Imipramine Blocks Rapidly Activating and Delays Slowly Activating K⁺ Current Activation in Guinea Pig Ventricular Myocytes

Carmen Valenzuela, José Sánchez-Chapula, Eva Delpón, Alejandro Elizalde, Onésima Pérez, Juan Tamargo

Abstract Imipramine is a tricyclic antidepressant drug that also exhibits antiarrhythmic effects and whose clinical spectrum of activity is similar to that of quinidine. It has been previously demonstrated that imipramine inhibits the aggregate time-dependent outward K⁺ current (Iₓ). Iₓ is composed of at least two components: a slowly activating La⁺-resistant delayed rectifying current (Iₓₓ) and a rapidly activating La⁺-sensitive current (Iₓₛ). To assess the effects of imipramine on Iₓₓ and Iₓₛ, single guinea pig ventricular myocytes were studied using the nystatin-perforated patch-clamp technique in the absence and in the presence of La⁺++. Imipramine inhibited Iₓₓ and Iₓₛ in a concentration-dependent manner. The effects of imipramine on the aggregate time-dependent outward current were more marked than those on Iₓₛ alone. Thus, 1 µmol/L imipramine decreased the tail currents elicited on return to −30 mV after long depolarizing pulses (5 seconds, from −40 to +50 mV) in the absence and in the presence of La⁺⁺ by 27±4% and 15±3% (n=6), respectively. Moreover, the inhibition induced by imipramine was greater after short (0.5-second) pulses than after 5-second depolarizing pulses, both in the absence and in the presence of La⁺⁺ (53±3% and 30±5%, respectively, n=6; P<.05). Imipramine did not significantly modify either the activation midpoint or the slope factor of the aggregate Iₓ and Iₓₛ activation curves. The reduction of Iₓₛ by imipramine was voltage dependent and was more marked at negative membrane potentials. In the presence of 1 µmol/L imipramine, the ratio of tail current to time-dependent current remained constant at 0.37±0.03, regardless of the test pulse duration at +50 mV. Thus, the envelope-of-tails test was satisfied in the presence of 1 µmol/L imipramine, which indicates that imipramine, at this concentration, blocks Iₓₛ. Imipramine (1, 5, and 10 µmol/L) had no effect on the kinetics of the later phase of Iₓ activation but delayed the beginning of the activation of Iₓₛ by 62±22, 74±23, and 155±53 milliseconds in the presence of 1.5, and 10 µmol/L imipramine, respectively. These results suggest that imipramine preferentially blocks rapidly activating K⁺ channels. In addition, experiments performed in the presence of 30 µmol/L La⁺⁺ suggest that the drug preferentially binds, but maybe not exclusively, to a closed state of the slowly activating K⁺ channel. (Circ Res. 1994;74:687-699.)

Key Words • imipramine • ventricular myocytes • K⁺ currents • voltage dependence • antiarrhythmic agents • K⁺ channels • nystatin-perforated patch clamp

Imipramine is a tricyclic antidepressant drug that inhibits the maximum rate of depolarization of the action potential in multicellular cardiac preparations, and it has been classified as a class Ia antiarrhythmic agent. In isolated guinea pig ventricular cells, imipramine produces a voltage- and frequency-dependent inhibition of the Na⁺ inward current (Iₐ). Its clinical spectrum of activity is similar to that of quinidine. Furthermore, imipramine also blocks the time-dependent outward K⁺ (Iₓ) and Ca²⁺ (Iₓₛ) currents in guinea pig ventricular myocytes. However, its effects on action potential duration are variable, depending on the animal species under study. Imipramine shortens the action potential duration in guinea pig and bovine papillary muscles and guinea pig ventricular myocytes, and it lengthens the action potential in rabbit and rat atrium. In rabbit atrial myocytes, this lengthening has been attributed to the inhibition of the transient outward current (Iₒ). Which, in these preparations, is a major current responsible for repolarization.

It has been described that in guinea pig ventricular myocytes, imipramine reduces the outward delayed rectifier current, Iₓ, in a voltage-independent manner. Nevertheless, its time dependence of block remains unknown. In many cells, Iₓ has at least two components, which can be separated by their sensitivity to La⁺⁺ and E-4031. Sanguinetti and Jurkiewicz have reported two components of the time-dependent Iₓ in these cells. They describe a small, “drug-sensitive,” rapidly activating component that they term Iₓₛ, as well as a slowly activating, greater component, Iₓₓ. However, whereas Sanguinetti and Jurkiewicz describe a noninactivating, inward rectifying K⁺ current, Bailer et al. describe a partial inactivation of Iₓₓ. The controversy concerning whether Iₓₓ inactivates could be attributed to the different experimental conditions used by both groups of investigators. The addition of La⁺⁺ eliminates the discrepancy between the time courses of increasing time-dependent Iₓ during positive clamp steps and the corresponding envelope of tail current amplitudes. Hence, La⁺⁺ can be used as a tool to isolate the delayed rectifier current (Iₓ), which may correspond to Iₓₛ.
Fig 1. A, Aggregate time-dependent outward K+ current (I\textsubscript{k}) recorded in the long-lasting perforated nystatin configuration of the patch-clamp technique. The I\textsubscript{k} amplitude was measured with reference to the zero current level. Tail current (I\textsubscript{tail}) was measured as the difference between the maximum outward current immediately after the step to \(-30\) mV (after the inward capacity artifact) and the steady-state current measured after 5 seconds at \(-30\) mV. Neither I\textsubscript{k} nor I\textsubscript{tail} was significantly altered for up to 70 minutes. I\textsubscript{k} indicates membrane current. B, Slowly activating I\textsubscript{k} (I\textsubscript{ka}) measured as the maximum activated I\textsubscript{k} at the end of the 5-second depolarizing pulse from \(-40\) to \(+50\) mV and as I\textsubscript{tail} measured in the same way as in panel A. The figure shows the control values for this current, its sensitivity to 10 \(\mu\text{mol/L}\) imipramine, and its washout.

Materials and Methods

Isolation of Cardiac Myocytes

Experiments were performed on guinea pig ventricular myocytes isolated by enzymatic dissociation by following a previously described procedure.\(^7\)\(^8\) Hearts from guinea pigs (200 to 300 g) were rapidly removed, mounted on the cannula of a Langendorff perfusion apparatus, and initially perfused for 1 to 2 minutes with a modified Tyrode’s solution that contained 1.8 mmol/L CaCl\(_2\). Then, the hearts were perfused for 5 minutes at 10 to 15 mL/min with a Ca\(^2+\) -free solution at 37°C, followed by an 8-minute perfusion with the same solution supplemented with 0.12 mg/mL collagenase (type Ia, Sigma Chemical Co, London, England) and 0.03 mg/mL protease (Sigma type XIV). The hearts were then washed with a high-K\(^+\), low-Cl\(^-\) solution or KB solution for 4 minutes. The hearts were then removed from the Langendorff apparatus, and the ventricles were dissected and cut into small pieces, which were placed in a beaker containing KB solution and gently shaken to disperse the isolated cells. The resulting cell pellet was stored in KB medium for 1 to 2 hours before beginning the experiment. The composition of the Tyrode’s solution was (mmol/L) NaCl 114, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, taurine 20, glucose 10, NaHCO\(_3\) 24, and NaH\(_2\)PO\(_4\) 0.42; pH was adjusted to 7.4 by the addition of NaOH. The high-K\(^+\), low Cl\(^-\) solution (KB solution) contained (mmol/L) glutamic acid 70, taurine 10, KCl 20, KH\(_2\)PO\(_4\) 10, MgCl\(_2\) 1.0, succinic acid 5.0, creatine 5.7, dextrose 10.0, K\(^+-\)EGTA 0.2, and K\(^+-\)HEPES 5.0; pH was adjusted to 7.4 by addition of KOH.

A small aliquot of a suspension of dissociated cells was transferred to a 0.5-mL chamber placed on the stage of an inverted phase-contrast microscope (model TMS, Nikon Co, Tokyo, Japan). After settling to the bottom of the chamber, cells were superfused with Tyrode’s solution at 1 mL/min. To measure I\textsubscript{k}, the external solution contained (mmol/L) NaCl 136, KCl 5.4, CaCl\(_2\) 1.0, MgCl\(_2\) 1.0, CoCl\(_2\) 2.0, tetrodotoxin 0.03, glucose 10, and HEPES 10; the pH was adjusted to 7.4 by the addition of NaOH. The pipette solution was composed of (mmol/L) potassium aspartate 80, KCl 42, KH\(_2\)PO\(_4\) 10, Mg\(^2+\) 1.0 ATP 4, phosphocreatine 3, K\(^+-\)EGTA 5, and K\(^+-\)HEPES 5. The pH was adjusted to 7.25 with KOH. All experiments were performed at room temperature (22°C to 23°C) on Ca\(^2+\)-tolerant healthy ventricular myocytes identified by their rod-like, striated appearance. Cells were typically 80 to 110 \(\mu\text{m}\) in length.

Recording Techniques

Membrane currents were measured with the nystatin-perforated patch configuration of the patch-clamp technique\(^7\)\(^8\) using an Axopatch-IC clamp amplifier (Axon Instruments, Foster City, Calif). The choice of the nystatin method in the present experiments was guided by previous studies using this compound to permeabilize cardiac myocytes and record I\textsubscript{k} without intracellular dialysis.\(^9\)\(^10\) Under these experimental situations, intracellular conditions appeared unaltered, avoiding “rundown,” which this current exhibits with the normal whole-cell recording. Fig 1A shows the stability of the aggregate time-dependent I\textsubscript{k} during long-lasting measurements using the nystatin-perforated patch configuration. I\textsubscript{k} magnitude remained constant for >70 minutes. Fig 1B illustrates the stability of I\textsubscript{k} recorded in the presence of 30 \(\mu\text{mol/L}\) La\(^3+\), its inhibition during exposure to 10 \(\mu\text{mol/L}\) imipramine, and its recovery during the 18-minute washout period in an experiment that lasted 70 minutes. Thus, currents measured by the nystatin method were sufficiently stable to permit I\textsubscript{k} and I\textsubscript{ka} to be recorded for periods exceeding 70 minutes. Patch pipettes were constructed from borosilicate capillary tubes (model GD-1, Narishige Co, Ltd, Tokyo, Japan) by use of a programmable patch micropipette puller (model P-87 Brown-Flaming, Sutter Instruments Co, Novato, Calif). The pipettes had resistances of 2 to 4 M\(\Omega\) when filled with the internal solution and immersed in the Tyrode’s solution. The effective access resistance was calculated from the ratio r\(_i\)/C\(_m\), where r\(_i\) is the time constant of uncompensated capacitive transient and C\(_m\) is the cell capacitance obtained by integration of uncompensated capacitive transients, as previously described by Sala et al.\(^26\) and ranged between 8 and 12 M\(\Omega\) (n=9). Since the series resistance was compensated by \(\approx 80\%\) and the largest currents recorded were \(<1\) nA (495.0±79.6 pA), the mean voltage error has an upper limit of 2.4 mV. During the experiments, membrane potential and current data were displayed on a storage oscilloscope (model 7854, Tektronix, Beaverton, Ore). Series resistance was compensated by the analog circuit of the Axopatch amplifier. Fully activated I\textsubscript{k}, ie, aggregate time-
dependent current, was measured relative to the zero current level. Voltage-clamp command pulses were generated by a 12-bit digital-to-analog converter. Membrane currents were filtered at 1 kHz (−3 dB) by a four-pole Bessel filter before sampling was done at 2 kHz by a 12-bit analog-to-digital converter. A Hewlett-Packard Vectra QS/16S computer was used to generate voltage-pulse protocols and to acquire and analyze data with PCLAMP 5.5.1 software (Axon Instruments).

To inactivate $I_{Na}$, the holding potential was maintained at −40 mV. In addition, the external solution contained 30 μmol/L tetrodotoxin and 2 mmol/L CoCl₂ to block the $I_{m}$ and the L-type $I_{Ca}$, respectively. LaCl₃ (30 μmol/L) was added to the external solutions in some experiments to pharmacologically isolate $I_{K,S}$, respectively. After a 15-minute period for control measurements, the perfusate was changed to one containing imipramine hydrochloride (Sigma), and data were collected again after 7 to 10 minutes.

Chemicals

Imipramine hydrochloride (Sigma) was dissolved in deionized water to make 1 mmol/L stock solutions.

Voltage and Time Dependence

Steady-state voltage-dependent drug effects were evaluated in two ways. The relation between transmembrane voltage and steady-state current was assessed directly by clamping from −40 mV to several depolarizing test potentials for 5 s. The total outward current developing during a positive clamp step was measured for increasing test potentials from −30 to +70 mV. A second measure of $I_{K}$ activation was obtained by clamping to −30 mV immediately after the long clamp steps at the positive test potentials. The amplitude of the deactivating current tail ($I_{Na}$) was measured as the difference between the maximum outward current immediately after the step to −30 mV (after the capacitance artifact) and the steady-state current measured after 5 seconds at −30 mV.

Time-dependent drug effects were quantified by measuring the amplitudes of $I_{m}$ elicited by depolarization pulses from −40 to +50 mV of variable durations (between 0.1 and 10 seconds). The relation between tail amplitude and pulse duration was fitted with a monoexponential function of time after exclusion of the first data points in the absence of La³⁺ and in the presence of 30 μmol/L LaCl₃ to obtain the dominant time constant of the activation and the delay in the activation without making any assumption about the gating kinetic model of the slowly activating K⁺ ($K_{s}$) channel (see below). Under these conditions, the zero-current time intercept of the monoexponential fit was used as an index of the delay in $I_{m}$ activation.

Data Analysis and Curve Fitting

Data obtained under control conditions were compared with those obtained after drug exposure in a paired manner. For comparisons at a single voltage or time point, differences were analyzed using Student’s t test. To analyze block at multiple voltages or drug concentrations, two-way ANOVA was used. All data are presented as mean ± SEM.

$I_{K}$ and $I_{K,S}$ activation curves were fitted by a Boltzmann distribution using a least-squares fitting routine described by Marquardt:22

$$I_{K}/I_{K_{max}}=\frac{1}{1+\exp [(V_{h}\text{-}V)/k]}$$

where $I_{K_{max}}$ is maximal $I_{K}$, $V_{h}$ is the half-point of activation (in millivolts), $V$ is the test potential, and $k$ represents the slope factor for the activation curve (in millivolts).

The activation kinetics of $I_{K,S}$ has been described as a sigmoidal process, assuming that the channel has multiple closed states.31 However, in the present study, to describe the dominant time constant of this process and the effects of imipramine on it, the exponential analysis was used as an operational approach, fitting the activation and the deactivation kinetics to an equation of the following form:

$$y=A_{1}\exp(-t/\tau_{1})+A_{2}\exp(-t/\tau_{2})+A_{3}\exp(-t/\tau_{3})+C$$

where $\tau_{1}$, $\tau_{2}$, and $\tau_{3}$ are the system time constants, $A_{1}$, $A_{2}$, and $A_{3}$ are the amplitudes of each component of the exponential, and $C$ is the baseline value. Therefore, in the present study, we used this approach in which the later part of the activating current and also the $I_{m}$ amplitudes were fitted with a single exponential to obtain the dominant time constant of activation as well as the delay in the activation process of the current.24,25

The advantage of this procedure is that it does not assume any gating kinetic model. The curve-fitting procedure used a nonlinear least-squares (Gauss-Newton) algorithm. Fits were displayed in linear and semilogarithmic format together with the difference plot. Goodness of the fit and of the required number of exponential components was judged by comparing $\chi^{2}$ values statistically (F test). We also inspected for systematic nonrandom deviations in the difference plot.26

Results

Effects of Imipramine on the Current-Voltage Relations of $I_{K}$

The left panel of Fig 2 shows original recording of $I_{K}$ in the control condition and in the presence of 5 μmol/L
imipramine. Outward currents were elicited by applying 5-second depolarizing pulses from a holding potential of -40 to +50 mV, followed by repolarization to -30 mV for 5 seconds. Pulses were applied once every 30 seconds. Under control conditions, depolarizing pulses from -40 mV to a potential of +50 mV evoked a slowly rising outward current and, on repolarization to -30 mV, an outward I_tail that decayed after a biexponential process (fast time constant $\tau_f=308\pm61$ milliseconds; slow time constant $\tau_s=1552\pm189$ milliseconds; $n=8$) was observed. These tail currents have been described as the deactivation of the delayed rectifier.13,14,27,28 As it can be observed, imipramine (5 $\mu$mol/L) decreased the maximum-aggregate time-dependent I_K induced by the depolarizing pulses from 855 to 779 pA and the I_tail elicited with deactivation at -30 mV from 307 to 207 pA. This effect was almost completely reversed within 10 to 15 minutes after perfusion with drug-free external solution. However, and in agreement with previous reports,12 imipramine had little or no effect on the holding current at -40 mV at any concentration tested. The amplitude of the maximum-aggregate I_K measured at the end of the test potentials ranging from -30 to +70 mV is plotted in the right panel of Fig 2 as a current-voltage relation. In 12 cells during the predrug control condition, the amplitude of the maximum-aggregate I_K elicited during a 5-second depolarizing pulse to +50 mV averaged 495±79 pA. Imipramine decreased the amplitude of the aggregate time-dependent I_K measured at the end of 5-second depolarizing pulses from -40 to +50 mV in a concentration-dependent manner (15±8% [P<.05] and 24±8% [P<.01] in the presence of 1 and 5 $\mu$mol/L imipramine, respectively; n=6).

In the present study, the effects of imipramine were studied under two different experimental conditions: (1) in the absence of La3+, recording the aggregate time-dependent I_K, and (2) in the presence of 30 $\mu$mol/L La3+, recording the slow-activating component of I_K (I_KS). The activation of the aggregate I_K is the result of the activation of two different types of channels (I_Kr and I_Ks). Although the voltage dependence or the activation kinetics of the aggregate I_K do not allow us to obtain a final conclusion about the effects of imipramine on each component of I_K (I_Kr and I_Ks), these results, together with those obtained in the presence of La3+ (when only I_Ks is recorded), can provide us information about the effects of imipramine on I_Kr. Moreover, they allow us to obtain the imipramine-sensitive current and thus to compare it with the E-4031-sensitive current, i.e., I_Kr.

Fig 3 shows typical recordings obtained both in the absence and in the presence of 30 $\mu$mol/L La3+. The holding potential was maintained at -40 mV, and 5-second depolarizing pulses were applied to +50 mV. In the absence of La3+, the time course of I_K shows a “hump” at the beginning of the depolarizing pulse, which is absent when the same experimental protocol was applied after perfusing the cell with a La3+ external solution. This apparent hump observed in the absence of La3+ represents the activation of the I_Kr component and is responsible for the faster time constant of activation kinetics as compared with that observed in La3+-perfused cells (0.93±0.12 versus 1.99±0.10 seconds, respectively; $P<.01$). The difference current shown in Fig 3 was obtained by digital subtraction of the two current tracings and represents the La3+-sensitive rapidly activating component of I_K.13,14

Fig 4 compares the time course of the imipramine-sensitive current in a cell not exposed to La3+ (Fig 4A) with that of a La3+-exposed cell (Fig 4B). The membrane potential was maintained at -40 mV, and 5-sec-

---

**Fig 3.** Effects of La3+ on outward K+ currents. Current was measured in response to a depolarizing pulse from -40 to +50 mV for 5 seconds. The voltage-pulse protocol is shown above the current tracings. The La3+-sensitive current (difference current) was obtained by digital subtraction of the two tracings shown in the figure. The dotted line represents the zero current level.

**Fig 4.** Tracings showing activation of the time-dependent outward K+ current after 5-second depolarizing steps to +50 mV from a holding potential of -40 mV before and during exposure to 5 $\mu$mol/L imipramine. Imipramine-sensitive current is shown superimposed. A shows an experiment in the absence of La3+; B, an experiment in the presence of 30 $\mu$mol/L La3+. The dotted lines represent the zero current level.
ond depolarizing pulses to +50 mV were applied every 30 seconds. In the absence of La⁺³, 5 μmol/L imipramine decreased the aggregate time-dependent current during the depolarizing step by 24±8%. Digital subtraction of the current tracings obtained in the absence and in the presence of imipramine revealed that the blockade induced by imipramine was fully developed at <1 second. Furthermore, imipramine eliminated the initial hump present under control conditions, suggesting that it blocks the rapidly activating component of I_K (I_Kr). In the presence of La⁺³ (Fig 4B), the activation process of the outward current exhibited a longer delay, and only the slow component of I_K (I_Ks) was recorded. This finding is also consistent with the interpretation that La⁺³ blocks I_Kr. Imipramine-induced block in the presence of La⁺³ was smaller than that observed in the absence of La⁺³ (7±2% and 13±4% in the presence of 1 and 5 μmol/L imipramine, respectively; n=5; P<.05), which suggests that imipramine inhibits both components of I_K. Under these experimental conditions, when only I_Ks is being recorded, imipramine-induced block developed more slowly, reaching a maximum level at ≈1.5 seconds and decreasing again during the depolarizing pulse. Hence, these findings suggest that imipramine blocks two components of the aggregate time-dependent I_K (Fig 4). Unfortunately, it was impossible to assess the effects of imipramine in a single cell in the absence and presence of La⁺³, since La⁺³ could not be washed out throughout the duration (40 to 50 minutes) of the experiment. In fact, it has been shown that long exposures (>20 minutes) to La⁺³ induce an accumulation of this cation intracellularly.²⁹,³⁰

Voltage-Dependent Effects of Imipramine on I_K and I_Ks

The effects of imipramine on the voltage dependence of the aggregate time-dependent I_K were assessed by measuring the amplitude of I_tail observed on return to −30 mV after 5-second depolarizing pulses to different test potentials (from −30 to +70 mV, in 10-mV steps) applied every 30 seconds from a holding potential of −40 mV. The currents were normalized relative to the maximum control I_tail amplitude for each experiment (value of 1.0), and the resulting values were plotted as a function of membrane potential. Under control conditions in the absence of La⁺³, the amplitude of I_tail recorded at −30 mV after a depolarizing pulse from −40 to +50 mV averaged a value of 192±38 pA (n=6), which was reduced by 27.4±9% and 36±9% in the presence of 1 and 5 μmol/L imipramine, respectively. Under these experimental conditions, I_K was activated at potentials positive to −30 mV, and its amplitude increased at more depolarized levels. Under control conditions, the steady-state activation curve of I_K had an activation midpoint of 12.9±0.6 mV and a slope factor of 11.6±0.4 mV (n=6). At 1 and 5 μmol/L imipramine, imipramine decreased the maximal amplitude of I_tail at all potentials tested but did not change the activation midpoint (11.1±0.9 and 12.3±0.8 mV, respectively; n=6; P>.05) and slope factor (12.6±1.2 and 12.1±1.5 mV, respectively; n=5; P>.05).

To study the voltage dependence of imipramine-induced block on the slow component of I_K (I_Ks), cells were perfused with a 30 μmol/L La⁺³-containing external solution (Fig 5). The voltage dependence of imipramine on this current was assessed in the same way as described above. Fig 5A shows typical I_tail recordings elicited on return to −30 mV after 5-second depolarizing pulses from −40 to +10, +30, +50, and +70 mV before and after exposure of the cells to 1 μmol/L imipramine. Under these experimental conditions, the amplitude of I_tail recorded at −30 mV after a 5-second depolarizing pulse from −40 to +50 mV averaged a value of 154±22 pA (n=9), which was decreased by 15±3%, 22±4%, and 40±3% in the presence of imipramine at 1, 5, and 10 μmol/L, respectively. Although the time course of I_tail was not modified by 1 μmol/L imipramine, the I_tail peaks in the presence of the drug are preceded by a brief “delay.” Fig 5B shows the I_Ks activation curve both in the absence and in the presence
of 1 μmol/L imipramine as well as the percentage of imipramine-induced I_k, block at the different membrane potentials. Under control conditions, the midpoint of the activation curve averaged in six cells was 39.9±4.3 mV, and the slope factor was 14.6±0.1 mV. Imipramine at 1 μmol/L did not modify either the activation midpoint (45.5±1.2 mV, P>.05) or the slope factor (15.9±0.6 mV, P>.05). However, the percentage of imipramine-induced I_k, block was significantly higher at more negative than at more depolarized membrane potentials (88±12% at −10 mV versus 19±13% at +70 mV, P<.01). Imipramine at 5 and 10 μmol/L also failed to change either the midpoint (35.2±2.1 versus 37.1±3.5 mV and 37.4±2.7 versus 38.6 mV) or the slope factor (14.2±0.1 versus 14.3±0.2 mV and 14.8±0.3 versus 14.8±0.5 mV) of the activation curve, although the percentage of I_k, block was again higher at negative than at positive potentials (91±7 at −10 mV versus 37±2% at +70 mV in the presence of imipramine 10 μmol/L, n=5, P<.01).

Envelope-of-Tails Test

The envelope-of-tails test33 predicts that if I_k represents the activation of a single type of channel, then the magnitude of I_{tail} after a given depolarizing pulse of variable duration should increase parallel to the outward current during the depolarizing pulse. Therefore, the ratio of the tail current to the time-dependent current (ΔI_{tail}/ΔI_k) should be constant, regardless of the duration of the pulse. Test pulses were applied from a holding potential of −40 to +50 mV for durations ranging from 0.1 to 10 seconds. ΔI_{tail}/ΔI_k was calculated in the absence and in the presence of 1 μmol/L imipramine. In cells perfused with La^{3+}-free solution, ΔI_{tail}/ΔI_k was dependent on the duration of the pulse. Thus, activation of I_k during short depolarizing pulses (<0.5 second) is characterized by tail currents that are equal to or larger than the time-dependent outward currents during the depolarizing pulse. However, as the duration of the test pulse was lengthened, the magnitude of ΔI_{tail} became less than that of ΔI_k measured during the test pulses to +50 mV, and so for pulses ≥1 second, ΔI_{tail}/ΔI_k of 0.41±0.05 (n=6) was obtained. These results agree with previous reports3,14 that demonstrate the existence of two different components of I_k in guinea pig ventricular myocytes as the result of the activation of two different types of K' channels: a rapidly activating K' channel, K_s, and a slowly activating one, K_r. If imipramine completely blocks the fast activating component of I_k, as was expected from the above experimental results, it would be predicted that the envelope-of-tails test should be satisfied after cell exposure to imipramine; i.e., ΔI_{tail}/ΔI_k will be constant regardless of the duration of the pulse. Time-dependent current during the test pulse (ΔI_k) was measured at the end of the depolarizing pulse, and it was subtracted from the current measured immediately after settling of the initial capacity spike (Fig 6A). Pooled data (six cells) are shown in Fig 6B, where ΔI_{tail}/ΔI_k is represented as a function of the test-pulse duration. In the absence of imipramine, the envelope-of-tails test showed that I_k consisted of more than one component, whereas after exposure to 1 μmol/L imipramine, the rapidly activating component of outward current, I_k,s, was blocked and the remaining outward current was composed of a single current type.

In the presence of 1 μmol/L imipramine, only the I_k,s component remained, and ΔI_{tail}/ΔI_k was constant at 0.37±0.03, regardless of the test pulse duration at +50 mV. This value is quite close to the predicted one (0.4) from the ratio of the driving force at +50 and −30 mV for a nonrectifying, K'-selective, outward current.14 Fig 6C shows the envelope-of-tails test obtained in the absence and in the presence of 30 μmol/L La^{3+}. When the effects induced by 30 μmol/L La^{3+} and 1 μmol/L imipramine are compared, it can be observed that at the concentrations used in the present study, both La^{3+} and imipramine completely block I_k,s.

In the absence of imipramine, the time course of I_{tail} at −30 mV after short (0.5-second) depolarizing pulses was well fitted by a biexponential function (τ=196±19 milliseconds and τ=1072±203 milliseconds, n=6). On the contrary, in the presence of 1 μmol/L imipramine, the time course of I_{tail} measured at −30 mV after 0.5-second depolarizing pulses was well fitted by a monoexponential function with a time constant of 595±40 milliseconds (n=6). Therefore, these results strongly suggest that imipramine blocks the rapidly activating component of I_k. Fig 6D shows I_k recorded before and after exposure to 1 μmol/L imipramine when applying a test pulse from −40 to +50 mV for 0.4 second. The imipramine-sensitive current, i.e., difference current, was obtained by digital subtraction of currents recorded in the presence of the drug from control recordings. The small size of the I_k component blocked by imipramine at +50 mV relative to the large amplitude of I_{tail} reflects the marked inward rectification properties of the imipramine-sensitive current.14,15

Voltage-Dependent Characteristics of the Imipramine-Sensitive Current

The experimental results obtained in the absence of La^{3+} strongly suggest that 1 μmol/L imipramine blocks I_k,s. It has been described that the activation curve of I_k,s exhibits a midpoint of activation at −21 mV and a slope factor of 7 mV.14 If imipramine blocks this component of the aggregate time-dependent I_k, it would be expected that the imipramine-sensitive current should exhibit this voltage-dependence. The imipramine difference activation curve was obtained from activation curves in which a short pulse (0.5 second) from −40 mV to different potentials (between −30 and +20 mV) was applied. I_{tail} was measured at −30 mV and plotted versus the test potential. Fig 7 shows the normalized imipramine-sensitive activation curve. Data were fitted by a Boltzmann equation obtaining an activation midpoint of −17.3±2.5 mV and a slope factor of 7.3±0.5 mV (n=4). This voltage dependence is quite similar to that reported for the E-4031-sensitive current.15

Time-Dependent Effects of Imipramine

The effects of imipramine on the time course of I_k activation kinetics were studied by generating envelopes of tail currents. In Fig 8 current tail amplitudes at −30 mV after depolarizing pulses from −40 to +50 mV of increasing duration (from 0.1 to 10 seconds) applied every 30 seconds were compared in the absence and presence of imipramine. Since it is known that I_k is composed by at least two components of outward current (I_k,s and I_k,r), we performed the envelope-of-tails test in cells perfused with La^{3+}- and with La^{3+}-
containing external solution. Fig 8 shows the effects of 1 µmol/L imipramine on the activation kinetics of the aggregate time-dependent $I_K$. Panel A shows typical recordings elicited after depolarizing pulses to +50 mV of different durations (between 0.1 and 5 seconds) before and after exposure of the cells to imipramine. Fig 8B shows that imipramine not only reduced the magnitude of the $I_K$ activated after long pulses but also slowed the activation kinetics of the aggregate current. The later part of the activation kinetics was fitted by a monoeponential function to obtain the dominant time constant of the process. In the absence of imipramine, the time constant averaged 0.93±0.12 seconds, which was lengthened to 1.44±0.24 seconds in the presence of 1 µmol/L imipramine ($P<.05$, $n=6$). Moreover, the block of $I_K$ by imipramine was greater with short (0.5-second) than with long (5-second) depolarizing pulses (53±3% versus 27±4%, respectively; $n=6$; $P<.05$), suggesting that at this concentration, imipramine was almost a selective $I_K$ blocker. The time course of the deactivating tail currents after 5-second depolarizing pulses in the absence of imipramine was fitted by a biexponential function ($\tau_1=308±61$ milliseconds and $\tau_2=1552±189$ milliseconds, $n=8$). In the presence of 1 µmol/L imipramine, the time course of this process was also well fitted with a biexponential function ($\tau_1=270±40$ milliseconds and $\tau_2=1326±176$ milliseconds, $n=6$, $P>.05$).

In cells perfused with a $\La^{3+}$-containing external solution, the later part of the activation kinetics of the outward current was fitted by a monoeponential function of time to obtain the dominant time constant of the
process (Fig 9), which was slower than that observed in the absence of La\textsuperscript{3+} (1.9±0.1 seconds, n=6, P<.01). Imipramine did not change the time constant of this process (2.1±0.2 seconds, n=6, P>.05), although it delayed the beginning of the activation of this component by 62±22 milliseconds (from 18±14 to 81±19 milliseconds, n=5, P<.05), measured as the x intercept of the exponential fit (Fig 9, bottom). We also measured the time constant and the delay of the activation process from the recordings obtained in the absence and in the presence of imipramine (1 μmol/L). The dominant time constant remained unchanged (1.6±0.5 versus 1.7±0.6 seconds, n=6, P>.05, in the absence and in the presence of 1 μmol/L imipramine, respectively), and the delay in the activation was 75±8 milliseconds in the presence of imipramine versus the control situation, which is very close to that obtained when using the I\textsubscript{tail} amplitudes (62±22 versus 75±8 milliseconds, P>.05).

In the presence of La\textsuperscript{3+}, the imipramine-induced block was also more evident after short (0.5-second) than after long (5-second) depolarizing steps (30±5% versus 15±3, n=6, P<.05) (Fig 9, middle). Under these experimental conditions, imipramine did not modify the activating I\textsubscript{tail} kinetics at −30 mV after 5-second depolarizing pulses to +50 mV, which was fitted by a biexponential function (see “Materials and Methods”) (τ\textsubscript{a}=274±45 milliseconds and τ\textsubscript{b}=1301±149 milliseconds versus τ\textsubscript{a}=256±61 milliseconds and τ\textsubscript{b}=1303±62 milliseconds, n=5, P>.05). After 0.5-second depolarizing pulses, the time course of the activating I\textsubscript{tail} was fitted by a monoeponential function, which in the absence of imipramine averaged 561±29 milliseconds. Imipramine (1 μmol/L), under these experimental conditions, did not modify this time course (time constant of 535±25 milliseconds, n=5, P>.05). The marked block at short pulses, the delay in the I\textsubscript{Ks} activation observed in our experiments, and the lack of effect on the deactivation kinetics are consistent with the binding of the drug to a closed state of the K\textsubscript{s} channel.

To further characterize the proposed closed-channel interaction, the effects of 5 and 10 μmol/L imipramine were also tested on the I\textsubscript{Ks} activation kinetics. It would be expected that if imipramine binds with high affinity to a closed state of the K\textsubscript{s} channel, higher concentrations of imipramine would emphasize this delay. Fig 10, top, shows typical recordings obtained after 5-second depolarizing pulses to +50 mV in the absence and in the presence of 10 μmol/L imipramine, where the delay at the beginning of the activation is evident. The delay in the activation kinetics of I\textsubscript{Ks}, measured as the x intercept of the I\textsubscript{tail} magnitude obtained when returning to −30 mV from a test pulse to +50 mV, plotted against the duration of the test pulse was significantly prolonged from 62±22 milliseconds (1 μmol/L imipramine) to 74±23 milliseconds (P<.05) and 155±53 milliseconds (P<.01) in the presence of 5 and 10 μmol/L imipramine, respectively. Fig 10, middle, shows the I\textsubscript{Ks} activation kinetics obtained in five cells, both in the absence and in the presence of 10 μmol/L imipramine. Imipramine did not significantly change either the time constant of this process (2.15±0.2 versus 1.55±0.16 seconds, n=5, P>.05) or the deactivation I\textsubscript{Ks} process measured on returning to −30 mV from a 5-second test pulse to +50 mV (τ\textsubscript{Ks}=274±45 versus 256±6 milliseconds, n=5, P>.05; τ\textsubscript{Ks}=1302±149 versus 1005±62 milliseconds, n=5, P>.05). Fig 10, bottom, shows the averaged delay, measured as the x intercept of the exponential fit, observed with this concentration of imipramine. However, although at this concentration the imipramine-induced I\textsubscript{Ks} block was greater after short than after long depolarizing pulses, it was not statistically different. Moreover, the I\textsubscript{Ks} block observed after long depolarizations was statistically greater than that observed in the presence of 1 μmol/L imipramine (13±4% versus 44±8% after 10-second depolarizing pulses, in the presence of 1 and 10 μmol/L imipramine, respectively; P<.05). Similar results were obtained when the activated outward current was measured at different times (35.1±0.3% versus 8.2±0.5% after 0.1- and 10-second depolarizing pulses in the presence of 1 μmol/L imipramine and 62.2±0.6% versus 32.6±0.5% after 0.1- and 10-second depolarizing pulses in the presence of 10 μmol/L imipramine).

Effects of Imipramine on I\textsubscript{K} and I\textsubscript{Ks} in the Presence of Nisoldipine

The previous results presented in the present study were obtained in the presence of Co\textsuperscript{2+} as an I\textsubscript{Ks} blocker. The
Fan and Hiroaka\textsuperscript{32} have previously reported that Co\textsuperscript{2+} blocks the aggregate I\textsubscript{K} and shifts the activation curve for aggregate I\textsubscript{K} in a concentration-dependent manner in guinea pig ventricular myocytes. Although the experiments were performed in a Na\textsuperscript{+}-, K\textsuperscript{+}-, and Ca\textsuperscript{2+}-free solution and thus they may not be quantitatively applicable to the case in a Na\textsuperscript{+}-, K\textsuperscript{+}-, and Ca\textsuperscript{2+}-containing solution, the time- and voltage-dependent effects of imipramine on I\textsubscript{K} and I\textsubscript{Ks} were also studied in other series of experiments in which 200 nmol/L nisoldipine instead of Co\textsuperscript{2+} was used to block I\textsubscript{Ca}. Fig 11A shows the envelope-of-tails test obtained in the absence and in the presence of 1 \textmu mol/L imipra-
Fig 11. Graphs showing voltage- and time-dependent effects of imipramine on outward K⁺ current (Iₖ) and slowly activating Iₖ recorded in the presence of 200 nmol/L nisoldipine instead of Co²⁺ as a Ca²⁺ current blocker. A. Envelope-of-tails test for Iₖ in the absence and in the presence of 1 μmol/L imipramine. Current ratio (ΔIₖ/ΔIₖ, where Iₖ is the tail current) is plotted as a function of pulse duration. The external solution contained 0 mmol/L La³⁺. B, Voltage dependence of the slowly activating component of Iₖ activation, measured as the peak Iₖ at -30 mV vs the previous depolarizing potential. The external solution contained 30 μmol/L La³⁺. The continuous line is the best fit obtained using a Boltzmann equation: Iₖ/Imax = 1/(1+exp[(Vₖ-Vₖm)/k]), where Imax is maximal Iₖ, Vₖ is half-activation voltage (35 mV in the control solution [●] and 41 mV in the presence of 1 μmol/L imipramine [○]), Vₖm is the test potential, and k is the slope factor (14 mV in the control situation and 13 mV in the presence of 1 μmol/L imipramine). The percentage of Iₖ block at each membrane potential (○) was measured as follows: [(Iₖtest-Iₖcontrol)/Iₖtest] × 100, where Iₖtest and Iₖcontrol are Iₖ for the steady-state test and control conditions, respectively. C, Envelope of Iₖ in the control condition and after 10 minutes of exposure to 1 μmol/L imipramine. The external solution contained 30 μmol/L La³⁺. Cells were held at -40 mV and pulsed every 30 seconds to +50 mV for variable periods (0.1, 0.3, 0.5, 1, 2, 3, 5, 7, and 10 seconds), followed by deactivation at -30 mV for 5 to 10 seconds. The Iₖ amplitudes are plotted vs the time of the depolarizing pulse.

Discussion

The results of the present study show that in isolated guinea pig ventricular myocytes, imipramine blocks the aggregate time-dependent Iₖ. Drug actions in the absence and in the presence of La³⁺ indicate that imipramine inhibits at least two components of this time-dependent Iₖ and is more potent in inhibiting the rapidly activating component (Iₖr) than in inhibiting the slowly activating one (Iₖs). As discussed below, with La³⁺-sensitive currents eliminated, the data in the present study suggest that imipramine preferentially associates with a closed state of the slowly activating K⁺ channel, although an open-channel interaction cannot be ruled out.
Effects of Imipramine on Aggregate Time-Dependent \( I_K \)

In guinea pig isolated ventricular myocytes perfused with normal external solution, imipramine decreased and delayed the activation of the aggregate time-dependent \( I_K \) in a concentration-dependent manner. However, imipramine did not change the midpoint or the slope factor of the activation curve of the current. These results confirm and extend results previously described by our laboratory. Recently, it was demonstrated that \( I_K \) in guinea pig ventricular myocytes represents the composite of at least two components that can be separated by their sensitivity to La\(^{3+}\): a rapidly activating La\(^{3+}\)-sensitive component and a slowly activating La\(^{3+}\)-insensitive component. In our experiments, an envelope-of-tails test confirmed that \( I_K \) was composed of at least two components. Furthermore, the results suggest that imipramine inhibits both components of \( I_K \). In fact, imipramine delayed the activation of both the aggregate \( I_K \) and \( I_{K_r} \) when the rapidly activating component was blocked with 30 \( \mu \)mol/L La\(^{3+}\).

It has been reported that Co\(^{2+}\) blocks the aggregate \( I_K \) and shifts the activation curve for aggregate \( I_K \) in the guinea pig in a concentration-dependent manner. However, in six additional experiments in which nisoldipine (200 nmol/L) was used as an \( I_{Ca} \) blocker, effects similar to those reported in the present study were observed (Fig 11).

Effects of Imipramine on the Slow Component of \( I_K \)

The voltage dependence of imipramine effects on this component of \( I_K \) was assessed by analyzing the actions of imipramine on cells perfused with 30 \( \mu \)mol/L La\(^{3+}\) to inhibit \( I_{K_r} \). As it was previously reported, concentrations of La\(^{3+}\) > 10 \( \mu \)mol/L screen and bind negative charges on the external surface of the cell membrane, and displace Ca\(^{2+}\) from binding sites on the surface of sarcoplasmic reticulum Ca\(^{2+}\) stores, and after long exposures (>20 minutes) result in intracellular accumulation of La\(^{3+}\). However, measurements of the external surface potential in the presence of different concentrations of La\(^{3+}\) (between 10\(^{-7}\) and 10\(^{-2}\) mol/L) indicate that the shift in the activation \( I_{K_s} \) curve cannot result exclusively from the availability of this ion to screen and bind external negative surface charges. Therefore, La\(^{3+}\) specifically blocks \( I_{K_r} \). Under these experimental conditions, imipramine (1, 5, and 10 \( \mu \)mol/L) failed to change the activation midpoint or the slope factor of the \( I_{K_s} \) activation curve. The reversible imipramine-induced block was always more evident after weak depolarizations (-30 to +30 mV) than after more positive steps (see Fig 5 and 11). If imipramine binds to an open state of the \( K_+ \) channel, one would expect an increased block of this channel to occur when stepping to more positive potentials, as the probability of opening increases at more positive membrane potentials. Nevertheless, at very positive potentials (+50 to +70 mV) and after long sustained depolarizations (>5 seconds), the imipramine-induced block was partially relieved (see Figs 4B, 5B, and 9, middle). Also, the deactivating process of \( I_{Na} \) is not modified by imipramine in La\(^{3+}\) perfused cells. The absence of an effect on the time course of \( I_{Na} \) deactivation indicates that imipramine blocks a closed state of the \( K_+ \) channel. However, the time course of unblocking may depend on the kinetics with which the drug interacts with the channel and may vary at different membrane potentials. Moreover, open-channel unblock would be most apparent at short times relative to channel closure and could appear primarily as an alteration in the peak \( I_{Na} \). Although in our experiments we were not able to see changes in the \( I_{Na} \) time course, the use of a global time constant measurement at a single potential could mask the possible effects of imipramine on an open state of the \( K_+ \) channel. In fact, a brief delay of the \( I_{Na} \) peak can be observed (Fig 5A), which could be consistent with an open-channel unblocking mechanism. The slow opening rate of the \( K_+ \) channels makes it difficult to study an open-channel interaction on the depolarizing pulse, unless the kinetics of drug binding was slower than the opening kinetics of these channels. On repolarization to -30 mV, if there is a large fraction of open channels blocked and the unbinding rate of the drug is fast enough, \( I_{Na} \) may display a delay of the \( I_{Na} \) peak, reflecting the open-channel unblocking mechanism.

The later part of the activation kinetics of the slow component of \( I_K \) was fitted with a single exponential to obtain the dominant time course of this process and its delay, without making any assumption about the gating mechanism of this channel. Imipramine (1 and 10 \( \mu \)mol/L) did not change the later time course of this process. However, it delayed the activation in a concentration-dependent manner by 62±22, 74±23, and 155±53 milliseconds in the presence of 1, 5, and 10 \( \mu \)mol/L imipramine. This delay in the activation suggests that imipramine binds to a closed state of the \( K_+ \) channel, which is also consistent with the results obtained from the voltage dependence of imipramine block. On the other hand, the imipramine-induced \( I_{K_s} \) block after long depolarizing pulses in the presence of high concentrations of imipramine (10 \( \mu \)mol/L) was not significantly smaller than that observed after short (0.5-seconds) depolarizing pulses, which can be interpreted as an open-state interaction with high concentrations of the drug. Thus, our experiments suggest that imipramine preferentially inhibits a closed state of the \( K_+ \) channel, although a possible interaction with its open state cannot be ruled out. Thus, further experiments at the single-channel level are necessary to further characterize the imipramine-\( K_+ \) channel interaction.

Effects of Imipramine on the Rapidly Activating Component of \( I_K \)

The effects of imipramine on \( I_{K_r} \) cannot be studied directly because there is no specific blocker of \( I_{K_r} \). However, there are several pieces of evidence indicating that imipramine blocks this component of \( I_K \). First, the envelope-of-tails test was satisfied in cells perfused with 1 \( \mu \)mol/L imipramine. Thus, in the presence of imipramine, the envelope-of-tails test demonstrated that the recorded outward current was composed by a single current, \( I_{K_r} \). Second, the activation curve of the imipramine-sensitive current exhibits a midpoint and a slope factor (-17.3±2.5 and 7.3±0.5 mV, respectively) that are very close to those previously reported for the E-4031-sensitive current. Furthermore, the imipramine-sensitive current obtained after short (0.4-second) depolarizing pulses shows inward rectification in the same way that the E-4031-sensitive current does. These results strongly suggest that imipra-
mine, at this concentration, completely inhibits the rapidly activating component of the time-dependent $I_{K_w}$ with little effect on the slow ($I_{K_s}$) component. Similar results have been obtained in the presence of E-4031, d-sotalol, and flecainide, 14,15,37,39 drugs that specifically block the rapidly activating component of $I_K$. Additional experiments are necessary to assess the underlying mechanism through which imipramine inhibits this $I_K$ component.

Relation of Imipramine Effects to Action Potential Duration

It is well known that ionic currents responsible for the repolarization of the cardiac action potential show important species dependence. Frog and guinea pig isolated ventricular myocytes exhibit a large maintained time- and voltage-dependent $I_{K_w}$38-40. However, this current is relatively small in atrial and ventricular myocytes isolated from rat, dog, rabbit, or human hearts, 11,12,37,42 44. In these latter four species, a larger transient (inactivating) outward $K^+$ current, $I_{K_s}$, is responsible to a major extent for repolarization of the action potential. Since imipramine inhibits both components of $I_K$ as well as $I_{K_w}$, 45 a lengthening of the action potential duration would be expected. However, a shortening of the action potential had been observed in guinea pig papillary muscles and isolated myocytes, 1,2,7,8. This shortening could be attributed to the inhibitory effects of imipramine on $I_{K_s}$7,8,10. However, in guinea pig ventricular cells, imipramine, even at concentrations that do not affect this current, is still able to shorten the action potential duration.19 Moreover, it has been demonstrated that imipramine fails to shorten the duration of action potentials elicited in the presence of tetrodotoxin. This effect of imipramine on the action potential duration has been attributed to the inhibitory effects of the drug on the late $I_{K_s}$ flowing during the plateau in guinea pig ventricular myocytes.7

We have investigated the effects of therapeutically relevant concentrations of imipramine (1 to 5 μmol/L)45 on the outward plateau currents of guinea pig ventricular myocytes. However, it is difficult to relate the in vivo plasma concentrations to those of drug-perfusing isolated cardiac myocytes, particularly when the drug is highly bound to plasma and tissue proteins. In fact, it has been found that in experimental animals and humans, imipramine, a highly lipophlic drug, has a selective affinity for cardiac muscle, where it can reach concentrations 20 to 200 times greater than the concentrations found in plasma.45-47 Therefore, the effects of these concentrations of imipramine on the time-dependent $I_K$ can be not only of experimental but also of clinical relevance.

Acknowledgments

This study was supported by CICYT SAF92-0157, FIS 91/0648, Salud2000, and Instituto de Cooperación Iberoamericana grants. The authors express their thanks to Drs Luc M. Hondeghem and Paul B. Bennett for reading the manuscript and their valuable suggestions.

References

26. Wiedt-Gallitelli M, Isenberg G. Extra- and intracellular lanthanum: modified calcium distribution, inward currents and con-
42. Hume J, Uehara A. Ionic basis of the different action potential configurations of single guinea-pig atrial and ventricular myocytes. *J Physiol (Lond).* 1985;358:525-544.
Imipramine blocks rapidly activating and delays slowly activating K+ current activation in guinea pig ventricular myocytes.
C Valenzuela, J Sánchez-Chapula, E Delpón, A Elizalde, O Pérez and J Tamargo

doi: 10.1161/01.RES.74.4.687

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/74/4/687

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/