Activation of purified cardiac ryanodine receptors by dihydropyridine agonists

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Sagawa, Toshio, Manabu Nishio, Kazuko Sagawa, James E. Kelly, Andrew J. Lokuta, John Tsai, Edward Kan, and J. Andrew Wasserstrom. Activation of purified cardiac ryanodine receptors by dihydropyridine agonists. Am J Physiol Heart Circ Physiol 280: H1201–H1207, 2001.—Prior observations have raised the possibility that dihydropyridine (DHP) agonists directly affect the sarcoplasmic reticulum (SR) cardiac Ca\(^{2+}\) release channel [i.e., ryanodine receptor (RyR)]. In single-channel recordings of purified canine cardiac RyR, both DHP agonists (-)-BAY K 8644 and (+)-SDZ202-791 increased the open probability of the RyR when added to the cytoplasmic face of the channel. Importantly, the DHP antagonists nifedipine and (-)-SDZ202-791 had no competitive blocking effects either alone or after channel activation with agonist. Thus there is a stereospecific effect of SDZ202-791, such that the agonist activates the channel, whereas the antagonist has little effect on channel activity. Further experiments showed that DHP agonists changed RyR activation by suppressing Ca\(^{2+}\)-induced inactivation of the channel. We concluded that DHP agonists can also influence RyR single-channel activity directly at a unique allosteric site located on the cytoplasmic face of the channel. Similar results were obtained in human purified cardiac RyR. An implication of these data is that RyR activation by DHP agonists is likely to cause a loss of Ca\(^{2+}\) from the SR and to contribute to the negative inotropic effects of these agents reported by other investigators. Our results support this notion that the negative inotropic effects of DHP agonists result in part from direct alteration in the activity of RyRs.

nifedipine; BAY K 8644; PN-202-791; calcium release channels; sarcoplasmic reticulum

CERTAIN DIHYDROPYRIDINE (DHP) derivatives, such as (±)-BAY K 8644 (BAYK) and (+)-SDZ202-791, are used widely as specific agonists for the L-type Ca\(^{2+}\) channel located in the surface membrane of many excitable cells, including the heart. Numerous studies (4, 16, 18, 22) demonstrate their ability to increase Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) magnitude, slow inactivation, and produce a positive inotropic action in cardiac tissues. Recent work (6, 12, 13, 17) also suggests that additional actions of BAYK might include an effect on the cardiac sarcoplasmic reticulum (SR) that could influence excitation-contraction coupling independent from its agonistic actions on sarcolemmal Ca\(^{2+}\) channels. These reports found that, aside from its direct positive inotropic effect, BAYK also suppresses SR Ca\(^{2+}\) release and contraction during postrest potentiation, producing a secondary negative inotropic effect. The conclusion from this work was that binding of BAYK to the DHP receptor on the L-type Ca\(^{2+}\) channel had an effect that was somehow translated, through a “functional linkage,” to the SR, resulting in a suppression of Ca\(^{2+}\) release (13). Because this action could arise as a consequence of the maintained leak of Ca\(^{2+}\) from the SR, a subsequent study examined the possibility that BAYK might have a direct action to activate the SR Ca\(^{2+}\) release channel or ryanodine receptor (RyR). However, despite an action of BAYK to increase SR Ca\(^{2+}\) leak, as indicated by increased Ca\(^{2+}\) spark frequency, no direct effect on RyR single-channel activity was observed using a crude vesicular SR preparation (17).

The purpose of the present study was to further investigate the possibility of a direct agonist effect on the cardiac RyR. We wanted to test for possible actions of DHP agonists on the RyR in the absence of other regulatory proteins because it is known that the type of RyR preparation used (native vesicles vs. purified channels) may influence the RyR response to a ligand (19). A crude microsome preparation is likely to contain factors in the form of both proteins and other signaling molecules that regulate the RyR. It is possible that, in the process of SR vesicle isolation, these factors may render the vesicular preparation insensitive to activators that might normally be effective under physiological conditions. In addition, it is not yet clear whether these additional regulatory factors operate in SR vesicles, as they would in intact cells, to influence the function and pharmacological sensitivity of RyRs. Thus our sin-
Single-channel studies were conducted using the purified RyRs, where it is easier to look for direct ligand/receptor interactions.

**METHODS**

**Single RyR channel studies.** Dog cardiac (n = 5) preparations were obtained from animals anesthetized with pentobarbital sodium (35 mg/kg iv) before removal of the heart. Human hearts were obtained from three normal patients who died as a result of illness unrelated to cardiac disease and whose hearts were donated for research purposes because they were unsuitable for transplant. All animal and human tissue use was subject to review and approval by the Internal Animal Care and Use Committee and the Internal Review Board, respectively.

Crude microsomes were obtained from the left ventricle using differential centrifugation. Purification of the canine cardiac SR Ca\(^{2+}\) release channel was performed using 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate solubilization of heavy SR vesicles with subsequent reconstitution of purified protein into proteoliposomes (1, 9, 10, 20).

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Single-channel recordings were made using the planar lipid bilayer technique with 250 mmol/l KCl and 10 mmol/l HEPES (pH 7.4) on the cis (cytoplasmic) side and trans (luminal) sides of the bilayer. The trans side also contained 1 mmol/l Ca\(^{2+}\), while cis [Ca\(^{2+}\)] was measured using a Ca\(^{2+}\)-sensitive electrode as being 5 μM. Bilayer composition was 0.4 mg each of phosphatidylserine and phosphatidylethanolamine (Avanti Polar Lipids) suspended in 20 μl of n-decane. 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 (20).

Single-channel data were recorded (Axopatch 200 and 200A amplifiers, Axon Instruments) using pCLAMP version 6.0 software in data files of 30 s in duration at constant holding potentials (V\(_h\)) of ±30, ±40, and ±50 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.

In some experiments, [Ca\(^{2+}\)] was altered by addition of mixtures of EGTA and CaCl\(_2\) according to Calcium software. Concentrations of Ca\(^{2+}\) were varied over the range of 0.1 μM to 1 mM in this manner. Channel activity was established with free cis [Ca] = 50 μmol/l, and EGTA was then added to reduce [Ca] to 0.1 μmol/l. Data were recorded at −40 mV before [Ca] was increased in a cumulative fashion to 1–1,000 μM to obtain [Ca\(^{2+}\)]-open probability (P\(_o\)) curves. Separate experiments were performed in the absence and presence of 10–30 μM BAYK.

All chemicals were obtained from Sigma with the exception of BAYK (Calbiochem) and (+)- and (−)-SDZ202-791, which was a gift from Sandoz Pharmaceutical.

**Data analysis.** Data are presented as means ± SE. 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.

**RESULTS**

Effects of BAYK on single-channel activity of purified RyR. Figure 1A shows recordings from a single purified dog cardiac Ca\(^{2+}\) release channel before and during exposure to BAYK (10 μmol/l added to the cis side). BAYK increased single-channel P\(_o\) from 0.38 to 0.80 at a V\(_h\) of −40 mV and from 0.19 to 0.78 at a V\(_h\) of +40 mV. In eight experiments, P\(_o\) increased from 30.9 ± 6.8 to 76.9 ± 4.7 (P < 0.03) in the presence of 10 μM BAYK on the cis side at a V\(_h\) of −40 mV. The recording in Fig. 1A, bottom left, shows typical sensitivity of the channel to ryanodine (10 μmol/l), as demonstrated by the appearance of a stable subconductance state (~60% of the fully open conductance) after addition to the cis side.

Lifetime analysis of open and closed events indicated that channel P\(_o\) was increased by BAYK primarily as a result of increased long openings and decreased closings of all durations (Fig. 1B). Both open and closed events were best described by the sum of two exponentials. In this experiment (V\(_h\) = −40 mV), there was a modest increase in short open lifetimes and a large increase in long opening durations with a shift to a lower percentage of short openings. Both durations of closed times were decreased by BAYK, which also induced an increase in the proportion of shorter closings.

Summarized data include the open lifetime events (in ms; n = 9) in control as follows: τ\(_1\) = 0.90 ± 0.15 and τ\(_2\) = 2.96 ± 0.41 (where τ\(_1\), is fast time constant and τ\(_2\) is slow time constant), with the percentage of all openings represented by τ\(_1\) = 65 ± 7%. In the presence of 10 μmol/l BAYK, τ\(_1\) = 1.88 ± 0.54 (no significant difference compared with control) and τ\(_2\) = 7.89 ± 1.69 (n = 9, P < 0.05 compared with control); τ\(_1\) = 49 ± 7% (P < 0.05). The increase in P\(_o\) was not simply the result of changes in the characteristics of channel openings; under control conditions, closed lifetime events were summarized as follows (n = 10): τ\(_1\) = 1.21 ± 0.17 and τ\(_2\) = 6.15 ± 0.89; τ\(_1\) = 50 ± 6%. In the presence of BAYK, τ\(_1\) = 0.69 ± 0.08 (P < 0.01) and τ\(_2\) = 2.65 ± 0.37 (P < 0.001); τ\(_1\) = 65 ± 4% (P < 0.02). Thus both open and closed events were significantly altered by BAYK, with a resulting increase in overall P\(_o\).

Investigation of the relationship between concentration and P\(_o\) (Fig. 1C) demonstrated that the BAYK concentration at which 50% of the maximal effect was achieved (EC\(_{50}\)) was ~3 μmol/l. These effects of BAYK occurred in the absence of any change in current magnitude (Fig. 1D) and in the absence of any obvious voltage dependence (Fig. 1E).

Enantiomeric-specific effects of SDZ202-791 on single-channel activity. From the preceding experiments, it was clear that BAYK activated the cardiac Ca\(^{2+}\) release channel as a result of increasing longer openings and promoting shorter closings. We also studied the effects of another DHP agonist, SDZ202-791, whose (+) and (−)-enantiomers allowed the separation of agonist and antagonist actions, respectively (5, 7, 22). As with BAYK, addition of the agonist (+)-SDZ202-791 (100 μmol/l) increased P\(_o\) from 0.29 to 0.90 (Fig. 2A). Subsequent addition of the antagonist (−)-SDZ202-791 (100 μmol/l) did not affect the agonist action of the (+)-enantiomer and, in fact, produced a slight increase in P\(_o\). In nine experiments, P\(_o\) was 0.13 ± 0.48 in control and 0.58 ± 0.11 in the presence of 100 μmol/l (+)-SDZ202-791 (P < 0.01). Subsequent addition of the
antagonist \((-\)SDZ202-791 caused a small additional increase in $P_o$, probably reflecting a partial agonist effect, increasing $P_o$ from $0.41 \pm 0.16$ to $0.58 \pm 0.13$ ($P < 0.03, n = 5$) after addition of \((-\)SDZ202-791 in the maintained presence of \(+)\)-SDZ202-791. If there were a competitive interaction between the two enantiomers at a common receptor site, we would have expected a reduction in $P_o$ under these conditions, which was not observed. Identical results were obtained with BAYK and subsequent exposure to nifedipine (up to $100 \mu\text{mol/l}$, $n = 4$; data not shown).

It is possible that the antagonist effect of \((-\)SDZ202-791 precluded by prior occupancy of an allosteric site during exposure to the \((+)\)-enantiomer. When the order of drug application was reversed (Fig. 2B), \((-\)SDZ202-791 had no antagonist action on the purified cardiac $\text{Ca}^{2+}$ release channel and again induced a modest activation of the channel. Subsequent addition of \((+)\)-SDZ202-791 then produced the typical agonist effect even after prior exposure to the \((-)\)-enantiomer. This result was confirmed in two additional experiments. High concentrations of both \((-\)SDZ202-791 and nifedipine ($100 \mu\text{mol/l}$) were responsible for a partial agonist effect, causing an average increase in $P_o$ of $0.21 \pm 0.04$ when added alone ($n = 10$ or 5 of each type, $P < 0.001$). These results demonstrate that the response of the cardiac $\text{Ca}^{2+}$ release channel is selective for specific stereoisomers of the DHP agonists and that there is little response to traditional antagonists. These observations suggest that there is a unique $\text{Ca}^{2+}$ channel agonist effect on the purified cardiac $\text{Ca}^{2+}$ release channel.
Additional experiments were performed to identify the location of the modulatory site on the channel protein. After addition of (-)SDZ202-791 (100 μmol/l) to the trans (luminal) side, $P_o$ was unchanged (Fig. 2C). However, subsequent addition to the cis side caused the typical increase in $P_o$ from 0.02 to 0.47. Three additional experiments yielded the same results; four experiments with BAYK also yielded an increase in $P_o$ only after introduction to the cis side. There was little effect of agonist when applied to the luminal side, and the large increase in $P_o$ occurred only when agonist was applied to the cytoplasmic side of the channel. These results indicate that the putative binding site resides on the cytoplasmic face of the channel.

**DHP agonists and sensitivity of purified RyR to Ca$^{2+}$ concentration.** In an attempt to identify the mechanism by which DHP agonists activate the Ca$^{2+}$ release channel, we studied the relationship between $P_o$ and Ca$^{2+}$ concentration in the absence and presence of DHP agonists (Fig. 3). Under control conditions, the purified Ca$^{2+}$ release channel showed the typical response to changes in cis [Ca$^{2+}$]; activation of the channel occurred when [Ca$^{2+}$] was increased above 1 μmol/l; maximal activation occurred at a [Ca$^{2+}$] of ~100 μmol/l. This portion of the relationship reflects the activation of the channel by Ca$^{2+}$, presumably as a result of Ca$^{2+}$ binding to an activation site on the cytoplasmic side of the channel, whereas the decrease in $P_o$ when [Ca$^{2+}$] is increased above 300 μmol/l reflects the binding of Ca$^{2+}$ to an inactivation site on the channel (3, 11, 14). When this experiment was performed in the presence of DHP agonists, Ca$^{2+}$-dependent activation of $P_o$ was not affected, but there was a dramatic increase in maximal $P_o$ with little indication of Ca$^{2+}$-induced inactivation at high [Ca$^{2+}$]. When the $P_o$-[Ca$^{2+}$] relationship is adjusted to maximal $P_o$ (Fig. 3, bottom), there was almost no change in affinity for Ca$^{2+}$ (EC$_{50} = 6.8$ and 9.6 μmol/l in the absence and presence of BAYK, respectively). There was also a modest increase in the Hill coefficient (from 1.45 to 1.98) in the presence of agonist, suggesting an increase in cooperativity for Ca$^{2+}$-induced activation. However, the most striking implication of these results is the suggestion that Ca$^{2+}$ channel agonists bring about Ca$^{2+}$ release channel activation by suppressing Ca$^{2+}$-induced inactivation of the channel.

**Effects of DHP agonists on purified human RyR.** We also investigated the possibility that the agonist action of DHPs might occur in the human ventricular Ca$^{2+}$ release channel to determine whether the effect is specific to the dog cardiac RyR and whether this action might have wider implications by its presence in the human heart. Figure 4 shows the effects of BAYK (10 μmol/l) on a single purified Ca$^{2+}$ release channel from a normal human heart. $P_o$ increased from 0.11 to 0.47. Subsequent addition of nifedipine (100 μmol/l) had no antagonist action on $P_o$ (data not shown), and, in fact, there was a slight increase in activity during exposure to the antagonist. Summarized results ($n = 3$) indicated that $P_o$ was 0.23 ± 0.06 in control and 0.74 ± 0.13 in the presence of BAYK. These results demon-
strate that the agonist effect occurs in the human ventricular Ca\textsuperscript{2+} release channel as well as in the dog.

**DISCUSSION**

**DHP agonists and antagonists of L-type Ca\textsuperscript{2+} channels.** It has been known for many years that DHPs act as antagonists to L-type Ca\textsuperscript{2+} channels. It was subsequently found that some of these agents have optically active stereoisomers that can act as agonists to I\textsubscript{Ca} (7, 16, 22). Thus, in contrast to closely related DHP antagonists, agonists like BAYK increased I\textsubscript{Ca} magnitude and slowed inactivation, causing increased Ca\textsuperscript{2+} influx with resultant positive inotropy. Single Ca\textsuperscript{2+} channel recordings in cardiac cells suggested that the changes in whole cell current were the result of BAYK binding to and stabilizing the highly active state (mode 0) of the Ca\textsuperscript{2+} channel, where \( P_{o} \) is very low.

Sanguinetti et al. (16) subsequently found that there were complex voltage and concentration dependencies to the effects of BAYK; most notably, I\textsubscript{Ca} in cardiac Purkinje fibers was increased by BAYK at negative potentials, whereas an antagonistic action was found at depolarized test potentials. They and others (2) demonstrated that this behavior was the result of preferential binding of the two enantiomers of BAYK under the different experimental conditions; the l- or (-)-enantiomer was a pure agonist, whereas the r- or (+)-enantiomer was an antagonist. Thus a racemic mixture may give conflicting results depending on the concentration and voltage dependencies of the two forms of the drug. Exactly the opposite behavior was observed with another compound, SDZ202-791, whose (+)-enantiomer was an agonist, whereas the (-)-enantiomer was an antagonist (5, 7, 22).

We found that the effects of the pure agonists on purified SR Ca\textsuperscript{2+} release channels were similar to those on I\textsubscript{Ca}. These agents behaved as if they were stabilizing the channel in mode 2 or the long opening state, just as with I\textsubscript{Ca}. Thus channel openings were prolonged by reduction in closed times with a simultaneous increase in open times. In striking contrast, the

![Fig. 3. Top: [Ca\textsuperscript{2+}] dependence of \( P_{o} \) for the purified SR Ca\textsuperscript{2+} release channel in the absence (●) and presence (○) of DHP agonists [●, results of BAYK = 10 \( \mu \)M; ○ and ○, 2 experiments with (+)-202-791 = 30 \( \mu \)mol/l]. Each point indicates results from 5–12 experiments. Bottom: \( P_{o} \) adjusted to maximal \( P_{o} \) in the absence and presence of BAYK for the Ca\textsuperscript{2+}-induced activation phase only.](image1.png)

![Fig. 4. Effects of BAYK (10 \( \mu \)mol/l) on single-channel activity of human purified cardiac RyR. Single-channel activity under control conditions 1 min after addition of BAYK to the cytoplasmic face of the channel is shown.](image2.png)
transmitted via a functional linkage to the SR, causing through intracellular Ca\(^{2+}\) was found to increase the frequency of local changes in high-affinity Ca\(^{2+}\) site inducing channel closure (3, 14, 17). We found that BAYK enhanced ryanodine binding, suggesting an in-

potentiation of contraction after rest to a decay in both Ca\(^{2+}\) concentration. DHP antagonists did not directly reduce ac-

tivity nor did they antagonize the increase in channel activity resulting from prior exposure to agonists. These results suggest that the antagonists do not oc-

cupy a binding site on the RyR and thus do not affect channel activity directly or displace previously bound agonist. Even more important is the fact that there is a clear stereospecificity to the agonist action, which supports the notion that channel activation is a result of a specific interaction with the RyR and not of nonspecific drug effects.

One of the most intriguing results reported here is that the effect of DHP agonists may involve a suppres-

sion of Ca\(^{2+}\)-induced inactivation. This property of the RyR has been well documented and is thought to reflect binding of Ca\(^{2+}\) at high concentrations to a low-affinity site inducing channel closure (3, 14, 17). We found that high-affinity Ca\(^{2+}\)-induced activation of channel activ-

ity was nearly unaffected by agonists, whereas inactiv-

ation was largely suppressed. This unusual observa-

tion is among the first to suggest the possibility that pharmacological activation of the cardiac RyR by cer-
	ain agents might occur through selective inhibition of Ca\(^{2+}\)-induced inactivation.

Calcium channel agonists and cardiac excitation-

contraction coupling. The possibility that the effect of BAYK on excitation-contraction coupling might involve an action on the SR as well as on the L-type Ca\(^{2+}\) channel came from work by Bers and co-workers (6, 12, 13, 17). BAYK produced an increase of Ca\(^{2+}\) influx through \(I_{\text{Ca}}\), but also accelerated the decline of the SR Ca\(^{2+}\) content during rest, thus converting the typical potentiation of contraction after rest to a decay in both the dog and ferret ventricle.

One of the previous studies (13) also found that BAYK enhanced ryanodine binding, suggesting an in-

crease in RyR channel \(P_{o}\). These investigators con-

cluded that the effect of BAYK occurs as a result of a modification of the L-type Ca\(^{2+}\) channel, which is then transmitted via a functional linkage to the SR, causing alterations in SR Ca\(^{2+}\) release. More recently, BAYK was found to increase the frequency of local changes in intracellular \([Ca^{2+}]_{\text{intra}}\) (Ca\(^{2+}\) sparks) without altering their spatial or temporal characteristics (17). This action was strikingly similar to the effects on Ca\(^{2+}\) sparks of low concentrations of ryanodine, which locks the RyR channel in a permanently open state. Virtu-

ally all of these results support the idea that BAYK might induce a reduction in SR Ca\(^{2+}\) content, possibly as the result of a ryanodine-like action to promote long-lasting activation of the RyR channels. When this possibility was directly studied in crude SR vesicular Ca\(^{2+}\) release channels isolated from the ferret heart (17), BAYK was found to have no effect on single-

channel activity. The authors concluded that BAYK activates SR Ca\(^{2+}\) release at rest but that the effect of BAYK is indirect via an action on the DHP receptor on L-type Ca\(^{2+}\) channels that is transmitted by an unknown mechanism to the SR Ca\(^{2+}\) release channel.

In contrast with the results obtained by Satoh et al. (17), our observations suggest that this effect on SR Ca\(^{2+}\) release may be the result of a direct action on the Ca\(^{2+}\) release channel itself and may occur independently of any interactions with sarcolemmal proteins, such as the L-type Ca\(^{2+}\) channel. It is well known that DHPs are very lipophilic, often with a partition coeffi-

cient (oil:water) of -3 (8, 21), so it is highly likely that these agents can easily cross the sarcolemma. The intracellular accumulation of DHPs has in fact been directly demonstrated in ventricular tissue (15). Once in the cytoplasm, they can then gain access to the recog-
nition site on the SR Ca\(^{2+}\) release channel with the resulting functional response of the channel de-

pending on the regulatory factors and mechanisms involved. A direct action of DHP agonists on purified RyRs is consistent with the observations of Satoh et al. (17), who found significant alterations in Ca\(^{2+}\) spark frequency, as would be expected for an agent that increases single RyR channel activity. In addition, a direct effect offers a reasonably straightforward expla-
nation for their findings as well as for the other re-

ported suppressant effects of BAYK on excitation-con-

traction coupling (6, 12, 13, 17).

It is possible that our single-channel data differ from those of Satoh et al. (17) because of differences in experimental conditions. For example, the different response to BAYK of our purified RyRs could arise as a consequence of something as simple as a difference in charge carrier (K\(^+\) in the current study compared with Cs\(^+\) in Ref. 17). However, there have been few reported differences in pharmacological or physiological sensi-
tivities using different monovalent cationic species (3, 14). A more likely explanation may lie in the possibility that the purification process could alter the pharmacological sensitivity of the RyR. The difference in response to DHP agonists suggests that there are factors associated with the crude channel, possibly but not necessarily proteinaceous in nature, that either pre-

clude access of an agonist to its receptor or regulate the response to the activated receptor in some fashion. We do not yet know which form of the channel more accu-

rately reflects channel behavior under physiological conditions, so it is difficult at this point to determine the role of these regulatory factors in influencing the pharmacological responses of the channel in vivo.

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