Non-specific action of methoxamine on I_{to}, and the cloned channels hKv 1.5 and Kv 4.2

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1 The α_1-adrenoceptor agonist methoxamine acted independently of receptor activation to reduce I_{to} and the sustained outward current in rat ventricular myocytes, and hKv 1.5 and Kv 4.2 cloned K^+ channel currents. Two hundred μM methoxamine reduced I_{to} by 36% in the presence of 2 μM prazosin, and by 37 and 38% after preincubation of myocytes with either N-ethylmaleimide or phenoxybenzamine ($n = 6$). The EC_{50} values at $+60$ mV for direct reduction of I_{to}, hKv 1.5, and Kv 4.2 by methoxamine were 239, 276, and 363 μM, respectively, with Hill coefficients of 0.87–1.5.

2 Methoxamine accelerated I_{to} and Kv 4.2 current inactivation in a concentration- and voltage-dependent manner. Apparent rate constants for methoxamine binding and unbinding gave K_d values in agreement with EC_{50} values measured from dose-response relations. The voltage-dependence of block supported charged methoxamine binding to a putative intracellular site that sensed $\sim 20\%$ of the transmembrane electrical field.

3 In the presence of methoxamine, deactivating Kv 4.2 tail currents displayed a distinct rising phase, and were slowed relative to control, such that tail current crossover was observed. These observations support a dominant mechanism of open channel block, although closed channel block could not be ruled out.

4 Single-channel data from hKv 1.5 patches revealed increased closed times with blank sweeps and decreased burst duration in the presence of drug, and a reduction of mean channel open time from 1.8 ms in control to 0.4 ms in 500 μM methoxamine. For this channel, therefore, both open and closed channel block appeared to be important mechanisms for the action of methoxamine.

Keywords: Methoxamine; transient outward current; hKv 1.5; Kv 4.2; channel block

Abbreviations: CTL, control; δ, fraction of transmembrane electric field sensed by a singly-charged drug molecule at its receptor site; Mox, methoxamine; NEM, N-ethylmaleimide; PE, phenylephrine; PhBz, phenoxybenzamine; Pz, prazosin; τ_{block}, time constant of block of current by drug; $\tau_{deactivation}$, time constant of deactivation of tail currents; τ_{decay}; time constant of current decay in the presence of drug; τ_{inact}; time constant of control current inactivation

Introduction

Inhibition of repolarizing K^+ currents in cardiac muscle in response to α-adrenoceptor stimulation by hormones, neuro-transmitters, and drugs is one mechanism of positive inotropy in the heart, which becomes increasingly important pathophysiologically when α_1-adrenoceptors are increased in proportion to other adrenoceptor subtypes, such as during heart failure (Hwang *et al.*, 1996). Many previous studies of α_1-adrenoceptor-mediated responses in cardiac tissues have used the α-adrenoceptor-specific agonist methoxamine (Fedida *et al.*, 1990), since methoxamine lacks activity at β-adrenoceptors. Structurally, methoxamine is related to phenylephrine, but has greater specificity for the α_1-adrenoceptor subtypes. Several studies, however, have demonstrated that methoxamine, especially at high concentrations, can produce pharmacological responses that differ from those observed in response to phenylephrine. In sinoatrial nodal cells, for example, methoxamine reduced the slow inward (I_{si}) and the hyperpolarization-activated (I_h) currents, whereas phenylephrine, in the presence of pindolol, increased both of these currents (Satoh & Hashimoto, 1988). The authors speculated that methoxamine may have had non-specific actions in addition to its activity at α_1-adrenoceptors. Here we have investigated prazosin-insensitive actions of methoxamine on I_{to} in isolated rat ventricular cardiac myocytes, and in further experiments we have studied methoxamine block of current carried by two cloned potassium channels, hKv 1.5 and Kv 4.2. The results suggest that methoxamine can inhibit voltage-gated K^+ currents independently of α_1-adrenoceptor activation.

Methods

Myocyte isolation and cell culture

Hearts were rapidly removed from male rats weighing between 150 and 250 g previously anaesthetized with sodium pento-barbitone (80 mg kg^{-1}, i.p.), and ventricular cells were isolated as previously described for rabbit myocytes (Braun *et al.*, 1990). hKv 1.5 or Kv 4.2 were stably or transiently transfected into human embryonic kidney 293 (HEK) cells using the vector pCDNA3 (Invitrogen, San Diego, CA, U.S.A.). Transfected cells were plated on glass coverslips in 25 mm Petri dishes, and maintained in Modified Eagle's Medium (MEM) at 37°C in an air/5% CO_2 incubator until use. Cells transiently expressing hKv 1.5 or Kv 4.2 were detected by co-transfection with pHook-1 (Invitrogen), as previously described (Zhang *et al.*, 1997).

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Electrophysiological solutions

For whole-cell recording from myocytes, the pipette filling solution contained (in mM): KCl, 20; K-aspartate, 120; EGTA, 1; MgCl₂, 1; HEPES, 5; Na₂ATP, 4; GTP, 0.1; pH 7.2 with KOH. For HEK cells, K-aspartate was substituted by KCl. The bath solution for both cell types contained (in mM): NaCl, 135; KCl, 5; sodium acetate, 2.8; MgCl₁, 1; HEPES, 10; CaCl₂, 1; pH 7.4 with NaOH. For cell-attached recordings, the pipette was filled with this bath solution and the membrane potential was zeroed with a solution that contained (in mM): KCl, 135; MgCl₂, 1; HEPES, 10; CaCl₂, 1; dextrose, 10; pH 7.4 with KOH. For myocyte experiments, bath solutions were continuously bubbled with 100% O₂, and contained 5 μM propranolol to block β-adrenergic stimulation, and 300 μM CdCl₂ to block calcium and calcium-activated currents. Methoxamine (Mox), phenylephrine (PE), phenoxybenzamine (PhBz), and N-ethylmaleimide (NEM) were prepared in water just prior to use, at appropriate stock concentrations. Prazosin (Pz) was dissolved in ethanol at 2 mM, and protected from light. The concentration of ethanol in the bath solution never exceeded 0.1%. When cells were exposed to methoxamine, effects on membrane currents usually reached a steady-state within 1–2 min so that drug exposure times rarely exceeded 5 min. All chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Electrophysiological procedures and analysis

All experiments were carried out at 22–23°C. Whole-cell recordings were made using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, U.S.A.). Patch electrodes were pulled from thin-walled borosilicate glass (TW150, World Precision Instruments, FL, U.S.A.) and had resistances of 1.5–3.0 MΩ. Capacity compensation and 75–85% series resistance compensation were used in all measurements. In some experiments involving HEK cells, leak subtraction was applied to data. Data were filtered at 5–10 kHz before digitization and stored on a microcomputer for later analysis using pClamp6 software (Axon Instruments). The transient outward current (I$_{to}$) amplitude from rat ventricular myocytes was taken to represent the peak outward current measured during depolarizing voltage steps, as described previously (Apkon & Nerbonne, 1988). The dose-response curves (Figures 2B, 4D, and 5B) for current inhibition produced by methoxamine were computer-fitted to a Hill equation:

$$f = \max/[1 + (EC_{50}/[D]^{n_H})] \qquad (1)$$

where f is the percentage current block (f = [1 − I$_{drug}$/I$_{control}$] × 100%) at drug concentration [D], max is the maximum fitted level of block, EC$_{50}$ is the concentration producing half-maximal inhibition, and n_H is the Hill coefficient.

In rat ventricular myocytes, outward currents evoked upon depolarization contain at least two kinetic components, the first of which activates and inactivates rapidly (I$_{to}$), while the kinetics of the second are at least 10 fold slower, as previously described (Apkon & Nerbonne, 1991). Thus, peak outward current was taken to represent predominantly I$_{to}$, with only a negligible contribution from the second, slowly-activating sustained component of the outward current. I$_{to}$ and the sustained current component also differ in their voltage-dependence of steady-state inactivation, and this can be used to separate them (Apkon & Nerbonne, 1991). After a 1 s conditioning step to −100 mV, outward currents evoked at a test potential of +60 mV exhibited both transient and

sustained components (Figure 3A). After a conditioning step to −60 mV the sustained current was preferentially inactivated with only minimal effects on the peak outward current, whereas a prepulse to 0 mV inactivated both currents, and left only a small residual current representing leak and other non-inactivating currents. I$_{to}$ was isolated by subtracting current traces recorded using a conditioning potential of 0 mV from those recorded using a conditioning potential of −60 mV. Single-exponential fits to this difference current, measured under control conditions, were taken to represent the time constant of inactivation (τ_{inact}) of I$_{to}$. For Kv 4.2, control current traces were better fit to a second order exponential function, and the fast inactivation time constant in control was denoted τ_{inact}. In the presence of drug, I$_{to}$ and Kv 4.2 currents were fit to second-order exponentials, and the fast time constants of current decay in the presence of each concentration of drug were denoted τ_{decay}, since they represented both inactivation and block of current by drug. We extracted time constants of methoxamine block (τ_{block}) as an approximation of the drug-channel interaction kinetics as previously described (Slawsky & Castle, 1994; Snyders & Yeola, 1995), using:

$$1/\tau_{decay} = 1/\tau_{block} + 1/\tau_{inact} \qquad (2)$$

and values of τ_{inact} measured as indicated above. In addition we calculated first order rate constants for block, using:

$$1/\tau_{block} = k_{+1}\,[D] + k_{-1} \qquad (3a)$$

and

$$K_d = k_{-1}/k_{+1} \qquad (3b)$$

in which k_{+1} and k_{-1} are the apparent rate constants of binding and unbinding for the drug, respectively, and K_d is the equilibrium dissociation constant. For single channel analysis, blank sweeps were averaged and subtracted from all current traces to compensate for leak and capacity currents. Data were filtered at 1 kHz for presentation and analysis. Experimental values are given as means ± s.e.mean. For the data in Figures 1E, 4C and 5C, repeated measures ANOVA was used to determine significance. In all other cases, paired or unpaired *t*-tests were used, as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

Methoxamine can reduce outward currents independently of α_1-adrenoceptor activation

In isolated rat ventricular myocytes, methoxamine reduced the amplitude of the outward currents, and this effect had both prazosin-sensitive and prazosin-insensitive components, as illustrated by the data in Figure 1A–D. Addition of 200 μM methoxamine (B) reduced the amplitude of transient and sustained outward currents compared with control (A) across the entire potential range where currents could be measured. After wash, 2 μM prazosin was added to the bath solution (C), which has been shown to inhibit α-adrenergic responses in isolated cardiac myocytes (Fedida *et al.*, 1991; Li *et al.*, 1996). Even in the presence of prazosin, however, 200 μM methoxamine was still able to reduce current amplitude (D), although this effect was not as great as that observed with methoxamine alone. Specifically, at +60 mV, 200 μM methoxamine in the presence of prazosin was able to reduce peak outward current (I$_{to}$, see Methods) to 69.5 ± 0.4% ($n = 6$) of control. After washout, some cells were exposed for a second time to the same concentration of methoxamine and prazosin, which resulted in a reduction of I$_{to}$ to 69.2 ± 0.7% of control ($n = 5$),
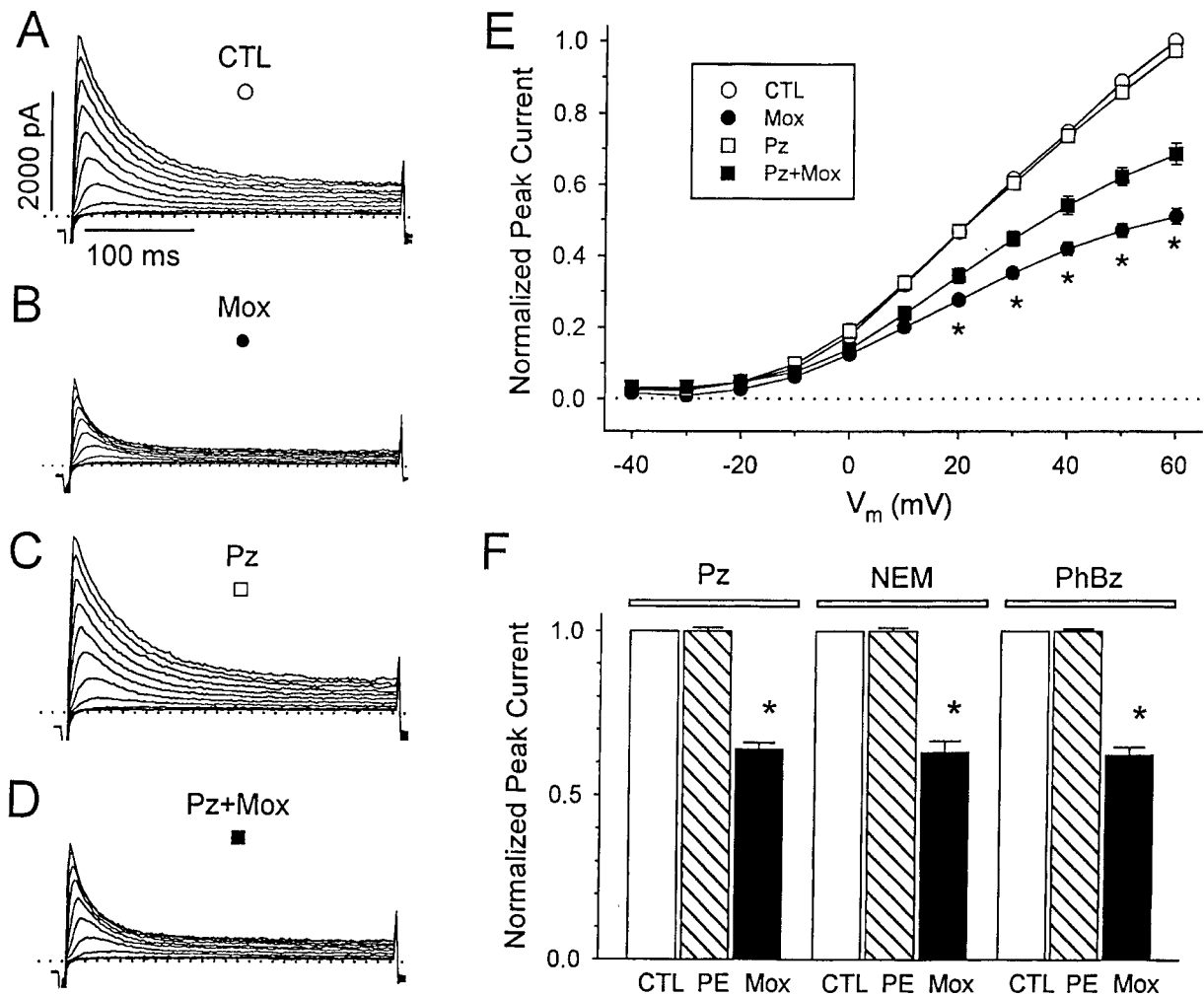
Figure 1 Methoxamine directly blocks outward currents in rat ventricular myocytes. A–D, currents from a representative myocyte during depolarizations from -80 mV to between -20 and $+60$ mV, in 10 mVsteps at a pulse frequency of 0.2 Hz. Dotted lines denote zero current level. Control (CTL, A) and with 200 μM methoxamine (Mox, B) after 5 min exposure. After 15 min of wash in control solution the cell was exposed to 2 μM prazosin (Pz, C) and then 200 μM methoxamine plus 2 μM prazosin (Pz + Mox, D). (E) mean peak I_{to} current-voltage relations in control and during exposure to prazosin or methoxamine, or both ($n=6$). For each cell, I_{to} was normalized to peak current in control solution measured at $+60$ mV. Symbols are as marked in panels A–D. * Indicates significant differences between current amplitudes in methoxamine alone compared with both methoxamine and prazosin, $P<0.05$. (F) block of signal transduction does not prevent the action of methoxamine. Effects of 100 μM phenylephrine (PE) and 200 μM methoxamine (Mox) on I_{to} amplitude in the presence of 2 μM prazosin (Pz), or after 30 min pretreatment with 50 μM N-ethylmaleimide (NEM) or 1 μM phenoxybenzamine (PhBz), as indicated. Currents were measured at $+60$ mV, and were normalized to I_{to} amplitude in the absence of α-agonist, but in the presence of Pz, NEM, or PhBz. Data are means $\pm$ s.e.mean ($n=6$). * indicates a significant difference from control, $P<0.05$.

which was not significantly different from that produced by the first exposure. Phenylephrine is approximately ten times more potent than methoxamine in stimulating α_1-adrenoceptors (Yang & Endoh, 1994), and addition of 100 μM phenylephrine resulted in a significant decrease in I_{to} amplitude that was completely blocked by 2 μM prazosin ($n=5$).

Mean current-voltage relations for peak current reduction in response to methoxamine are shown in Figure 1E. Both the prazosin-sensitive and -insensitive actions of methoxamine affected current at all positive potentials. However, while the prazosin-sensitive action of methoxamine was relatively potential-independent, the prazosin-insensitive inhibition of I_{to} increased at the more positive potentials studied, greater than $+20$ mV. These data suggest that methoxamine reduced I_{to} in a manner that was both dependent upon, and independent of α-adrenoceptor activation. The increasing block of I_{to} at more positive potentials caused by methoxamine in the presence of prazosin was fitted with a Woodhull

equation (Woodhull, 1973) which allowed calculation of the fraction of the electric field (δ) sensed by a singly charged drug molecule at its receptor site. From data in Figure 1E, a value of $\delta=0.18$ was obtained.

Prazosin is a competitive antagonist of α-adrenoceptors. To explore the possibility that methoxamine was merely overcoming prazosin block, cells were exposed to the alkylating agents NEM or PhBz (Figure 1F). NEM, which alkylates sulphhydryl groups, has been shown to inhibit GTP-binding proteins, including G-proteins (Ueda et al., 1990). There is substantial molecular homogeneity between cardiac α_1-adrenoceptors and the primary sequences of other members of the G-protein-binding superfamily (Harrison et al., 1991), and it appears that in mammalian cardiac cells, the effects of α_1-adrenoceptor stimulation are primarily transduced via G-proteins (Im & Graham, 1990; Wu et al., 1992; Nakaoka et al., 1994), and it has been demonstrated that NEM can inhibit α_1-adrenoceptor-mediated responses (Liebau et al., 1989). PhBz

irreversibly alkylates α-adrenoceptors, with relative specificity for the α_1 subtypes, and its use as an α-adrenoceptor antagonist has been well described (Minneman *et al.*, 1994).

Preincubation of isolated cardiac myocytes for 30 min with either 50 μM NEM or 1 μM PhBz failed to prevent the reduction of peak outward current observed in response to methoxamine (Figure 1F). The mean reduction of I_{to} in response to 200 μM methoxamine in NEM- or PhBz-pretreated cells was $37.0 \pm 3.5\%$ ($n=6$, mean $\pm$ s.e.mean), and $37.8 \pm 2.4\%$ ($n=6$) respectively, as shown by the solid bars in Figure 1F. These results are comparable with, and not significantly different from, the mean reduction observed in response to methoxamine in the presence of prazosin, which was $36.0 \pm 1.9\%$ ($n=6$). In contrast to methoxamine, all three agents antagonized the action of 100 μM phenylephrine. These data also suggest that methoxamine resulted in an inhibition of I_{to} that was not mediated through α_1-adrenergic receptor pathways.

Inhibition of I_{to} by methoxamine is concentration dependent

The non-specific action of methoxamine was concentration-dependent, as illustrated in Figure 2. Increasing concentrations of methoxamine, in the presence of 2 μM prazosin, decreased both the peak and steady-state levels of the outward currents recorded on depolarization (Figure 2A). As described previously for adult rat ventricular myocytes, depolarization-activated outward currents are composed of at least two kinetically distinct components: one that activates and inactivates rapidly (I_{to}), and one that activates and inactivates slowly (Apkon & Nerbonne, 1991). Since the more rapid of these components activates approximately 10 fold faster and inactivates approximately 30 fold faster than the slowly-activating component, the peak outward current predominantly reflects I_{to}, whereas the plateau current remaining after 100 ms should largely reflect the contribution of the second slower component. For purposes of analysis, therefore, we have considered only the effects of methoxamine on the peak transient current as a reflection of drug effects on I_{to}. In the dose-response relation in Figure 2B, data points correspond to percentage current block by methoxamine of the peak current in response to a depolarizing voltage step to $+60$ mV from a holding potential of -80 mV. The solid line was fitted to the data using equation [1]. The EC_{50} at $+60$ mV was 239 μM, and n_H was 1.5. The threshold for non-specific inhibition of I_{to} by methoxamine was between 10 and 50 μM, and the peak amplitude of I_{to} was then decreased by methoxamine in a concentration-dependent manner. The concentration of methoxamine that resulted in a maximum reduction of I_{to} was difficult to determine, as concentrations greater than 1 mM were often toxic to the myocytes. Based on the results of the fit to the Hill equation, it is likely that a maximum reduction of I_{to} of about 75% could be achieved at concentrations of methoxamine greater than 2–5 mM.

Methoxamine accelerates I_{to} inactivation in a concentration and voltage-dependent manner

In addition to decreasing I_{to} amplitude, increasing concentrations of methoxamine also apparently accelerated the rate of I_{to} decay. However, methoxamine reduced not only I_{to}, but also the sustained outward current component as well. Therefore, in order to measure the rate of I_{to} decay alone, we separated these two current components using a voltage protocol based on the differences in voltage-dependence of
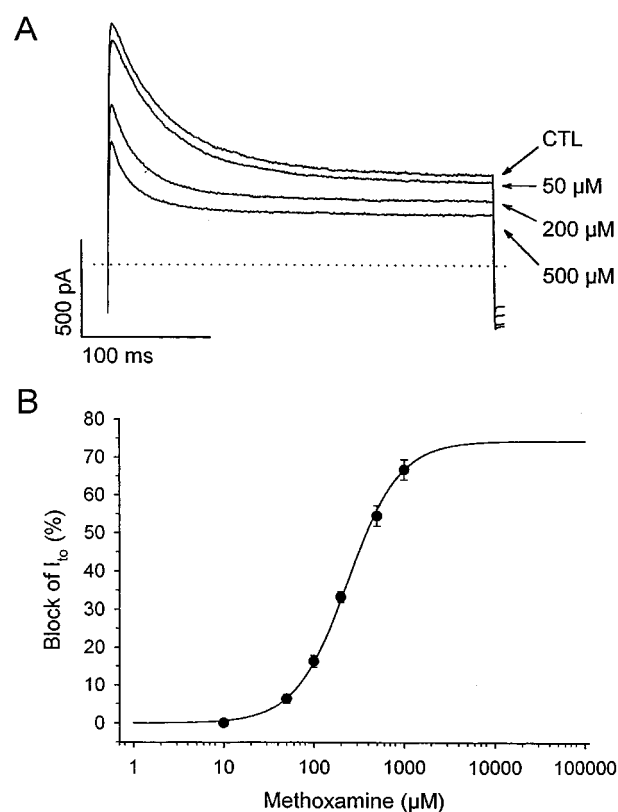

Figure 2 Concentration-dependence of non-specific action of methoxamine on I_{to} in rat ventricular myocytes. (A) currents recorded during steps to $+60$ mV from -80 mV in the presence of 2 μM prazosin (CTL), and in response to 50, 200, and 500 μM methoxamine in the presence of prazosin, as indicated. (B) dose-response curve for the non-specific action of methoxamine. Data are means $\pm$ s.e.mean from 6–14 cells at each concentration. Solid line was fit to data using equation [1]. The EC_{50} value was 239 μM and the Hill coefficient, n_H, was 1.5, with a maximum expected block of 75%.

steady-state inactivation of I_{to} and the sustained current component. Outward currents were measured at $+60$ mV after 1 s conditioning pulses to -100, -60, or 0 mV, as shown in Figure 3A. After a -100 mV prepulse, outward current comprised both transient and sustained components, but after a conditioning prepulse to -60 mV, the sustained component was largely inactivated with little effect on peak current. Both components were inactivated by a 0 mV prepulse. I_{to} and the sustained current component were isolated by subtraction as described in the Methods, and are illustrated in the lower panel of Figure 3A. The current traces in the inset to Figure 3B show I_{to} under control conditions and in the presence of 200 and 500 μM methoxamine, as indicated. Control currents were fitted with a single exponential function, whereas in the presence of drug, currents have been fitted to a double exponential decay (solid lines). There is a clear concentration-dependent acceleration of current decay in the presence of the drug. In controls, during 250 ms depolarizations, I_{to} inactivation at $+60$ mV had a time constant, τ_{inact} of 34.6 ± 1.2 ms ($n=9$). In the presence of methoxamine and prazosin the rapid time constant of current decay, τ_{decay}, accelerated with increasing concentrations of methoxamine. From these data we extracted the concentration-dependence of $(1/\tau_{block})$ as described in the Methods and shown in Figure 3B. $1/\tau_{block}$ increased linearly over a wide range of drug concentrations, and τ_{block} was taken as an approximation of the time course of drug-channel interaction (Slawsky & Castle, 1994). From

equation [3a] the best least-squares fit to $1/\tau_{block}$, as indicated by the solid line in Figure 3B, resulted in an apparent association rate constant, k_{+1}, of $8.9 \times 10^4 \, M^{-1} \, s^{-1}$, and an apparent dissociation rate constant, k_{-1}, of $32.9 \, s^{-1}$. Using equation [3b], the calculated K_d was 307 μM, which is in agreement with the EC_{50} value of 239 μM obtained from the dose-response curve (Figure 2B).

τ_{block} also decreased in a voltage-dependent manner, while time constants obtained from monoexponential fits to control current inactivation (τ_{inact}) showed little voltage-dependence over the same potential range (Figure 3C, $\square$). On the other hand, τ_{decay}, measured in the presence of 200 μM methoxamine, decreased from a value of 28.8 ± 1.9 ms at $+10$ mV to a value of 11.6 ± 1.0 ms at $+60$ mV. From these measurements we extracted values for τ_{block} (Figure 3C, $\bullet$), which decreased from 103 ± 13.9 ms at $+10$ mV to 15.9 ± 1.9 ms at $+60$ mV. The values indicate that an increase in the electrical gradient

accelerated block, which supports the idea that τ_{block} reflects the time course of channel block by a positively charged form of methoxamine. In addition, the data support the observations described in Figure 1, where the degree of block increased at the more positive potentials studied.

Methoxamine inhibits hKv 1.5 and Kv 4.2 currents

Data presented in Figure 1 suggested that methoxamine reduced both peak (I_{to}) and sustained outward currents recorded from myocytes, and it was therefore of interest to examine individual cloned channel currents to find out which, if any, were preferentially blocked by the non-specific action of methoxamine. Both Kv 4.2 and hKv 1.5 are thought to be important components of outward current in cardiac tissue. Kv 4.2 and Kv 4.3 are thought to underlie I_{to} in rat and human ventricular myocytes (Barry et al., 1995;
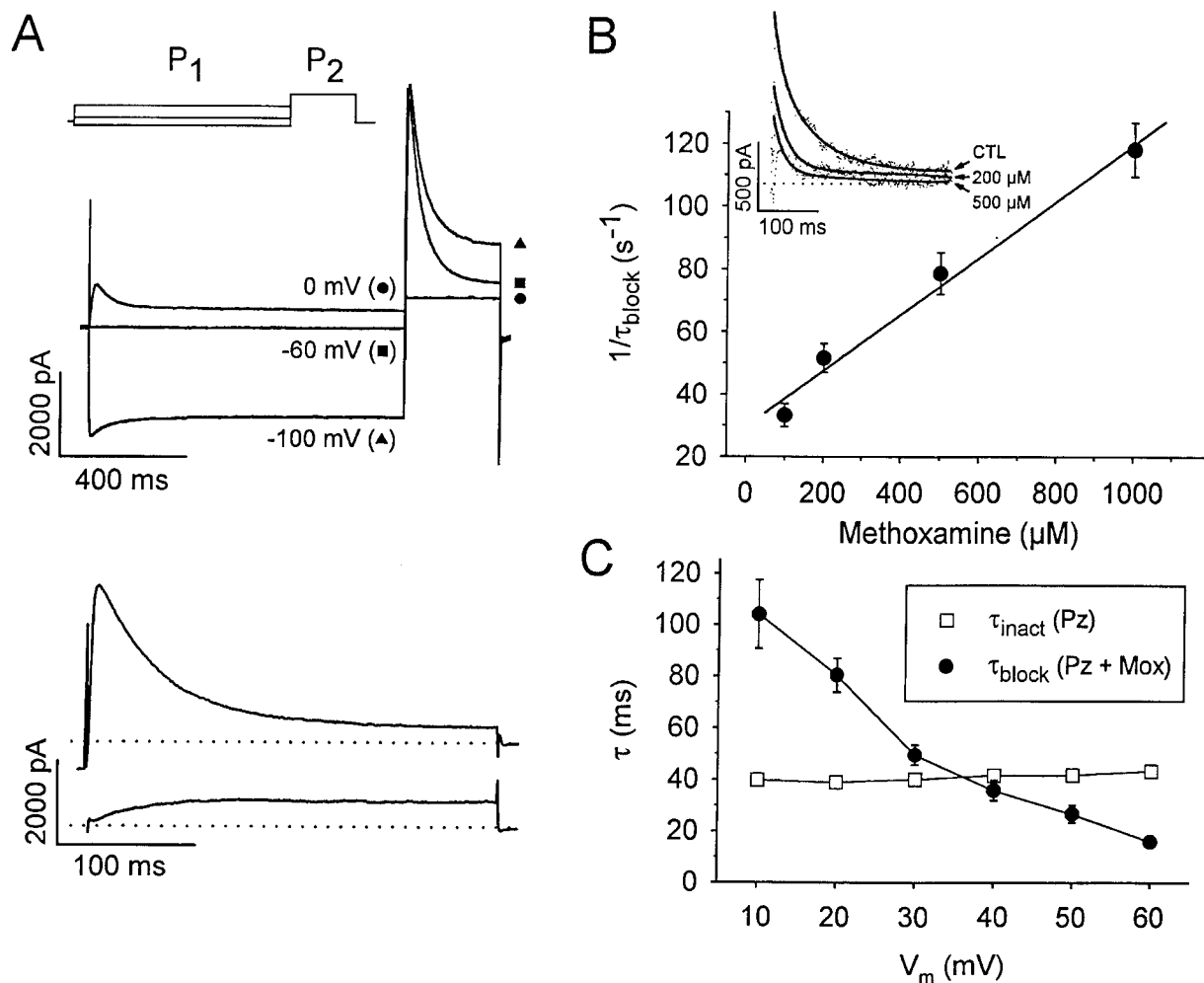

Figure 3 Concentration- and voltage-dependent kinetics of I_{to} block due to the non-specific action of methoxamine. (A) outward currents evoked from rat ventricular myocytes during a step to $+60$ mV (P_2), preceded by a 1 s conditioning prepulse (P_1) to -100 mV, -60 mV, or 0 mV, as indicated in the top panel. The lower panel shows difference currents isolating I_{to} (above) and the sustained outward current (below), derived as described in the Methods. Dotted lines indicate zero current level. (B) inset shows isolated I_{to} currents at $+60$ mV in control (CTL), and in the presence of 200 and 500 μM methoxamine, as indicated. Control currents were fit to a single exponential function, and currents in the presence of drug were fit to a double exponential (solid lines). The time constant for drug-channel interaction (τ_{block}) was extracted as described in the Methods. In the main panel, $1/\tau_{block}$ values have been plotted vs methoxamine concentration. The solid lines fit to data using equation [3a] gave apparent association (k_{+1}) and dissociation (k_{-1}) rate constants of $8.9 \times 10^4 \, M^{-1} \, s^{-1}$ and $32.9 \, s^{-1}$ respectively. The K_d value from equation [3b] was 307 μM. Data are means $\pm$ s.e.mean from 5-10 cells at each concentration of methoxamine, and 2 μM prazosin was continuously present. (C) voltage-dependent acceleration of I_{to} inactivation by methoxamine in rat ventricular myocytes. Inactivation time constants were obtained from fits to the relaxation phase of I_{to} (see Methods) during 300 ms voltage steps from -80 mV. Control current inactivation was fit to a single exponential function, yielding τ_{inact}. In 2 μM prazosin and 200 μM methoxamine, the current decay was accelerated and τ_{block} was extracted as described in the Methods. τ_{block} values between $+10$ to $+60$ mV were accelerated, but τ_{inact} were not voltage dependent. Data are means $\pm$ s.e.mean ($n = 5-10$ at each voltage).

Dixon *et al.*, 1996). hKv 1.5, cloned from human heart (Tamkun *et al.*, 1991; Fedida *et al.*, 1993) is thought to be a component of sustained current (Fedida *et al.*, 1993; Wang *et al.*, 1993), perhaps I_{Kur} in human myocytes (Feng *et al.*, 1997). hKv 1.5 and Kv 4.2 were heterologously expressed in HEK cells, which lack α_1-adrenoceptors (Theroux *et al.*, 1996), and currents expressed in these cells failed to show any response to phenylephrine ($n = 6$). Figure 4A illustrates current traces obtained from an HEK cell transfected with hKv 1.5, in response to depolarizing voltage steps to $+60$ mV, during exposure to a range of methoxamine concentrations. hKv 1.5 currents are relatively slowly inactivating and the effect of methoxamine was to reduce both peak and sustained hKv 1.5 current amplitude with less of an acceleration of the rate of current decay than seen for I_{to} in myocytes. Current traces obtained in the presence of methoxamine activated initially as in control (see Figure 4A), but reached a lower peak and subsequently decayed more quickly. Untransfected HEK cells are known to contain a small endogenous voltage-activated delayed

rectifier current. The average magnitude of this endogenous current, measured in untransfected HEK cells at $+60$ mV and at 300 ms, was 274 ± 32 pA ($n = 10$), and current tracings from a typical cell, obtained in response to a series of depolarizations over the range of -80 to $+80$ mV, are shown in the top panel of Figure 4B. The lower panel of Figure 4B illustrates typical drug-sensitive endogenous currents, in the presence of 200 μM and 1 mM methoxamine. This endogenous current was insensitive to concentrations of methoxamine < 200 μM ($n = 6$), and partial inhibition was noted at concentrations > 500 μM. This suggests that the reduction of sustained currents observed in hKv 1.5-transfected cells with < 500 μM methoxamine must represent solely block of hKv 1.5 current. Even with 1 mM methoxamine, the average magnitude of the methoxamine-sensitive current, measured at $+80$ mV, was 89 pA. Nonetheless, to minimize the contributions of this endogenous current, our analyses of expressed hKv 1.5 cloned channel currents included only those cells that contained relatively large currents, such that the methoxamine-sensitive endogenous
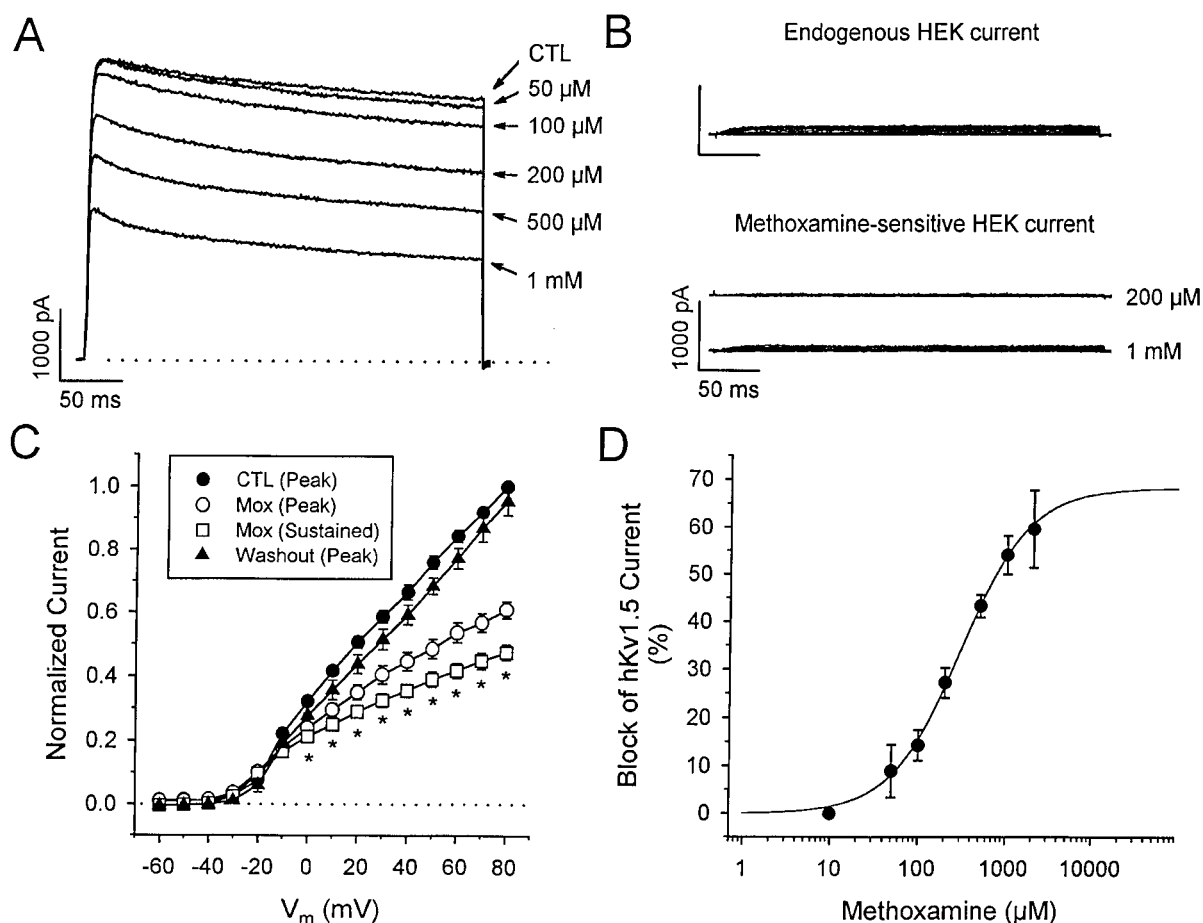

Figure 4 Reduction of hKv 1.5 current by methoxamine. (A) transfected HEK cell hKv 1.5 currents at $+60$ mV in control (CTL) and during exposure to a range of methoxamine concentrations as indicated. 2 μM prazosin was present in the bath at all times. The dotted line indicates zero current level. (B) endogenous HEK current in untransfected cells. The top panel shows typical endogenous currents in response to depolarizations over the range of -80 to $+80$ mV, from a holding potential of -80 mV. The lower panel illustrates the small methoxamine-sensitive endogenous currents obtained by subtracting currents in the presence of the indicated concentration of methoxamine from the corresponding currents in the absence of drug. The voltage-step protocol is the same as that described above. (C) normalized I-V relationships for peak control ($n = 5$), peak ($n = 5$) and sustained ($n = 5$) hKv 1.5 currents in the presence of 500 μM methoxamine and peak current after washout ($n = 4$), as indicated. Peak and sustained (at 300 ms) hKv 1.5 currents were individually normalized to peak and sustained current, respectively, measured at $+80$ mV under control conditions. *denotes statistically significant difference of both peak and sustained current amplitudes from control, $P < 0.05$. (D) dose-response for the block of sustained hKv 1.5 current at $+60$ mV by methoxamine. The data are means $\pm$ s.e.mean ($n = 5$). The solid line represents a fit of the data to equation [1]. At $+60$ mV, the EC_{50} was 276 μM with $n_H = 1.2$, and a maximum expected block of 65.5%.

current would be expected to contribute less than 3% of the total measured methoxamine-sensitive hKv 1.5 current. Mean peak ($\bigcirc$) and sustained ($\square$) current-voltage relations for cells exposed to 500 μM methoxamine are shown in Figure 4C, and illustrate the large effects of methoxamine on peak hKv 1.5 current, as well as reversibility of the drug action on washout ($\blacktriangle$). Block of hKv 1.5 current by methoxamine was voltage- (Figure 4C) and concentration-dependent (Figure 4D). Fit to current-voltage data positive to $+20$ mV gave a value for $\delta = 0.18$, similar to that determined for I_{to} with methoxamine and prazosin (see text to Figure 1). Data points in Figure 4D were well fit by equation [1], which yielded an EC_{50} value, at $+60$ mV, of 276 μM, and $n_H = 1.2$, with a maximum level of block of 65.5% predicted from the fit.

The response of Kv 4.2 channel currents to methoxamine was similar to that observed for I_{to}. Threshold drug actions were observed at 50 μM and consisted predominantly of an acceleration of the rate of current decay. Higher concentrations of methoxamine not only accelerated the rate of Kv 4.2 current decay, but also reduced peak current, with little effect of methoxamine on the initial activation of the currents relative to control (Figure 5A). As for hKv 1.5 data, at drug

concentrations greater than 500 μM, the methoxamine-sensitive endogenous current was small, and would be expected to compose less than 5% of the total methoxamine-sensitive current in all Kv 4.2-transfected cells included in analyses. The EC_{50} for steady-state Kv 4.2 current reduction was 363 μM, with $n_H = 0.87$, with a maximum expected block of 95% predicted from the fit of the data in Figure 5B. I-V relationships for cells in control solution (Figure 5C, $\bullet$, $n = 10$) and in the presence of 100 μM ($\square$, $n = 6$) and 500 μM ($\bigcirc$, $n = 7$) methoxamine again illustrate a voltage-dependence of block, and a fit of the current-voltage data positive to $+20$ mV in the presence of 500 μM methoxamine yielded a value of $\delta = 0.21$. For Kv 4.2 the control current inactivation was bi-exponential with fast time constants that were relatively voltage-independent in control (data not shown). The time constant for drug-channel interaction (Figure 5D; $1/\tau_{block}$, $\bullet$) was again concentration-dependent as for I_{to}. From equation [3a] the best least-squares fit to these data, as indicated by the solid line in Figure 5D, gave an apparent k_{+1} of 3.2×10^4 $M^{-1}s^{-1}$, and $k_{-1} = 8.3$ s^{-1}. From equation [3b], the calculated K_d was 388 μM, which is in close agreement with the EC_{50} value of 363 μM obtained from the dose-response curve (Figure 5B).
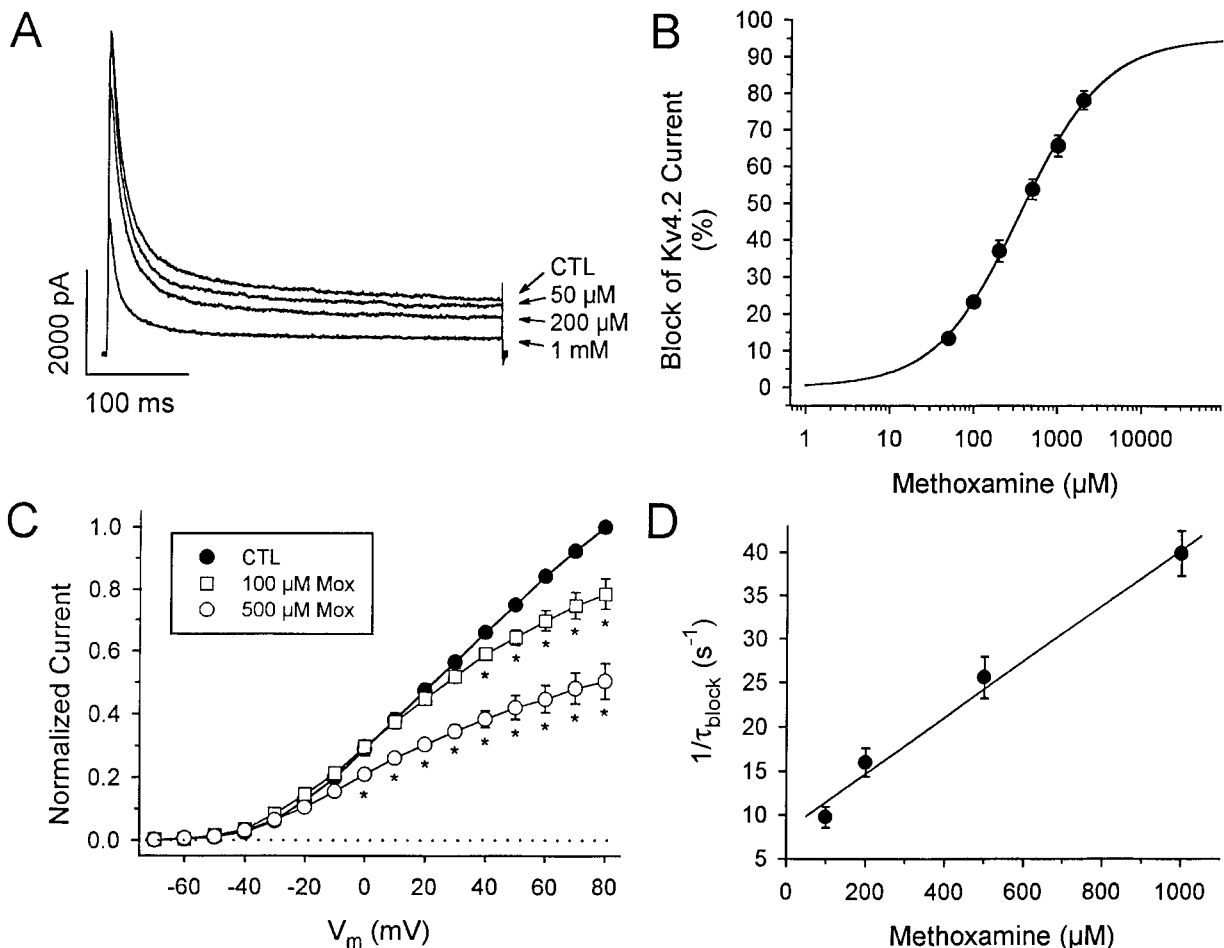

Figure 5 Reduction of Kv 4.2 current by methoxamine. (A) Kv 4.2 transfected HEK cell currents at $+60$ mV in control (CTL), and in the presence of 50, 200 μM, and 1 mM methoxamine, as indicated. (B) dose-response relation for Kv 4.2 current block by methoxamine. Current was measured at 300 ms and data points are means$\pm$s.e.mean ($n = 6$ at each concentration). The solid line was fit to data using equation [1]. The EC_{50} was 363 μM with $n_H = 0.87$, and a maximum expected block of 95%. (C) normalized mean I-V relations for Kv 4.2 current in control ($n = 6$), 100 μM ($n = 5$), and 500 μM ($n = 6$) methoxamine, as indicated. Currents were normalized to control currents at $+80$ mV, measured at 300 ms. *denotes statistically significant difference from control current amplitude, $P < 0.05$. (D) $1/\tau_{block}$ values plotted vs methoxamine concentration. τ_{block} values were extracted as described in the Methods from fits to the rapid phase of Kv 4.2 decay in the presence of methoxamine. From equation [3a] the best least-squares fit to these data, as indicated by the solid line in Figure 7D, gave an apparent k_{+1} of 3.2×10^4 M^{-1} s^{-1}, and $k_{-1} = 8.3$ s^{-1}. From equation [3b], the calculated K_d was 388 μM. Data are means$\pm$s.e.mean ($n = 5-9$ at each concentration).

Methoxamine modifies Kv 4.2 tail currents

Under control conditions, at -40 mV, Kv 4.2 current deactivated with a time constant ($\tau_{\text{deactivation}}$) of 24.4 ± 1.6 ms ($n = 6$), which represents the closing of channels from the open state. In the presence of methoxamine, the peak tail current amplitude was reduced relative to control, but also displayed an initial rising phase that was not observed in the absence of drug ($n = 8$). This rising phase was more pronounced with higher doses of methoxamine, and represents unblock of open channels previously inhibited by methoxamine. Figure 6A shows representative tail currents obtained at -40 mV, after 100 ms depolarizations in control conditions, and in the presence of 100, 500 μM or 1 mM methoxamine. Not only was the peak more reduced and the rising phase more pronounced at higher methoxamine concentrations, but subsequent decay of the tail currents was significantly slowed in the presence of methoxamine, and this effect was dose-dependent (Figure 6B). This had the result that tail currents recorded at the higher concentrations of methoxamine crossed over the control tail current (Figure 6A, inset). These data indicate that upon repolarization, methoxamine dissociated from blocked channels, allowing these channels to become conducting and resulting in the rising phase of tail current. Subsequently, a dynamic equilibrium was reached, wherein a fraction of these open, unblocked channels became blocked again, delaying the irreversible closing of these channels and resulting in the slowed rate of tail current decay relative to control.
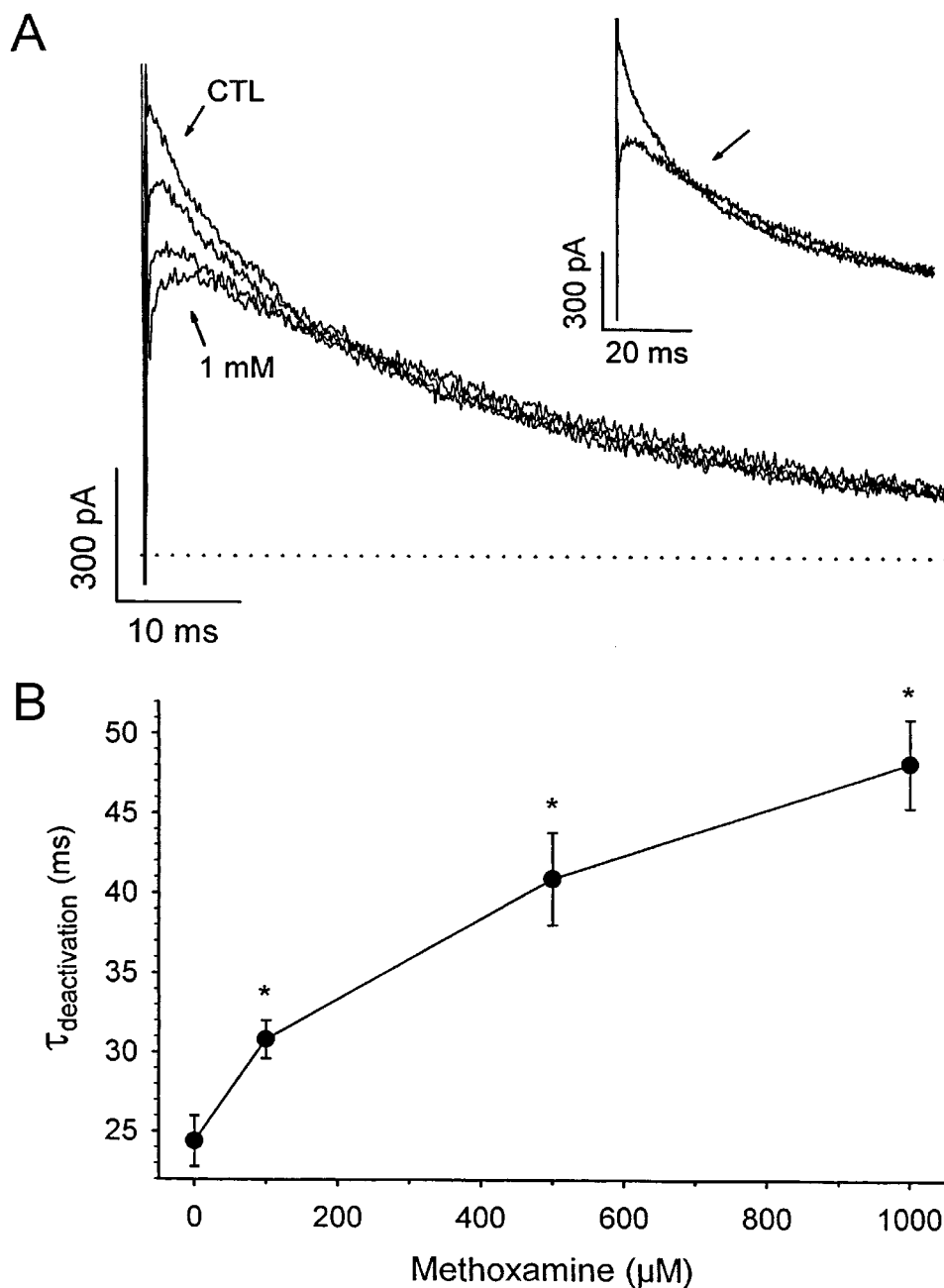

Figure 6 Methoxamine modifies Kv 4.2 deactivating tail currents. (A) typical deactivating tail currents in control (CTL), 100 μM, 500 μM, and 1 mM methoxamine. Peak tail current amplitudes decreased with increasing dose. The cell was held at -80 mV, and tails were recorded at -40 mV after a 100 ms depolarization to $+50$ mV. Dotted line indicates zero current level. Inset panel shows crossover of tail in control and 1 mM methoxamine and crossover point is arrowed. (B) slowing of rate of decay of tail currents with increasing doses of methoxamine. Tail currents in control and in the presence of drug were obtained as in (A) and were computer-fitted to a single exponential function to obtain the deactivation time constant, $\tau_{\text{deactivation}}$, at each concentration of drug. Data are means $\pm$ s.e.mean ($n = 6$). *denotes significant difference from control (i.e. 0 μM methoxamine), $P < 0.05$.

Methoxamine block of single Kv channels

For single-channel recording, HEK cells were depolarized in high K⁺ solution containing 2 μM prazosin. Pipettes contained 5 mM K⁺ solution and patches containing one or a few hKv 1.5 channels were held at a transmembrane potential of -80 mV

and depolarized to $+60$ mV, at 0.5 Hz. Representative control sweeps are presented in Figure 7A, and tracings recorded with 500 μM methoxamine in the bath solution are illustrated in Figure 7B. Under control conditions, hKv 1.5 channels opened in multiple bursts that were characterized by frequent brief closings. These kinetics for hKv 1.5 have been reported
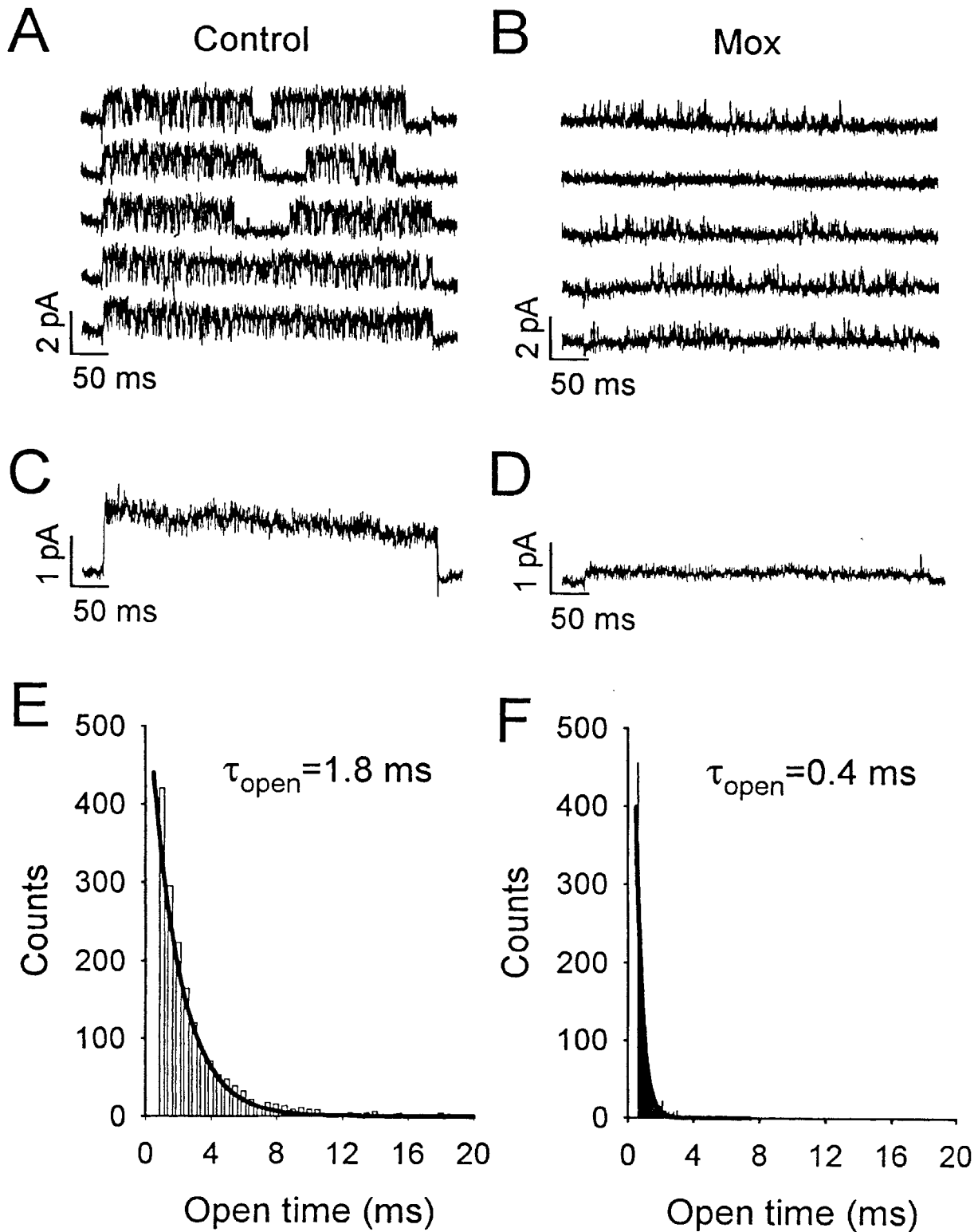

Figure 7 Effects of methoxamine on hKv 1.5 single channels in cell-attached patches with 140 mM K⁺ in the bath (see Methods). (A and B) cells were held at -80 mV and single-channel currents obtained at $+60$ mV, in control and in 500 μM methoxamine. Each sweep was recorded during 500 ms depolarizations. (C and D) ensemble averages ($n=20$) in control and in 500 μM methoxamine. (E and F) open-channel dwell time distributions in control and 500 μM methoxamine. Bin widths were 0.5 ms in E and 0.1 ms in F. Solid lines are single-exponential fits to data, with $\tau_{open}=1.8\pm0.01$ ms in control, and 0.4 ± 0.06 ms in methoxamine.

previously from our laboratory (Chen & Fedida, 1998) and are quite different from the maintained openings with few closings that characterize the endogenous current in HEK cells (data not shown). In the presence of methoxamine, burst duration was greatly reduced, rapid closings within bursts became even more frequent, and the channel rarely opened to its full amplitude. The mean single channel current in control solution, as determined by a Gaussian fit to an all-points amplitude histogram (data not shown) was 1.1 ± 0.01 pA, which corresponds to a single channel conductance of 9 pS, consistent with that reported previously for hKv 1.5 (Grissmer *et al.*, 1994). In the presence of methoxamine, however, the mean single channel current amplitude was significantly reduced to 0.3 ± 0.01 pA. These two actions of 500 μM methoxamine had the effect of reducing the ensemble-average current by 73% (Figure 7D) relative to control (Figure 7C). This may be compared with the 43% reduction of hKv 1.5 current observed in the whole cell configuration (Figure 4C). In addition, methoxamine significantly decreased the mean channel open time of hKv 1.5. Fits to open channel dwell time distributions for a cell in control solution (Figure 7E) and following addition of methoxamine (Figure 7F) yielded time constants (τ_{open}), corresponding to mean channel open times, of 1.8 ± 0.01 ms and 0.4 ± 0.06 ms, respectively. Similar results were seen in four other cells.

Discussion

Methoxamine has both α_1-adrenoceptor-dependent and α_1-adrenoceptor-independent actions

Methoxamine and phenylephrine have both been widely used in studies of α-adrenergic effects on cardiac ionic currents. In our studies, both phenylephrine and methoxamine reduced peak I_{to} amplitude in rat ventricular myocytes, but the response to phenylephrine was prevented by prazosin, whereas prazosin could reduce, but not abolish, the response to methoxamine (Figure 1). Similarly, preincubation of ventricular cells with prazosin, NEM, or PhBz prevented the action of phenylephrine, but failed to prevent the reduction in I_{to} observed during exposure to 200 μM methoxamine (see Figure 1F). This suggests that although prazosin, NEM, and PhBz act to disrupt different stages of the α-adrenergic signal transduction pathway, the non-specific action of methoxamine was not dependent on receptor activation, or on signal transduction by heterotrimeric G-proteins.

Methoxamine reduced both peak (I_{to}) and sustained outward currents in rat ventricular myocytes, independently of α_1-adrenoceptor stimulation, so we examined the action of methoxamine on two classes of Kv channels. These were Kv 4.2, a rapidly inactivating channel thought to underlie I_{to} (Barry *et al.*, 1995; Dixon *et al.*, 1996) and hKv 1.5, a slowly-inactivating delayed rectifier-type of channel thought to contribute to sustained outward current (Van Wagoner *et al.*, 1996; Feng *et al.*, 1997). Our data demonstrated that methoxamine could reduce the whole-cell currents recorded from hKv 1.5 and Kv 4.2 in HEK cells (Figures 4 and 5) which express no detectable endogenous α_1-adrenoceptors (Theroux *et al.*, 1996).

### Concentration- and voltage-dependence of receptor-independent action of methoxamine

In the presence of prazosin, methoxamine decreased the amplitudes of I_{to}, hKv 1.5, and Kv 4.2 in a concentration- and voltage-dependent manner. The $EC_{50}s$ for observed fractional block were similar for all three currents, and Hill coefficients were also similar, suggesting a single binding site model in each case. In myocytes, methoxamine characteristically increased the rate of decay of I_{to} (Figure 3). This was similar to data obtained for Kv 4.2 (Figure 5), but appeared to be much less important an effect for hKv 1.5 (Figure 4). We extracted a methoxamine-dependent time constant for block, τ_{block} (Figures 3 and 5), which decreased with increasing drug concentrations. The calculated K_d values, at $+60$ mV, were in good agreement with EC_{50} values obtained from dose-response relations (Figures 2B and 5B).

The observed voltage-dependence of I_{to} (Figure 1E), hKv 1.5 (Figure 4C) and Kv 4.2 (Figure 5C) inhibition, increasing with potential, is expected if the positively charged form of methoxamine (at pH 7.4, with $pK_a = 9.2$) blocks the channel and therefore responds to changes in membrane potential. Uncharged methoxamine could cross the plasma membrane and become protonated and charged within the cell, and then reach a putative intracellular binding site. This is supported by single channel data in Figure 7 where methoxamine was added to the bath solution and the external face of the membrane at the recording site was shielded by the pipette. The fraction of the membrane electrical field (δ) sensed by the drug at its receptor site was determined to be 0.18 for I_{to} and for hKv 1.5, and 0.21 for Kv 4.2. These values are similar to those for quinidine (Fedida, 1997) and quaternary ammonium compounds (Choi *et al.*, 1993), and suggests that positively charged agents may bind to a common intracellularly-accessible site (Snyders & Yeola, 1995).

Non-specific action of methoxamine is consistent with both open- and closed-channel block

Methoxamine accelerated the rate of decay of I_{to} and Kv 4.2 without markedly affecting the initial time course of activation (Figures 2A and 5A), which is generally suggestive of a mechanism of open-channel block (Slawsky & Castle, 1994). In the presence of higher concentrations of methoxamine, the reduction in peak I_{to}, hKv 1.5 and Kv 4.2 currents can be explained by rapid drug-channel interaction according to the first-order rate constant given by $k_{+1}[D]$. In the presence of methoxamine, deactivating Kv 4.2 tail currents showed a distinct rising phase that was not observed under control conditions, and decay of the current tails was significantly slowed in the presence of drug (Figure 6). These observations are consistent with a simple open channel block scheme for methoxamine and Kv 4.2:

$$ C \underset{4\beta}{\overset{\alpha}{\rightleftharpoons}} O \underset{k_{-1}}{\overset{k_{+1}[D]}{\rightleftharpoons}} OB $$
$$ \downarrow $$
$$ I $$
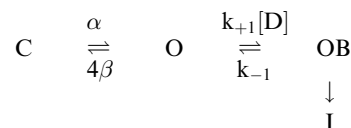
In this model, α and 4β refer to standard opening and closing rate constants, respectively, between the last closed (C) and open (O) states in a Hodgkin-Huxley four-subunit model, (I) is the inactivated state, and k_{+1} and k_{-1} are the apparent binding and unbinding rates for methoxamine and Kv 4.2, which were calculated to be 3.2×10^4 M^{-1} s^{-1} and 8.3 s^{-1}, respectively (Figure 5D). Since the unbinding rate is relatively fast, upon repolarization tail currents in the presence of drug should exhibit a rising phase indicative of the OB→O transition. Subsequently, these unblocked open channels can reblock, which slows tail current decay according to the relative values of 4β and $k_{+1}[D]$. If drug binding is sufficiently rapid relative

to channel closing, a 'crossover' of tail currents can be observed, as is the case for quinidine (Snyders *et al.*, 1992; Fedida, 1997), and was demonstrated by data in Figure 6.

In addition to accelerating the rate of decay of whole cell current, methoxamine decreased the mean hKv 1.5 single channel open time, consistent with rapid association and dissociation of the drug and the channel, and supportive of open-channel block. However, mean single channel current was also reduced, which may reflect brief openings not visualized at our bandwidth (1 kHz), or alternatively, alteration of the channel conductance by methoxamine. The number of blank sweeps also increased during methoxamine, and this increase in channel closed times strongly suggests an additional interaction with resting or closed states of hKv 1.5.

Implications for previous studies

Methoxamine has been a widely-used agonist to study the role of α_1-adrenergic modulation of cardiac function, and the concentrations of drug used in these studies vary widely over the range of $10^{-6} - 10^{-2}$ M. In rat ventricular myocytes a 25 – 50% reduction in the magnitude of the outward K$^+$ currents was observed in response to 20 μM methoxamine (Apkon & Nerbonne, 1988). At this concentration of methoxamine, the non-specific component of I$_{to}$ inhibition is negligible (Figure 2B) compared to the α_1-adrenoceptor-mediated response. Many studies, however, have used higher concentrations (e.g. >100 μM methoxamine), at which there is likely to be a significant non-specific component to the observed action that cannot be overlooked. In the absence of prazosin, 100 μM methoxamine resulted in ~35% reduction of I$_{to}$ in rat ventricular cells (Fedida & Bouchard, 1992), at which concentration our results indicate that I$_{to}$ would be reduced

by ~15% due to a non-specific action alone. Yang *et al.* (1996) routinely used 100 μM methoxamine to examine positive inotropy in isolated rabbit myocardium, and methoxamine concentrations up to 1 mM have been used to demonstrate modulation of I$_{to}$ in hyperthyroid rabbit myocytes (Shimoni & Banno, 1993). Interestingly, in rabbit atrium, 200 μM methoxamine has also been shown to decrease the I$_{to}$ mean single channel open time (Braun *et al.*, 1990). This result is similar to our α-adrenoceptor-independent action on single hKv 1.5 channels, although we noted additional closed channel effects (Figure 7). All these results suggest that great caution must be exercised in the use of methoxamine as an α-agonist in systems which possess significant numbers of voltage-gated K$^+$ channels or where ion channel modulation itself is being directly studied.

Interestingly, other compounds that interact with adrenoceptors have also been shown to have channel-blocking properties. The α-antagonist phentolamine can block ATP-sensitive K$^+$ (K$_{ATP}$) channels in rabbit ventricle (Wilde *et al.*, 1994) and pancreatic β-cells (Plant & Henquin, 1990), as can the specific α_2-antagonist yohimbine. Dicentrene, an alkaloid α-antagonist, inhibits I$_{to}$, I$_K$, and the sodium current (I$_{Na}$) in guinea-pig ventricular cells independently of adrenoceptor binding (Su *et al.*, 1994). To our knowledge, however, this is the first report of an α-adrenoceptor agonist with K$^+$ channel-blocking properties.

References

APKON, M. & NERBONNE, J.M. (1988). α_1-adrenergic agonists selectively suppress voltage-dependent K$^+$ currents in rat ventricular myocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8756 – 8760.

APKON, M. & NERBONNE, J.M. (1991). Characterization of two distinct depolarization-activated K$^+$ currents in isolated adult rat ventricular myocytes. *J. Gen. Physiol.*, **97**, 973 – 1011.

BARRY, D.M., TRIMMER, J.S., MERLIE, J.P. & NERBONNE, J.M. (1995). Differential expression of voltage-gated K$^+$ channel subunits in adult rat heart: Relation to functional K$^+$ channels. *Circ. Res.*, **77**, 361 – 369.

BRAUN, A.P., FEDIDA, D., CLARK, R.B. & GILES, W.R. (1990). Intracellular mechanisms for α_1-adrenergic regulation of the transient outward current in rabbit atrial myocytes. *J. Physiol. (Lond.)*, **431**, 689 – 712.

CHEN, F.S.P. & FEDIDA, D. (1998). On the mechanism by which 4-aminopyridine occludes quinidine block of the cardiac K$^+$ channel, hKv 1.5. *J. Gen. Physiol.*, **111**, 539 – 554.

CHOI, K.L., MOSSMAN, C., AUBÉ, J. & YELLEN, G. (1993). The internal quaternary ammonium receptor site of *Shaker* potassium channels. *Neuron*, **10**, 533 – 541.

DIXON, J.E., SHI, W.M., WANG, H.S., MCDONALD, C., YU, H., WYMORE, R.S., COHEN, I.S. & MCKINNON, D. (1996). Role of the Kv 4.3 K$^+$ channel in ventricular muscle-A molecular correlate for the transient outward current. *Circ. Res.*, **79**, 659 – 668.

FEDIDA, D., SHIMONI, Y. & GILES, W.R. (1990). α-Adrenergic modulation of the transient outward current in rabbit atrial myocytes. *J. Physiol. (Lond.)*, **423**, 257 – 277.

FEDIDA, D., BRAUN, A.P. & GILES, W.R. (1991). α_1-Adrenoceptors reduce background K$^+$ current in rabbit ventricular myocytes. *J. Physiol. (Lond.)*, **441**, 673 – 684.

FEDIDA, D., WIBLE, B., WANG, Z., FERMINI, B., FAUST, F., NATTEL, S. & BROWN, A.M. (1993). Identity of a novel delayed rectifier current from human heart with a cloned K$^+$ channel current. *Circ. Res.*, **73**, 210 – 216.

FEDIDA, D. (1997). Gating charge and ionic currents associated with quinidine block of human Kv 1.5 delayed rectifier K$^+$ channels. *J. Physiol. (Lond.)*, **499**, 661 – 675.

FEDIDA, D. & BOUCHARD, R.A. (1992). Mechanisms for the positive inotropic effect of α_1-adrenoceptor stimulation in rat cardiac myocytes. *Circ. Res.*, **71**, 673 – 688.

FENG, J.L., WIBLE, B., LI, G.R., WANG, Z.G. & NATTEL, S. (1997). Antisense oligodeoxynucleotides directed against Kv 1.5 mRNA specifically inhibit ultrarapid delayed rectifier K$^+$ current in cultured adult human atrial myocytes. *Circ. Res.*, **80**, 572 – 579.

GRISSMER, S., NGUYEN, A.N., AIYAR, J., HANSON, D.C., MATHER, R.J., GUTMAN, G.A., KARMILOWICZ, M.J., AUPERIN, D.D. & CHANDY, K.G. (1994). Pharmacological characterization of five cloned voltage-gated K$^+$ channels, types Kv 1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol. Pharmacol.*, **45**, 1227 – 1234.

HARRISON, J.K., PEARSON, W.R. & LYNCH, K.R. (1991). Molecular characterization of α_1- and α_2-adrenoceptors. *TIPS*, **12**, 62 – 67.

HWANG, K.C., GRAY, C.D., SWEET, W.E., MORRAVEC, C.S. & IM, M.-J. (1996). α_1-adrenergic receptor coupling with G$_h$ in the failing human heart. *Circulation*, **94**, 718 – 726.

IM, M.-J. & GRAHAM, R.M. (1990). A novel guanine nucleotide-binding protein coupled to the α_1-adrenergic receptor. I. Identification by photolabeling of membrane and ternary complex preparations. *J. Biol. Chem.*, **265**, 18944 – 18951.

LI, G.R., FENG, J.L., WANG, Z.G., FERMINI, B. & NATTEL, S. (1996). Adrenergic modulation of ultrarapid delayed rectifier K$^+$ current in human atrial myocytes. *Circ. Res.*, **78**, 903 – 915.

LIEBAU, S., HOHLFELD, J. & FORSTERMANN, U. (1989). The inhibition of α_1-adrenoceptor-mediated contraction of rabbit pulmonary artery by Ca^{2+}-withdrawal, pertussis toxin, and N-ethylmaleimide. *Naun. Schmied. Arch. Pharmacol.*, **339**, 496–502.

MINNEMAN, K.P., THEROUX, T.L., HOLLINGER, S., HAN, C. & ESBENSHADE, T.A. (1994). Selectivity of agonists for cloned α_1-adrenergic receptor subtypes. *Mol. Pharmacol.*, **46**, 929–936.

NAKAOKA, H., PEREZ, D.M., BAEK, K.J., DAS, T., HUSAIN, A., MISONO, K., IM, M.-J. & GRAHAM, R.M. (1994). G_h: a GTP-binding protein with transglutaminase activity and receptor signalling function. *Science,* **264**, 1593–1595.

PLANT, T.D. & HENQUIN, J.C. (1990). Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β-cells. *Br. J. Pharm.,* **101**, 115–120.

SATOH, H. & HASHIMOTO, K. (1988). Effect of α_1-adrenoceptor stimulation with methoxamine and phenylephrine on spontaneously beating rabbit sino-atrial node cells. *Naun. Schmied. Arch. Pharmacol.,* **337**, 415–422.

SHIMONI, Y. & BANNO, H. (1993). α-Adrenergic modulation of transient outward current in hyperthyroid rabbit myocytes. *Am. J. Physiol. Heart Circ. Physiol.,* **264**, H74–H77.

SLAWSKY, M.T. & CASTLE, N.A. (1994). K^+ channel blocking actions of flecainide compared with those of propafenone and quinidine in adult rat ventricular myocytes. *J. Pharmacol. Exp. Ther.,* **269**, 66–74.

SNYDERS, D.J., KNOTH, K.M., ROBERDS, S.L. & TAMKUN, M.M. (1992). Time-, voltage-, and state-dependent block by quinidine of a cloned human cardiac potassium channel. *Mol. Pharmacol.,* **41**, 322–330.

SNYDERS, D.J. & YEOLA, S.W. (1995). Determinants of antiarrhythmic drug action - Electrostatic and hydrophobic components of block of the human cardiac hKv 1.5 channel. *Circ. Res.,* **77**, 575–583.

SU, M.J., NIEH, Y.C., HUANG, H.W. & CHEN, C.C. (1994). Dicentrine, an α-adrenoceptor antagonist with sodium and potassium channel blocking activities. *Naunyn-Schmiedebergs Arch. Pharm.,* **349**, 42–49.

TAMKUN, M.M., KNOTH, K.M., WALBRIDGE, J.A., KROEMER, H., RODEN, D.M. & GLOVER, D.H. (1991). Molecular cloning and characterization of two voltage-gated K^+ channel cDNAs from human ventricle. *FASEB J.,* **5**, 331–337.

THEROUX, T.L., ESBENSHADE, T.A., PEAVY, R.D. & MINNEMAN, K.P. (1996). Coupling efficiencies of human α_1-adrenergic receptor subtypes: Titration of receptor density and responsiveness with inducible and repressible expression vectors. *Mol. Pharmacol.,* **50**, 1376–1387.

UEDA, H., MISAWA, H., KATADA, T., UI, M., TAKAGI, H. & SATOH, M. (1990). Functional reconstitution of purified G_i and G_o with μ-opioid receptors in guinea-pig striatal membranes pretreated with micromolar concentrations of N-ethylmaleimide. *J. Neurochem.,* **54**, 841–848.

VAN WAGONER, D.R., KIRIAN, M. & LAMORGESE, M. (1996). Phenylephrine suppresses outward K^+ currents in rat atrial myocytes. *Am. J. Physiol. Heart Circ. Physiol.,* **271**, H937–H946.

WANG, Z., FERMINI, B. & NATTEL, S. (1993). Sustained depolarization-induced outward current in human atrial myocytes: Evidence for a novel delayed rectifer K^+ current similar to Kv 1.5 cloned channel currents. *Circ. Res.,* **73**, 1061–1076.

WILDE, A.A., VELDKAMP, M.W., VAN GINNEKEN, A.C. & OPTHOF, T. (1994). Phentolamine blocks ATP sensitive potassium channels in cardiac ventricular cells. *Cardiovasc. Res.,* **28**, 847–850.

WOODHULL, A.M. (1973). Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.,* **61**, 687–708.

WU, D., KATZ, A., LEE, C.-H. & SIMON, M.I. (1992). Activation of phospholipase C by α_1-adrenergic receptors is mediated by the α subunits of G_q family. *J. Biol. Chem.,* **267**, 25798–25802.

YANG, H. & ENDOH, M. (1994). Dissociation of the positive inotropic effect of methoxamine from the hydrolysis of phosphoinositide in rabbit ventricular myocardium: a comparison with the effects of phenylephrine and the subtype of the α_1 adrenoceptor involved. *J. Pharm. Exp. Ther.,* **269**, 732–742.

YANG, H.T., NOROTA, I., ZHU, Y. & ENDOH, M. (1996). Methoxamine-induced inhibition of the positive inotropic effect of endothelin via α_1-adrenoceptors in the rabbit heart. *Eur. J. Pharmacol.,* **296**, 47–54.

ZHANG, X., ANDERSON, J.W. & FEDIDA, D. (1997). Characterization of nifedipine block of the human heart delayed rectifier, hKv 1.5. *J. Pharmacol. Exp. Ther.,* **281**, 1247–1256.