A Novel Benzodiazepine that Activates Cardiac Slow Delayed Rectifier K$^+$ Currents

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ABSTRACT

The slowly activating delayed rectifier K$^+$ current, I\textsubscript{Ks}, is an important modulator of cardiac action potential repolarization. Here, we describe a novel benzodiazepine, (3\text{-}R)-1,3-dihydro-5-(2-fluorophenyl)-3-(1H-indol-3-ylmethyl)-1-methyl-2H-1,4-benzodiazepin-2-one (R-L3), that activates I\textsubscript{Ks} and shortens action potentials in guinea pig cardiac myocytes. These effects were additive to isoproterenol, indicating that channel activation by R-L3 was independent of \beta-adrenergic receptor stimulation. The increase of I\textsubscript{Ks} by R-L3 was stereospecific; the S-enantiomer, S-L3, blocked I\textsubscript{Ks} at all concentrations examined. The increase in I\textsubscript{Ks} by R-L3 was greatest at voltages near the threshold for normal channel activation, caused by a shift in the voltage dependence of I\textsubscript{Ks} activation. R-L3 slowed the rate of I\textsubscript{Ks} deactivation and shifted the half-point of the isochronal (7.5 sec) activation curve for I\textsubscript{Ks} by $\sim$16 mV at 0.1 \mu M and $\sim$24 mV at 1 \mu M. R-L3 had similar effects on cloned KvLQT1 channels expressed in Xenopus laevis oocytes but did not affect channels formed by coassembly of KvLQT1 and hminK subunits. These findings indicate that the association of mink with KvLQT1 interferes with the binding of R-L3 or prevents its action once bound to KvLQT1 subunits.

Repolarization from the plateau phase of the AP in ventricular myocytes is controlled by a delicate balance between inward and outward currents in the setting of a high membrane resistance. Important outward currents that determine repolarization are I\textsubscript{K1} and I\textsubscript{Ks} (Sanguinetti and Jurkiewicz, 1990). Several class III antiarrhythmic agents block I\textsubscript{K1} and thereby prolong APD and the QT interval on the electrocardiogram. Excessive APD prolongation by these drugs causes LQT, which is associated with torsades de pointes, a ventricular tachyarrhythmia that can degenerate into ventricular fibrillation and cause sudden death.

LQT can also be inherited. The finding that mutations in HERG, the gene that encodes I\textsubscript{K1} channels, cause inherited LQT\textsuperscript{1} (Curran et al., 1995; Sanguinetti et al., 1995, 1996a) provided a mechanistic link between acquired LQT and one form of inherited LQT. The most common form of LQT is caused by mutations in KvLQT1, a novel K$^+$ channel gene (Wang et al., 1996). Expression of KvLQT1 in either Xenopus laevis oocytes or mammalian cell lines induced a K$^+$ current with biophysical properties unlike any known cardiac K$^+$ current. Coexpression of KvLQT1 with minK induced a current that was essentially identical to cardiac I\textsubscript{Ks}, indicating that KvLQT1 and minK proteins coassemble to form I\textsubscript{Ks} channels (Barhanin et al., 1996; Sanguinetti et al., 1996b). Thus, dysfunction of either I\textsubscript{K1} or I\textsubscript{Ks} can increase the risk of cardiac arrhythmia and sudden death.

An activator of I\textsubscript{K1} or I\textsubscript{Ks} channels might be useful for the treatment of LQT that results from excessive pharmacological block of these channels or from mutations in the genes that encode the channel proteins. We previously described the properties of L-735,821, a benzodiazepine that is a potent and selective stereospecific blocker of cardiac I\textsubscript{Ks} (Salata et al., 1996). In this study and one preliminary report (Salata et al., 1997), we describe another 1,4 benzodiazepine, R-L3 (L-364,373), that is a stereospecific activator of cardiac I\textsubscript{Ks}.

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ABBREVIATIONS: AP, action potential; APA$\textsubscript{50}$, action potential amplitude measured at 50 msec after the upstroke; APD$\textsubscript{90}$, action potential duration at 90\% repolarization; APD$\textsubscript{220}$, action potential duration at 50\% repolarization; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline; I\textsubscript{Ca}, L-type Ca$^{2+}$ current; I\textsubscript{K}, delayed rectifier K$^+$ current; I\textsubscript{K1}, rapidly activating component of delayed rectifier K$^+$ current; I\textsubscript{K1\textsubscript{m}}, slowly activating component of delayed rectifier K$^+$ current; I\textsubscript{K1\textsubscript{i}}, inward rectifier K$^+$ current; I\textsubscript{K\textsubscript{tail}}, tail current; I\textsubscript{K\textsubscript{tail\textsubscript{max}}}, maximum amplitude I$\textsubscript{K}$ tail current; I$\textsubscript{iso}$, isoproterenol; I-V, current-voltage; [K$^+$]$\textsubscript{o}$, extracellular K$^+$ concentration; LQT, long QT syndrome; R-L3, (3\text{-}R)-1,3-dihydro-5-(2-fluorophenyl)-3-(1H-indol-3-ylmethyl)-1-methyl-2H-1,4-benzodiazepin-2-one; V$\textsubscript{h}$, holding potential; V$\textsubscript{t}$, test potential.

Experimental Procedures

Isolation of guinea pig ventricular myocytes. Guinea pig ventricular myocytes were isolated as described previously (Salata et al., 1995). After isolation, the cells were stored in HBS containing 132 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES, and 10 mM glucose, pH 7.2, at 24–26°C until used in our experiments, T-type Ca²⁺ channels most likely contributed negligible current; therefore, the I_Ca measured in the current study was considered to be L-type Ca²⁺ current.

Culture and voltage clamp of AT-1 cells. The original mouse atrial tumor (AT-1) mouse colonies were established by Dr. Loren Field (Krannert Institute of Cardiology, Indianapolis, IN) (Field, 1988), and the lineage at Merck Research Laboratories was established from tumor cells that were provided by Dr. Dan Roden (Vanderbilt University, Nashville, TN). AT-1 cells were propagated in vivo, and their isolation and culturing were conducted as described previously (Delcarpio et al., 1991; Yang et al., 1994; Jurkiewicz et al., 1996).

For voltage-clamp studies, AT-1 cells were trypsinized to remove them from the culture dishes and stored in PC-1 culture medium (22–24°C). Outward K⁺ currents were recorded in normal HBS at 22–24°C using standard whole-cell voltage-clamp techniques within 14 hr of isolation. Pipettes were filled with a solution containing 110 mM KCl, 5 mM K-BAPTA, 1 mM MgCl₂, and 10 mM HEPES, pH 7.2, and had resistances of 3–7 MΩ (average, 5.5 ± 0.3 MΩ). All cells were round in appearance, had large outward tail currents and resting membrane potentials (RMP) negative to ~35 mV, and did not beat spontaneously. I_Ca and T-type calcium currents were inactivated by voltage-clamping the cells to a V_h of ~40 mV. I_Ca was blocked with 0.4 μM nisoldipine.

cRNA injection and voltage-clamp of oocytes. The isolation and maintenance of X. laevis oocytes, in vitro transcription of KvLQT1 and hminK cRNA, and its injection into oocytes were performed as described previously (Sanguinetti et al., 1995, 1996b). Stage V and VI oocytes were injected with 11.5 ng of KvLQT1 cRNA (46 nl of a 250 ng/μl solution) alone or co.injected with 11.5 ng of hminK cRNA plus 1.25 or 0.1 ng of hminK cRNA. Currents were recorded 2–4 days later using standard two-microelectrode voltage-clamp techniques and a Dagan TV-200 amplifier. Oocytes were bathed at room temperature (22–25°C) in a solution containing 94 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, and 5 mM HEPES, pH 7.6.

Materials. R-L3 (Fig. 1) was prepared as described previously by Evans et al. (1987). Its enantiomer, S-L3, was prepared by the same procedure described for R-L3, with L-tryptophan acid chloride hydrochloride used in place of the D-isomer: ¹H NMR (CDCl₃) identical to that of R-L3. The chemical and chiral purity of R-L3 and S-L3 were determined to be >99%. R-L3: high performance liquid chromatography (Vydac C-18, 15 × 0.46 cm, 16 min gradient 95:5 to 95:0.1% H₂PO₄/H₂O/CH₃CN, 1.5 ml/min, 215 and 254 nm) retention time (rt) = 11.0 min, >96%, coelutes with R-L3. R-L3 high performance liquid chromatography (Chiralcel absorbance 25 × 0.46 cm, 90/10 hexane/ EtOH, 1.5 ml/min, 280 nm) rt = 9.95 min, 98.2%, contains <1% of R-L3 (R-L3: rt = 10.94 min, 99.6%, contains <0.5% of R-L3), TLC (silica, 10% Et₂O in CH₂Cl₂): single component, Rf = 0.43, coelutes with R-L3. Calc. for C₂₂H₄₀F₂N₂O₂: C 75.55, H 5.07, N 10.57; found: C 75.57, H 5.17, N 10.46.

Fig. 1. Chemical structure of R-L3.
Compounds were dissolved in dimethylsulfoxide at a stock concentration of 1 or 10 mM and diluted directly into test solutions. Serial dilutions were used to achieve the final test concentrations. Dimethylsulfoxide at the concentrations used had no significant effect on any of the parameters measured in these studies. Nisoldipine (a gift from Miles Pharmaceuticals, New Haven, CT) was prepared as a 4 mM stock solution in dimethylsulfoxide and diluted as needed.

Statistics. Data are expressed as mean \pm standard error. Concentration-dependent changes in AP parameters and individual ionic currents were assessed by repeated-measures analysis of variance. Post hoc comparison of the treatment with the control mean values were made with Dunnett’s t test to determine significant changes between the control and test group mean values. Statistical comparisons for the time constants of IKs activation and deactivation were made using a paired t test. A one-tailed probability (p < 0.05) was considered significant.

Results

R-L3 decreases APD of cardiac myocytes. R-L3 (0.1–1.0 μM) caused a concentration-dependent shortening of APD. Fig. 2A shows a representative example of APs recorded at a stimulus frequency of 1 Hz. R-L3 significantly decreased APD_{90} and APD_{90} without significantly affecting other AP parameters (Table 1). Shortening of APD was maximal at 1 μM; APD_{90} was decreased at concentrations of 1 and 10 μM R-L3 by 14.2 \pm 1.6% and 13.8 \pm 4.0%, respectively.

Stimulation of β-adrenergic receptors can also shorten AP of cardiac myocytes (Carmeliet and Vereecke, 1969; Sanguinetti et al., 1991). Therefore, we determined whether the decrease in APD by R-L3 was mediated through the same or parallel pathway. At a concentration of 30 nM, Iso decreased APD_{90} and APD_{90} without significantly affecting other AP parameters (Table 1). Shortening of APD was maximal at 1 μM; APD_{90} was decreased at concentrations of 1 and 10 μM R-L3 by 14.2 \pm 1.6% and 13.8 \pm 4.0%, respectively.

Iso also increased APD_{90} by 12.9 \pm 2.9%. Iso also increased APD_{90} by 12.9 \pm 2.9% (Fig. 2B), presumably by enhancement of L-type Ca^{2+} current (Kass and Wiegens, 1982). The addition of 1 μM R-L3 in the presence of 30 nM Iso decreased APD_{90} further (26.9 \pm 1.4%) and diminished the increase in APD_{90}. Block of β-adrenergic receptors with 100 nM timolol did not alter configuration (Fig. 2C) but prevented the effects of 30 nM Iso (data not shown). In the continued presence of timolol, the addition of 1 μM R-L3 decreased APD_{90} by 14.6 \pm 2.2%, very similar to the effect observed in the absence of timolol. Thus, the decrease in APD by R-L3 is additive to the effect mediated by β-adrenergic stimulation.

R-L3 increases I_{Ks} of guinea pig myocytes in a concentration-dependent and stereospecific manner. R-L3 is structurally related to L-735,821, a benzodiazepine that selectively blocks IKs and prolongs APD of guinea pig ventricular myocytes (Salata et al., 1996). Therefore, we reasoned that R-L3 might shorten APD of guinea pig myocytes by activating IKs.

The effect of R-L3 on IKs was measured under voltage-clamp conditions using 3-sec depolarizations to a test potential (V_{t}) of −10 mV from a V_{h} of −50 mV (Fig. 3A). In contrast to the previously reported effect of L-735,821, R-L3 increased IKs. R-L3 enhanced IKs measured at −10 mV at concentrations as low as 30 nM and had a maximal effect at 1 μM. At this concentration, IKs was increased by a factor of 17 ± 5 (six cells). At a concentration of 3 or 10 μM, the percentage increase in IKs by R-L3 was less than that observed for 1 μM (Fig. 3B). This diminished response at high concentrations was caused by a time- and voltage-dependent block of IKs that was most obvious during long pulses. For example, IKs was increased by 10 μM R-L3 during the first few seconds of a 7.5-sec pulse to +50 mV. However, when the depolarization exceeded ~3 sec, the current measured in the presence of R-L3 was reduced compared with control (Fig. 3C). R-L3 increased time-dependent IKs at the end of 7.5-sec pulses to potentials <+10 mV but decreased IKs at more positive potentials (Fig. 3D). Thus, the biphasic concentration-response relationship for the effects of R-L3 on IKs for 3-sec pulses to −10 mV (Fig. 3B) reflects the dual effects of the drug: activation that predominates at low concentrations and voltage-dependent block at higher concentrations.

The increase of IKs by R-L3 was stereospecific. S-L3 blocked IKs at all concentrations (1–10 μM) and test potentials (−10 to +50 mV) examined. At concentrations of 1 and 10 μM, S-L3 blocked IKs measured at the end of 3-sec test pulses to +50 mV by an average of 14.8 ± 4.3% and 68.8 ± 3.4% (five cells).

Fig. 2. Effects of R-L3 on action potentials of guinea pig isolated ventricular myocytes. A–C, APs were recorded during stimulation at 1 Hz during control (A) and after 10 min superfusion with R-L3 at 0.1, 1, and 10 μM in normal HBS; after 30 nM Iso alone and after the addition of 1 μM R-L3 (B); and after 100 nM timolol alone and after the addition of 1 μM R-L3 (C). Bar graphs, percentage changes in APD_{90} and APD_{90}. Data are mean \pm standard error.
R-L3 shifts the voltage dependence of activation and slows deactivation of I\textsubscript{ks}. The voltage dependence of I\textsubscript{ks} activation was estimated using 7.5-sec depolarizing steps from a V\textsubscript{h} of −50 mV (Fig. 4A). The amplitude of the tail currents was normalized relative to the maximum amplitude and fit to a Boltzmann function (Fig. 4B). Because I\textsubscript{ks} does not achieve a steady state, even during extremely long pulses (Hice et al., 1994), the activation curves are isochronal. In control, the V\textsubscript{1/2} was 19.2 ± 1.6 mV, and k for this relationship was 11.0 ± 1.2 mV (five cells). In these same cells, R-L3 shifted V\textsubscript{1/2} to 3.0 ± 0.8 mV at 0.1 μM and 2.4.9 ± 3.4 mV at 1 μM but had no effect on k. The maximally activated I\textsubscript{ks} measured at a V\textsubscript{t} of +60 mV (896 ± 196 versus 953 ± 183 pA, 1 μM) was slightly but not significantly increased by R-L3. Likewise, after pretreatment with 100 nM timolol, 1 μM R-L3 shifted the V\textsubscript{1/2} by −19 mV without affecting k, indicating that its effect was independent of β-adrenergic stimulation. These results suggest that the primary mechanism of the increase in I\textsubscript{ks} by R-L3 is an effect on channel gating.

The onset of I\textsubscript{ks} activation, after a short delay, was best described by a two-exponential function. The fast time constants of activation were slightly, but not significantly, faster in the presence of 1 μM R-L3. This effect was likely due to the negative shift in the voltage dependence of channel activation. In contrast to the modest effect on the kinetics of activation, R-L3 greatly slowed the rate of I\textsubscript{ks} deactivation (Fig. 4A). The kinetics of deactivation were determined at potentials of −60 to −10 mV after a 3-sec prepulse to +30 mV from a V\textsubscript{h} of −50 mV. The deactivation of I\textsubscript{ks} was best described by a two-exponential function before and after the addition of R-L3. R-L3 significantly increased the fast (\tau\textsubscript{fast}) and the slow (\tau\textsubscript{slow}) time constants of deactivation (Fig. 5). The slowing of

<table>
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<th>1</th>
<th>10</th>
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<td>APD\textsubscript{90} (msec)</td>
<td>199.7 ± 7.6</td>
<td>193.0 ± 6.8</td>
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<td>APD\textsubscript{50} (msec)</td>
<td>166.5 ± 7.7</td>
<td>160.9 ± 7.6</td>
<td>157.1 ± 5.0</td>
<td>124.4 ± 9.9</td>
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<tr>
<td>APD\textsubscript{at0} (msec)</td>
<td>128.3 ± 9.8</td>
<td>119.1 ± 11.2</td>
<td>104.4 ± 7.4</td>
<td>90.1 ± 12.2</td>
<td>0.127</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>121.1 ± 3.6</td>
<td>119.8 ± 4.5</td>
<td>120.6 ± 4.2</td>
<td>118.1 ± 6.5</td>
<td>0.994</td>
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<td>RMP (mV)</td>
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<td>−89.1 ± 1.4</td>
<td>−86.9 ± 1.2</td>
<td>−88.0 ± 1.4</td>
<td>0.844</td>
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<tr>
<td>APA\textsubscript{50ms} (mV)</td>
<td>111.6 ± 2.9</td>
<td>110.9 ± 3.6</td>
<td>109.0 ± 2.9</td>
<td>105.7 ± 5.0</td>
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APD\textsubscript{90} - action potential duration at 0 mV.
Data are mean ± standard error.

![Image](attachment:image.png)

Fig. 3. Modulation of I\textsubscript{ks} by R-L3 is concentration dependent in guinea pig isolated ventricular myocytes. A, Superimposed currents from a single cell before and after the addition of 0.03 and 0.3 μM R-L3 during a 3-sec voltage step from −50 to −10 mV. B, Percent increase in I\textsubscript{ks} at +V\textsubscript{t} of −10 mV by R-L3 (six or more cells). C, Superimposed currents before and after the addition of 10 μM R-L3 during a 7.5-sec voltage step from −50 to +50 mV. D, I-V relationship for the time-dependent I\textsubscript{ks} measured at the end of 7.5-sec pulses (six cells).
the rate of deactivation by R-L3 represents an additional mechanism that would increase outward current during repolarization of a cardiac AP.

**R-L3 activates I_{Ks} independent of β-adrenergic receptor activation.** I_{Ks} was activated by 0.5-sec pulses to a V_t ranging from −40 to +50 mV. Iso (10 nM) alone increased I_{Ks} by 1.75-fold. The addition of 1 μM R-L3 produced a further increase in I_{Ks}, slowed the rate of deactivation, and shifted the threshold for current activation to more negative potentials (Fig. 6). The effects of R-L3 persisted after washout of the Iso. Similar effects were observed when exposure of cells to R-L3 preceded the addition of Iso. Thus, similar to the decrease in APD caused by R-L3, the increase in I_{Ks} by R-L3 was additive to that caused by β-adrenergic receptor stimulation.

**R-L3 activates cloned human KvLQT1 channels expressed in X. laevis oocytes.** At a concentration of 1 μM, R-L3 increased KvLQT1 elicited with 2-sec pulses to potentials ranging from −70 to +60 mV (Fig. 7, A and B). This increase can partially be accounted for by a −10 mV shift in the voltage dependence of activation caused by the drug (Fig. 7C). R-L3 also slowed the kinetics of KvLQT1, an effect that is easily observed when the time-dependent currents recorded before and after exposure to 1 μM R-L3 are superimposed and scaled to match peak current (Fig. 8A). Activation of KvLQT1 current is best described by a two-exponential function. The effect of R-L3 on these two components varied with V_t. R-L3 slowed the rate of the fast component at V_t = −30 mV (Fig. 8B), but increased the rate of the slow component of activation at V_t = +30 mV (Fig. 8C). The net effect of R-L3 was to slow the rate of KvLQT1 activation because of a reduction in the relative amplitude of the fast component of activation over the entire voltage range that was examined (Fig. 8D). R-L3 also slowed the rate of KvLQT1 deactivation when assessed at voltages negative to −40 mV (Fig. 8E). Thus, R-L3 increased the magnitude of KvLQT1, shifted the voltage dependence of its activation, and slowed the rates of activation and deactivation. These effects of R-L3 on KvLQT1 current are similar to those observed for I_{Ks} recorded in guinea pig ventricular myocytes.

**R-L3 activates cloned human I_{Ks} currents depending on hminK/KvLQT1 ratio.** I_{Ks} currents are formed by coassembly of KvLQT1 and minK subunits (Barhanin et al., 1996; Sanguinetti et al., 1996b). Therefore, in addition to its effects on KvLQT1 channels, we determined the effects of R-L3 on KvLQT1 plus hminK (I_{Ks}) currents expressed in X. laevis oocytes. When oocytes were injected with 11.5 ng of KvLQT1 and 1 ng of hminK cRNAs, amounts similar to previous studies, we expected to observe an increase in the magnitude of cloned I_{Ks} similar to that described above for I_{Ks} recorded from guinea pig myocytes. Surprisingly, we found that R-L3 had no obvious effect on channels formed by coassembly of KvLQT1 plus hminK (Fig. 9). The only statistically significant effect of the drug was a slowing of the rate of deactivation. At −50 mV, deactivation was 470 ± 18 msec in control and 507 ± 12 msec after 1 μM R-L3 (p < 0.05). We observed

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**Fig. 4.** R-L3 shifts the voltage-dependence of I_{Ks} activation in guinea pig isolated ventricular myocytes. A, Currents recorded at the indicated V_t before (control) and after the addition of 1 μM R-L3. B, Isochronal activation curves were determined from the normalized amplitudes of tail currents after 7.5-sec pulses. Data were fitted to a Boltzmann function to determine the V_{1/2} and slope factor (k) for the relationship. The lines were fitted to a Boltzmann function to determine the V_{1/2} and slope factor (k) for the relationship. The V_{1/2} was 19.2 ± 1.6 mV in control and 3.0 ± 0.8 mV in 1.0 μM R-L3, respectively (five cells). The k value was 11.0 ± 1.2 mV in control and was not significantly changed by R-L3.

**Fig. 5.** R-L3 slows the rate of I_{Ks} deactivation in guinea pig isolated ventricular myocytes. Time constants for deactivation were determined for tail currents on return to a variable potential after a 3-sec activating pulse from −50 to +30 mV (four or more cells). * Significantly different from control (p < 0.05) by paired t test.
a similar lack of effect of R-L3 on expressed Ik in either human or guinea pig minK cRNA alone was injected into X. laevis oocytes (data not shown). Presumably, these currents represent channels formed from coassembly of exogenous minK and endogenous KvLQT1. These data indicate that association of KvLQT1 with minK subunits prevents the activation of channel activity by R-L3 that occurs when only KvLQT1 channels are overexpressed in oocytes.

To test this hypothesis further, the ratio of hminK/KvLQT1 subunits was reduced by injecting oocytes with 11.5 ng of KvLQT1 and 0.1 ng of hminK cRNA. Under these conditions (Fig. 10), the induced current activated at a rate faster than Ik but slower than KvLQT1 alone (compare with Figs. 7 and 9), suggesting that not all channels were heteromultimeric. In this case, R-L3 caused an increase in current in all cells (seven cells). On average, R-L3 increased peak outward current by 28% at +40 mV.

**R-L3 blocks Ik and Ica but not Ik.** To determine the selectivity of R-L3, we measured its effects on three other currents, Ik, Ik, and Ica, that modulate cardiac APD. Ik and Ica were measured in guinea pig isolated ventricular myocytes. R-L3 at 10 μM had no significant effect on Ik. For example, Ik at -60 mV was 5.5 ± 1.1 pA/pF in control and 5.7 ± 1.2 pA/pF after the addition of R-L3 (five cells). R-L3 had no significant effect on Ica at 1 μM, but at 10 μM it reduced peak Ica at +20 mV by 43.6 ± 6.6% (nine cells; Fig. 11). The block of Ica was not use-dependent. In two cells, block of Ica by 10 μM R-L3 during trains of 30 pulses applied to +20 mV at rates of 1 and 3 Hz initially was 57% and 61%, respectively, and was unchanged (59% and 60%) at the end of the pulse trains.

Because R-L3 caused a negative shift in the voltage-dependence of Ik, activation, Ik could not satisfactorily be measured in isolation from Ik in guinea pig myocytes. Therefore, the effects of R-L3 on Ik were determined in mouse AT-1 myocytes (Fig. 12). These cells have a large Ik but no measurable Ik (Yang et al., 1994). R-L3 blocked Ik tail currents after a 1-sec test pulse to +20 mV by 21 ± 6% and 53 ± 3% (three cells) at concentrations of 1 and 10 μM, respectively.

**Discussion**

At concentrations of ≤1 μM, R-L3 shortened APD of cardiac myocytes by selective activation of Ik. At membrane potentials and pulse durations typical for a cardiac AP, the most important mechanisms of action of R-L3 were a negative shift

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**Fig. 6.** Effects of Iso and R-L3, alone and in combination, on the I-V relationship of Ik in guinea pig isolated ventricular myocytes. A, Traces were recorded during control, after exposure to 10 nM Iso alone, and after the addition of 1 μM R-L3 and then after washout of Iso but in the continued presence of R-L3. Currents were elicited by 0.5-sec pulses from a Vh of -50 mV. B, I-V relationship for time-dependent Ik for each condition (five cells).

**Fig. 7.** R-L3 activates cloned KvLQT1 channels expressed in X. laevis oocytes. A, Currents were measured in response to 2-sec pulses from a Vh of -80 mV to a Vt of +40 mV, applied in 20-mV increments. Tail currents were measured at -70 mV. B, I-V relationships for peak KvLQT1 current during 2-sec pulses to the indicated test potential before and after 1 μM R-L3. C, Voltage dependence of KvLQT1 activation. Tail current amplitudes were determined from extrapolating a single exponential fit of deactivating currents to the onset of membrane repolarization. Isochronal activation curves were determined by fitting normalized tail current amplitudes to a Boltzmann function. In control, the V1/2 was -28 mV and the slope factor (k) was 11 mV for this relation. In the presence of 1 μM R-L3, the V1/2 was -40 mV and k was 13 mV (eight cells).
in the voltage dependence of activation and a slowing of \(I_{Ks}\) deactivation. R-L3 also caused a modest increase in \(I_{Ks}\) beyond what could be explained by these two mechanisms. The molecular mechanism of these effects on \(I_{Ks}\) current-gating is not known, but it is not mediated through the \(\beta\)-adrenergic receptor activation pathway.

At concentrations of >1 \(\mu\)M, the effect of R-L3 on APD would reflect multiple mechanisms, including activation of \(I_{Ks}\) (especially at potentials of <0 mV), block of \(I_{Kr}\), and block of L-type \(I_{Ca}\). Block of \(I_{Kr}\) would lengthen APD, whereas block of \(I_{Ca}\) would contribute to a shortening of APD. Because \(APD_{90}\) was unchanged, whereas \(APD_{50}\) was further short-

**Fig. 8.** R-L3 slows the rates of activation and deactivation of KvLQT1 expressed in Xenopus oocytes. A, To illustrate the change in KvLQT1 kinetics induced by R-L3, the peak current activated by a 2-sec pulse to +40 mV was scaled to match the peak current recorded after the addition of 1 \(\mu\)M R-L3. Tail current was measured at −70 mV. B, Time constants for fast component of KvLQT1 activation. C, Time constants for slow component of KvLQT1 activation. D, Relative amplitude of the fast component of KvLQT1 activation. E, Time constants of KvLQT1 deactivation (eight cells for all graphs).
Fig. 9. R-L3 does not activate cloned $I_{sc}$ currents expressed in X. laevis oocytes. A, Currents recorded in an oocyte during 7.5-sec pulses applied to $V_t$ between −20 mV and +40 mV. Oocyte was injected with 11.5 ng of KvLQT1 and 1 ng of hminK cRNAs, and currents were recorded in control and 7 min after the addition of 1 μM R-L3. B, I-V relationships for peak currents (eight cells).

Fig. 10. R-L3 activates current induced by injection of KvLQT1 and hminK cRNAs when amount of minK cRNA is limiting. A, Currents recorded in an oocyte during 7.5-sec pulses applied in 20-mV increments to a $V_t$ between −40 and +40 mV. Oocyte was injected with 11.5 ng of KvLQT1 and 0.1 ng of hminK cRNAs, and currents were recorded in control and 7 min after the addition of 1 μM R-L3. B, I-V relationships for peak currents (seven cells).
ened and the plateau height (APA50ms) was reduced when R-L3 was increased from 1 to 10 μM, it is likely that the block of ICa was more important than the block of IKr at 10 μM in guinea pig ventricular myocytes. A high concentration (10 μM) of R-L3 also caused a block of IKr after long pulses to very positive potentials. However, even at 10 μM, R-L3 would be expected to cause only an increase in IKr during the limited time of depolarization of a cardiac AP. We could not test the effects of R-L3 at concentrations of >10 μM because of its limited aqueous solubility.

We compared and contrasted the effects of R-L3 and Iso on configuration. These studies revealed a similar shortening of APD90 but other important differences, especially on plateau height. Effects of β-adrenergic receptor stimulation on APs are difficult to interpret because of the multitude of effects on ion channels and pumps. Nevertheless, the persistent effects of R-L3 in the presence of timolol, a β-adrenergic receptor blocker, demonstrate that its effects are not mediated via this signaling pathway.

The findings that R-L3 activated IKr at low concentrations but blocked IKr at high concentrations, as did the S-enantiomer, suggest that there may be multiple binding sites for L-3 on the IKr channel. Multiple binding sites have been proposed to explain the dual action of dihydropyridines such as Bay K 8644 on the L-type Ca2+ channel (Brown et al., 1986; Kokubun et al., 1986).

R-L3 increased the magnitude of cloned KvLQT1 current, but its activation of human IKr channels formed by coassembly of KvLQT1 and hminK subunits depended on their ratio. When minK and KvLQT1 were coexpressed at relatively high ratio, such that the expression of minK was not limiting, R-L3 had no significant effect on IKr. However, when coexpressed at a 10-fold lower ratio, where minK was limiting, R-L3 increased IKr. The resulting currents at the low minK/KvLQT1 ratio were larger and activated at a rate slower than that of the current induced by KvLQT1 alone but were faster than those induced by the high subunit ratio. The stoichiometry of IKr channels is unknown. Because KvLQT1 alone or coexpressed with minK can form functional channels, it may be possible for minK subunits to coassemble with individual KvLQT1 subunits or KvLQT1 tetramers in variable ratios.

Fig. 11. R-L3 blocks ICa in guinea pig isolated ventricular myocytes at 10 μM. ICa was measured at room temperature during 100-msec pulses from a Vh of −50 mV to Vt between −40 and +60 mV during control and after the addition of 1 and 10 μM R-L3.

Fig. 12. R-L3 blocks IKr in AT-1 cells. Currents were measured at room temperature during 1-sec pulses from a Vh of −40 mV to Vt between −30 and +50 mV during control and after the addition of 1 and 10 μM R-L3.
and thereby impart differing biophysical characteristics and pharmacological sensitivity. Alternatively, there may be only one functional IsK channel type with a fixed stoichiometry of KvLQT1 and minK subunits, and these may coexist in variable ratios with KvLQT1 homotetramers. A clearer understanding of this stoichiometry may help to explain why the effects of the drug on guinea pig IKr are better mimicked by cloned KvLQT1 channels, whereas the biophysical properties of IKr are more closely mimicked by channels formed by coassembly of KvLQT1 + hminK. Nevertheless, these findings indicate that the binding site for R-L3 is located on the KvLQT1 subunit and that coassembly with hminK diminishes or abolishes the agonist activity of R-L3. These studies are consistent with, but not proof of, the ideas that minK and R-L3 bind to a common region of KvLQT1 subunits and that association of minK with KvLQT1 precludes the drug-induced alteration of gating kinetics observed when only KvLQT1 channels are overexpressed in oocytes. Regardless of the exact mechanism, these findings suggest the possibility that IKr recorded in cardiac myocytes represents the sum of current mediated by KvLQT1 homotetrameric channels and heteromultimeric channels formed by coassembly of KvLQT1 plus minK subunits.

This and all studies of IKs face the possible same limitations, including the potential for K\(^+\) accumulation during long depolarizing pulses (Boyd et al., 1980, but see also Sanguinetti and Jurkiewicz, 1990), a lack of steady state activation of IKs and interference or overlap with other cardiac currents. Our voltage-clamp protocols and conditions were designed to reduce or eliminate these concerns. To measure activation of IKs channels, relatively long pulse durations of 7.5 sec were used as in previous studies (Sanguinetti and Jurkiewicz, 1990) to approach steady state activation, whereas longer pulses were avoided to minimize the potential for K\(^+\) accumulation. IKs blockers were used in excess (100 \times IC_{50} values at 4 ms [K\(^+\)]) to completely block IKs. Although an increase in [K\(^+\)] was reported to decrease the potency of IKs blockers (e.g., dofetilide; (Yang and Roden, 1996), the 100-fold excess used to block IKs in this study would not be overcome by potential elevations of [K\(^+\)]. Most currents other than IKs were also eliminated by pharmacological blockade or inactivation (or both) with V\(_{h}\).

A drug that activates IKs could be beneficial for the treatment of certain arrhythmias. The proarrhythmic potential of class III antiarrhythmic drugs that block IKr prompted the search for agents that would produce more modest prolongation of APD at high doses. The proarrhythmic liability associated with block of IKr currents was recently confirmed by the finding that mutations in HERG, the gene encoding IKr channel subunits, can cause inherited LQT (Curran et al., 1995). Recently, IKs blockers have been described that lengthen APD and have antiarrhythmic effects in animal models. Chromanol 293B (Busch et al., 1996) and the more potent L-735,821, a 1,4-benzodiazepine (Salata et al., 1996), inhibit IKs and cause a self-limiting prolongation of APD that is less than that produced by IKr blockers. However, mutations in KvLQT1 can also cause inherited LQT (Wang et al., 1996; Neyroud et al., 1997). This latter finding could dampen enthusiasm for the development of IKs blockers for prophylactic treatment of ventricular tachyarrhythmias. If IKs or IKr blockers are used as antiarrhythmic drugs, it would be useful to have a channel activator that could reverse the effects of overdose. Moreover, pharmacological activation of IKs might also be useful for treatment of inherited LQT and abnormally delayed repolarization associated with heart failure (Beuckelmann et al., 1993; Tomasselli et al., 1994). In summary, we described the properties of R-L3, a novel benzodiazepine that selectively activates IKs in myocytes at low concentrations. This compound represents a new pharmacological probe for the study of IKs in cardiac myocytes. In addition, R-L3 can be used as a probe to define the physiologic role of IKs and KvLQT1 currents in other tissues, such as the pancreas (Wang et al., 1996) and stria vascularis of the inner ear (Neyroud et al., 1997).

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References


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