heart and skeletal (gastrocnemius) preparations were obtained in the same manner. Human hearts were obtained from three normal patients who died from noncardiac disease whose hearts were donated for research purposes. Protocols for animal and human tissue use were reviewed and approved by the Institutional Animal Care and Use Committee and the Internal Review Board at Northwestern University, respectively.

Crude microsomes were obtained from the skeletal muscle and left ventricle with differential centrifugation. Purification of Ca$^{2+}$ release channels was performed using 3-[N-cholamidopropyl(dimethylammonio)]-1-propanesulfonate solubilization of heavy SR vesicles with subsequent reconstitution of purified protein into proteoliposomes (21).

Recordings of activity of purified channels were obtained using the planar lipid bilayer technique with 250 mM KCl and 10 mM HEPES (pH 7.2) on the cis (cytoplasmic) and trans (luminal) sides of the bilayer. The trans side also contained 1 mM CaCl$_2$. [Ca$^{2+}$] in the cis chamber was measured by a Ca$^{2+}$-sensitive electrode to be 5 nM and was unchanged in most experiments unless stated otherwise. In some experiments, admixtures of CaCl$_2$ and EGTA were used to set [Ca$^{2+}$] in the cis compartment according to Calcium software. Bilayer composition was 0.4 mg each of phosphatidylserine and phosphatidylethanolamine (Avanti Polar Lipids) suspended in 20 μl of n-decane.

Channel incorporation took place in the presence of a 250:20 mM KCl concentration gradient. Once a stable bilayer was formed, 3 μl of microsomes or liposomes were added to the cis side of the bilayer during constant stirring. Channel activity indicated successful fusion of a vesicle with the bilayer. In most experiments, the ionic gradient was then abolished by addition of the appropriate amount of 3 M KCl to the trans side of the bilayer. Recordings (usually 2 or 3 in each condition of 30-s duration each) were initiated after 2 to 3 min of stable activity. Pharmacological agents were added directly to either compartment of the bilayer apparatus after control recordings were obtained and the experimental protocol was repeated after 1 min of stirring.

Single channel activity was also recorded from channels in heavy SR vesicles. Procedures were identical to those described above for purified channels with the exception of Cs$^{+}$-methanesulfonate substitution for KCl to avoid interference from K$^+$ and Cl$^-$ channels native to crude SR vesicles. Dog cardiac SR channels were recorded in the absence of a Na$^+$ gradient at different holding potentials. Dog skeletal SR channels were recorded in the maintained presence of a Cs$^{+}$ gradient at 0 mV.

Single channel data were recorded (Axopatch 200 and 200A amplifiers, Axon Instruments) using pCLAMP version 6 software in data files 30 s in duration at constant holding potentials ($V_h$) of $\pm$40 mV in most experiments. Data were filtered with an eight-pole Bessel filter (model 902, Frequency Devices) at 2 kHz, digitized at 5 kHz, and analyzed off-line using half-amplitude threshold algorithms. All chemicals were obtained from Sigma, with the exception of FPL, which was obtained from BioMol.

$[^3H]$ryanodine binding studies. Aliquots (80 μg) of microsomal protein were added to an incubation medium containing 7 nM $[^3H]$ryanodine, 0.2 M KCl, 20 mM 3-(N-morpholin- o)propanesulfonic acid (pH 7.2), 1 mM EGTA, and different amounts of CaCl$_2$ to set free [Ca$^{2+}$] in the range of 0.01–10,000 μM. Experimental modulators were added to reaction mixtures from 10× stocks. The incubation took place in a volume of 0.1 ml containing ~0.3 pM of the receptor at 37°C for 90 min. After incubation, bound and free $[^3H]$ryanodine were separated by rapid filtration onto Whatman GF/B or GF/C glass fiber filters and the filters were washed three times with cold distilled water with a Millipore manifold. The filters were placed in liquid scintillation cocktail and counted in a Beckman LS6500 β-counter. Nonspecific $[^3H]$ryanodine binding was determined in the presence of 5 μM unlabeled ryanodine and has been subtracted from all reported values.

Data analysis. Data are presented as means ± SE. Only data obtained from bilayers containing a single channel were included in this study, so $P_r$ refers to the activity of a single channel. Data were compared using paired or unpaired Student’s t-tests or a one-way analysis of variance (with secondary comparisons made using a Student-Newman-Keuls test). Differences between sample means were considered significant if $P < 0.05$, unless indicated otherwise, and NS refers to comparisons in which differences did not achieve statistical significance.

RESULTS

Effects of FPL on purified human RyR2. The effect of FPL on single channel activity using purified RyR2 channels from the human ventricle is shown in Fig. 1A. When FPL (100 μM) was added to the cis (cytoplasmic) side of the bilayer, channel open times increased, as did $P_o$. Subsequent increases in drug concentration caused further increases in $P_o$ and long open times.
with the result being a nearly continuously open channel at 200 μM (\(P_o = 0.913\)).

The concentration dependence of FPL effects on \(P_o\) are summarized in Fig. 1B. The threshold for increasing \(P_o\) occurred \(-100\) μM. Because drug solubility becomes problematic \(>200\) μM, this was considered the maximal usable concentration, giving an estimated EC\(_{50}\) of \(\sim 150\) μM. This increase in channel activity occurred in the absence of a change in single channel conductance (591 ± 24 pS in control vs. 601 ± 22 pS in the presence of 150 μM FPL, NS, \(n = 7\)).

One of the questions raised by the ability of an agent to activate RyR2 pertains to the specificity of the action on the channel protein. Figure 2 shows the effects of FPL when added to the trans (luminal) side of the bilayer after control measurements. There was little effect on single channel activity (increase in \(P_o\) from 0.03 to 0.06). When the same concentration of drug was then added to the cis side of the bilayer, channel activity showed the dramatic increase also illustrated in Fig. 1. In a total of four channels, \(P_o\) was 0.079 ± 0.036 in control and 0.103 ± 0.066 after addition of FPL to the trans chamber (NS). These results demonstrate that the action of FPL to activate single RyR2 activity is likely to be the result of a specific interaction with a receptor located on the cytoplasmic face of the channel rather than the result of a nonspecific interaction with either the channel protein or the lipid bilayer itself.

**Effects of FPL on channel activity in dog SR vesicles.** In addition to an effect on purified RyR2, we also investigated the effects of FPL on single channels from crude SR vesicles. The effects of FPL on RyR2 single-channel activity in dog SR vesicles are shown in Fig. 3A. Under control conditions, channel openings were brief and relatively infrequent, giving an overall \(P_o\) of 0.08. After addition of FPL to the cis side of the bilayer, channel activity was increased (\(P_o = 0.58\)) as a result of prolonged open times. The results obtained under these conditions are summarized in Fig. 3B and show that FPL activated cardiac RyR2 channels whether or not they are in the purified or vesicular form, an observation that is in distinct contrast to that of BAYK effects on RyR2 (18).

**Effects of FPL on open and closed characteristics.** To understand the basis for the activating effects of FPL on RyR2, we analyzed the changes in gating characteristics of dog SR channels induced by FPL. The histograms shown in Fig. 4 summarize the open duration times (Fig. 4, A and B) and the closed duration times (Fig. 4, C and D) before and during exposure to FPL in one experiment. Under control conditions, open time...
duration of all openings and reduces the duration of all closings and 2) the drug shifts the channel to a mode of long open and short closed time durations. The net result is an increase in single channel $P_o$ as a result of greatly increased dwell times in the open state.

Dependence of FPL activation of RyR2 by cis [Ca$^{2+}$]. One of the most common mechanisms by which different agents affect single RyR2 channel activity is by altering the sensitivity of the channel to [Ca$^{2+}$] on the cis side of the bilayer. We therefore tested this possibility regarding the actions of FPL on RyR2. Figure 6A shows the effect of FPL on [3H]ryanodine binding to dog cardiac SR vesicles at different cis [Ca$^{2+}$]. In the absence of FPL, [3H]ryanodine binding is low at 0.1 μM cis [Ca$^{2+}$], increases to a maximum at 10–100 μM, and then declines at 1–10 mM. This biphasic reliance of [3H]ryanodine binding on [Ca$^{2+}$] is thought to represent channel activation by calcium over the range of 0.1–100 μM with subsequent development of inactivation occurring >100 μM. In the presence of FPL, [3H]ryanodine binding is unchanged at 0.1 μM [Ca$^{2+}$] but there is an increase in [3H]ryanodine binding at all subsequent [Ca$^{2+}$], which is significant at >10 μM. These results suggest that FPL might increase channel activity at low concentrations of Ca$^{2+}$ as well as reduce channel sensitivity to Ca$^{2+}$–induced inactivation at higher concentrations. However, at a [Ca$^{2+}$] of 10 mM,

The effects of FPL on gating characteristics are summarized in Fig. 5. FPL increased $\tau_{O1}$ and $\tau_{O2}$ durations (Fig. 5A, top) as well as the proportion of $\tau_{O2}$ (Fig. 5A, bottom). Both long and short closed time durations were significantly decreased (Fig. 5B, top) and the contribution of long closings to total closed time was diminished (Fig. 5B, bottom).

These results provide an explanation for the observed effects of FPL on RyR2: 1) FPL increases the

---

**Fig. 3. Effects of FPL on single channel activity of crude dog RyR.** A: original recordings of single channel activity from a crude preparation of heavy sarcoplasmic reticulum (SR) vesicles from the dog ventricle are shown before (control) and during exposure to FPL. B: histograms summarize the results of 6 experiments in which $P_o$ was measured both in control and during exposure to FPL (150 μM). ** P < 0.01. $V_h$ = +40 mV.

**Fig. 4. Effects of FPL on single dog crude channel gating: open and closed time characteristics.** This figure shows the results of a single experiment in which open times (A and B) and closed times (C and D) were measured in control (left) and during exposure to FPL (right). The data for channel openings were described as the sum of two exponentials (solid line) representing time constants for both short ($\tau_{C1}$) and long ($\tau_{C2}$) closings. Channel closings are best described as the sum of two exponentials, with the shorter closed time durations accounting for 25% of the total. During exposure to FPL, both short ($\tau_{O1}$) and long ($\tau_{O2}$) open time durations are increased but there is an especially large increase in $\tau_{O2}$. In addition, there is a shift in the proportion of the two open states such that FPL causes a decrease in the percentage of $\tau_{O1}$ (to 20% of total) with a concomitant increase in the percentage of $\tau_{O2}$ compared with control recordings.

FPL had the opposite effect on closed times. Under control conditions, closed times were also described as the sum of two exponentials, with the shorter closed durations accounting for 41% of total openings. During exposure to FPL, both short ($\tau_{O1}$) and long ($\tau_{O2}$) open time durations are increased but there is an especially large increase in $\tau_{O2}$. In addition, there is a shift in the proportion of the two open states such that FPL causes a decrease in the percentage of $\tau_{O1}$ (to 20% of total) with a concomitant increase in the percentage of $\tau_{O2}$ compared with control recordings.

The effects of FPL on gating characteristics are summarized in Fig. 5. FPL increased $\tau_{O1}$ and $\tau_{O2}$ durations (Fig. 5A, top) as well as the proportion of $\tau_{O2}$ (Fig. 5A, bottom). Both long and short closed time durations were significantly decreased (Fig. 5B, top) and the contribution of long closings to total closed time was diminished (Fig. 5B, bottom).

These results provide an explanation for the observed effects of FPL on RyR2: 1) FPL increases the
the inactivation again becomes manifest, as though high concentrations of Ca$^{2+}$ were able to successfully compete with the effects of FPL, thus restoring Ca$^{2+}$-dependent inactivation.

These results were confirmed with single channel measurements of human purified RyR2. Figure 6B summarizes data obtained under control conditions (shown as solid circles) and in the presence of 150–200 μM FPL (shown as open circles) in which [Ca$^{2+}$] was increased in the cis compartment over the range of 0.1 μM to 10 mM. Just as with [3H]ryanodine binding in SR vesicles, there was no effect of FPL at 0.1 μM but single channel activity (as measured by $P_o$) increased significantly across the rest of the concentration range tested in these experiments (up to 10 mM) but also showed the decline at $>$1 mM.

These results suggest that the mechanism by which FPL activates cardiac RyRs involves both an increase in channel activity at activating concentrations of Ca$^{2+}$ without a shift in threshold and by a decreased sensitivity of inactivation to high concentrations of Ca$^{2+}$.

**Lack of effect of FPL on skeletal channels.** To determine whether or not the activation of cardiac RyRs by FPL is unique to that channel isoform, we also investigated the effects of FPL on single channel activity in crude dog skeletal RyR channels (RyR1). In a total of 4 channels, there was little change in $P_o$ ($0.039 \pm 0.005$ in control vs. $0.030 \pm 0.006$ in 150 μM FPL, NS). Similarly, we found that mean channel open time was $1.07 \pm 0.08$ ms in control and $1.11 \pm 0.14$ ms during exposure to FPL (NS).

These results demonstrate that RyR2 is sensitive to activation by FPL, whereas RyR1 is not. In addition, they demonstrate that channel activation by FPL is not a nonspecific interaction with the channel protein, because it only occurs with RyR2 and not with RyR1 under identical recording conditions.
DISCUSSION

Mechanisms of RyR2 channel activation by FPL. Our previous study showed that DHP activation of the RyR2 channel occurred primarily in the range of \([Ca^{2+}]\) responsible for RyR inactivation (\(>100 \mu M\)). We proposed that the agonist action was primarily the result of an effect to suppress \(Ca^{2+}\)-induced inactivation and that this was responsible for channel activation. In the present study, we found that FPL stimulated both \([H]\)ryanodine binding and single channel activity at \([Ca^{2+}] \geq 1 \mu M\). One possible explanation is that the primary effect of FPL is to reduce the sensitivity of inactivation to \(Ca^{2+}\). Because there is likely to be extensive overlap in \(Ca^{2+}\)-dependent activation and inactivation, a greater effect on inactivation might be obvious with little apparent effect on activation. This might explain why the threshold for activation is not shifted to lower \([Ca^{2+}]\) with the net result being that of enhanced channel activity in the activating portion of the curve. It is also possible but less likely that FPL acts on both \(Ca^{2+}\)-induced activation and inactivation to increase single channel activity. These effects would explain the apparent \(Ca^{2+}\)-sensitizing effect on activation in the \([Ca^{2+}]\) range of 1–100 \(\mu M\) and the suppression of inactivation occurring in the range of 1–10 mM but would not, however, explain why there is no increase in sensitivity at pCa 7. It is interesting that the activating effect results from an increase in the slope of the \([Ca^{2+}]\)-activity relationship rather than a leftward shift as might be observed with caffeine (17). This result suggests that there may be an increase in cooperativity between calcium ions instead of a true sensitization of channel activity to low \([Ca^{2+}]\). However, the best explanation for the effects of FPL to increase RyR2 channel activity is likely to reside in a suppression of inactivation, possibly by reducing its sensitivity to \(Ca^{2+}\).

One of the most interesting features of FPL effects on RyR2 inactivation is that it seems to be the result of a competitive interaction with high \([Ca^{2+}]\) because it can be largely overcome at the highest \([Ca^{2+}]\) tested (i.e., 10 mM). Both ryanodine binding and \(P_o\) decline over the range of pCa \(> 4\), suggesting that inactivation remains intact and that the effects of FPL would likely be overcome entirely at high enough \([Ca^{2+}]\). One interesting implication of these results is that the prolongation of open times (and concomitant reduction in closed times) might be primarily the result of this suppression of inactivation. Therefore, normal channel gating may be heavily influenced by \(Ca^{2+}\)-induced inactivation, causing channel closure. When inactivation is suppressed by occupation of the agonist receptor by FPL (or for that matter DHP agonists), channel gating behavior is altered, giving much longer openings and briefer closings. It is even possible that closings under these conditions reflect native channel closing behavior uninfluenced by the ordinarily overlapping effect of inactivation. These ideas were not tested directly in these experiments but represent a compelling implication of the data and an area for future study.

Is the effect of FPL the result of specific interaction with RyR2? The fact that the FPL concentrations required to activate RyR2 are \(~500\)-fold higher than for \(I_{Ca,L}\) (1, 14) raises the possibility that the effects of FPL on RyR2 are nonspecific. Several observations argue against this point. First, channel activation occurs only when FPL is added to the \(cis\) side of the bilayer, suggesting not only a specific interaction with the protein but also binding to a site on the cytoplasmic face of the channel. Second, the inability of FPL to activate RyR1 implies that the interaction with the cardiac isoform is a specific one. Third, the fact that stimulation of both single channel activity and \([H]\)ryanodine binding by FPL is dependent on cytoplasmic \([Ca^{2+}]\) supports the notion that the drug effect is a result of a specific interaction between drug and channel.

It is also not clear why such high drug concentrations are required, given the higher sensitivity of the \(I_{Ca,L}\) to FPL. It is important to consider, however, that drug concentration in the bulk solution (plasma or in vitro superfusate) may be very different from intracellular accumulation. For example, the ratio of total tissue concentration to that in the medium for the lipid soluble calcium channel antagonist bepridil varied between 20- and \(~50\)-fold, depending on the cardiac preparation (12). Thus cardiac tissues are capable of concentrating lipophilic agents and it is possible that high concentrations of FPL might exist in bilayers because this agent is very lipophilic. The low water solubility might lead to an accumulation of FPL in SR membranes, thus providing higher local concentrations of drug in the vicinity of RyR2 than is present in bulk aqueous solution.

It is also extremely difficult to relate physiological effects to underlying pharmacological interactions between ligand and receptor. The literature is full of disparities between concentrations of drug required to produce a physiological response and those measured to characterize the molecular interaction between a drug and its receptor. For example, it is not clear why cardiac glycosides only affect cardiac cell function in intact tissues and ventricular myocytes at concentrations in the micromolar range (8, 9, and S. Ruch et al., unpublished observations), but the cardiac glycosides are known to inhibit the sarcoplasmic \(Na^+/K^+\)-ATPase with dissociation constant values in the range of 2–7 nM (2). The disparity between results from biochemical binding assay and from physiological measurements has still not been explained satisfactorily, even after decades of study. However, it is still assumed that \(Na^+\) pump inhibition is the basis for all of the inotropic and toxic actions of cardiac glycosides. These results serve to indicate that measured sensitivity of pharmacological or biochemical interactions between drug and receptor cannot always be translated to sensitivity of physiological response to that drug. Further study is required to resolve this issue.

Effects of different classes of \(Ca^{2+}\) channel agonists. In our previous study of purified RyR2 activation by DHP agonists (18), we found very similar effects to those reported here for an entirely different class of
agonist represented by FPL. We observed that BAYK activated the channel with an EC₅₀ of ~3 μM, a much higher efficacy than FPL. However, nearly 100 μM of another agonist, (+)-SZ-202791, was necessary to produce activation, a concentration quite similar to FPL. The actions of these DHP agonists were not influenced by either previous or subsequent addition of equal or higher concentrations of antagonist [i.e., nifedipine and (+)-SZ-202791]. These results suggested that if a binding site does exist on the cytoplasmic face of the RyR2, then it is specific for DHP agonists because antagonists could neither displace agonists nor prevent their access to the receptor with prior exposure. In addition, the Ca²⁺ dependence of single channel Pₒ revealed that both agents suppress inactivation to a greater extent than enhancing activation. These observations in turn suggested the presence of a specific agonist receptor on the RyR2. One of the primary goals of the current study was to determine whether or not channel activation extended to another known class of Iₘ₉₉₉₉₉₉₉ agonists represented by FPL. Our current results demonstrate striking similarities in the actions of BAYK and FPL.

If the two classes of agonist share a binding site on RyR2, is there any basis for such overlap in the L-type channel? This in turn raises the issue of whether or not there are separate binding sites responsible for the agonist and antagonist actions of DHPs. The fact that mutations in domains III and IV of the α-subunit produced similar shifts in binding for (+)-PN 200–110 and (+)-BAYK is taken as a strong indication that both agonist and antagonist effects rely on drug binding to a common receptor (7, 13). In contrast, other studies (5, 7, 19, 20, 22) provide strong evidence suggesting distinct binding sites for agonists and antagonists. Thus it is not clear whether or not DHP agonists share a common receptor with antagonists on Iₘ₉₉₉₉₉₉₉.

Given the possibility that there may in fact be a unique receptor for activation of Iₘ₉₉₉₉₉₉₉, it is then possible that other agonists besides DHPs might occupy and activate it. FPL is structurally unrelated to DHPs and is thought to bind to a separate site from DHPs on the Iₘ₉₉₉₉₉₉₉ in a noncompetitive manner (1, 4, 15, 16, 23). However, several reports (11, 15, 22) suggest that there is an allosteric interaction between the binding sites for FPL and DHPs even if they do not share a common receptor on the channel protein itself. Thus there is already evidence for interactions between binding sites in Iₘ₉₉₉₉₉₉₉ for the two classes of agonist.

In RyR2, it appears that FPL has more pronounced effects on single channel activity than BAYK but does so only at higher drug concentrations. The fact that both agents activate the channel without any effect by DHP antagonists (either directly on the channel or by competitive interaction with DHP agonists) suggests that the RyR2 binds only agonists and responds to binding with channel activation. It is not yet clear whether agonist-binding sites on the two Ca²⁺ channels share structural similarities which could explain the efficacy of both agonist types to modify channel function. We cannot be certain as to the exact primary sequence on the RyR2 protein that might serve as a docking region for these agonists. Comparisons between primary structures of the Iₘ₉₉₉₉₉₉₉, and the RyR2 show some similarities, but because of the limited ability to extrapolate primary structures to three-dimensional conformations, we must await additional work to determine if there really is a shared binding site for Ca²⁺ channel agonists on the two proteins. However, physiological evidence suggests that there may be a binding site specific for both classes of Ca²⁺ channel agonists on the cardiac channel. This putative “Ca²⁺ channel agonist receptor” is not present on RyR1 and may be partially occluded by ancillary proteins such as triadin and/or junctin because the purified cardiac channel (but not the crude channel) was activated by DHP agonists. Additional experiments are required to determine why FPL gains access to this receptor unimpeded in the crude channel and why DHP agonists require stripping of these secondary proteins before gaining access to the receptor.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-30724 (to J. A. Wasserstrom), an American Heart Association Scientist Development Award (to A. J. Lokuta), and a medical student summer research fellowship from Northwestern University Medical School Division of Cardiology (to S. T. Reddy).

REFERENCES

10. Katoh H, Scholthauer K, and Bers DM. Transmission of information from cardiac dihydropyridine receptor to ryano-
tors with single L-type Ca\(^{2+}\) channels. Naunyn Schmiedebergs Arch Pharm 360: 122–128, 1999.


