0026-895X/98/050220-11$3.00/0

MOLECULAR PHARMACOLOGY, **53:**220–230 (1998).

MOL PHARM

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

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Received July 10, 1997; Accepted January 29, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

The slowly activating delayed rectifier K^+ current, I_{Ks}, is an important modulator of cardiac action potential repolarization. Here, we describe a novel benzodiazepine, [L-364,373 [(3-*R*)-1,3-dihydro-5-(2-fluorophenyl)-3-(1*H*-indol-3-ylmethyl)-1-methyl-2*H*-1,4-benzodiazepin-2-one] (R-L3), that activates I_{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 β-adrenergic receptor stimulation. The increase of I_{Ks} by R-L3 was stereospecific; the *S*-enantiomer, S-L3, blocked I_{Ks} at all concentrations examined. The increase in I_{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_{Ks} activation. R-L3 slowed the rate of I_{Ks} deactivation and shifted the half-point of the isochronal (7.5 sec) activation curve for I_{Ks} by −16 mV at 0.1 μM and −24 mV at 1 μM. 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_{Kr} and I_{Ks} (Sanguinetti and Jurkiewicz, 1990). Several class III antiarrhythmic agents block I_{Kr} 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_{Kr} channels, cause inherited LQT (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_{Ks}, indicating that KvLQT1 and minK proteins coassemble to form I_{Ks} channels (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996b). Thus, dysfunction of either I_{Kr} or I_{Ks} can increase the risk of cardiac arrhythmia and sudden death.

An activator of I_{Kr} or I_{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_{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_{Ks}.

This study was supported in part by U.S. Public Health Service Grant RO1-HL55236 (M.C.S.).

ABBREVIATIONS: AP, action potential; APA_{50ms}, action potential amplitude measured at 50 msec after the upstroke; APD_{90}, action potential duration at 90% repolarization; APD_{50}, action potential duration at 50% repolarization; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline; I_{ca}, L-type Ca^{2+} current; I_K, delayed rectifier K^+ current; I_{kr}, rapidly activating component of delayed rectifier K^+ current; I_{Ks} slowly activating component of delayed rectifier K^+ current; I_{K1}, inward rectifier K^+ current; I_{ktail}, I_K tail current; $I_{ktail\text{-}max}$, maximum amplitude I_K tail current; Iso, isoproterenol; I-V, current-voltage; $[K^+]_o$, extracellular K^+ concentration; LQT, long QT syndrome; R-L3, (3-*R*)-1,3-dihydro-5-(2-fluorophenyl)-3-(1*H*-indol-3-ylmethyl)-1-methyl-2*H*-1,4-benzodiazepin-2-one; V_h, holding potential; V_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 $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.2, at 24–26° until studied, usually within 8 hr after isolation.

Action potential studies. Transmembrane potentials were recorded using conventional microelectrodes filled with 3 M KCl (tip resistances, 30–50 MΩ) using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) as described previously (Fermini *et al*., 1995). Cells were superfused with HBS maintained at 37° at a rate of 2 ml/min APs were evoked with brief current pulses (1 msec, 1.2 times threshold) delivered at a frequency of 1 Hz through the recording electrode using an active bridge circuit. Only cells showing normal AP configurations and resting membrane potentials equal to or more negative than −85 mV were used in this study. Cells were studied after a ≥5-min control period and after ≥5 min of superfusion with each concentration of drug. After reaching a steady state effect under each condition, 20 individual APs were sampled and digitally averaged. The APA_{50ms} and APD_{90} were measured from the digitally averaged records. Concentration-response relationships were determined by measuring APs or currents in each cell under control conditions and during superfusion with successively increasing concentrations of a given drug.

Voltage-clamp of guinea pig ventricular myocytes. Whole-cell voltage-clamp studies were performed using a List EPC-7 (Medical Systems, Greenvale, NY) or Axopatch 200A (Axon Instruments) amplifier as described previously (Fermini *et al*., 1995). Pipettes were made from square bore (1.0 mm outer diameter) borosilicate capillary tubing (Glass Company of America, Bargaintown, NJ). Pipettes were filled with 0.5 M K^+ gluconate, 25 mM KCl, and 5 mM K_2ATP to minimize "rundown" (Giles and Shibata, 1985) and had resistances of 3–7 MΩ (average, 5.5 ± 0.3 MΩ). Series resistance was compensated 40–70%. Currents were low-pass filtered (−3 dB at 0.2 kHz) before digitization at 1 kHz. I_{Ks} was measured during superfusion of the cells at a rate of 2–3 ml/min with normal HBS (35°) containing 0.4–1 μM nisoldipine to block L-type Ca^{2+} current and standard selective I_{Kr} blockers in 100-fold excess of the IC_{50} (e.g., 3μM MK-499) to completely block I_{Kr} (Sanguinetti and Salata, 1996). Cells were voltage clamped at a V_h of −50 mV to inactivate I_{Na}. Time-dependent I_{Ks} amplitude was measured as the difference from the initial instantaneous current, after the settling of the capacity transient, to the final current level at the end of a depolarizing pulse. I_{Ktail} was measured as the difference from the holding current level to the peak tail current amplitude on return to V_h. I_{Ktail} was normalized to the maximum measured amplitude ($I_{Ktail\text{-}max}$) after 7.5-sec pulses. Averaged data were fit to a Boltzmann distribution of the form: $I_{Ktail}/I_{Ktail\text{-}max} = 1/(1 + \exp[(V_{1/2} - V_t)/k])$ with a nonlinear least-squares fitting routine (Origin; Microcal Software, Northampton, MA) to estimate the half-point ($V_{1/2}$) and slope factor (k) for this relationship. The time courses of I_{Ks} activation and deactivation were fit with a double exponential relationship of the form: $I_t = A_0 + A_1 e^{-t/\tau}1 + A_2 e^{-t/\tau}2$ using a Chebeshev noniterative fitting technique (pCLAMP; Axon Instruments).

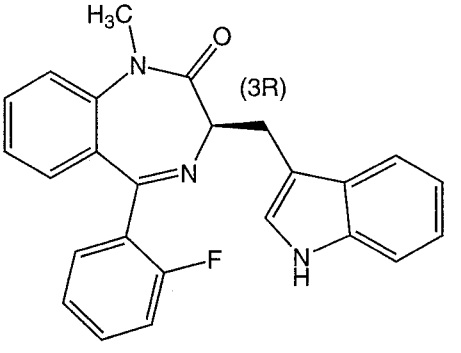
I_{Ca} was recorded using external and internal (pipette) solutions designed to minimize currents through Na^+ and K^+ channels and reduce rundown (Xu and Lee, 1994). Cells were superfused with a solution containing 157 mM TEACl, 5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM HEPES, pH adjusted to 7.4 with CsOH (24–26°). Microelectrodes were fashioned from borosilicate capillary tubing (1.5 mm absorbance and filled with the following solution: 151 mM CsOH, 10 mM L-aspartic acid, 20 mM taurine, 20 mM TEACl, 10 mM EGTA, and 5 mM glucose, pH adjusted to 7.5 with H_3PO_4; 0.4 mM Na_2GTP and 5 mM MgATP were added just before use. The filled pipettes had resistances ranging from 2 to 4 MΩ. Series resistance was compensated by 80%. Because we did not observe bimodal I-V relations in our experiments, T-type Ca^{2+} channels most likely contributed negligible current; therefore, the I_{Ca} measured in the current study was considered to be L-type Ca^{2+} 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°). Outward K^+ currents were recorded in normal HBS at 22–24° 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, 5 mM K_2ATP, 1 mM $MgCl_2$, 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_{Na} 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 coinjected with 11.5 ng of *KvLQT1* 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 TEV-200 amplifier. Oocytes were bathed at room temperature (22–25°) in a solution containing 94 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 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 ($CDCl_3$) identical to that of R-L3. The chemical and chiral purity of R-L3 and S-L3 were determined to be >99%. S-L3: high performance liquid chromatography (Vydac C-18, 15 × 0.46 cm, 16 min gradient 95:5 to 5:95 0.1% H_3PO_4/H_2O:CH_3CN, 1.5 ml/min, 215 and 254 nM) retention time (rt) = 11.0 min, >96%, coelutes with 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 S-L3). TLC (silica, 10% Et_2O in CH_2Cl_2): single component, Rf = 0.43, coelutes with R-L3. Calc. for $C_{25}H_{20}FN_3O$: C 75.55, H 5.07, N 10.57; found: C 75.57, H 5.17, N 10.46.



L-364,373

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 ± 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 I_{Ks} 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_{50} 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 ± 1.6% and 13.8 ± 4.0%, respectively.

Stimulation of β-adrenergic receptors can also shorten APD 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} by 12.9 ± 2.9%. Iso also increased APA_{50ms} by 6.6 ± 2.9% (Fig. 2B), presumably by enhancement of L-type Ca^{2+} current (Kass and Wiegers, 1982). The addition of 1 μM R-L3 in the presence of 30 nM Iso decreased APD_{90} further (26.9 ± 1.4%) and diminished the increase in APA_{50ms}. 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 ± 2.2%, very similar to the effect observed as 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 I_{Ks} 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 I_{Ks}.

The effect of R-L3 on I_{Ks} 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 I_{Ks}. R-L3 enhanced I_{Ks} measured at −10 mV at concentrations as low as 30 nM and had a maximal effect at 1 μM. At this concentration, I_{Ks} was increased by a factor of 17 ± 5 (six cells). At a concentration of 3 or 10 μM, the percentage increase in I_{Ks} 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 I_{Ks} that was most obvious during long pulses. For example, I_{Ks} 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 I_{Ks} at the end of 7.5-sec pulses to potentials <+10 mV but decreased I_{Ks} at more positive potentials (Fig. 3D). Thus, the biphasic concentration-response relationship for the effects of R-L3 on I_{Ks} 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 I_{Ks} by R-L3 was stereospecific. S-L3 blocked I_{Ks} at all concentrations (1–10 μM) and test potentials (−10 to +50 mV) examined. At concentrations of 1 and 10 μM, S-L3 blocked I_{Ks} measured at the end of a 1-sec test pulse to +50 mV by an average of 14.8 ± 4.3% and 68.8 ± 3.4% (five cells).

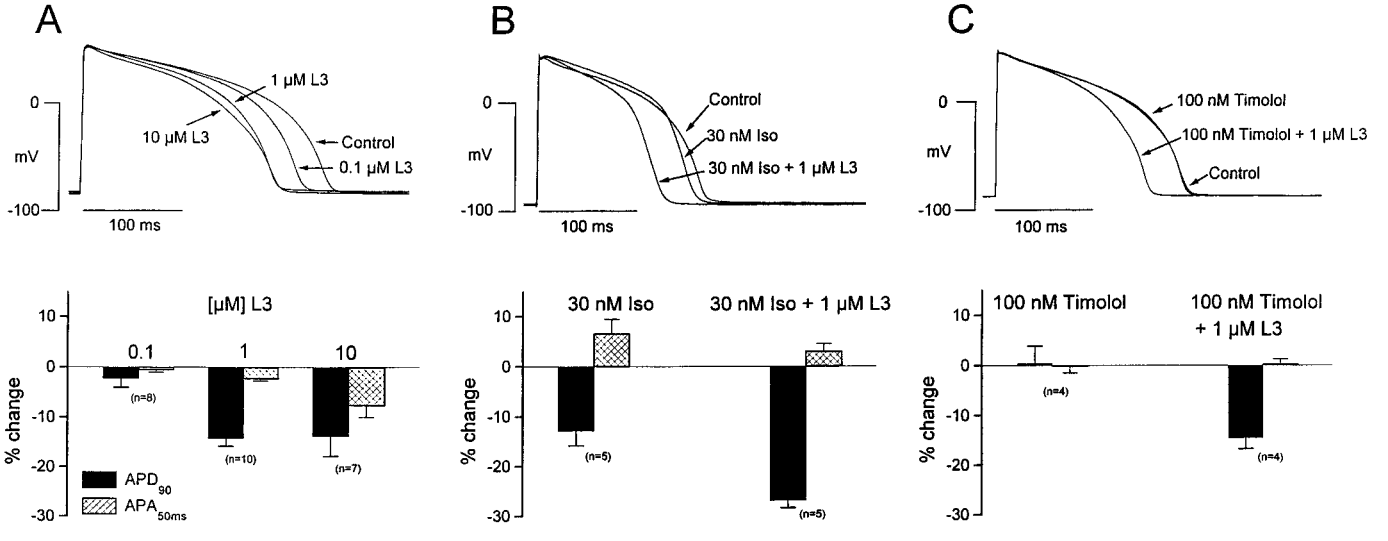


Fig. 2. Effects of R-L3 on action potentials of guinea pig isolated ventricular myocytes. A–C, APs were recorded during stimulation at 1 Hz during control (A) and after 10 min superfusion with R-L3 at 0.1, 1, and 10 μM in normal HBS; after 30 nM Iso alone and after the addition of 1 μM R-L3 (B); and after 100 nM timolol alone and after the addition of 1 μM R-L3 (C). *Bar graphs*, percentage changes in APD_{90} and APA_{50ms}. Data are mean ± standard error.

R-L3 shifts the voltage dependence of activation and slows deactivation of I_{Ks}. The voltage dependence of I_{Ks} activation was estimated using 7.5-sec depolarizing steps from a V_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_{Ks} does not achieve a steady state, even during extremely long pulses (Hice *et al.*, 1994), the activation curves are isochronal. In control, the $V_{1/2}$ 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 $V_{1/2}$ to 3.0 ± 0.8 mV at 0.1 μM and −4.9 ± 3.4 mV at 1 μM but had no effect on k. The maximally activated I_{Ks} measured at a V_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 $V_{1/2}$ 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_{Ks} by R-L3 is an effect on channel gating.

The onset of I_{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_{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_h of −50 mV. The deactivation of I_{Ks} was best described by a two-exponential function before and after the addition of R-L3. R-L3 significantly increased the fast (τ_{fast}) and the slow (τ_{slow}) time constants of deactivation (Fig. 5). The slowing of

TABLE 1
Effect of [3-R]L-364,373 (R-L3) on action potential (AP) parameters of guinea pig isolated ventricular myocytes

AP parameter	Control	R-L3			ANOVA
		0.1	1	10	*p*
			μM		
APD_{90} (msec)	199.7 ± 7.6	193.0 ± 6.8	170.6 ± 4.7	171.1 ± 8.3	0.012
APD_{50} (msec)	166.5 ± 7.7	160.9 ± 7.6	137.1 ± 5.0	124.4 ± 9.9	0.002
$APD_{@0}$ (msec)	126.3 ± 9.8	119.1 ± 11.2	104.4 ± 7.4	90.1 ± 12.2	0.127
APA (mV)	121.1 ± 3.6	119.8 ± 4.5	120.6 ± 4.2	118.1 ± 6.5	0.994
RMP (mV)	−88.25 ± 1.3	−89.1 ± 1.4	−86.9 ± 1.2	−88.0 ± 1.4	0.844
APA_{50ms} (mV)	111.6 ± 2.9	110.9 ± 3.6	109.0 ± 2.9	105.7 ± 5.0	0.783
No. of cells	10	8	10	7	

$APD_{@0}$ - action potential duration at 0 mV.
Data are mean ± standard error.

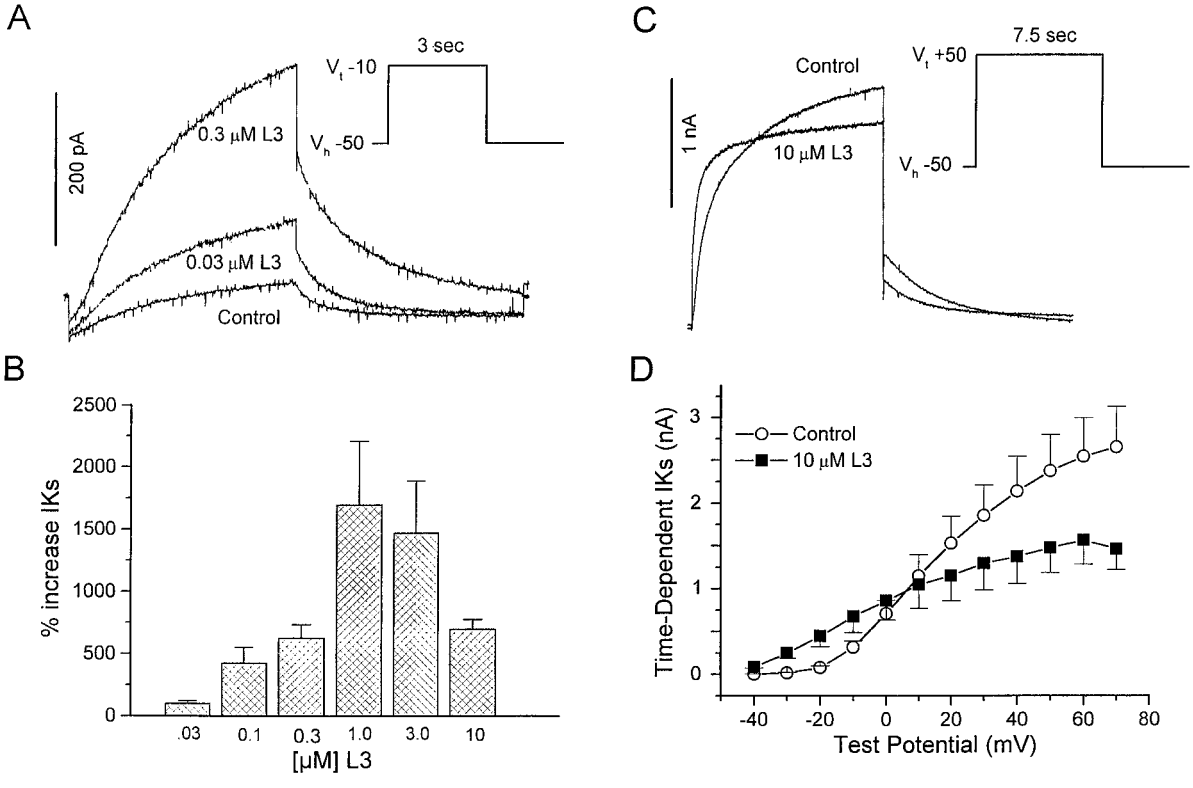


Fig. 3. Modulation of I_{Ks} by R-L3 is concentration dependent in guinea pig isolated ventricular myocytes. A, Superimposed currents from a single cell before and after the addition of 0.03 and 0.3 μM R-L3 during a 3-sec voltage step from −50 to −10 mV. B, Percent increase in I_{Ks} at a V_t of −10 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_{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 $V_t \geq 0$ mV (Fig. 8B), but increased the rate of the slow component of activation at $V_t \geq -30$ 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

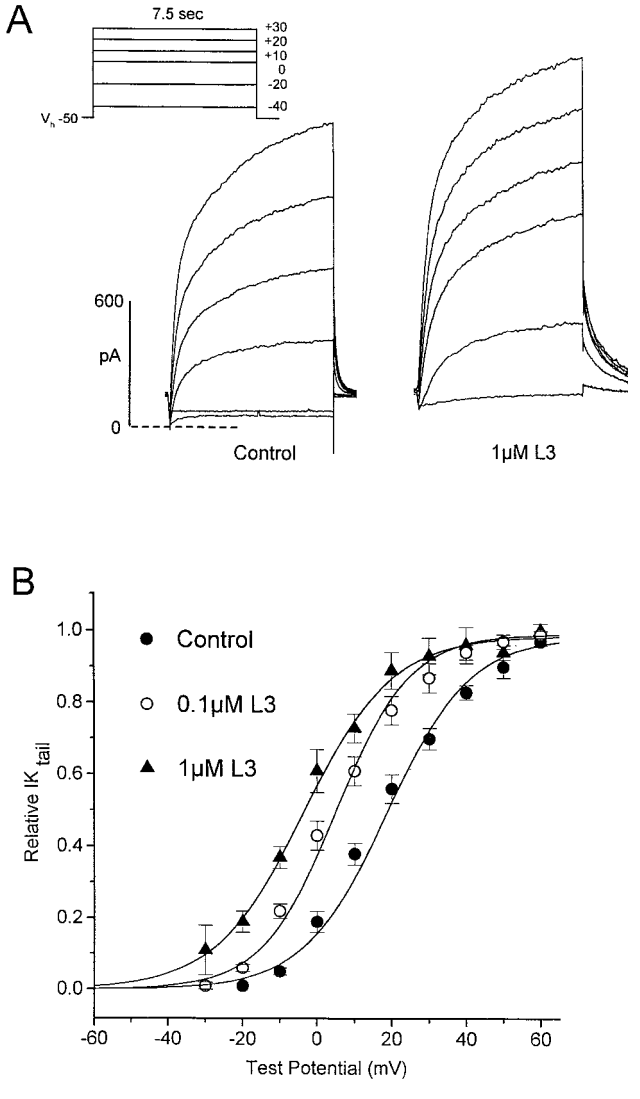


Fig. 4. R-L3 shifts the voltage-dependence of I_{Ks} activation in guinea pig isolated ventricular myocytes. A, Currents recorded at the indicated V_t before (*control*) and after the addition of 1 μM R-L3. B, Isochronal activation curves were determined from the normalized amplitudes of tail currents 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 $V_{1/2}$ was 19.2 ± 1.6 mV in control and 3.0 ± 0.8 mV and −4.9 ± 3.4 mV at 0.1 and 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.

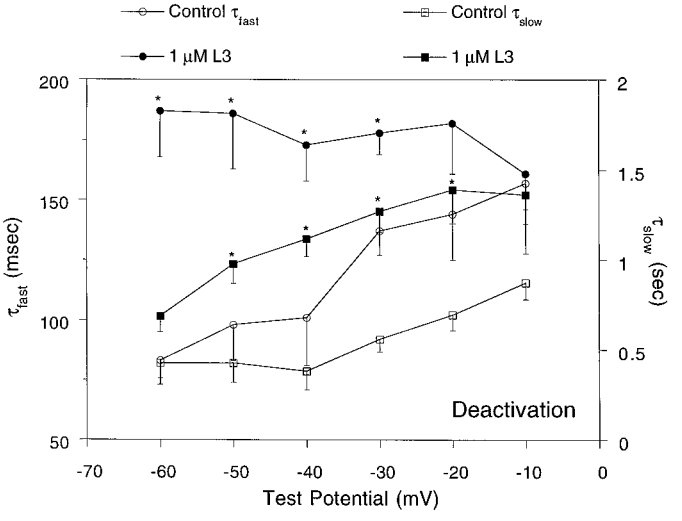


Fig. 5. R-L3 slows the rate of I_{Ks} deactivation in guinea pig isolated ventricular myocytes. Time constants for deactivation were determined for tail currents on return to a variable potential after a 3-sec activating pulse from −50 to +30 mV (four or more cells). ∗, Significantly different from control ($p < 0.05$) by paired t test.

a similar lack of effect of R-L3 on expressed I_{Ks} when 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 I_{Ks} 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 I_{Kr} and I_{Ca} but not I_{K1}. To determine the selectivity of R-L3, we measured its effects on three other currents, I_{Kr}, I_{K1}, and I_{Ca}, that modulate cardiac APD.

I_{K1} and I_{Ca} were measured in guinea pig isolated ventricular myocytes. R-L3 at 10 μM had no significant effect on I_{K1}. For example, I_{K1} 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 I_{Ca} at 1 μM, but at 10 μM it reduced peak I_{Ca} at +20 mV by 43.6 ± 6.6% (nine cells; Fig. 11). The block of I_{Ca} was not use-dependent. In two cells, block of I_{Ca} 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 I_{Ks} activation, I_{Kr} could not satisfactorily be measured in isolation from I_{Ks} in guinea pig myocytes. Therefore, the effects of R-L3 on I_{Kr} was determined in mouse AT-1 myocytes (Fig. 12). These cells have a large I_{Kr} but no measurable I_{Ks} (Yang *et al.*, 1994). R-L3 blocked I_{Kr} 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.

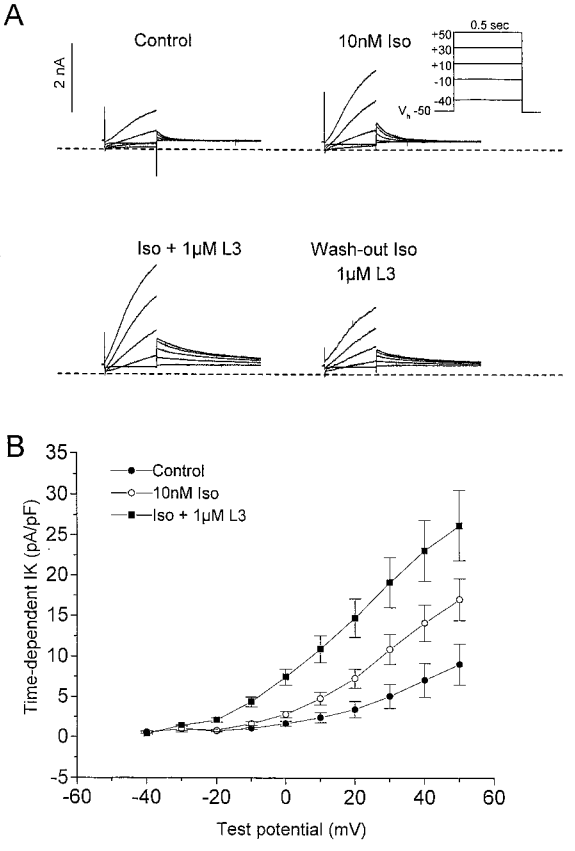


Fig. 6. Effects of Iso and R-L3, alone and in combination, on the I-V relationship of I_{Ks} in guinea pig isolated ventricular myocytes. A, *Traces* were recorded during control, after exposure to 10 nM Iso alone, and after the addition of 1 μM R-L3 and then after washout of Iso but in the continued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

Discussion

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

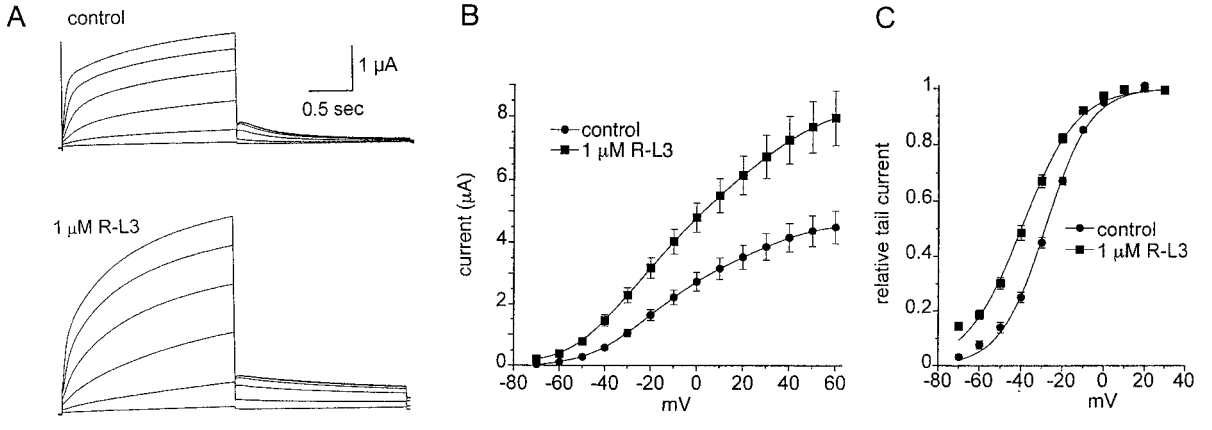


Fig. 7. R-L3 activates cloned KvLQT1 channels expressed in *X. laevis* oocytes. A, Currents were measured in response to 2-sec pulses from a V_h of −80 mV to a V_t of −60 mV to +40 mV, applied in 20-mV increments. Tail currents were measured at −70 mV. B, I-V relationships for peak KvLQT1 current during 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 $V_{1/2}$ was −28 mV and the slope factor (k) was 11 mV for this relation. In the presence of 1 μM R-L3, the $V_{1/2}$ 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 β-adrenergic receptor activation pathway.

At concentrations of >1 μM, 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-

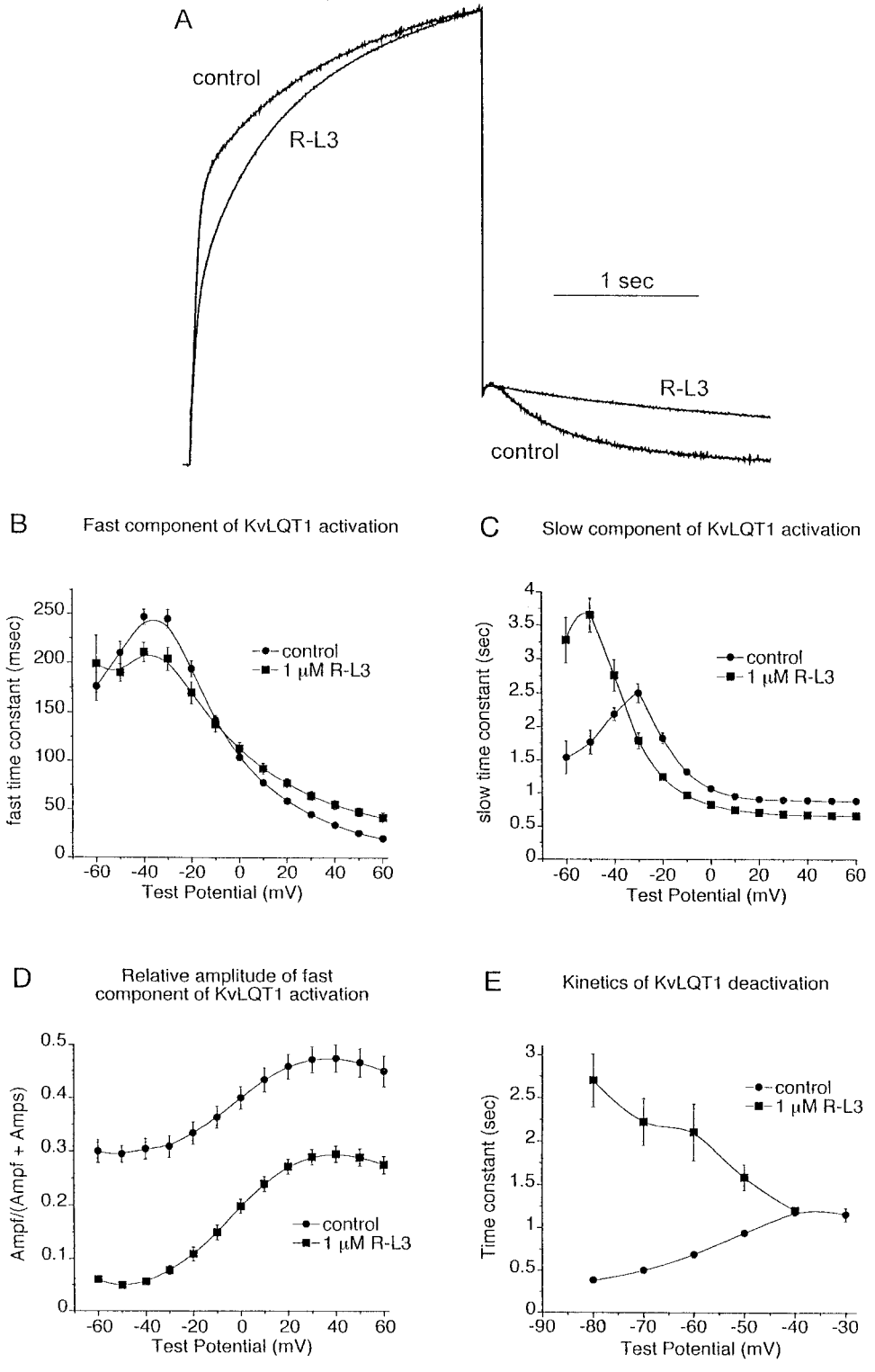


Fig. 8. R-L3 slows the rates of activation and deactivation of KvLQT1 expressed in *Xenopus* oocytes. A, To illustrate the change in KvLQT1 kinetics induced by R-L3, the peak current activated by a 2-sec pulse to +40 mV was scaled to match the peak current recorded after the addition of 1 μM R-L3. Tail 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).

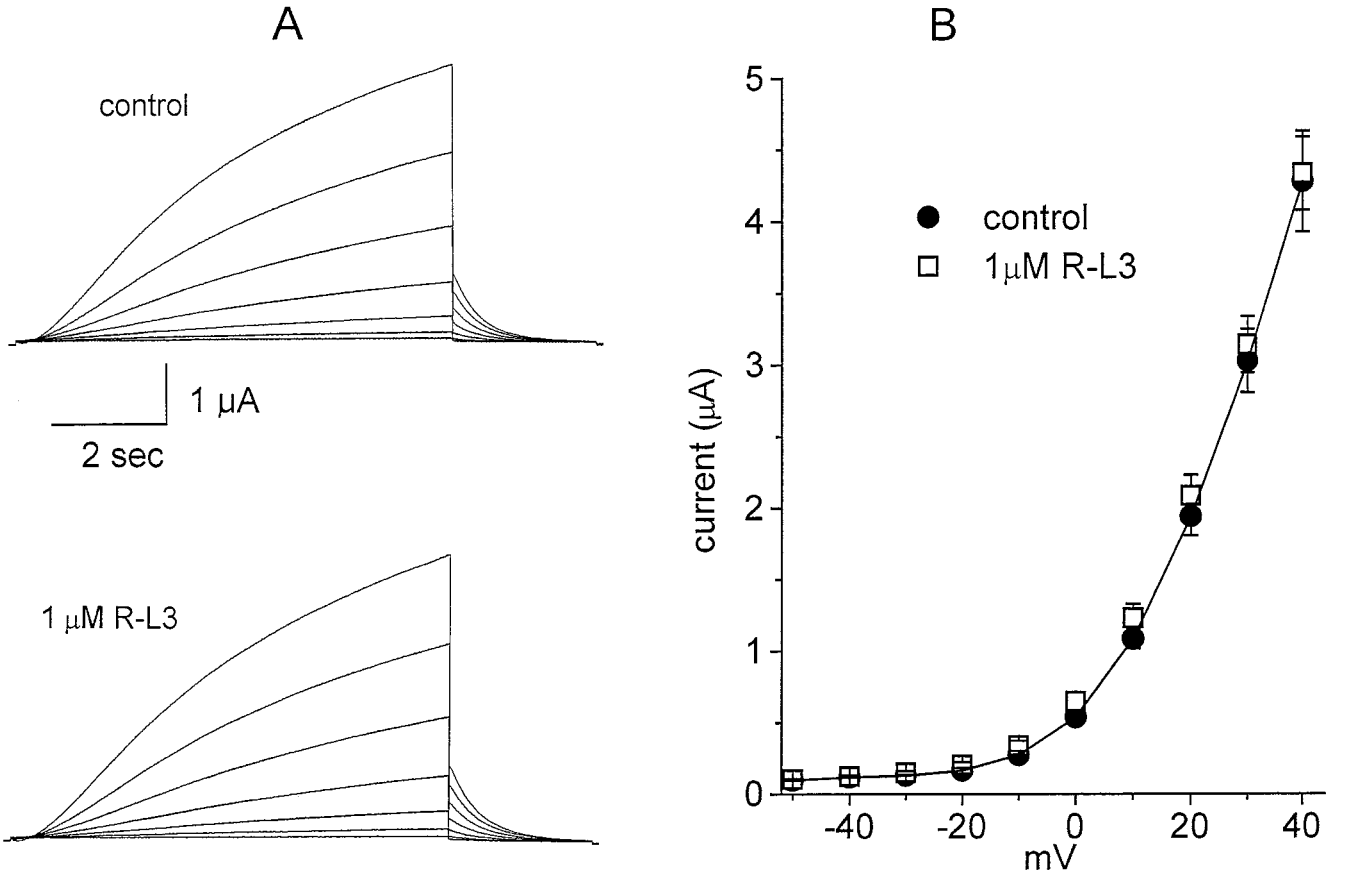


Fig. 9. R-L3 does not activate cloned I_{Ks} currents expressed in *X. laevis* oocytes. A, Currents recorded in an oocyte during 7.5-sec pulses applied to V_t between −20 mV and +40 mV. Oocyte was injected with 11.5 ng of *KvLQT1* and 1 ng of *hminK* cRNAs, and currents were recorded in control and 7 min after the addition of 1 μM R-L3. B, I-V relationships for peak currents (eight cells).

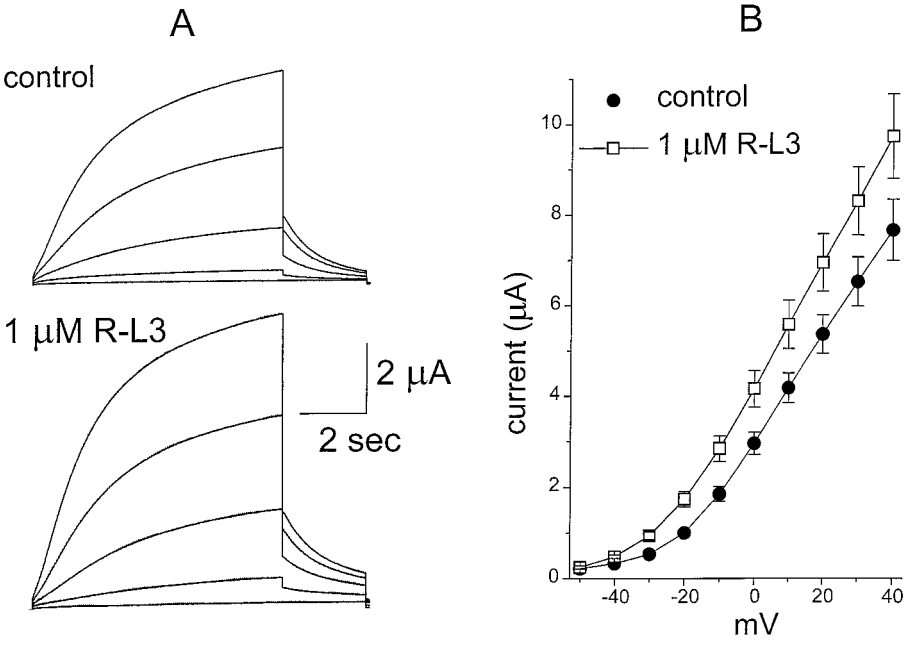


Fig. 10. R-L3 activates current induced by injection of *KvLQT1 and hminK* cRNAs when amount of *minK* cRNA is limiting. A, Currents recorded in an oocyte during 7.5-sec pulses applied in 20-mV increments to a V_t between −40 and +40 mV. Oocyte was injected with 11.5 ng of *KvLQT1* and 0.1 ng of *hminK* 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 (APA_{50ms}) was reduced when R-L3 was increased from 1 to 10 μM, it is likely that the block of I_{Ca} was more important than the block of I_{Kr} at 10 μM in guinea pig ventricular myocytes. A high concentration (10 μM) of R-L3 also caused a block of I_{Ks} after long pulses to very positive potentials. However, even at 10 μM, R-L3 would be expected to cause only an increase in I_{Ks} 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 APD_{90} 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 I_{Ks} at low concentrations but blocked I_{Ks} at high concentrations, as did the *S*-enantiomer, suggest that there may be multiple binding sites for L-3 on the I_{Ks} channel. Multiple binding sites have been proposed to explain the dual action of dihydropyridines such as Bay K 8644 on the L-type Ca^{2+} channel (Brown *et al.*, 1986; Kokubun *et al.*, 1986).

R-L3 increased the magnitude of cloned KvLQT1 current, but its activation of human I_{Ks} 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 I_{Ks}. However, when coexpressed at a 10-fold lower ratio, where minK was limiting, R-L3 increased I_{Ks}. 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 I_{Ks} 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

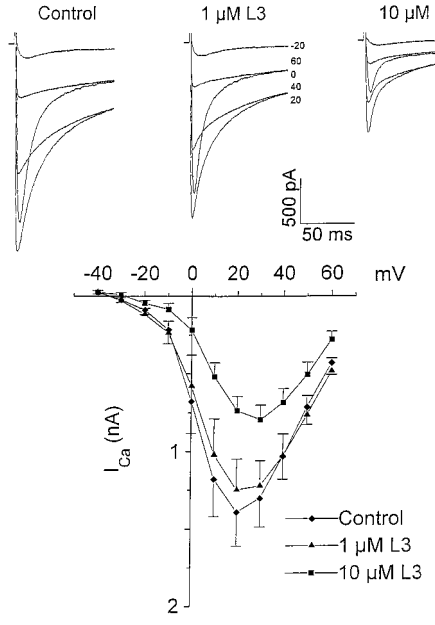


Fig. 11. R-L3 blocks I_{Ca} in guinea pig isolated ventricular myocytes at 10 μM. I_{Ca} was measured at room temperature during 100-msec pulses from a V_h of −50 mV to V_t between −40 and +60 mV during control and after the addition of 1 and 10 μM R-L3.

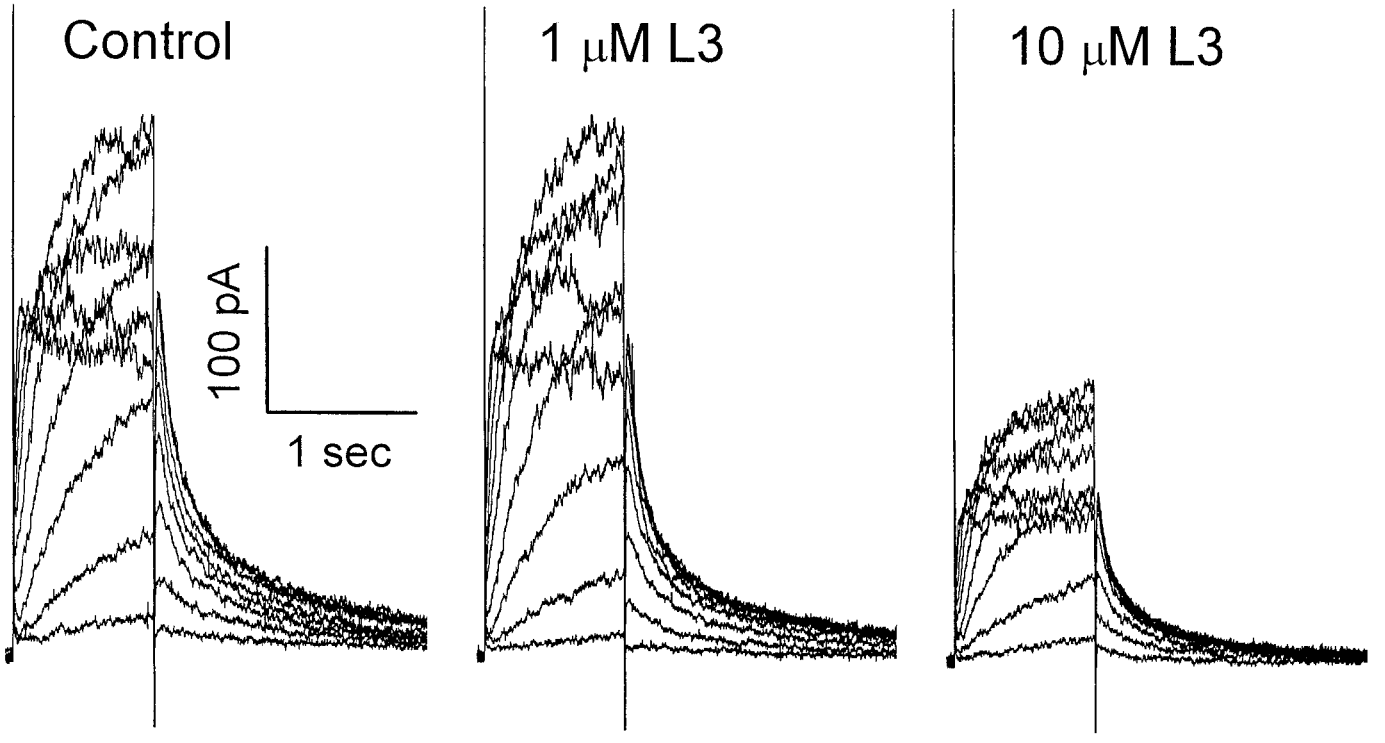


Fig. 12. R-L3 blocks I_{Kr} in AT-1 cells. Currents were measured at room temperature during 1-sec pulses from a V_h of −40 mV to V_t between −30 and +50 mV during control and after the addition of 1 and 10 μM R-L3.

and thereby impart differing biophysical characteristics and pharmacological sensitivity. Alternatively, there may be only one functional I_{Ks} 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 I_{Ks} are better mimicked by cloned KvLQT1 channels, whereas the biophysical properties of I_{Ks} 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 I_{Ks} 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 I_{Ks} face the same possible limitations, including the potential for K^+ accumulation during long depolarizing pulses (Boyett *et al*., 1980, but see also Sanguinetti and Jurkiewicz, 1990), a lack of steady state activation of I_{Ks}, 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 I_{Ks} 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. I_{Kr} blockers were used in excess ($100 \times IC_{50}$ values at 4 mM $[K^+]_o$) to completely block I_{Kr}. Although an increase in $[K^+]_o$ has been reported to decrease the potency of I_{Kr} blockers (e.g., dofetilide; (Yang and Roden, 1996), the 100-fold excess used to block I_{Kr} in this study would not be overcome by potential elevations of $[K^+]_o$. Most currents other than I_{Ks} were also eliminated by pharmacological blockade or inactivation (or both) with V_h.

A drug that activates I_{Ks} could be beneficial for the treatment of certain arrhythmias. The proarrhythmic potential of class III antiarrhythmic drugs that block I_{Kr} prompted the search for agents that would produce more modest prolongation of APD at high doses. The proarrhythmic liability associated with block of I_{Kr} currents was recently confirmed by the finding that mutations in *HERG*, the gene encoding I_{Kr} channel subunits, can cause inherited LQT (Curran *et al*., 1995). Recently, I_{Ks} 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 I_{Ks} and cause a self-limiting prolongation of APD that is less than that produced by I_{Kr} 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 I_{Ks} blockers for prophylactic treatment of ventricular tachyarrhythmias. If I_{Kr} or I_{Ks} 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 I_{Ks} might also be useful for treatment of inherited LQT and abnormally delayed repolarization associated with heart failure (Beuckelmann *et al*., 1993; Tomaselli et al., 1994).

In summary, we described the properties of R-L3, a novel benzodiazepine that selectively activates I_{Ks} in myocytes at low concentrations. This compound represents a new pharmacological probe for the study of I_{Ks} in cardiac myocytes. In addition, R-L3 can be used as a probe to define the physiological role of I_{Ks} 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).

Acknowledgments

We thank Mr. Carl Homnick for conducting the chiral high performance liquid chromatography studies and Qing Xu for preparing the cRNA.

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