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State-Dependent Block of Rabbit Vascular Smooth Muscle Delayed Rectifier and Kv1.5 Channels by Inhibitors of Cytochrome P450-Dependent Enzymes

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ABSTRACT

The effects of the cytochrome P450 inhibitors clotrimazole, ketoconazole, and 1-aminobenzotriazole (1-ABT) on native delayed rectifier (K_{DR}) and cloned Kv1.5 (RPV Kv1.5) K^+ channels of rabbit portal vein (RPV) myocytes were determined using whole-cell and single channel patch-clamp analysis. Clotrimazole reduced K_{DR} and RPV Kv1.5 whole-cell current with respective K_d values of 1.15 ± 0.39 and 1.99 ± 0.6 μM. Clotrimazole acted via an open state blocking mechanism based on the following: 1) the early time course of K_{DR} current activation was not affected, but inhibition developed with time during depolarizing steps and increased the rate of decay in current amplitude; 2) the inhibition was voltage-dependent, increasing steeply over the voltage range of K_{DR} activation; and 3) mean open time of RPV Kv1.5 channels in inside-out patches was decreased significantly. Ketoconazole reduced K_{DR} current amplitude with a K_d value of 38 ± 3.2 μM. However, ketoconazole acted via a closed (resting) state blocking mechanism: 1) K_{DR} amplitude was reduced throughout the duration of depolarizing steps and the rate of decay of current was unaffected, 2) there was no voltage dependence to the block by ketoconazole over the K_{DR} activation range, and 3) ketoconazole did not affect mean open time of RPV Kv1.5 channels in inside-out membrane patches. 1-ABT between 0.5 and 3 mM did not affect native K_{DR} or RPV Kv1.5 current of rabbit portal vein myocytes. Clotrimazole and ketoconazole, but not 1-ABT, suppress vascular K_{DR} channels by direct, state-dependent block mechanisms not involving the modulation of cytochrome P450 enzyme activity.

Vascular smooth muscle K^+ channel activity is critical for the control of arterial tone and blood pressure. K^+ channels regulate the level of resting membrane potential and, thereby, the open probability of L-type Ca^{2+} channels, Ca^{2+} influx, and contraction. At least four different types of K^+ channels, including Ca^{2+}-activated channels of large (BK_{Ca}), intermediate, and small conductance; ATP-sensitive channels (K_{ATP}); inward rectifier channels, and voltage-gated, delayed rectifier channels (K_{DR}) appear to be involved in control of membrane potential, with the contribution of each conductance dependent on the vascular bed, vessel size, physiological condition, and presence of vasoactive agonists (Nelson and Quayle, 1995; Cole and Clément-Chomienne, 2000).

The identification of K^+ channels involved in endothelium-dependent relaxation of vascular smooth muscle has received considerable recent attention. Effects on K^+ channel activity of three different endothelium-derived relaxing factors have been inferred through the use of channel-selective blockers. These factors include 1) nitric oxide, 2) prostacyclin, and 3) an as yet ill-defined endothelium-derived hyperpolarizing factor (EDHF) (Triggle et al., 1999). With regard to the identity of EDHF, several candidate compounds and/or mechanisms have been advanced to account for endothelium-dependent hyperpolarization and relaxation not involving nitric oxide or prostacyclin (Triggle et al., 1999). Epoxide metabolites of cytochrome P450-dependent monooxygenase breakdown of arachidonic acid, epoxyeicosatrienoic acids (EETs), were recently proposed to be EDHFs (Hecker et al., 1994; Campbell et al., 1996; Popp et al., 1996). This conclusion was based on evidence showing that 1) acetylcholine-evoked endothelium-dependent relaxation was suppressed by inhibitors of

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ABBREVIATIONS: BK_{Ca}, large conductance Ca^{2+}-activated K^+ channel; K_{ATP}, ATP-sensitive K^+ channel; K_{DR}, delayed rectifier K^+ channel; EDHF, endothelium-derived hyperpolarizing factor; EET, epoxyeicosatrienoic acid; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channel; Kv, voltage-gated K^+ channel; I-O, inside-out; 1-ABT, 1-aminobenzotriazole; RPV, rabbit portal vein; RPV Kv1.5, rabbit portal vein Kv1.5; HEK, human embryonic kidney; I-V, current-voltage.

cytochrome P450-dependent enzymes (Félétou and Vanhoutte, 1996; Triggle et al., 1999); and 2) application of exogenous EETs affects vascular smooth muscle K^+ channel activity and/or induces hyperpolarization in intact arteries (Campbell et al., 1996; Li et al., 1997). Additionally, EETs have been suggested to participate in the regulation of pulmonary arterial tone by PO_2: decreased cytochrome P450 enzyme activity and reduced EET production during hypoxia were postulated to result in a lower open probability of vascular smooth muscle K_{DR} channels, and thereby, depolarization (Yuan et al., 1995).

Recent studies have raised the possibility, however, that cytochrome P450 inhibitors may suppress ion channel activity in endothelial and/or vascular smooth muscle cells via mechanisms not involving effects on cytochrome P450-dependent enzymes (Alvarez et al., 1992; Villalobos et al., 1992; Edwards et al., 1996; Hatton and Peers, 1996; Rittenhouse et al., 1997; Yamanaka et al., 1998; Vanheel et al., 1999; Wulff et al., 2000). Whole-cell currents due to BK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}), K_{ATP}, and/or Kv channels were shown to be depressed by several P450 inhibitors, and in the case of BK_{Ca}, a decrease in open probability of channels in inside-out (I-O) membrane patches was described. These observations suggest possible nonspecific blocking action of the drugs, but the mechanism responsible was not conclusively determined and the effect on the kinetics of the channels at the whole-cell and unitary current levels was not investigated.

In this study, we used whole-cell and single channel patch-clamp analyses to study the effect of three structurally different inhibitors of cytochrome P450-dependent enzymes, specifically, clotrimazole, ketoconazole, and 1-aminobenzotriazole (1-ABT) on K_{DR} channels of freshly isolated rabbit portal vein (RPV) myocytes and on recombinant Kv channels due to the expression of cDNA encoding rabbit portal vein Kv1.5 (RPV Kv1.5). We had two goals: first, to determine the effect of the P450 inhibitors on the biophysical properties of the whole-cell and microscopic currents; and second, to determine the change in channel activity that accounted for any differences in current amplitude or kinetics. Kv1.5 is expressed by vascular smooth muscle cells of several vessels (Roberds and Tamkun, 1991; Overturf et al., 1994; Wang et al., 1994; Mays et al., 1995; Yuan et al., 1998; Clément-Chomienne et al., 1999) and whole-cell currents due to RPV Kv1.5 closely resemble the properties of native K_{DR} current (Clément-Chomienne et al., 1999). Our results indicate for the first time that clotrimazole and ketoconazole, but not 1-ABT, directly block native and cloned vascular voltage-gated K^+ channels by different state-dependent mechanisms not involving suppression of cytochrome P450 enzyme activity. A preliminary account of some of these data was published previously (Waldron et al., 1999).

Materials and Methods

Rabbit Portal Vein Myocyte Isolation. Rabbits (2–2.5 kg) were killed by an overdose of sodium pentobarbitone (1 ml/kg) injected in the ear vein according to a research protocol consistent with the standards of the Canadian Council on Animal Care and approved by the local Animal Care Committee of the University of Calgary. Single smooth muscle cells of rabbit portal vein were enzymatically dissociated as previously described (Aiello et al., 1995).

Transfection and Cell Culture. RPV Kv1.5 cDNA was obtained as previously described (Clément-Chomienne et al., 1999) and then subcloned and ligated into a mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, CA) using *Kpn*I and *Bam*HI restriction enzymes for subsequent transient transfection into mouse connective tissue L cells or stable transfection of HEK293 cells (American Type Culture Collection, Manassas, VA). To facilitate identification of successful transient transfection, L cells were cotransfected with cDNAs encoding RPV Kv1.5 and a mutant form of green fluorescent protein coupled to a CAG promoter using lipofectin (Life Technologies Gibco BRL, Rockville, MD) (Clément-Chomienne et al., 1999). Transfected HEK293 and L cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) under a 10% CO_2 atmosphere. Transiently transfected cells were stored at 37°C and used within 72 h.

Electrophysiological Measurements. Rabbit portal vein myocytes, L cells, or HEK293 cells were placed in a 300-μl constant flow bath containing physiological saline solution at room temperature (20–22°C) on the stage of an epifluorescent inverted microscope Diaphot-TMD (Nikon, Natick, MA) for study by patch clamp as previously described (Aiello et al., 1995, 1996; Clément-Chomienne et al., 1996, 1999). L cells expressing green fluorescent protein were detected using an HMX Lamphouse (Nikon) with a blue excitation filter (B2, 450–490 nm), a dichroic mirror cutting at 510 nm, and a barrier filter at 520 nm. Single cells were voltage clamped, and whole-cell membrane currents or single channel currents were, respectively, measured using conventional whole-cell and inside-out patch-clamp techniques (Hamill et al., 1981). Pipettes of 1 to 3 and 4 to 5 MΩ for the whole-cell and single channel experiments, respectively, were prepared from capillary glass (7052 glass; Richland Glass Co., Richland, NJ) with a Sutter P-87 puller (Sutter Instruments Co., Novato, CA) and an MF-83 microforge (Narashige Co., Tokyo, Japan). For the whole-cell experiments, the pipette solution contained 110 mM potassium gluconate, 30 mM KCl, 0.5 mM $MgCl_2$, 5 mM HEPES, 5 mM Na_2ATP, 1 mM GTP, and 10 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 7.2, to provide for strong buffering of internal Ca^{2+} and minimal contamination with large Ca^{2+}-activated K^+ and Cl^- currents in the RPV myocytes. The bath solution contained 120 mM NaCl, 3 mM $NaHCO_3$, 4.2 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10 mM glucose, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. For the inside-out patch experiments, the Sylgard-coated pipettes contained 140 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4 with KOH. The bath solution contained 140 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM Na_2ATP, 10 mM HEPES, pH 7.2 with KOH, and was nominally Ca^{2+} free (i.e., no added Ca^{2+}; free Ca^{2+} approximately 1 μM).

Recordings of whole-cell or single channel currents were obtained using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Pipette potential was nulled and a 10 to 15 GΩ seal formed with the cell membrane via application of negative pressure to the pipette interior and pipette capacitance compensated. For the whole-cell voltage-clamp experiments, membrane rupture was achieved by application of additional negative pressure to the pipette interior and series resistance and membrane capacitance (70–80%) were compensated. A series resistance of less than 8 MΩ and leak currents of <25 and <75 pA at -60 mV following membrane rupture were deemed appropriate for the myocytes and L cells, which had input resistance values in the 1 to 3 GΩ range. No leak compensation was used and all myocytes/cells displaying a change in leak of $>40\%$ during the experiments were discarded. Whole-cell voltage-clamp protocols were applied using pClamp 6.0 software (Axon Instruments). Data were filtered at 1 to 2 kHz by an on-board eight-pole Bessel filter before digitization (3–10 kHz) with a Digidata 1200 A/D

convertor (Axon Instruments) and storage on the hard disk of a Pentium PC clone.

Whole-cell current records were displayed and analyzed using pClamp software (Axon Instruments). A consistent value of 10 mV for the junction potential (the difference between the tip potential of 15 pipettes nulled in pipette solution and then immersed in bath solution) was used to correct all whole-cell voltage-clamp protocols. Current-voltage (I-V) relations for end-pulse and tail currents were obtained in the following manner: end-pulse current amplitude was measured at the end of 250-ms command pulses to voltages between −80 and +30 mV. Tail current amplitude was calculated as the difference current between the peak amplitude of the tail and the sustained level of current at −40 mV. All macroscopic current values were normalized for cell capacitance and expressed in pA/pF ± S.E.M. Cell capacitance was determined by integration of the capacity transient. The average values of membrane capacitance of the myocytes and L cells used in this study were 38.8 ± 1.2 pF (n = 63) and 12.5 ± 2.1 pF (n = 37), respectively. Parameters of native currents of freshly isolated portal vein myocytes or whole-cell currents due to expression of RPV Kv1.5 in L cells ± cytochrome P450 inhibitors were compared by paired Student's t test. To assess the closed (resting) state dependence of inhibition of K_{DR} or RPV Kv1.5 whole-cell currents, cells were held at −60 mV for 5-min treatment with clotrimazole or ketoconazole prior to the application of repeated 250-ms depolarizing steps from −60 to +10 mV.

Single channel data were filtered at 2 kHz by an on-board eight-pole Bessel filter before digitization (10 kHz) with a Digidata 1200 A/D convertor (Axon Instruments) and stored to hard disk in a 486 PC clone. Data were displayed and analyzed using pClamp (Axon Instruments): open probability was determined from amplitude histograms determined from recording periods of identical duration (30–60 s) ± cytochrome P450 inhibitor.

Analysis of Whole-Cell and Single Channel Data. Activation and inactivation curves determined from standard voltage-clamp protocols were fitted with Boltzmann equations defined, respectively, as follows:

$$Y\infty = \{1 + \exp[(V_{0.5} - V)/k]\}^{-1} \quad (1a)$$

and

$$Y\infty = \{1 + \exp[(V - V_{0.5})/k]\}^{-1} \quad (1b)$$

where V is membrane voltage, $V_{0.5}$ is the voltage of half-maximal activation or inactivation, and k is the slope constant (mV).

A first order blocking scheme was assumed for the drug-channel interactions as described previously (Snyders and Yeola, 1995) and the following equation was used to determine values for the Hill coefficient (n) and apparent affinity constant (K_d) from concentration-response data:

$$f = 1/\{1 + (K_d/[D]^n)\} \quad (2)$$

where f is the fractional inhibition of current ($f = 1 - I_{drug}/I_{control}$) at a test potential (+20 mV) and [D] is the concentration of drug used. Additionally, a second independent measure was used to estimate the K_d for clotrimazole for comparative purposes. In this case, rate constants were determined by plotting the reciprocal of the time constant (τ_D) for the clotrimazole-induced decay in current amplitude during 250-ms steps to +20 mV against the concentration of the drug. The slope and intercept of the least-squares fit of the data yield the association (k_{+1}) and dissociation (k_{-1}) constants according to the following equation:

$$1/\tau_D = k_{+1}[D] + K_{-1} \quad (3)$$

and the value of K_d is

$$K_d = k_{-1}/k_{+1} \quad (4)$$

To investigate the voltage dependence of block by clotrimazole and ketoconazole we calculated the fractional inhibition produced at potentials positive to −30 mV. These data were then used to determine the voltage dependence according to the Woodhull equation (Woodhull, 1973):

$$f = [D]/\{[D] + K_d(+20\text{ mV}) \times \exp(-z\delta FV/RT)\} \quad (5)$$

where z, F, V, R, and T have their usual thermodynamic meaning; δ is the fractional electrical distance (i.e., the fraction of the transmembrane electrical field sensed by a single charge at its binding site within the channel); and K_d (+20 mV) is the apparent affinity constant at the reference potential of +20 mV.

Analysis of the single channel data were conducted as follows: the number of channels in each patch was unknown, so open probability (P_O) was expressed as NP_O [number of channels (N) × mean P_O of the single channels] determined from the amplitude histograms according to the following equation:

$$NP_o = (A_1 + 2A_2 + 3A_3 + \ldots + nA_n)/(A_o + A_1 + A_2 + A_3 + \ldots + A_n) \quad (6)$$

where A_0, A_1, A_2, A_3, and A_n are the areas under each histogram peak with the channels closed, one open, and simultaneous openings of two to n channels, respectively.

Dwell time analysis of RPV Kv1.5 channel open state was accomplished using idealized traces based on recordings of equal duration (75 s) ± clotrimazole or ketoconazole (a bin width of 0.1 ms was used and events of less than 0.1-ms duration were ignored) at a voltage of +40 mV. Dwell time histograms were fitted using pClamp software (Axon Instruments).

Drugs. Three structurally different inhibitors of cytochrome P450 were used: clotrimazole at concentrations between 0.5 and 30 μM, ketoconazole at 2 to 200 μM, and 1-ABT at 0.5 to 3 mM. All drugs were obtained from Sigma Chemical Co. (St. Louis, MO), prepared in dimethyl sulfoxide or ethanol, and diluted to the desired final concentration in bath solution. Neither ethanol nor dimethyl sulfoxide had an effect on native K_{DR} or RPV Kv1.5 current of five myocytes or L cells at the concentrations used (data not shown).

Results

To determine the effect of treatment with inhibitors of cytochrome P450-dependent enzymes on K_{DR} currents of rabbit portal vein myocytes and currents due to expression of RPV Kv1.5 in L cells, a whole-cell voltage-clamp protocol was used to activate the channels over a range of membrane potentials in the absence and presence of drugs (Fig. 1A). Figure 1 shows the effect of clotrimazole (2.5 μM) on representative families of native K_{DR} (A) and RPV Kv1.5 (C) whole-cell currents evoked between −80 and +30 mV. Clotrimazole depressed the amplitude of current evoked at all potentials positive to −30 mV, and in both cases, it produced an increase in the rate of decay in current amplitude during depolarizing steps to voltages positive to −10 mV. Figure 1, B and D, show average I-V relations for end-pulse and peak tail current amplitudes for the two conductances determined (as described under *Materials and Methods*) from the families of whole-cell currents in five myocytes and four L cells recorded in the absence and presence of drug. Native K_{DR} and RPV Kv1.5 currents showed a statistically significant level of inhibition of end-pulse and tail current amplitude by clotrimazole at all potentials tested positive to −30 mV. These data indicate that clotrimazole was an effective inhibitor of native K_{DR} and RPV Kv1.5 channels, and that an open block mechanism might be involved based on the presence of an in-

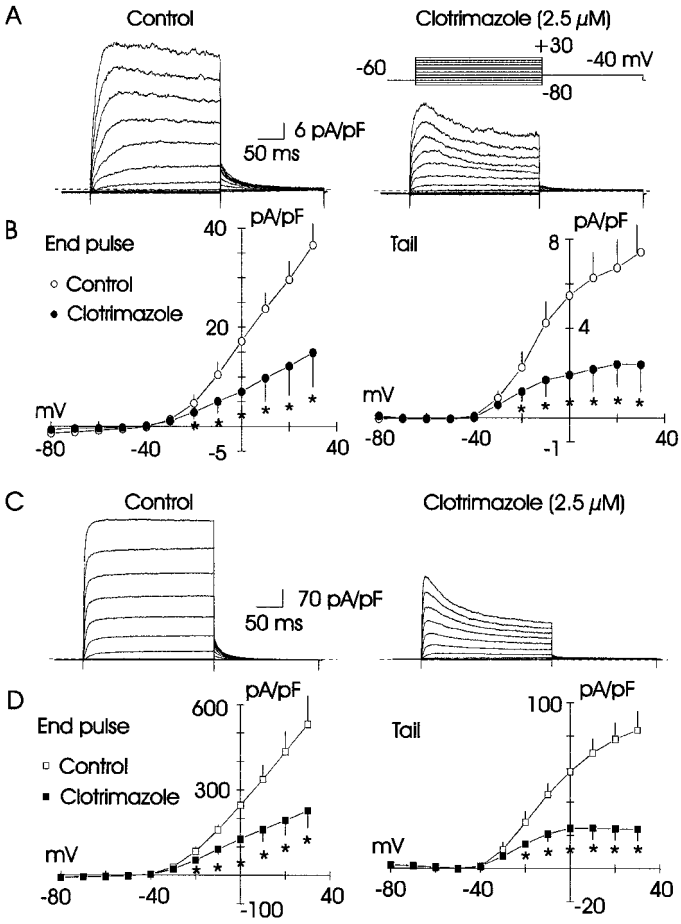


Fig. 1. Inhibition of native K_{DR} and RPV Kv1.5 currents by clotrimazole. A, representative families of whole-cell K_{DR} currents evoked by the indicated voltage-clamp protocol in the absence (control) and presence of 2.5 μM clotrimazole. Scale bar indicates the current density (pA/pF) and time in milliseconds. Dashed line here and in all figures indicates zero current. Note the inhibition of current at all potentials positive to -30 mV and the marked increase in rate of decay of current during command steps to potentials positive to -10 mV. B, average I-V relations for end-pulse and tail current amplitude determined from families of K_{DR} currents and normalized to cell capacitance (as described under *Materials and Methods*) to express the values as current density (pA/pF) versus command step voltage in the absence (control) and presence of 2.5 μM clotrimazole ($n = 5$ myocytes). $*$, the current density in the presence of clotrimazole was significantly different from control by a paired Student's t test ($P < 0.05$). C, representative families of RPV Kv1.5 currents evoked by an identical voltage-clamp protocol, as indicated in A before (control) and after treatment with 2.5 μM clotrimazole. Note that the inhibition of RPV Kv1.5 current by clotrimazole was also accompanied by a marked increased in rate of current decay during the command steps. D, average I-V relations for end-pulse and tail current density due to RPV Kv1.5 channels $\pm$ clotrimazole (2.5 μM) normalized to cell capacitance and expressed as current density (pA/pF) versus command step voltage ($n = 4$ L cells expressing RPV Kv1.5). $*$, the current density in the presence of clotrimazole was significantly different from that in control conditions as determined by a paired Student's t test ($P < 0.05$).

creased rate of decay in current amplitude during depolarizing steps.

Rabbit portal vein K_{DR} currents were also suppressed by ketoconazole. Figure 2A shows a representative example of the effect of ketoconazole (200 μM) on families of whole-cell K_{DR} currents evoked by a similar voltage-clamp protocol as was used for determination of the effect of clotrimazole. Ketoconazole produced a marked inhibition of native K_{DR} current amplitude, but a change in the rate of current decay during pulses to voltages positive to -10 mV was not observed (in contrast to the effect of clotrimazole). The depression of K_{DR} current by ketoconazole was determined for four myocytes and the average changes in the I-V relations for end-pulse and tail current amplitude plotted as a function of the step potential were determined (Fig. 2B). Ketoconazole produced a significant decline in end-pulse and tail current amplitude at all voltages positive to -10 mV, similar to that observed for clotrimazole. These data indicate that this structurally different cytochrome P450 inhibitor suppressed K_{DR} channel activity, but the lack of a change in the rate of decay in current amplitude during the command steps suggested that a different mechanism of channel block might be involved compared with clotrimazole.

Suppression of K_{DR} and RPV Kv1.5 currents by clotrimazole and ketoconazole required approximately 5 min to reach equilibrium, and although the effects of both agents were completely reversed upon washout with control solution, this also required considerable time (>10 min; data not shown). This prolonged time to achieve steady-state block and washout is consistent with an internal site of action.

Figure 3A shows that 1-ABT was without effect on representative whole-cell native K_{DR} currents, and on average in three myocytes, there was no change in the I-V relations for end-pulse or tail current amplitude in the presence compared with the absence of the drug. Similar results were also obtained in three additional myocytes during application of 0.5, 1, and 3 mM 1-ABT and in three L cells expressing RPV Kv1.5 channels (1 mM 1-ABT; data not shown). These data indicate that this structurally different, irreversible blocker of cytochrome P450-dependent enzymes did not affect native K_{DR} or RPV Kv1.5 channels. This finding is in direct contrast to the results obtained for clotrimazole and ketoconazole and suggested that their effect on rabbit portal vein K_{DR} and RPV Kv1.5 channels

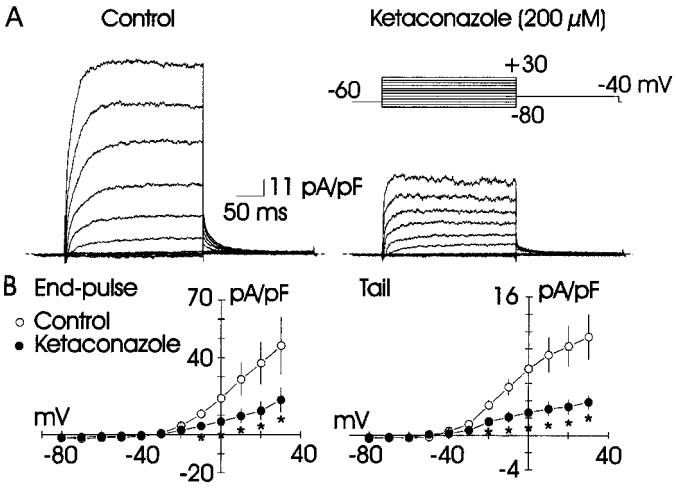


Fig. 2. Inhibition of native K_{DR} current by ketoconazole. A, representative families of whole-cell K_{DR} currents evoked by the indicated voltage-clamp protocol in the absence (control) and presence of 200 μM ketoconazole. Note the lack of change in current decay during the command steps in the presence of ketoconazole. B, average I-V relations for end-pulse and tail current amplitude due to native K_{DR} channels before (control) and during treatment with 200 μM ketoconazole, normalized to cell capacitance, and expressed as current density (pA/pF) ($n = 4$ myocytes). $*$, the value for the current density in the presence of ketoconazole was significantly different from control by paired Student's t test ($P < 0.05$).

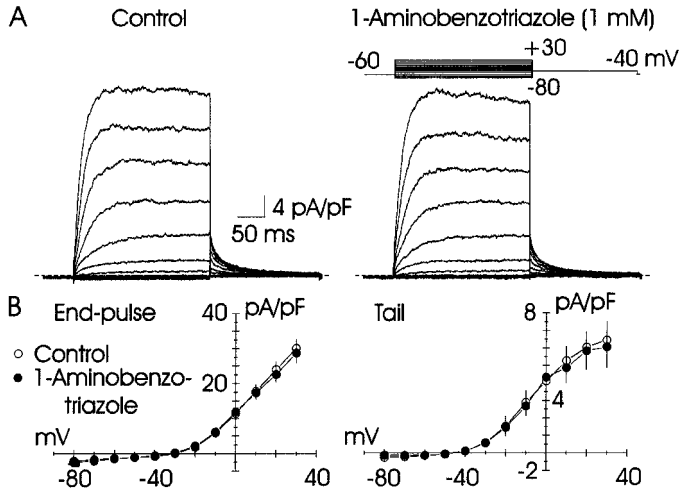


Fig. 3. Lack of effect of 1-ABT on native K_{DR} current. A, representative families of whole-cell K_{DR} currents evoked by the indicated voltage-clamp protocol in the absence (control) and presence of 1 mM 1-aminobenzotriazole. Note the lack of change in current in the presence of this inhibitor of cytochrome P450-dependent enzymes. B, average I-V relations for end-pulse and tail current amplitude due to native K_{DR} channels in the absence (control) and presence of 1 mM 1-ABT normalized to cell capacitance and expressed as current density (pA/pF) (n = 3 myocytes).

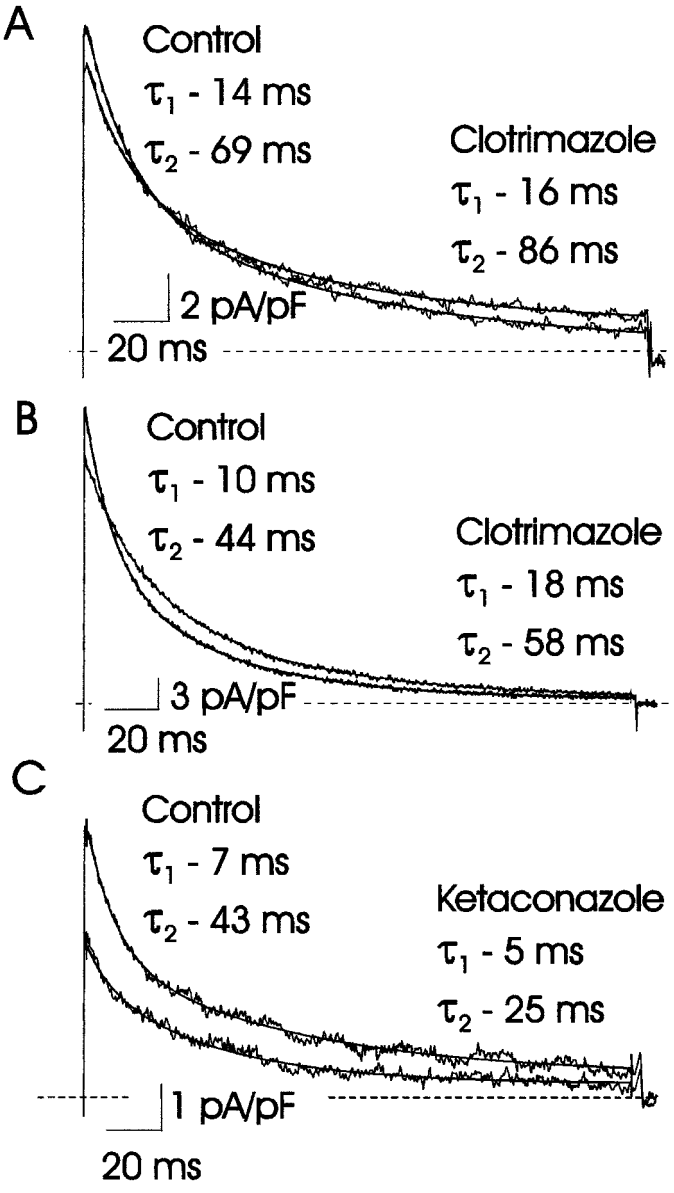


Fig. 4. Clotrimazole but not ketoconazole slows deactivation of native K_{DR} and RPV Kv1.5. A, representative tail currents due to native K_{DR} channels recorded at −40 mV after steps to +30 mV in the absence (control) and presence of 0.5 μM clotrimazole. Values for the fast (τ_1) and slow (τ_2) time constants determined from fitting the decay in tail current density with a two exponential function (the solid lines overlaid on the current traces) are indicated. Note the increase in the value of the second time constant in the presence of clotrimazole. B, representative tail currents due to RPVKv1.5 channels recorded at −40 mV after steps to +30 mV in the absence (control) and presence of 0.5 μM clotrimazole. The indicated values for the fast (τ_1) and slow (τ_2) time constants were determined from fits to the current traces (solid lines) as described for the data in A. Note that the slow time constant of decay in RPV Kv1.5 tail current density showed a similar increase in the presence of clotrimazole as described for the native K_{DR} channels. C, representative tail currents due to native K_{DR} channels recorded at −40 mV after steps to +30 mV in the absence (control) and presence of 50 μM ketoconazole. The indicated values for the fast (τ_1) and slow (τ_2) time constants were determined from fits to the current traces (solid lines) as described for the data in A. Note the absence of any change in the rate of decay in tail current density or the slow time constant in the presence of ketoconazole.

were probably unrelated to an inhibition of cytochrome P450. For this reason, we considered the possibility that a direct inhibition of the channels could be responsible for the actions of clotrimazole and ketoconazole.

Direct block of voltage-dependent K^+ channels can occur via interaction with the channels while in the closed (resting), open, and/or inactivated states. The differences in the rate of decay during depolarizing steps in the presence of clotrimazole versus ketoconazole suggested that different state-dependent mechanisms of block might be involved. For this reason, we performed several analyses of the effects of clotrimazole and ketoconazole on the kinetics of native K_{DR} and RPV Kv1.5 activation, deactivation, and inactivation to provide evidence that would permit us to identify the different state dependencies of channel block by these compounds. To determine whether an open block of the channels might be involved, we analyzed the effects of clotrimazole and ketoconazole on the rate of decay in tail current amplitude upon repolarization to −50 mV, which reflects the kinetics of channel deactivation (closure). Figure 4 shows representative effects of clotrimazole (0.5 μM) and ketoconazole (2 μM) on the time course of K_{DR} or RPV Kv1.5 tail currents recorded at −40 mV following depolarizing steps to +30 mV. Clotrimazole slowed the time course of current decay upon repolarization of the membrane potential, and at lower concentrations of the drug (<1 μM), this produced an obvious "crossover" of K_{DR} and RPV Kv1.5 tail currents: peak amplitude was depressed but the level of current after 200 ms was greater in the presence compared with the absence of drug. In contrast, ketoconazole reduced peak tail current amplitude without affecting the time course of decay of native K_{DR} tail currents at all concentrations that were used (Fig. 4 shows data for 50 μM ketoconazole). The decay of K_{DR} and RPV Kv1.5 tail currents under control conditions was best fitted with a two-exponential function with fast and slow time constants, as previously reported (Clément-Chomienne et al., 1999). Clotrimazole caused a significant increase in the slow time constant (58 ± 14 to 104 ±17 ms at 0.5 μM; $P <$ 0.05), but did not affect the fast time constant of K_{DR} deactivation (13 ± 2 and 16 ± 2 ms; $P > 0.05$). RPV Kv1.5

currents were similarly affected: the slow time constant increased from 44 ± 4 to 70 ± 14 ms ($P < 0.05$), but there was no change in fast time constant [12 ± 2 and 16 ± 2 ms ($P > 0.05$)]. Neither ketoconazole ($n = 3$ or 4) nor 1-ABT ($n = 3$) altered deactivation [ketoconazole before and after: 37 ± 8 and 31 ± 5 ms at 2 μM, 49 ± 6 and 32 ± 5 ms at 50 μM, 57 ± 6 and 43 ± 11 at 200 μM ($P > 0.05$); 1-ABT: 64 ± 13 and 57± 9 ms at 1 mM ($P > 0.05$)]. A similar depression of whole-cell currents due to expression of cDNA encoding cardiac Kv1.5 channel protein in *Xenopus laevis* oocytes by ketoconazole was previously reported, but the effects of clotrimazole and 1-ABT were not examined (Dumaine et al., 1998). The slower decay of native K_{DR} and RPV Kv1.5 tail currents in the presence of clotrimazole is consistent with the view that this drug blocks the channels in the open state, but must first dissociate from its binding site before the channels can close. The lack of change in deactivation in the presence of ketoconazole was consistent with the view that the channels were not affected by this drug while in the open state.

The voltage dependence of activation and inactivation of K_{DR} were, respectively, determined using eqs. 1a and 1b defined under *Materials and Methods*. For determination of the former, normalized values of peak tail current were measured in the absence and presence of clotrimazole (1 μM) or ketoconazole (50 μM). No change in the value of membrane voltage required for half-maximal activation was detected in the presence of either compound: the respective values for the $V_{0.5}$ (−14.4 ± 3.4 and −14.3 ± 4.3 mV) and k (10.1 ± 1 and 9.9 ± 0.9 mV) for activation in control and clotrimazole were not different ($n = 6$; $P > 0.05$) nor were the respective values of $V_{0.5}$ (−16.7 ± 1.7 and −19.3 ± 1.5 mV) and k (12.1 ± 1.4 and 10.2 ± 1.5 mV) before and after ketoconazole treatment ($n = 6$; $P > 0.05$). Similarly, no changes in activation were evident as determined by a comparison of the amplitude of current evoked at +20 mV following 10-s steps to a range of voltages between −110 and +20 mV in the absence and presence of drug. There was no difference in the respective values of $V_{0.5}$ (−42.0 ± 1.3 and −44.8 ± 2.4 mV) and k (7.2 ± 0.5 and 7.4 ± 0.4 mV) of inactivation in control and clotrimazole ($n = 6$; $P > 0.05$ in both cases) or for $V_{0.5}$ (−40.5 ± 2.2 and −41.9 ± 7.2 mV) and k (8.5 ± 0.4 and 8.2 ± 1.2 mV) before and after ketoconazole ($n = 4$; $P > 0.05$).

The time course of inactivation of K_{DR} currents during 10-s pulses was best fitted with a two-exponential function (Clément-Chomienne et al., 1999). The decay of current was notably faster in the presence of clotrimazole (10 μM; $n = 4$); there was a significant decrease in the fast time constant from 678 ± 154 to 133 ± 22 ms ($P < 0.05$), but the slow time constant was unaffected (3001 ± 335 and 3343 ± 450 ms; $P > 0.05$). In contrast, the decay of current was best fitted by a single exponential during ketoconazole (50 μM; $n = 3$) treatment and the time constant (2692 ± 182) had a value similar to that of the slow time constant in control conditions (3393 ± 632; $P > 0.05$). These data suggest that the rate of association of clotrimazole with the channels was considerably more rapid than their rate of inactivation. The absence of the first component of inactivation in the presence of ketoconazole reflects the absence of an influence on the channels after they were activated. Significantly, no difference in the recovery kinetics was observed in the presence of clotrimazole or ketoconazole (data not shown). These data suggest that clotrimazole and ketoconazole probably do not interact with the channels while in the inactivated state.

The concentration dependence of inhibition of native K_{DR} and/or RPV Kv1.5 currents by clotrimazole (between 0.5 and 10 μM) and ketoconazole (between 2 and 200 μM) is demonstrated in Fig. 5. Note the increased rate of current decay during depolarizing steps at all concentrations of clotrimazole used, but lack of a similar effect of ketoconazole at any concentration used. Figure 5B shows concentration-response curves determined for three to six myocytes or L cells exposed to each concentration of clotrimazole or ketoconazole by normalizing end-pulse current amplitude in the presence of drug to the control amplitude. Inhibition of K_{DR} and RPV Kv1.5 currents by clotrimazole occurred with similar respective values for K_d of 1.15 ± 0.39 and 1.99 ± 0.6 μM ($P > 0.05$), and Hill coefficient of 1.1 ± 0.2 and 1.2 ± 0.2 ($P > 0.05$). RPV Kv1.5 current was completely inhibited at 25 μM, but the native current was only depressed to a maximum level of approximately 30% of that in control conditions, indicating the presence of a clotrimazole-resistant component(s) of outward current. By comparison, ketoconazole was less potent, with a K_d of 38 ± 3.2 μM (Hill coefficient of 1.2 ± 0.4) in RPV myocytes, however, the maximal level of inhibition (~70%) was similar to that observed with clotrimazole. The inability of clotrimazole and ketoconazole to completely suppress whole-cell outward current is consistent with previous observations showing a residual component of noninactivating outward current of portal vein myocytes that is insensitive to 4-aminopyridine (Aiello et al., 1996; Clément-Chomienne et al., 1996). The identity of this con-

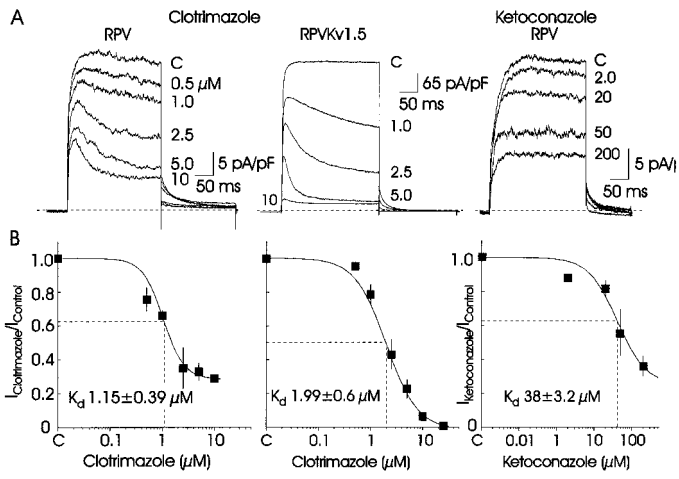


Fig. 5. Concentration dependence of inhibition of native K_{DR} and RPV Kv1.5 channels by clotrimazole and ketoconazole. A, representative currents due to native K_{DR} (RPV) or RPV Kv1.5 channel activity recorded during 250-ms test pulses to +10 mV from a holding potential of −60 mV before (C) and in the presence of varied concentrations of clotrimazole (0.5–10 μM) or ketoconazole (2–200 μM). B, concentration-response relations for inhibition of currents due to native K_{DR} or RPV KV1.5 channels by clotrimazole or ketoconazole. Each data point is the average of values of end-pulse current amplitude recorded during depolarizing steps to +10 mV from more than three myocytes or L cells in the presence of clotrimazole or ketoconazole and normalized to the amplitude of current recorded in the absence of drug (C). Average values for the apparent affinity constant (K_d) for the P450 inhibitor-sensitive component of whole-cell current were determined from the best fit of eq. 2 under *Materials and Methods* to the data and are indicated in each plot.

ductance remains to be defined. The analysis of Hill coefficient suggests that a single binding site was responsible for the inhibition produced by clotrimazole, as well as by ketoconazole.

Values for the time constant of the increase in decay of native and RPV Kv1.5 current during steps to +20 mV in the presence of varied concentrations of clotrimazole (0.5–10 μM) were determined by fitting the decay in each trace with a single exponential. Average values of the reciprocal of these time constants of current decay were calculated for four to five myocytes at each concentration and then used to derive an independent estimate of the values for K_d, and association and dissociation rate constants by creating plots of the average value of the reciprocal of the time constant for current decay versus concentration of drug (Fig. 6). The values of the apparent association (k_{+1}) and dissociation (k_{-1}) rate constants were 2.31 ± 0.28 μM^{-1} s^{-1} and 6.37 ± 1.45 s^{-1} based on the slope and *y*-intercept of the linear regression line through the data points (eq. 3 under *Materials and Methods*). These values where then used to yield a derived value for the K_d of 2.7 μM according to eq. 4 under *Materials and Methods*. This value is consistent with the value determined from the concentration-response curve shown in Fig. 5. Similar values of 2.60 ± 0.28 μM^{-1} s^{-1}, 7.81 ± 0.35 s^{-1}, and 2.98 μM for k_{+1}, k_{-1}, and K_d, respectively, for clotrimazole block of RPV Kv1.5 were obtained (Fig. 6).

The increased rate of decay in current amplitude and crossover of tail currents in the presence of clotrimazole, but not ketoconazole, suggested that different state-dependent mechanisms of channel block might be involved in the inhibition of K_{DR} and RPV Kv1.5 channels by these drugs.

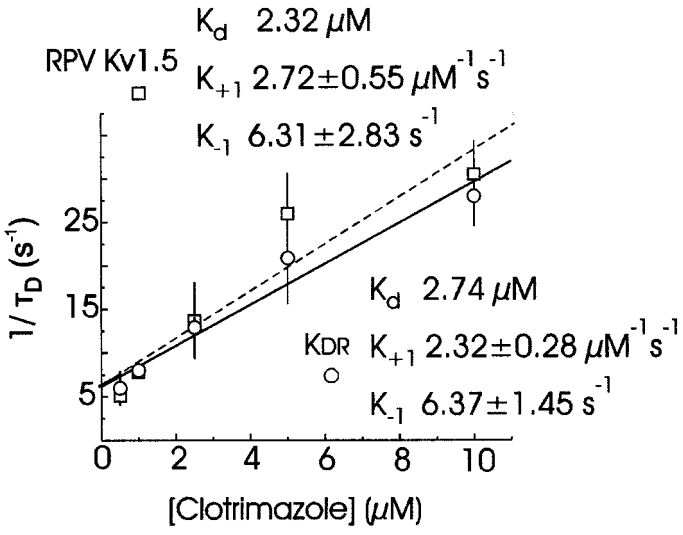


Fig. 6. Time constant of decay in evoked current due to clotrimazole as a function of drug concentration. Values for the time constant (τ_D) of decay of current density recorded from myocytes or L cells expressing RPV Kv1.5 during command steps to +20 mV in the presence of varied concentrations of clotrimazole were determined by fitting current traces with a single exponential function. The reciprocal of the time constant ($1/\tau_D$) in seconds^{-1} was determined for each recording and average values for three to four myocytes or L cells expressing RPV Kv1.5 were plotted against the concentration of clotrimazole. The apparent association (k_{+1}) and dissociation (k_{-1}) rate constants were calculated from the slope and *y*-intercept, respectively, of the least-squares fit of the data for the myocytes (solid line) and L cells (dashed line) according to eq. 3 under *Materials and Methods*. The indicated K_d values were then determined from these values for k_{+1} and k_{-1} according to eq. 4 under *Materials and Methods*.

Specifically, that clotrimazole was interacting with the channels after activation, whereas they were inhibited by ketoconazole while in the resting state. Three additional approaches were therefore used to assess the state dependence of action of clotrimazole and ketoconazole: 1) the voltage dependence of block was determined, 2) a voltage-clamp protocol was applied to assess the extent of closed (resting) channel block of K_{DR} currents at −60 mV by the drugs, and 3) the effect of the drugs on mean open time of RPV Kv1.5 channels was determined. Figure 7 shows the average relative inhibition ($1 - I_{drug}/I_{control}$) plotted against voltage of the command step for concentrations of clotrimazole or ketoconazole that produced approximately half-maximal inhibition of whole-cell current. Block by clotrimazole increased steeply in a voltage-dependent manner over a range of potentials coinciding with the voltage dependence for K_{DR} channel activation. The superimposed dashed line in Fig. 7 is the activation curve for K_{DR} current in control conditions as determined from plots of normalized tail current amplitude versus command step voltage that were fitted with eq. 1a under *Materials and Methods*. In contrast, the inhibition by ketoconazole did not exhibit any voltage dependence over the same range of membrane potentials. Similar results were obtained for RPV Kv1.5 currents in the presence of clotrimazole and

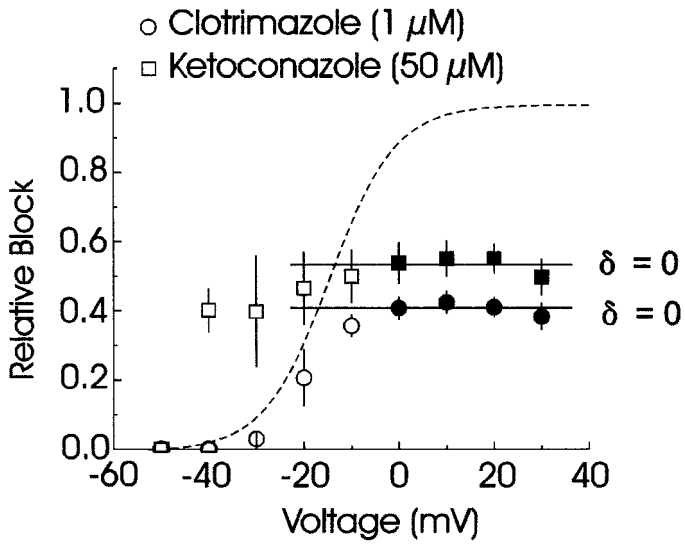


Fig. 7. Voltage dependence of inhibition of native K_{DR} channels by clotrimazole and ketoconazole. The extent of inhibition (relative block) of end-pulse K_{DR} current density by clotrimazole (circles) and ketoconazole (squares) applied to myocytes at concentrations approximately equivalent to the IC_{50} values determined in Fig. 5B was determined for a range of command step potentials between −50 and +30 mV and plotted as a function of membrane voltage for each step. The relative block by clotrimazole increased sharply between −40 and −10 mV coincident with the voltage range of K_{DR} channel activation as is indicated by the dashed, sigmoidal line, which represents the steady-state activation curve for native K_{DR} current determined from the best fit of a Boltzmann function (eq. 1a under *Materials and Methods*) to a plot of normalized K_{DR} tail current amplitude versus voltage of the command pulse under control conditions. This indicates that clotrimazole block was associated with channel activation. In contrast, the relative block by ketoconazole did not change over this range of K_{DR} channel activation. The solid lines in the plot represent the best fits of the Woodhull equation (eq. 5 under *Materials and Methods*) to the filled data points positive to 0 mV at which K_{DR} current activation is essentially complete. The slope of the lines represents the fraction of the electrical field of the membrane, δ, which is sensed by the drugs at their binding site within the channel. The lack of change in extent of block yields δ values of zero.

ketoconazole (data not shown). These data are consistent with the view that clotrimazole and ketoconazole act via open and closed channel block mechanisms, respectively. Neither drug produced additional block of current positive to 0 mV and least-squares curve fitting (solid lines in Fig. 7) of these data points (filled symbols) yielded a slope value of zero. This suggests that there was no effect of the electrical field on the interaction of clotrimazole or ketoconazole with the channels (i.e., $\delta = 0$ in both cases according to eq. 5) and, therefore, that the non-ionized form of the drug probably interacted with the channel.

Figure 8 shows representative data from two myocytes exposed to clotrimazole and ketoconazole. Three-step depolarizations to +20 mV were applied under control conditions prior to 5 min of drug treatment at −60 mV followed by the application of three subsequent depolarizing steps in the presence of drug (at a frequency of 0.1 Hz). The 5-min interval at a sustained holding potential of −60 mV would allow accumulation within the intracellular compartment and permit block to develop if the inhibitors affected K_{DR} channels while in the resting state. A steady-state level of reduction in end-pulse current amplitude was achieved during the first pulse in the presence of both drugs, however, the early time course of current activation was differentially affected. The expanded time base recordings of Fig. 8 indicate that K_{DR} current activated over an identical time course during the first 15 to 20 ms of depolarization ± clotrimazole; i.e., early activation of K_{DR} was unaffected and block developed during the pulse. In contrast, the depression in current amplitude by ketoconazole was evident throughout the depolarizing steps. Similar results were obtained in three additional myocytes for each drug.

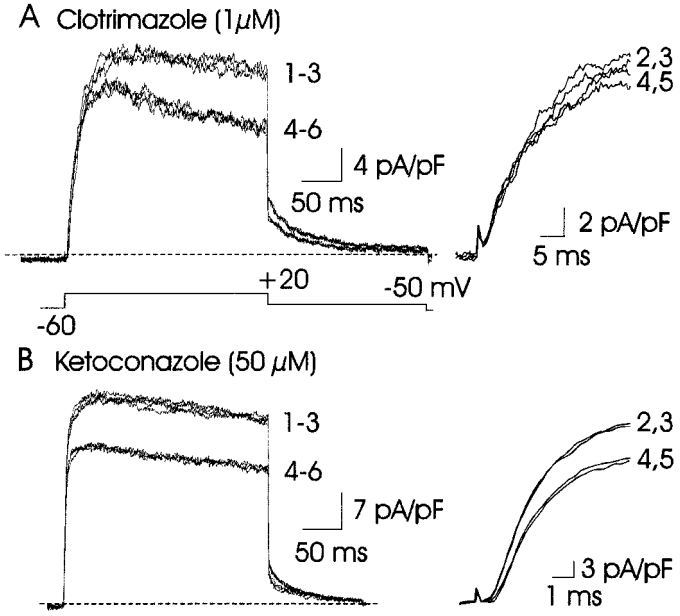


Fig. 8. Distinct effect of clotrimazole and ketoconazole on K_{DR} current activation. A, traces on the left are representative recordings of K_{DR} currents evoked by depolarizing steps to +20 mV applied at a frequency of 0.1 Hz before (traces 1–3) and after (traces 4–6) 5-min treatment with clotrimazole (1 μM) at a constant holding potential of −60 mV. The traces on the right are expanded versions of selected traces shown on the left to show the first 30 ms of two depolarizing steps in the absence (2, 3) and presence of clotrimazole (4, 5). Note that the initial activation of K_{DR} current during the first 30 ms after the start of the command step (note residual capacitance transient) was unaffected by clotrimazole, but block developed during the command step and the end-pulse amplitude indicated in the traces on the left was depressed, consistent with an open channel block mechanism. B, traces on the left are representative recordings of K_{DR} currents evoked by depolarizing steps to +20 mV applied at a frequency of 0.1 Hz before (traces 1–3) and after (traces 4–6) 5-min treatment with ketoconazole (50 μM) at a constant holding potential of −60 mV. The traces on the right are expanded versions of selected traces shown on the left to show the first 7 ms of the depolarizing steps in the absence (2, 3) and presence of ketoconazole (4, 5). Note that the inhibition of K_{DR} current by ketoconazole immediately after the start of the command step and the activation of channel activity, consistent with a closed state block mechanism.

Figures 9 and 10 show representative recordings of RPV Kv1.5 channel activity at +40 mV and open dwell time histograms for cells treated with clotrimazole and ketoconazole, respectively. Multiple RPV Kv1.5 channels were consistently present in the inside-out patches of the HEK293 cells, precluding an analysis of channel closed time. Under the symmetrical 140/140 mM KCl recording conditions used, both 50 μM clotrimazole and 100 μM ketoconazole reduced RPV Kv1.5 channel open probability: average values of NPo were 0.118 ± 0.039 and 0.049 ± 0.025 before and during clotrimazole ($P < 0.05$; $n = 4$), and 0.350 ± 0.101 and 0.068 ± 0.026 before and during ketoconazole ($P < 0.05$; $n = 3$), respectively. In contrast to the inhibition produced in the whole-cell experiments, the block of RPV Kv1.5 channels in the I-O membrane patches was rapid and evident within 10 to 15 s of the application of the drugs to the bath solution, consistent with the view that the drugs bind to an internal site on the channel.

Clotrimazole caused an obvious decrease in the duration of transitions to the open state (Fig. 9), and mean open time decreased from 4.62 ± 0.20 to 2.68 ± 0.30 ms ($P <$

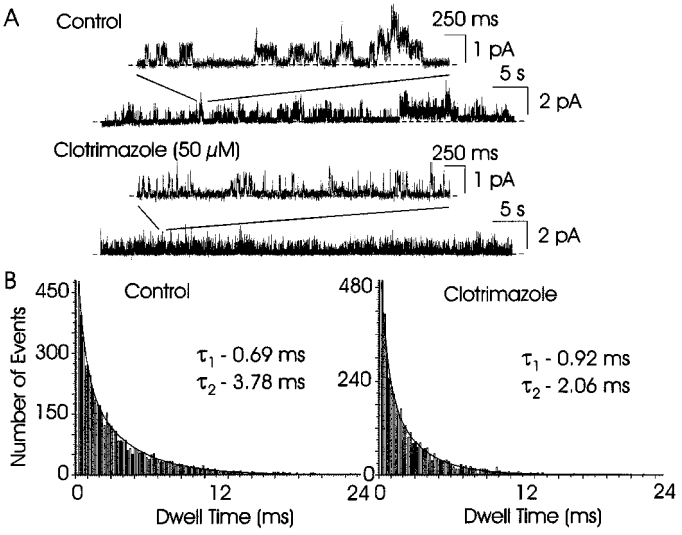


Fig. 9. Effect of clotrimazole on single RPV Kv1.5 channel activity. A, representative recordings of RPV Kv1.5 channel activity in an inside-out membrane patch containing three channels before (control) and during treatment with clotrimazole (50 μM) at +40 mV under symmetrical 140/140 mM KCl recording conditions are shown. Expanded traces from the indicated regions of the recordings are displayed above each record. Deflections in the upward direction reflect transition to open state from the closed level indicated by the dashed line. Note the very transient nature of these transitions in the presence of clotrimazole. B, open state dwell time histograms for RPV Kv1.5 channels determined from identical 75-s recording periods before (control) and during clotrimazole (clotrimazole) treatment for the experiment shown in A. The histograms were best fitted with a two-exponential function (solid line) with the indicated time constants.

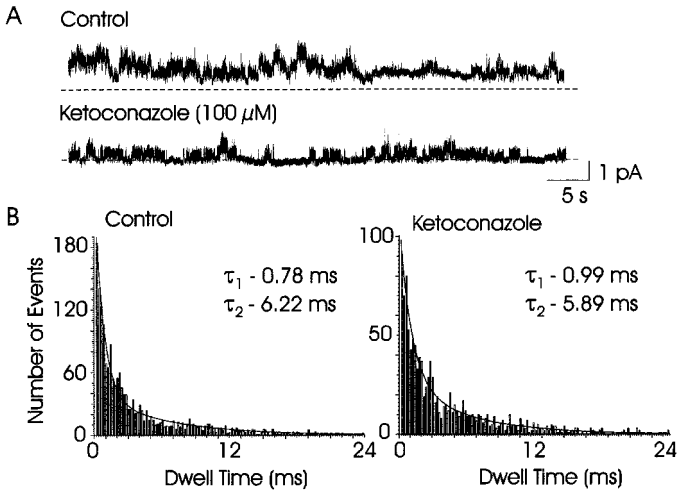


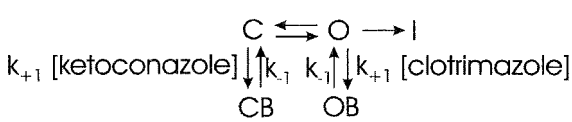
Fig. 10. Effect of ketoconazole on single RPV Kv1.5 channel activity. A, representative recordings of RPV Kv1.5 channel activity in inside-out membrane patch containing four channels before (control) and during treatment with ketoconazole (100 μM) at +40 mV under symmetrical 140/140 mM KCl recording conditions. Note the decline in level of current consistent with a decline in open probability, but lack of change in the duration of the openings that occurred in the presence of ketoconazole. B, open state dwell time histograms for RPV Kv1.5 channels determined from identical 75-s recording periods before (control) and during ketoconazole (ketoconazole) treatment for the experiment shown in A. The histograms were best fitted with a two-exponential function (solid line) with the indicated time constants.

0.05; $n > 300$ transitions in four patches in each condition). In contrast, openings of relatively long duration were still observed, and mean open time did not change in the presence of ketoconazole: 5.02 ± 0.68 compared with 5.01 ± 0.54 ms ($P > 0.05$; $n > 200$ transitions in three patches in each condition) (Fig. 10). Figures 9B and 10B show that the open dwell time histograms were best fitted by a double exponential function, consistent with the view that RPV Kv1.5 channels can occupy at least two open states with different transition rates to the closed (resting or inactivated) state, as was described for the human cardiac Kv1.5 isoform (Rich and Snyders, 1998). Figure 10 shows that ketoconazole was without effect on time constants for transition to the closed state: average values of τ_1 and τ_2 in the absence and presence of drug were 0.67 ± 0.08 and 0.76 ± 0.06 ms and 6.26 ± 0.78 and 4.81 ± 0.89 ms, respectively ($P > 0.05$ in both cases). This is consistent with the absence of a blocking interaction with channels while they are in the open state. Clotrimazole treatment did not affect the value of τ_1 [0.82 ± 0.06 versus 0.74 ± 0.09 ms ($P > 0.05$)], but it did produce a significant decline in τ_2 from 4.31 ± 0.35 to 2.07 ± 0.08 ms ($P < 0.05$) (Fig. 9). This increased rate of transition to a closed (blocked) state is consistent with the view that clotrimazole has a preferential interaction with the channel while it is in the open state.

Discussion

This study provides novel evidence that vascular K_{DR} channels are inhibited by drugs that affect cytochrome P450 by mechanisms not involving suppression of enzyme activity and reduced levels of epoxides. The data indicate for the first time that vascular K_{DR} channels are blocked by clotrimazole and ketoconazole, but not by 1-ABT, via distinct mechanisms involving direct open and closed (resting) state inhibition, respectively. The data have important implications for the interpretation of pharmacological studies using cytochrome P450 inhibitors for identification of epoxides as modulators of vascular smooth muscle K_{DR} channel activity and tone.

This study shows that clotrimazole, ketoconazole, and 1-ABT have different effects on native K_{DR} and cloned Kv1.5 channels of rabbit portal vein myocytes at concentrations consistent with those used to inhibit cytochrome P450-dependent enzymes in cell-free conditions (for a review of cytochrome P450 inhibition, see Halpert, 1995) and shown to depress endothelium-dependent vascular relaxation (Hecker et al., 1994; Campbell et al., 1996; Popp et al., 1996). Moreover, we found that clotrimazole and ketoconazole affected K_{DR} and RPV Kv1.5 channels via different mechanisms, involving a use-dependent, open state block and a non-use-dependent, closed (resting) state block, respectively. The evidence indicating the involvement of divergent mechanisms of channel block is as follows: clotrimazole did not affect the early time course of K_{DR} current activation, but it increased the rate of decay of current during depolarizing steps, slowed the time course of deactivation producing a crossover of tail current, and decreased mean open time of RPV Kv1.5 channels in inside-out membrane patches. Additionally, the block by clotrimazole was voltage-dependent and increased steeply over a range of membrane potentials coinciding with the activation of K_{DR} and RPV Kv1.5 channels. These data indicate that the channels were blocked by clotrimazole after transition to the open state during each pulse, and that unblock was required for channel closure, similar to the situation described for classical open channel blockers of Kv1.5 and other voltage-gated K^+ channels by drugs such as quinidine (Snyders and Yeola, 1995; Yeola et al., 1996). In contrast, ketoconazole caused a stable depression in the early time course of K_{DR} current activation and it did not affect the rate of decay of K_{DR} current during depolarizing steps, the decay of K_{DR} tail currents, or mean open time of RPV Kv1.5 channels. Moreover, there was no voltage dependence in the extent of block over the range of membrane potentials associated with the activation of the channels. This indicates that ketoconazole affected the channels prior to activation, likely via an interaction with the channels in the closed (resting state). A similar conclusion with regards to mechanism of block of cardiac Kv1.5 channels by ketoconazole was reached by Dumaine et al. (1998) based on an analysis of whole-cell current data. We cannot entirely rule out the possibility that the drugs also interacted with the channels in the inactivated state, but the lack of change in voltage dependence or time course of recovery from inactivation suggests that this was unlikely. On the basis of these observations, the actions of clotrimazole and ketoconazole can be interpreted by the kinetic scheme as follows:



(7)

where C represents the resting state of the channel (this simplification corresponds to the four independent conformations C_0, C_1, C_2, and C_3 in a Hodgkin-Huxley model); O is the open state; I is the inactivated state; and OB and CB are the

drug-bound open and closed states produced by interaction with clotrimazole and ketoconazole, respectively.

The direct inhibition of native K_{DR} and RPV Kv1.5 channels by clotrimazole and ketoconazole via interactions with the open and closed states, respectively, contrasts with the lack of change observed in the presence of 1-ABT. If clotrimazole, ketoconazole, and 1-ABT were all acting via a common mechanism involving the suppression of cytochrome P450-dependent synthesis of an epoxide required for maintenance of channel activity, identical alterations in whole-cell current amplitude and kinetics due to a single state-dependent mechanism would have been expected. The disparate effects of these three inhibitors, and the clearly different state dependence of inhibition produced by clotrimazole and ketoconazole, are, therefore, not consistent with a role for an epoxide in the modulation of K_{DR} channels in rabbit portal vein or RPV Kv1.5 channels expressed in L cells. This conclusion is consistent with the reported inability of an epoxide, 11,12-EET to affect 4-aminopyridine-sensitive K_{DR} channels of cell-attached patches of coronary arterial myocytes (Li et al., 1997), but it is in direct contrast with the conclusion of Yuan et al. (1995) that cytochrome P450 enzyme activity is required for the maintenance of rat pulmonary arterial Kv currents. Inhibition by cytochrome P450 inhibitors of Kv currents of vascular myocytes isolated from other vessels has been described (Yuan et al., 1995; Edwards et al., 1996; Vanheel et al., 1999). However, the mechanism responsible for the suppression of channel activity was not firmly established: on the basis of the whole-cell experiments conducted it was not possible to differentiate between an epoxide-based mechanism or channel block due to non-specific actions of the cytochrome P450 inhibitors and no attempt was made to determine the change in channel kinetics or mechanism of channel block involved. However, the novel understanding of the actions of clotrimazole and ketoconazole provided by the present study gives insight into the mechanism of pulmonary arterial block by miconazole (Yuan et al., 1995) and rat portal vein Kv channel by proadifen and 17-octadecynoic acid (Edwards et al., 1996). The rate of decay in current amplitude during depolarizing steps was markedly enhanced by these P450 inhibitors, identical to the effect of clotrimazole on rabbit portal vein K_{DR} in the present study. For this reason, it is likely that a direct open channel block mechanism accounts for the effects of these other cytochrome P450 inhibitors on vascular Kv channels.

The binding site and structural moiety of clotrimazole and ketoconazole responsible for direct inhibition of native K_{DR} and cloned Kv1.5 channels of rabbit portal vein myocytes remain to be determined; however, some speculation with regards to these points based on the current data is warranted. The slow onset of inhibition in whole-cell experiments and rapid block of single RPV Kv1.5 channels in I-O membrane patches by bath applied clotrimazole and ketoconazole imply that an internal blocking site was probably involved. Consistent with this view, previous experiments using a membrane-impermeant derivative of clotrimazole showed that interaction with an internal binding site was required for inhibition of IK_{Ca} channels (Dunn, 1998). Imidazole-ring based compounds, such as clotrimazole and ketoconazole, produce a noncompetitive inhibition of cytochrome P450 enzymes by reversibly binding to and forming a complex with the heme moiety in the active center of the enzyme. However, the imidazole ring does not seem to be required for clotrimazole inhibition of ferret portal vein BK_{Ca} channels: a clotrimazole metabolite lacking the ring also produced channel block (Rittenhouse et al., 1997). Similarly, block of IK_{Ca} by a series of compounds based on clotrimazole was found to be independent of the imidazole ring, which was required for cytochrome P450 inhibition (Wulff et al., 2000). Clotrimazole and ketoconazole contain aromatic rings and in this respect they are similar in structure to well recognized K^+ channel blockers, such as phencyclidine and quinidine. These drugs are known to inhibit native vascular K_{DR} and/or Kv1.5 channels (Beech and Bolton, 1989; Yeola et al., 1996), and in the case of quinidine, the binding site has been identified to be within the internal vestibule of the Kv1.5 channel and to require channel opening to permit drug access to the binding site (Yeola et al., 1996). It is possible that the aromatic ring of clotrimazole also interacts with this inner vestibule binding site. It is difficult, however, to reconcile the closed channel block and considerably higher K_d value observed with ketoconazole with the idea that this drug also interacts with this inner vestibule binding site. However, both compounds are weak bases (pK_a values in the range of 7–8) and appear to interact with the channels in the non-ionized form at intracellular pH. This is suggested by the lack of any indication of voltage dependence in the fractional block produced by the drugs positive to +10 mV. Quinidine, on the other hand, demonstrates a slightly enhanced block at potentials positive to +10 mV consistent with the view that the charged species interacts with Kv1.5 (Snyders and Yeola, 1995). It is possible that in its uncharged form, ketoconazole may have access to the inner vestibule to elicit channel block in the closed state. Clearly, additional structure-activity studies combined with a mutation analysis of the Kv channel subunits, which constitute vascular Kv channels (e.g., Kv1.5), will be required to determine whether the aromatic moiety of the P450 inhibitors interacts with a binding site within the internal vestibule of the channels.

In summary, this study provides novel evidence that the activity of vascular K_{DR} channels can be suppressed by a direct, state-dependent interaction with inhibitors of cytochrome P450-dependent enzymes. These data combined with reports of non-specific effects of cytochrome P450 inhibitors on other types of K^+ channels that are known to be expressed in vascular myocytes and endothelial cells (e.g., BK_{Ca}, IK_{Ca}, K_{ATP}) suggest that considerable caution should be exercised in the interpretation of experiments involving inhibition of endothelium-dependent relaxation of intact vascular preparations by inhibitors of cytochrome P450-dependent enzymes.

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