Open Channel Block of A-type, Kv4.3 and Delayed Rectifier K⁺ Channels, Kv1.3 and Kv3.1 by Sibutramine

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   Kv, voltage-gated K⁺ channel; CHO, Chinese hamster ovary; CNS, central nervous system; IMDM, Iscoves’ modified Dulbecco’s medium
ABSTRACT

The effects of sibutramine on Kv4.3, Kv1.3 and Kv3.1, stably expressed in CHO cells, were investigated using the whole-cell patch-clamp technique. Sibutramine did not significantly decrease the peak Kv4.3 currents, but accelerated the rate of decay of current inactivation in a concentration-dependent manner. This phenomenon was effectively characterized by integrating the total current over the duration of a depolarizing pulse to +40 mV. The IC_{50} for the sibutramine block of Kv4.3 was 17.3 µM. Under control conditions, the inactivation of Kv4.3 currents could be fit to a biexponential function, and the time constants for the fast and slow components were significantly decreased after the application of sibutramine. The association (k_{+1}) and dissociation (k_{-1}) rate constants for the sibutramine block of Kv4.3 were 1.51 µM^{-1}s^{-1} and 27.35 s^{-1}, respectively. The theoretical K_d value, derived from k_{-1}/k_{+1}, yielded a value of 18.11 µM. The block of Kv4.3 by sibutramine displayed a weak voltage dependence, increasing at more positive potentials, and was use-dependent at 2 Hz. Sibutramine did not affect the time course for the deactivating tail currents. Neither steady-state activation and inactivation nor the recovery from inactivation was affected by sibutramine. Sibutramine caused the concentration-dependent block of the Kv1.3 and Kv3.1 currents with an IC_{50} of 3.7 and 32.7 µM, respectively. In addition, sibutramine reduced the tail current amplitude and slowed the deactivation of the tail currents of Kv1.3 and Kv3.1, resulting in a crossover phenomenon. These results indicate that sibutramine acts on Kv4.3, Kv1.3 and Kv3.1 as an open channel blocker.
Sibutramine is an anorectic agent for the management of obesity and exerts its pharmacological actions by inhibiting the reuptake of serotonin and norepinephrine in the CNS (Buckett et al., 1988; Jackson et al., 1997). An increase in these neurotransmitters in the brain induces satiety and decreases food intake (Halford et al., 2005). Sibutramine has a dual action and also increases the metabolic rate by enhancing peripheral norepinephrine functions (Finer, 2002). However, the concomitant increase in these monoamines in the central and peripheral nervous systems can lead to adverse CNS and cardiovascular effects. For example, headache, insomnia, and nervousness are the most common side effects of sibutramine (Luque and Rey, 1999). The use of sibutramine has also been associated with arrhythmia and hypertension (Luque and Rey, 1999; McMahon et al., 2000). Although sibutramine is known to be a relatively safe drug, its side effects are still under investigation.

The activity of Kv channels is critical in maintaining the resting membrane potential, regulating action potential duration and frequency, and determining pacemaker activity in a wide variety of excitable cells (Rudy, 1988). Kv4.3, one of the major transient outward Kv currents, is mainly expressed in a variety of tissues including neurons, cardiac myocytes and smooth muscle cells (Ohya et al., 1997; Birnbaum et al., 2004). This channel can contribute to distinct functional roles due to differences in the membrane potential of these cells. For example, in many neurons, Kv4.3 does not activate at the resting membrane potential but, in vascular smooth muscle cells, Kv4.3 is open in the window current range and is involved in the maintenance of membrane potential and the regulation of excitability (Amberg et al., 2003; Sergeant et al., 2005). Several studies have shown that some serotonin-norepinephrine reuptake inhibitors are effective blockers of Kv channels. Dexfenfluramine inhibits
delayed rectifier K⁺ channels in rat lingual taste cells (Hu et al., 1998) and rat vascular smooth muscle cells (Weir et al., 1996). Aminorex, phentermine, dexfenfluramine, sibutramine and fluoxetine also cause a concentration-dependent inhibition of the hKv1.5 current stably expressed in HEK cells (Perchenet et al., 2001). However, some of these drugs have been withdrawn from the market, due to primary pulmonary hypertension as a side effect. Although the mechanism by which these drugs cause pulmonary hypertension is not known, the inhibitory effect of the drug on Kv channels has been proposed as one of the important mechanisms responsible for causing vasoconstriction and initiating pulmonary hypertension (Weir et al., 1996). From a consideration of drugs that are involved in the modulation of Kv channels, the vasoconstriction of mesenteric arterial smooth muscle cells due to the block of these currents reduces mesenteric blood flow and diminishes the transport of absorbed nutrients (McDaniel et al., 2001). These potential mechanisms for inhibiting nutrient absorption can be considered to be a favorable anorectic effect. Therefore, to understand the therapeutic action of sibutramine and its side effects, the present study was designed to evaluate the effects of sibutramine on A-type, Kv4.3 and the delayed rectifier K⁺ channels, Kv1.3 and Kv3.1, which may play important roles in regulating the membrane potential of vascular smooth muscle cells, and to compare block of Kv4.3, and Kv1.3 and Kv3.1, by sibutramine.
Materials and Methods

**Cell Cultures.** The Kv4.3, Kv1.3 and Kv3.1 cDNA were stably transfected into CHO cells (ATCC, Rockville, MD, USA) using the lipofectamine reagent (Invitrogen Corporation, Grand Island, NY, USA) as previously described (Hahn et al., 1996; Choi et al., 1999; Ahn et al., 2006). CHO cells were maintained in IMDM (Invitrogen Corporation) supplemented with 10% fetal bovine serum, 0.1 mM hypoxanthine and 0.01 mM thymidine in a humidified 5% CO₂ incubator at 37°C. The transfected cells were exchanged with fresh IMDM containing 0.3 mg/ml of geneticin (Invitrogen) and passed at 2 - 3 day intervals using a brief trypsin-EDTA treatment. The cells were dissociated and seeded onto glass coverslips (diameter: 12 mm, Fisher Scientific, Pittsburgh, PA, USA) in a 35 mm dish 1 day before use. For electrophysiological experiments, coverslips with attached cells were transferred to a recording chamber (RC-13, Warner Instrument Corporation, Hamden, CT, USA). The chamber was superfused at a rate of 0.5 ml/min with normal external solution at room temperature (22 - 24°C).

**Electrophysiological Recordings.** Currents were recorded using the whole-cell patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were pulled from glass capillary tubing (PG10165-4, World Precision Instruments, Sarasota, FL, USA) on a programmable horizontal puller (Model P-97, Sutter Instrument Company, Novato, CA, USA). The tip resistances of the recording pipettes in the bath solution were 2 - 3 MΩ. The series resistances were approximately 4 to 8 MΩ. Whole-cell capacitive currents were compensated by analog compensation. Series resistance compensation (80%) was employed if the current exceeded 1 nA. The currents were low-pass filtered at 2
kHz (four-pole Bessel filter) and sampled at 5 kHz before being digitized. Data acquisition and analysis were performed with an IBM pentium computer, using the pClamp 9.01 software (Molecular Devices).

**Solutions and Drugs.** The external bath solution contained: 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM HEPES, and was adjusted to pH 7.3 with NaOH. The internal pipette solution contained: 140 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES and 10 mM EGTA, and was adjusted to pH 7.3 with KOH. The measured osmolarity of the solutions was 300 - 340 mOsm. Sibutramine (Hanmi Pharmaceutical, Seoul, Korea) was dissolved in the bath solution to give a 30 mM stock solution.

**Data Analysis.** The Origin 7.0 software program (OriginLab Corp., Northampton, MA, USA) was used for the analysis. The concentration-response data were fit to the following Hill equation:

$$y = \frac{1}{1 + ([D]/IC_{50})^n}$$

where $IC_{50}$ is the concentration of sibutramine required to produce a 50% block, [D] the concentration of sibutramine and $n$ the Hill coefficient. The voltage dependence of the fractional block ($f$) was fit to the following equation (Woodhull, 1973):

$$f = [D]/([D] + K_D(0) \times \exp (-z\delta FV/RT))$$

where $K_D(0)$ represents the apparent affinity at 0 mV, $z$ the charge valence of sibutramine, $\delta$ the fractional electrical distance, $F$ Faraday’s constant, $R$ the gas constant, and $T$ the absolute temperature. A value of 25.4 mV was used for $RT/F$ at 22°C in the present study. Kv4.3 currents were elicited by applying 500 ms depolarizing pulses from a holding potential of -80 mV to +40 mV at 10 s intervals. Steady-state activation curves were obtained by normalizing the tail currents.
measured at -60 mV after the application of 8 ms depolarizing pulses at potentials between -80 mV and +80 mV in 10 mV increments every 10 s from a holding potential of -80 mV in the absence and presence of sibutramine and were fit to the Boltzmann equation:

\[ y = \frac{1}{1 + \exp\left(-\frac{(V - V_{1/2})}{k}\right)} \]

where \( k \) represents the slope factor, \( V \) the test potential and \( V_{1/2} \) the potential at which the conductance was half-maximal. The voltage dependence for steady-state inactivation was investigated using a double pulse voltage protocol; currents were measured by 500 ms depolarizing pulses to +40 mV while 1 s preconditioning pulses were varied from -110 mV to +10 mV stepped by 10 mV at 10 s intervals in the absence and presence of the drug. The resulting steady-state inactivation data were fit to the Boltzmann equation:

\[ \frac{I - I_c}{I_{\text{max}} - I_c} = \frac{1}{1 + \exp\left(V - V_{1/2}\right)/k} \]

in which \( I_{\text{max}} \) represents the current measured at the most hyperpolarized preconditioning pulse, \( I_c \) a non-zero current which was not inactivated at the most depolarized preconditioning pulse, \( k \) the slope factor, \( V \) the test potential and \( V_{1/2} \) the potential at which the conductance was half-maximal. We eliminated the non-zero residual current by subtracting it from the actual value. Data are expressed as the mean ± S.E. The one-way analysis of variance, followed by Bonferroni test, was used to evaluate the statistical significance of the observed differences (Wallenstein et al., 1980). Statistical significance was considered at \( p < 0.05 \).
Results

Concentration-dependent Block of Kv4.3. Fig. 1 shows representative tracings of Kv currents from a CHO cell expressing Kv4.3 channels after the application of a 500 ms depolarizing pulse from -80 mV to +40 mV. Under control conditions, the Kv4.3 currents were activated to a peak and inactivated rapidly as previously reported (Ohya et al., 1997; Ahn et al., 2006). At the end of a 500 ms depolarizing pulse, Kv4.3 was almost completely inactivated and about 6% of the peak amplitude of Kv4.3 at +40 mV remained. In the presence of sibutramine, the peak amplitude of Kv4.3 was not affected at concentrations up to 10 µM, but an acceleration in the apparent rate of current decay was observed. For this reason, the concentration-dependent block of Kv4.3 was quantified as a reduction in the integral of the inactivating component of the Kv4.3 currents during the entire duration of a depolarizing pulse. Kv4.3 currents at +40 mV were reduced to 92, 89, 73, and 23% of the control after the application of 1, 3, 10 and 30 µM sibutramine, respectively. A nonlinear least-squares fit of the concentration-response equation to the individual data points yielded an apparent $IC_{50}$ of 17.3 ± 1.2 µM and a Hill coefficient of 2.0 ± 0.3 ($n = 7$). Furthermore, the acceleration in the inactivation rate of Kv4.3 by sibutramine was concentration-dependent (Fig. 2). Under control conditions, the time course for the inactivation of Kv4.3 was best fit to a biexponential function, and the time constants for the fast and slow components of inactivation were 25.1 ± 1.0 and 178.5 ± 30.6 ms ($n = 7$), respectively. The fast component of the inactivation current was predominant at +40 mV (87.5%) under control conditions. At 1, 3 and 10 µM, sibutramine decreased the fast time constants to 22.3 ± 1.4, 20.5 ± 1.0 and 18.2 ± 1.2 ms and the slow time constants to 137.9 ± 32.1, 116.7 ± 20.1 and 107.7 ± 22.5 ms ($n$
= 7), respectively. However, the relative contribution of the fast component to the total inactivation of Kv4.3 was not changed from 87.5% under control conditions to 86.4, 85.2 and 85.1% in the presence of 1, 3, and 10 µM sibutramine, respectively.

To assess the reversibility of the effect of sibutramine on Kv4.3, the single depolarizing pulse to +40 mV was repeated while 20 µM sibutramine was applied. The block of Kv4.3 by sibutramine appeared within 20 s of drug application and reached a steady state within 2 min. The washout of sibutramine by perfusion with a drug-free solution was complete within 1 min and the currents recovered to 95.5 ± 3.6% (n = 5) of the control. Furthermore, sibutramine did not affect the inactivation kinetics of Kv4.3 (fast time constant: 28.9 ± 3.2 ms, slow time constant: 152.8 ± 9.3 ms, n = 5) after washout. Thus, the effect of sibutramine on Kv4.3 is reversible.

**Time-dependent Block of Kv4.3 by Sibutramine.** The onset of the sibutramine block of Kv4.3 was time-dependent and appeared to develop slowly during depolarization. Because the acceleration in the current decay of Kv4.3 in the presence of the drug included both the current block by sibutramine and the intrinsic inactivation of the channel, the time course for sibutramine block was obtained by subtracting the current in the presence of the drug from the control current (Fig. 3A). This procedure was carried out at four different drug concentrations. The time course for the sibutramine-sensitive current was fit to a biexponential function that yielded the concentration-dependent fast and slow time constants. The fast components were taken as an approximation of the time course of the drug-channel interaction kinetics and plotted as a function of sibutramine concentration (Fig. 3B). A plot of the reciprocal of $\tau$ at +40 mV versus each concentration yielded an apparent association rate constant of $1.51 \pm 0.15 \mu M^{-1}s^{-1}(k_{+1})$ and a dissociation rate constant
of 27.35 ± 2.15 s\(^{-1}\) (\(k_+\)) (\(n = 7\)). Thus, the estimated \(K_d (k_+/k_-)\) is 18.11 ± 2.92 \(\mu\)M (\(n = 7\)), in good agreement with the \(IC_{50}\) of 17.3 \(\mu\)M calculated from the concentration-response curve. Furthermore, the exponential fits were extrapolated to zero block at the start of the depolarization pulse, suggesting that there was no block of Kv4.3 before activation.

**Voltage-dependent Block of Kv4.3.** Fig. 4A shows the current-voltage relationships in the absence and presence of 20 \(\mu\)M sibutramine in a typical experiment. The Kv4.3 currents began to activate at -50 mV and the control current-voltage relationship was almost linear for the depolarizations (Fig. 4B). Sibutramine reduced the Kv4.3 currents over the entire voltage range over which this current is activated. When the current block was expressed as a function of the test potential (Fig. 4C), the blocking effects of sibutramine on Kv4.3 were found to be voltage-dependent: the block increased between -20 and +20 mV, corresponding to the voltage range for channel opening (\(n = 8, p < 0.05\)). Furthermore, the reduction in the amount of charge crossing the membrane during depolarization increased in a shallow manner at membrane potentials where the maximal conductance was reached (+30 to +60 mV). This voltage dependence was fit to Woodhull’s equation (Materials and Methods) and yielded \(\delta = 0.16 ± 0.07\) (\(n = 8\)).

**Use-dependent Block of Kv4.3.** Fig. 5A shows Kv4.3 current traces elicited in the absence and presence of 20 \(\mu\)M sibutramine while applying a train of 10 depolarizing pulses of 20 or 200 ms duration at a frequency of 2 Hz. Because the peak amplitude of the Kv4.3 currents remained almost unaffected by repetitive pulses at 1 Hz under control conditions (data not shown), we studied use-dependent block by sibutramine at 2 Hz with variable pulse duration (20 and 200 ms). Under
control conditions, the peak amplitude of the Kv4.3 currents was not significantly reduced at the first pulse when 20 ms depolarizing pulses were applied, but declined with successive pulses, reaching a steady-state level equivalent to 93.9% of the control amplitude after 5 pulses. After increasing the pulse duration (200 ms), a further reduction in the peak currents was observed during the pulse trains, progressing to 22.2% block at the 5th pulse. In the presence of 20 µM sibutramine, the amplitude of the peak current was not significantly reduced at the first pulse indicating the absence of any tonic block by the drug. However, sibutramine produced use-dependent block with successive pulses when either 20 ms or 200 ms depolarizing pulses were applied. At the 10th pulse, the amplitude of the Kv4.3 currents for 20 ms pulse was 87.1% of the first pulse, whereas this percentage decreased to 68.1% for a 200 ms pulse duration. Although pulses of long duration (200 ms) resulted in a greater reduction in current compared to those of short duration (20 ms), the degree of block between the 20 ms and 200 ms pulses was not significantly changed by sibutramine, as shown in Fig. 5B.

**Effect of Sibutramine on the Time Course of Tail Currents.** In the absence of the drug, Kv4.3 currents were completely deactivated at -60 mV with a time constant of 10.0 ± 0.9 ms (n = 6) (Fig. 6). Sibutramine at a concentration of 20 µM reduced the tail current amplitude but failed to alter the time course for the deactivation of the tail current. The average value was 13.1 ± 2.4 ms (n = 6), not significantly different from the control value.

**Effect of Sibutramine on the Steady-state Activation and Inactivation of Kv4.3.** The voltage dependence for steady-state activation in the absence and presence of sibutramine was evaluated (Fig. 7A). The tail currents were recorded at -
60 mV after 8 ms depolarizing pulses between -80 mV and +80 mV in steps of 10 mV from a holding potential of -80 mV under control conditions and in the presence of 20 μM sibutramine. Activation curves were drawn by fitting the normalized tail currents to the Boltzmann equations. The potential for half-maximum activation (V_{1/2}) and the slope factor (k) were -7.7 ± 2.2 mV and 12.8 ± 0.7 mV for the control and -8.8 ± 3.7 mV and 12.3 ± 1.3 mV in the presence of 20 μM sibutramine (n = 8). These values were not statistically different. Thus, the steady-state activation curves remained unchanged in the presence of 20 μM sibutramine. Fig. 7B shows the steady-state inactivation curves of Kv4.3 in the absence and presence of 20 μM sibutramine. The steady-state inactivation curves were drawn by fitting the normalized peak currents to the Boltzmann equations. The potential of the half-inactivation point (V_{1/2}) and the slope factor (k) of the steady-state inactivation curves were -50.4 ± 1.9 mV and 4.2 ± 0.1 mV for the control, and -55.9 ± 2.0 mV and 4.3 ± 0.1 mV for 20 μM sibutramine, respectively (n = 8), and these changes of V_{1/2} and k were not statistically different.

**Effect of Sibutramine on the Recovery from Inactivation of Kv4.3.** Fig. 8 shows a typical example of the recovery kinetics of Kv4.3 in the absence and presence of 20 μM sibutramine. The peak currents elicited by the second depolarizing pulse were divided by those evoked by the first prepulse, and the normalized data were plotted against the interpulse interval. Under control conditions, the plotted data were fit well to a single exponential function with a time constant of 0.25 ± 0.03 ms (n = 7). In the presence of sibutramine, the time constant for recovery was 0.33 ± 0.04 ms (n = 7) and there was no significant drug effect in altering the recovery from the inactivation of Kv4.3.
Concentration-dependent block of Kv1.3 and Kv3.1. Fig. 9 shows the Kv1.3 (A) and Kv3.1 (B) currents expressed in CHO cells under control conditions and in the presence of various concentrations of sibutramine. Sibutramine decreased the peak amplitudes of Kv1.3 and Kv3.1 slightly, but increased the rate of current inactivation during depolarization. Thus, the peak amplitude of the currents was affected much less than the steady-state current amplitude at the end of the depolarizing pulses which was used as an index of the block. The steady-state currents of Kv1.3 and Kv3.1 were decreased by sibutramine in a concentration-dependent manner with an IC₅₀ of 3.7 ± 0.7 (n = 6) and 32.7 ± 5.0 µM (n = 5), respectively. The effects of sibutramine on the time constants for the deactivating tail currents of both channels were also analyzed. In the absence of the drug, the decay in tail currents was fit to a monoexponential function and the time constants for the tail currents of Kv1.3 and Kv3.1 averaged 33.9 ± 2.0 (n = 6) and 5.1 ± 0.8 ms (n = 5) at -40 mV, respectively. After exposure to sibutramine, the peak tail currents of Kv1.3 and Kv3.1 decreased and the subsequent time course for the tail currents was slower compared to the control. The average values were 81.8 ± 6.8 (n = 6) and 28.7 ± 2.7 ms (n = 5) for Kv1.3 and Kv3.1, respectively. Consequently, a crossover phenomenon of the tail currents in the absence and presence of sibutramine was observed.
DISCUSSION

The main finding of the present study is that sibutramine has two effects on Kv4.3: 1) an acceleration of the time-dependent decrease in current in a concentration-dependent manner and 2) use-dependent block. Similar blocking effects were also observed for Kv1.3 and Kv3.1, in terms of time- and concentration-dependent block. Furthermore, the deactivation kinetics of Kv1.3 and Kv3.1 upon repolarization was slowed, with a crossover in the tail currents. Thus, the effects of sibutramine on these currents may be explained by the block of these channels in the open states.

In the present study, the action of sibutramine on accelerating the rate of current inactivation of Kv4.3 in a concentration-dependent manner can be explained by an open channel block mechanism. Similar findings have previously been reported as characteristics of the block of Kv1.5 channels by sibutramine (Perchenet et al., 2001), bisindolylmaleimide (Choi et al., 2000), zatebradine (Valenzuela et al., 1996) and terfenadine (Yang et al., 1995). They are all characterized as open channel blockers: the acceleration in the current decay by the drugs is due to the slow binding of the drug to the channels after channel activation rather than the modulation of intrinsic inactivation. Thus, the mechanism of channel block by sibutramine in Kv4.3 and Kv1.5 channels is similar. Additional evidence of an open channel block is the voltage dependence of the sibutramine block. The block increased with channel opening over the voltage range of activation (-20 to +20 mV), suggesting the preferential binding to the open state of the channel. Because sibutramine is predominantly in the cationic form at physiological pH (Perchenet et al., 2001), the interaction of sibutramine and channels would be expected to experience a
transmembrane electrical field. Indeed, the block of Kv1.5 by sibutramine exhibits a shallow voltage-dependence which was described by an electrical distance ($\delta$) of 0.17 (Perchenet et al., 2001). At potentials positive to +30 mV where the maximal conductance is reached, the block of Kv4.3 increased with a shallow voltage dependence. Because the voltage-dependent block over this voltage range is generally considered to be an open channel block (Snyders et al., 1992; Valenzuela et al., 1996), this reflects the effects of the electrical field on the interaction between the charged form of sibutramine and the channel. We obtained a fractional electrical distance ($\delta$) of 0.16 for sibutramine, similar to that previously reported for sibutramine for Kv1.5 (Perchenet et al., 2001). This analysis suggests that sibutramine binds to a similar site in the internal mouth of the channel. Although the possibility that sibutramine increases the rate of intrinsic inactivation of Kv4.3 cannot be completely excluded, all of the actions of sibutramine may be explained by a single mechanism of open channel block.

Another characteristic of sibutramine block is its use dependence. The degree of block increased with repetitive depolarizations. However, the peak amplitude of Kv4.3 elicited by the first pulse was not significantly modified and a decrease in current amplitude was observed with subsequent pulses, indicating that the channels are not blocked before channel activation by depolarization. Furthermore, extrapolation to zero at the start of the pulse suggests that there is no block of Kv4.3 in the resting state. Use-dependent block may be accomplished by the binding of the drug either to the open or to the inactivated states of channels (Courtney, 1975; Butterworth and Strichartz, 1990; Wang et al., 1995; Valenzuela et al., 1996; Delpon
et al., 1997). To determine whether the binding of sibutramine to the open state of the channel was influenced by the process of inactivation of Kv4.3, we studied the use-dependent block using variable pulse durations. Although an enhancement in use dependence was observed with long duration pulses (200 ms) which allow adequate inactivation to occur compared to short duration pulses (20 ms) which limit the entry of Kv4.3 channels into the inactivated state, no change in the degree of block between the short and the long duration pulses in the presence of the drug was observed. Furthermore, sibutramine block was not affected by depolarizing prepulses over the inactivation voltage range as shown in the steady-state inactivation curves. These results suggest that the use-dependent block is due to channel activation but is independent of the channel inactivation of Kv4.3, and the interaction of sibutramine with the binding site was not influenced by the gating kinetics of inactivation.

Use-dependent block is generally associated with a slow rate of recovery from inactivation due to the slower dissociation of the drug from its binding site. However, in the present study, the recovery process in control and with sibutramine was the same: the rate of recovery of the blocked channel is similar to that of recovery from intrinsic inactivation. The lack of change in the time course for recovery from inactivation rules out the possibility that the drug interacts with Kv4.3 channels in the inactivated state. Furthermore, these results are similar to previous findings showing that mibebradil induced significant use-dependent block but paradoxically increased the rate of recovery from inactivation of Kv1.5 (Perchenet and Clement-Chomienne, 2000). According to this study, mibebradil exclusively competes with the inactivation gate in the channel pore. Thus, one possible explanation for the use-dependent block is that there is competition between the modulatory site of
inactivation and sibutramine, and the blocked channels cannot inactivate after drug binding. The drug is trapped at its binding site after the open channels are blocked and the use-dependent block develops when the rate of drug binding is equal to the rate of drug untrapping.

Sibutramine slowed the deactivation time course of Kv1.3 and Kv3.1, thus inducing a tail crossover. This phenomenon has also been reported for other open channel blockers of Kv1.5, such as sibutramine, terfenadine and zatebradine (Yang et al., 1995; Valenzuela et al., 1996; Perchenet et al., 2001), and provides further evidence for the open channel block mechanism. However, the tail crossover phenomenon which occurred in the release of the drug before channel closing was not observed in sibutramine block of Kv4.3. These results suggest that sibutramine is not able to dissociate from its binding site before the channels close and is trapped in the deactivating channels, as described above. Loratadine, which also acts as an open channel blocker, did not induce a tail crossover of Kv1.5 currents, suggesting that the channel can close with the drug bound (Lacerda et al., 1997). Propafenone did not induce a tail crossover in delayed rectifier K⁺ currents due to being trapped in the deactivating channels during closing or slow unblocking kinetics which produced use-dependent effects of the drug (Delpon et al., 1995). The differences in deactivation kinetics between both channels may be due to the slower dissociation rate constant observed for Kv4.3 than for Kv1.5 (Perchenet et al., 2001). Thus, this characteristic block of Kv4.3 by sibutramine differentiates it from other open channel blockers studied on delayed rectifier K⁺ channels, Kv1.3, Kv3.1 and Kv1.5.

Sibutramine is a centrally acting anorectic agent for the treatment of obesity (Buckett et al., 1988). While the precise mechanism by which sibutramine causes
weight loss remains unclear, the therapeutic effects of sibutramine are known to involve the selective inhibition of the reuptake of monoamines in the brain, thus inducing satiety and reducing feeding behavior (Halford et al., 2005). Since some anorexic agents, such as aminorex and fenfluramine that interfere with serotonin function have been associated with an increased incidence of pulmonary hypertension, these drugs have been withdrawn from the market. Sibutramine is known to be an efficacious and safe drug for the management of obesity and has not been shown to induce pulmonary hypertension although some cardiovascular dysfunctions have been reported in patients receiving sibutramine (Luque and Rey, 1999; McMahon et al., 2000). All of these drugs are known to inhibit Kv currents in vascular smooth muscle cells (Weir et al., 1996) and a dysfunctional Kv in pulmonary smooth muscle cells has been implicated in primary pulmonary hypertension (Yuan et al., 1998). Several types of Kv channels, including Kv1.3, Kv1.5, Kv3.1 and Kv4.3, have been identified in human artery smooth muscle cells and the functional expression of these channels may play a critical role in the regulation of membrane potential and pulmonary vascular tone (Amberg et al., 2003; Platoshyn et al., 2004). These observations suggest that sibutramine may regulate vascular tone through modulating Kv channel activity in pulmonary vascular smooth muscle cells. Thus, special attention to the possibility that pulmonary hypertension may occur is necessary in predisposed patients. Furthermore, since Kv4.3 and Kv1.5 are expressed at high levels in the heart and are responsible for repolarization of the cardiac action potential, cardiac arhythmias associated with the administration of sibutramine may be, at least partly, related to block of these channels in cardiac myocytes. On the other hand, anorexic effects of Kv channel blockers have been
reported in mesenteric arterial smooth muscle cells (McDaniel et al., 2001). Since Kv4.3, Kv1.3, Kv3.1 and Kv1.5 channels have been identified in vascular smooth muscle cells (McDaniel et al., 2001; Mandegar et al., 2002; Platoshyn et al., 2004; Sergeant et al., 2005), the inhibition of these channels in mesenteric arterial smooth muscle cells leads to membrane depolarization which, in turn, induces calcium entry and vasoconstriction. Thus, sibutramine can reduce mesenteric blood flow which transports nutrients to the liver and adipose tissue through its action on Kv4.3, Kv1.3 and Kv3.1 in the present study and Kv1.5 (Perchenet et al., 2001). Thus, we proposed that the block of Kv4.3, Kv1.3 and Kv3.1 by sibutramine involves a mechanism underlying another anorectic and metabolic action of this drug.

In conclusion, sibutramine blocked Kv4.3 binding to open channels in a concentration- and time-dependent manner. The open channel block of Kv4.3 by sibutramine is generally similar to that observed for Kv1.3, Kv3.1 and Kv1.5, as reported in previous studies, while its effects on current kinetics may vary depending on the channel being studied. Our results may help explain the mechanism underlying some of the therapeutic action of this drug and side effects associated with its use.
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Footnotes

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Fig. 1. Concentration dependence for sibutramine block of Kv4.3 whole-cell currents expressed in CHO cells. Kv4.3 currents were obtained by applying 500 ms depolarizing pulses from a holding potential of -80 mV to +40 mV at 10 s intervals in the absence and presence of 1, 3, 10, and 30 µM sibutramine. The dotted line represents the peak level of the Kv4.3 current in the control. Concentration-response curve for the block of Kv4.3 currents by sibutramine. The integral of a depolarizing pulse was normalized to the current recorded under control conditions. The normalized currents were plotted against sibutramine concentrations and fit to the Hill equation. Data are expressed as means ± S.E.

Fig. 2. Effects of sibutramine on the kinetics of inactivation of Kv4.3 currents. The inactivation time constants of the fast component ($\tau_{\text{fast}}$) and the slow component ($\tau_{\text{slow}}$) were obtained from a biexponential fit. Statistical significance versus control is indicated by *, $p < 0.05$ ($n = 7$). Data are expressed as means ± S.E.

Fig. 3. Time-dependent block of Kv4.3 by sibutramine. A, The sibutramine-sensitive currents were first subtracted from the control, and then normalized by the control. This procedure was carried out at four different drug concentrations. The time course was fit to a biexponential function that yielded the concentration-dependent time constants. B, The inverses of fast time constants were plotted versus sibutramine concentration. The solid line represents the least-squares fit of the data to the following equation: $1/\tau = k_+ [D] + k_- \tau$ in which $\tau$ is the drug-induced time...
Fig. 4. Effects of sibutramine on the Kv4.3 current-voltage relationship. 
A, Representative whole-cell Kv4.3 current traces under control conditions and in the presence of 20 µM sibutramine. Whole-cell currents were obtained by applying 500 ms depolarizing pulses from −80 mV to +60 mV in steps of 10 mV every 10 s from a holding potential of −80 mV. 
B, Current-voltage curves for sibutramine-induced Kv4.3 currents. The data were taken from the integral of the depolarizing pulses shown in Fig. 3A under control conditions and after the addition of 20 µM sibutramine, and normalized to the control. 
C, Voltage-dependent block of Kv4.3 currents by sibutramine was expressed as a relative current (I_{Sibutramine}/I_{Control}). The integral of currents in the presence of sibutramine was normalized to that at each voltage of the control. In the voltage range between -20 mV and +20 mV for channel opening, the block of Kv4.3 currents by sibutramine increased and was significantly different (n = 8, *, p < 0.05 versus data at -20 mV). The dotted line represents the activation curve of Kv4.3 under control conditions. Data are expressed as means ± S.E.

Fig. 5. Use-dependent block of Kv4.3 by sibutramine. 
A, Repetitive 20 or 200 ms depolarizing pulses of +40 mV from a holding potential of −80 mV were applied at the 2 Hz frequency under control conditions and after the application of 20 µM sibutramine. The peak amplitudes of the current at each pulse were normalized to that of the current obtained at the first pulse and then plotted versus pulse number (n
= 5). B, The current in the presence of sibutramine at 10th pulse was normalized to that in the absence of the drug. Data are expressed as means ± S.E.

Fig. 6. Effect of sibutramine on the time course of tail currents. Tail currents were induced at the repolarizing pulses to -60 mV after a 20 ms depolarizing pulse of +40 mV from a holding potential of -80 mV in the absence and presence of sibutramine.

Fig. 7. Effects of sibutramine on the steady-state activation of Kv4.3 A, Tail currents were recorded at -60 mV after 8 ms depolarizing pulses between -80 mV and +80 mV in steps of 10 mV from a holding potential of -80 mV under control conditions and in the presence of 20 µM sibutramine. The steady-state activation curves were drawn by fitting normalized tail currents to the Boltzmann equations (n = 8). Effects of sibutramine on the steady-state inactivation of Kv4.3 (B). The currents were evoked by 1 s prepulses that were varied from -110 mV to +10 mV stepped by 10 mV and a 500 ms depolarizing pulse to +40 mV in the absence and presence of sibutramine. Steady-state inactivation curves were shown as a plot of normalized peak currents during the depolarizing pulse as a function of the conditioning potential. The curves represent the best-fit Boltzmann equations (n = 8). Data are expressed as means ± S.E.

Fig. 8. Effects of sibutramine on the recovery from inactivation of Kv4.3. A double pulse protocol was used to characterize the recovery of Kv4.3 channels from inactivation: the first prepulse of a 500 ms depolarizing pulse of +40 mV from a holding potential of -80 mV was followed by a second identical pulse after
increasing the interpulse intervals between 5 and 5,000 ms at -80 mV. Typical Kv4.3 current traces for recovery from inactivation were shown under control conditions and in the presence of 20 μM sibutramine. The peak currents elicited by the second depolarizing pulse were divided by those evoked by the first prepulse and the normalized data were plotted against the interpulse interval. The plotted data were fit well to a single exponential function under control conditions and in the presence of 20 μM sibutramine (n = 7). Data are expressed as means ± S.E.

Fig. 9. Concentration dependence for sibutramine block of Kv1.3 (A) and Kv3.1 (B) whole-cell currents expressed in CHO cells. Kv1.3 currents were obtained by 200 ms depolarizing pulses from a holding potential of -80 to +40 mV at 30 s intervals in the absence and presence of 0.03, 0.3, 3, and 30 μM sibutramine. The steady-state currents of depolarizing pulses were normalized to the current recorded under control conditions. The normalized currents were plotted against sibutramine concentrations and fit to the Hill equation. The data yielded an IC₅₀ of 3.7 ± 0.7 μM and a Hill coefficient of 1.1 ± 0.2 (n = 6). Tail currents were recorded during 200 ms repolarizing pulses of -40 mV after 200 ms depolarizing pulses of +40 mV from a holding potential of -80 mV in the absence and presence of 3 μM sibutramine. Kv3.1 currents were obtained by 250 ms depolarizing pulses from a holding potential of -80 mV to +40 mV at 10 s intervals in the absence and presence of 3, 10, 30, and 100 μM sibutramine. The steady-state currents of depolarizing pulses were normalized to the current recorded under control conditions. The normalized currents were plotted against sibutramine concentrations and fit to the Hill equation. The data yielded an IC₅₀ of 32.7 ± 5.0 μM and a Hill coefficient of 1.6 ± 0.2 (n = 5). Tail
currents were recorded during 250 ms repolarizing pulses of –40 mV after 250 ms depolarizing pulses of +40 mV from a holding potential of –80 mV in the absence and presence of 30 µM sibutramine. The dotted lines in A and B represent zero current. Data are expressed as means ± S.E.
Figure 1

![Graph showing the effect of Sibutramine on normalized area vs concentration.](image-url)
Figure 2

- Top graph: 
  - Y-axis: $\tau_{\text{fast}}$ (ms)
  - X-axis: Sibutramine ($\mu$M)
  - Data points with error bars

- Bottom graph: 
  - Y-axis: $\tau_{\text{slow}}$ (ms)
  - X-axis: Sibutramine ($\mu$M)
  - Data points with error bars
Figure 6

Control

20 μM Sibutramine

50 pA

50 ms
Figure 8

Control

20 μM Sibutramine

Normalized current

Interpulse time (s)

Control

20 μM Sibutramine