0026-895X/99/061192-06$3.00/0

MOLECULAR PHARMACOLOGY, **56:**1192–1197 (1999).

Mibefradil Potently Blocks ATP-Activated K^+ Channels in Adrenal Cells

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Received July 16, 1999; accepted September 9, 1999

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

ABSTRACT

Mibefradil is a novel Ca^{2+} channel antagonist that preferentially blocks T-type Ca^{2+} channels in many cells. Using whole-cell and single-channel patch-clamp recording, we found that mibefradil also potently blocked an ATP-activated K^+ channel (I_{AC}) expressed by adrenal zona fasciculata cells. I_{AC} channels were inhibited by mibefradil with an IC_{50} value of 0.50 μM, a concentration 2-fold lower than that required to inhibit T-type Ca^{2+} channels under similar conditions in the same cells. The inhibition of I_{AC} by mibefradil was independent of the membrane potential. Mibefradil also reversibly blocked, with similar potency, unitary I_{AC} currents recorded in outside-out membrane patches. An analysis of dwell time histograms indicated the presence of two closed and one open state. Mibefradil (1 μM) increased the duration of the two closed time constants (τ_{c1} and τ_{c2}) from 2.30 $\pm$ 0.18 and 27.9 $\pm$ 4.7 ms to 4.32 $\pm$ 0.61 and 62.5 $\pm$ 13.8 ms, respectively, but did not alter the open time constant (τ_o). Mibefradil also failed to reduce the size of the unitary I_{AC} current. A voltage-gated A-type K^+ current was also inhibited by mibefradil at concentrations approximately 10-fold higher than those required to block I_{AC} (IC_{50} = 4.65 μM). These results identify mibefradil as a potent inhibitor of ATP-activated K^+ channels in adrenal zona fasciculata cells. It appears to function by stabilizing closed states of these channels. In contrast to its selective block of T-type Ca^{2+} channels, mibefradil may be a potent but less-selective K^+ channel blocker. In this regard, the block of K^+ channels may produce some of the toxicity associated with mibefradil in cardiovascular pharmacology.

Mibefradil is a new Ca^{2+} antagonist that is effective as an antianginal and antihypertensive agent (Noll and Lusher, 1998). Among Ca^{2+} channel blockers, mibefradil is distinctive in its favorable hemodynamic actions and lack of side effects that are frequently observed with other Ca^{2+} antagonists. At therapeutic concentrations, mibefradil reduces vascular resistance and heart rate without negative inotropy.

The favorable pharmacological profile of mibefradil and limited side effects appear to be related to selective block of T-type Ca^{2+} channels. Unlike other Ca^{2+} antagonists that are used clinically, mibefradil preferentially blocks T-type rather than L-type Ca^{2+} channels with 10- to 20-fold selectivity (Mehrke et al., 1994; Mishra and Hermsmeyer, 1994a; Ertel, and Ertel, 1997). Despite its desirable pharmacological and hemodynamic actions, mibefradil was removed from the market after it was shown to produce serious toxicity when taken in combination with a number of other drugs, including some H_1 antihistamine antagonists (Woosley, 1996).

Although a number of studies have been performed that characterize the effects of mibefradil on various Ca^{2+} channel subtypes and document its selective block of T-type channels, little is known about the effect of this drug on other types of ion-selective channels. In this regard, older Ca^{2+} antagonists, such as the dihydropyridines, that preferentially block L-type Ca^{2+} channels have also been shown to inhibit voltage-gated K^+ channels, albeit at considerably higher concentrations (Hume, 1985; Nerbonne and Gurney, 1987; Mlinar and Enyeart, 1994).

We studied the inhibition of K^+ channels by mibefradil in whole-cell and single-channel patch-clamp recordings from bovine adrenal zona fasciculata (AZF) cells. These cells express a novel ATP-activated K^+ channel (I_{AC}) that sets the membrane potential and couples adrenocorticotrophic hormone (ACTH) receptor activation to depolarization-dependent Ca^{2+} entry and cortisol secretion (Mlinar et al., 1993; Enyeart et al., 1997). Mibefradil potently blocks I_{AC} K^+ channels at concentrations below those required to inhibit T-type Ca^{2+} channels in the same cells (Gomora et al., 1999).

Materials and Methods

Tissue culture media, antibiotics, fibronectin, and FBS were obtained from Life Technologies (Grand Island, NY). Coverslips were

J.J.E. was supported by National Institute of Diabetes and Digestive and Kidney Grant DK47875.

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ABBREVIATIONS: AZF, bovine adrenal fasciculata; ACTH, adrenocorticotrophic hormone; I_A, rapidly inactivating A-type K^+ current; I_{AC}, noninactivating, ATP-activated K^+ current; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacidic acid; DPBP, diphenylbutylpiperidine.

from Bellco Glass (Vineland, NJ). Enzymes, MgATP, ACTH(1-24), and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Mibefradil was a gift from Hoffman La Roche (Basel, Switzerland).

Isolation and Culture of AZF Cells. Bovine adrenal glands were obtained from steers (age range, 1–3 years) within 30 min of slaughter at a local slaughterhouse. Fatty tissue was removed immediately, and the glands were transported to the laboratory in ice-cold PBS containing 0.2% dextrose. Isolated AZF cells were prepared as previously described (Enyeart et al., 1997). Cells were plated in Dulbecco's modified Eagle's medium/F-12+ in 35-mm dishes containing 9-mm^2 glass coverslips that had been treated with fibronectin (10 μg/ml) at 37°C for 30 min and then rinsed with warm, sterile PBS immediately before adding cells. Dishes were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Patch-Clamp Experiments. Patch-clamp recordings of K^+ channel currents were made in the whole-cell and outside-out patch configurations. For both recording configurations, the standard pipette solution consisted of 115 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, 11 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 200 μM GTP, with pH buffered to 7.2 using KOH. For whole-cell and single-channel patch recordings, pipette solutions contained 5 -and 2 mM MgATP, respectively. Pipette [Ca^{2+}] was 22 nM as determined using the Bound and Determined program (Brooks. and Storey, 1992). The external solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4 using NaOH. All solutions were filtered through 0.22-μm cellulose acetate filters.

AZF cells were used for patch-clamp experiments 2 to 12 h after plating. Typically, cells with diameters of $<$15 μm and capacitances of 8 to 12 pF were selected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume, 1.5 ml), which was continuously perfused by gravity at a rate of 3 to 5 ml/min. For whole-cell recordings, patch electrodes with resistances of 1.0 to 2.0 MΩ were fabricated from Corning 0010 glass (World Precision Instruments, Sarasota, FL). These routinely yielded access resistances of 1.5 to 4 MΩ and voltage clamp-time constants of $<$100 μs. For single-channel recordings, patch electrodes with higher resistances of 3 to 5 MΩ were used. K^+ currents were recorded at room temperature (22–25°C) according to the procedure of Hamill et al. (1981) with an Axopatch 1-D patch-clamp amplifier.

Pulse generation and data acquisition were done using a personal computer and pCLAMP software with a TL-1 interface (Axon Instruments, Inc., Burlingame, CA). Currents were digitized at 5 to 20 kHz after filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using scaled hyperpolarizing steps of one-third to one-fourth amplitude. Data were analyzed and plotted using pCLAMP 5.5 and 6.02 (CLAMPAN, CLAMPFIT, FETCHAN, and PSTAT) and SigmaPlot 4.0. Drugs were applied by bath perfusion, which was controlled manually with a six-way rotary valve.

Results

Bovine AZF cells express two types of K^+ currents: a voltage-gated, rapidly inactivating A-type current (I_A) and the noninactivating, ATP-activated current I_{AC} (Mlinar et al., 1993; Mlinar and Enyeart, 1993; Enyeart et al., 1997). I_{AC} consists of two components: an apparent instantaneous component and a time-dependent component (Enyeart et al., 1996). I_{AC} is only weakly voltage dependent with open probability (P_o) increasing by ~30% between voltages of −40 and +40 mV (Enyeart et al., 1997).

In whole-cell recordings, I_{AC} is present initially at low density but grows dramatically over a period of minutes, provided that ATP or other nucleotides are present at millimolar concentrations in the recording pipette (Enyeart et al., 1997). The absence of time and voltage-dependent inactivation allow I_{AC} to be easily isolated for measurement in whole-cell recordings using either of two voltage-clamp protocols. When voltage steps of 300-ms duration were applied from a holding potential of −80 mV to a test potential of +20 mV, I_{AC} was measured selectively near the end of a voltage step where the rapidly inactivating A-type current had completely inactivated (Fig. 1A, left traces). Alternatively, I_{AC} was selectively activated with an identical voltage step, after a 10-s prepulse to −20 mV had fully inactivated the A-type current (Fig. 1A, right traces).

Mibefradil applied to AZF cells externally through bath perfusion inhibited both noninactivating I_{AC} and rapidly inactivating I_A currents in AZF cells. Of these two currents, I_{AC} was more potently inhibited. The rapidly inactivating I_A current was also inhibited by mibefradil at ~10-fold higher concentrations.

In the experiment illustrated in Fig. 1, I_{AC} K^+ current grew to a stable amplitude over a 15-min period before the cell was superfused with mibefradil at concentrations between 0.1 and 5 μM. Over this range of concentrations, I_{AC} was preferentially inhibited in a concentration-dependent manner (Fig. 1, A and B). Inhibition was partially reversible with washing (Fig. 1B). Overall, mibefradil inhibited I_{AC} current with an IC_{50} value of 0.50 μM (Fig. 1C). The inhibition of I_{AC} by mibefradil was insensitive to changes in holding potential. Mibefradil (0.5 μM) was equally effective at inhibiting I_{AC}, activated from holding potential of −80 or −40 mV (data not shown).

ACTH (100 pM) selectively and completely suppresses the expression of I_{AC} in whole-cell recordings, allowing the rapidly inactivating A-type current to be studied in isolation (Mlinar et al., 1993; Enyeart et al., 1996; Fig. 2A). Under these conditions, mibefradil inhibits I_A with an IC_{50} value of 4.65 μM (Fig. 2, A and B). Inhibition of I_A by mibefradil was slowly reversible. Washing with control saline reduced I_A inhibition by 65.5 ± 9.3% (n = 6) after 15 to 20 min.

Block of Unitary I_{AC} Currents by Mibefradil. Mibefradil inhibited unitary I_{AC} K^+ currents recorded from excised outside-out patches without reducing the amplitude of the single-channel current. Figure 3 shows unitary currents recorded from an outside-out patch in response to depolarizing steps to +30 mV from a holding potential of −40 mV where I_A channels are inactivated (Mlinar and Enyeart, 1993). Under these conditions, a single type of K^+ channel was typically present in the membrane patch. After recording currents in control saline, unitary currents were recorded at several different mibefradil concentrations.

Amplitude histograms constructed from unitary currents recorded in response to 80 to 96 separate voltage steps of 400-ms duration in control saline included three peaks indi-

cating the presence of at least three active channels in the membrane patch (Fig. 3, right). Gaussian fits to the data showed that each of these peaks was spaced at approximately multiples of the unitary current amplitude (3.50 ± 0.08 pA).

Mibefradil inhibited unitary I_{AC} currents with the same potency observed in whole-cell recordings. Perfusion of mibefradil at concentrations of 1, 5, and 10 μM reduced channel activity in a concentration-dependent manner (Fig. 3). In the experiment illustrated, 10 μM mibefradil reduced channel P_o to nearly zero. Inhibition of I_{AC} channel activity was rapidly reversed on washing with control saline and showed the tendency of I_{AC} channel activity to increase spontaneously with time in outside-out patches (Fig. 3, bottom). In this experiment, analysis of amplitude histograms showed that four functional I_{AC} channels were present in the patch after reversal of the block by 10 μM mibefradil.

Figure 4A shows results from a similar experiment in which P_o is plotted against time. The calculated P_o was reduced from its control value of 0.21 to 0.07 and 0.02 by 1 and 5 μM mibefradil, respectively. At a concentration of 10 μM, channel openings were virtually eliminated. At each mibefradil concentration, inhibition was rapidly reversed on switching to control saline and revealed the underlying time-dependent increase in P_o. Results similar to these were obtained in each of eight cells.

Effect of Mibefradil on I_{AC} Dwell Times. Dwell time analysis of unitary I_{AC} currents showed that under control conditions, I_{AC} channel kinetics could be described by a single open time constant (τ_o) and two closed time constants (τ_{c1}, τ_{c2}) that differ by approximately one order of magnitude. Mibefradil increased both closed time constants but did not significantly alter the mean open time. In the experiment illustrated in Fig. 4B, mibefradil (1 μM) increased τ_{c1} and τ_{c2} from 1.56 and 17.9 ms to 4.09 ms and 70.6 ms, respectively. By comparison, the open time constant in control saline (τ_o = 1.73 ms) did not differ significantly from that determined in the presence of 1 μM mibefradil (τ_o = 1.51 ms). The effect of mibefradil on closed time constants was reversed on washing with control saline (Fig. 4B). Overall, mibefradil (1 μM) increased τ_{c1} and τ_{c2} from control values of 2.30 ± 0.18 and 27.9 ± 4.7 ms (n = 9) to 4.32 ± 0.61 and 62.50 ± 13.80 ms (n = 5), respectively. In contrast, in the presence of 1 μM mibefradil, τ_o was 1.25 ± 0.07 ms (n = 9 compared with a control value of 1.47 ± 0.07 ms (n = 9).

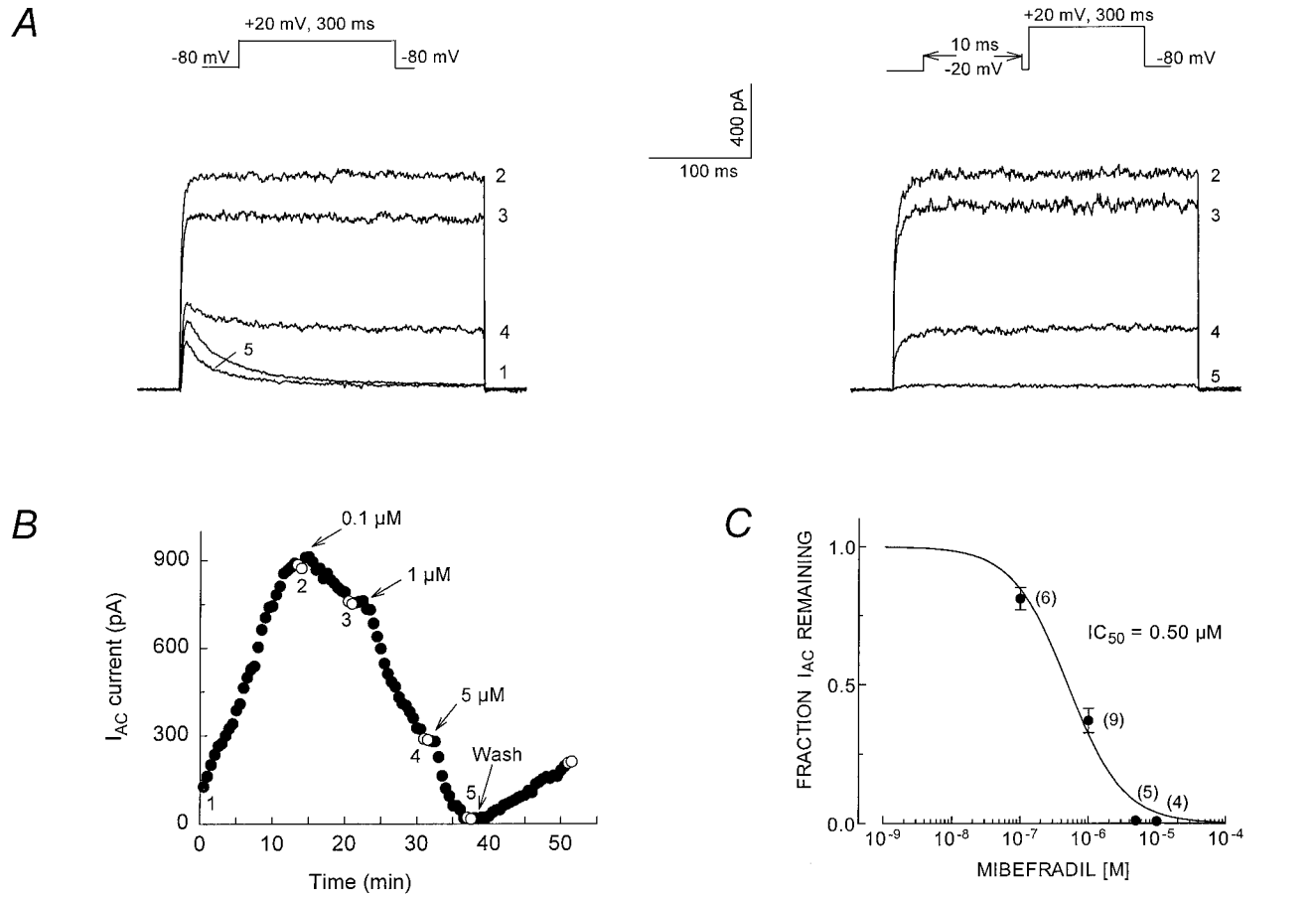

Fig. 1. Time- and concentration-dependent inhibition of I_{AC} K^+ current by mibefradil. Whole-cell K^+ currents were recorded from AZF cells at 30-s intervals in response to voltage steps to +20 mV applied from a holding potential of −80 mV with or without 10-s prepulses to −20 mV that inactivate A-type K^+ current. After I_{AC} reached a stable amplitude, cells were superfused with mibefradil at concentrations ranging from 0.1 to 5 μM. A, K^+ current records made with standard pipette solution supplemented with 5 mM MgATP and 200 μM GTP with (right) or without (left) 10-s prepulses to −20 mV. Numbers correspond to currents immediately after initiation of whole-cell recording (1); after I_{AC} reached a maximum amplitude (2); and after inhibition by 0.1 μM (3), 1 μM (4), or 5 μM (5) mibefradil. B, I_{AC} amplitudes recorded with (○) or without (●) depolarizing prepulses are plotted against time. Mibefradil was superfused as indicated. Numbers correspond to currents as in A. C, inhibition curve. Fraction of unblocked I_{AC} current is plotted against mibefradil concentration. Data were fit with an equation of the form: $I/I_{max} = 1/[1 + (B/IC_{50})^X]$, where B is the mibefradil concentration, IC_{50} is the concentration that reduces I_T by 50%, and X is the Hill coefficient. Values are mean ± S.E. of indicated number of determinations.

Discussion

In this study, it was discovered that the T-type Ca^{2+} channel antagonist mibefradil potently blocks ATP-activated I_{AC} K^+ channels in AZF cells. The inhibition of I_{AC} K^+ channels by mibefradil was voltage independent and, at the single-channel level, appeared to reduce channel P_o through stabilization of closed states. At 10-fold higher concentrations, mibefradil also inhibited voltage-gated, rapidly inactivating A-type K^+ channels.

Block of Ca^{2+} and K^+ Channels by Mibefradil. Mibefradil inhibits I_{AC} currents recorded in response to voltage steps from −80 with an IC_{50} value of 0.50 μM, a concentration 2-fold lower than that required to inhibit T-type Ca^{2+} channels in the same cells under similar conditions (IC_{50} = 1.0 μM; Gomora et al., 1999). Mibefradil inhibits T currents in other cells, including cerebellar neurons and vascular smooth muscle cells, with potency similar to that observed in AZF cells (Mishra and Hermsmeyer, 1994b; McDonough and Bean, 1998). In cells, including mouse spermatozoa, thyroid C cells, and rat sensory neurons, slightly higher IC_{50} values have been reported for T channel inhibition (Mehrke et al., 1994; Arnoult et al., 1998; Todorovic and Lingle, 1998). The relative potency of mibefradil as a T channel blocker is complicated because the drug displays prominent voltage and use dependence (see later). Regardless, in well-polarized cells, mibefradil blocks I_{AC}-type K^+ channels at concentrations lower than those required to block T-type Ca^{2+} channels in a variety of cells.

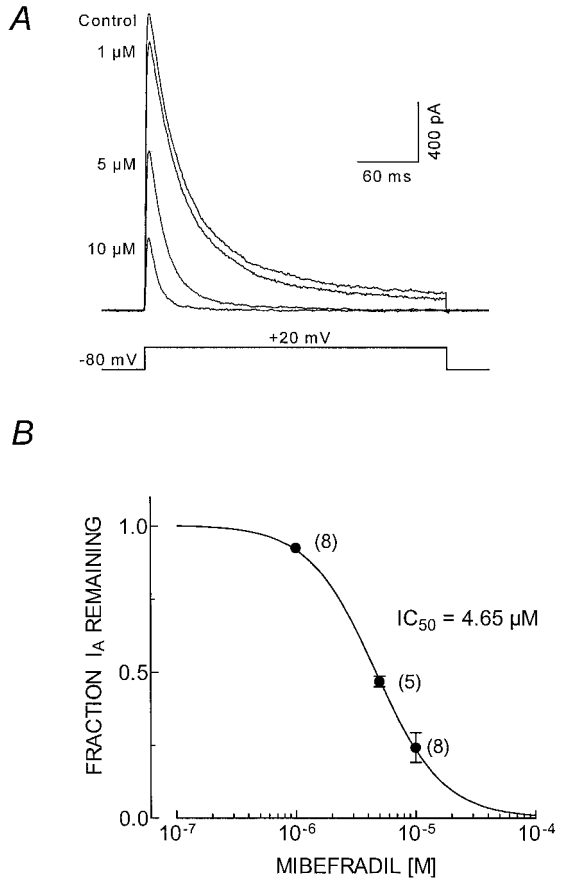

Fig. 2. Inhibition of voltage-gated I_A K^+ current by mibefradil. I_{AC} K^+ current was selectively inhibited by superfusing the cell with 200 pM ACTH for 5 min. I_A was activated by voltage steps to +20 mV, applied at 30-s intervals from a holding potential of −80 mV. A, I_A current records in control saline and after steady-state block by mibefradil at 1, 5, and 10 μM as indicated. B, inhibition curve. Fraction of unblocked I_A current is plotted against mibefradil concentration. Data were fit with an equation of the form: $I/I_{max} = 1/[1 + (B/IC_{50})^X]$, where B is the mibefradil concentration, IC_{50} is the concentration that reduces I_T by 50%, and X is the Hill coefficient. Values are mean ± S.E. of indicated number of determinations.

Mibefradil exhibits 10- to 25-fold selectivity for T-type over L-type Ca^{2+} channels (Mehrke et al., 1994; Bezprozvanny and Tsien, 1995; Ertel and Ertel, 1997) and as much as 200-fold selectivity for other Ca^{2+} channel subtypes (Bezprozvanny and Tsien, 1995; McDonough and Bean, 1998). By comparison, mibefradil blocks voltage-gated A-type K^+ channels with an IC_{50} value of 4.65 μM, a concentration only 10-fold greater than that which blocks I_{AC} channels. Overall, mibefradil may inhibit a variety of K^+ channels at concentrations similar to those that block T-type Ca^{2+} channels.

In this regard, the ATP-activated I_{AC} channel represents an interesting new type of K^+ channel whose relationship to other K^+ channels has not been determined. In developing a pharmacological profile of I_{AC} channels, we previously found that established organic K^+ channel blockers, including tetraethylammonium, 4-amino-pyridine, and quinidine, all inhibited I_{AC} channels with a potency similar to that observed in many other voltage-gated K^+ channels (Gomora and Enyeart, 1999). However, mibefradil was 50 to 10,000 times more potent than any of these agents as an inhibitor of I_{AC}. The sensitivity of other major K^+ channel subtypes, including inward rectifiers and dual-pore channels, to mibefradil has yet to be determined.

At the present, diphenylbutylpiperidine (DPBP) antipsychotic agents are the only other agents that block I_{AC} channels with a potency similar to that of mibefradil. DPBPs, including penfluridol, pimozide, and fluspirilene, inhibit I_{AC} with IC_{50} values between 0.19 and 0.35 μM (Gomora and Enyeart, 1999). Interestingly, DPBPs potently and preferentially block T-type Ca^{2+} channels in many cells, including those of the AZF (Enyeart et al., 1992, 1993). The molecular basis for this pharmacological similarity between T-type Ca^{2+} channels and I_{AC} K^+ channels is currently unknown.

Mechanism of Mibefradil Inhibition of I_{AC} K^+ Channels. Mibefradil-mediated inhibition of T-type Ca^{2+} channels shows prominent voltage and use dependence (McDonough and Bean, 1998; Gomora et al., 1999). As a result, its potency increases markedly in response to sustained or repeated depolarizations. According to the "modulated receptor hypothesis," voltage- and use-dependent block occurs when drugs preferentially bind to channels that have been opened or inactivated by depolarization (Hille, 1977; Hondeghem and Katzung, 1977).

In contrast to T-type Ca^{2+} channels, I_{AC} K^+ channel gating is primarily regulated by ATP and metabolic factors. These channels exhibit very little voltage-dependent activation, and they do not inactivate (Mlinar et al., 1993; Enyeart et al., 1996, 1997). Consequently, block of I_{AC} K^+ channels by mibefradil likely occurs through an entirely different mechanism.

Accordingly, at the whole-cell level, the potency of mibefradil as an inhibitor of I_{AC} K^+ channels did not vary when

the holding potential was switched from −80 to −40 mV. Furthermore, at the single-channel level, mibefradil increased τ_{c1} and τ_{c2} of I_{AC} channels but did not alter τ_o, suggesting that mibefradil binds to and stabilizes the closed states of the I_{AC} K^+ channel.

At the single-channel level, mibefradil does not reduce the size of unitary I_{AC} currents, as is frequently observed with blockers whose kinetics of binding and unbinding are very rapid (Moczydlowski, 1992). Mibefradil, therefore, blocks unitary I_{AC} currents with the characteristics of a slow or intermediate blocker.

Mechanism of Toxicity. Despite its beneficial hemodynamic effects, mibefradil has been withdrawn from the market due to toxicity associated with its use. The inhibition of cytochrome P-450 3A4 enzyme by mibefradil may result in numerous toxic drug interactions. Notably, when mibefradil is used in combination with certain antihistamines, such as astemizole, the Q-T interval is prolonged and ventricular arrhythmias may occur. The cellular mechanism probably involves inhibition of K^+ channels in the myocardium by elevated concentrations of the antihistamine (Woosley, 1996). The ability of mibefradil to potently block some types of K^+ channels suggests that in addition to altering the pharmacodynamics of antihistamines, the cardiovascular toxicity produced by the combination of these drugs could be due to their combined direct inhibition of K^+ channels in the heart.

In summary, mibefradil is a potent antagonist of ATP-activated K^+ channels in bovine AZF cells. This agent will be useful in determining the function of this novel K^+ channel in AZF cell physiology. Regardless of the ability of mibefradil to preferentially block T-type Ca^{2+} channels, the parallel inhibition of K^+ channels by this drug with equal or greater potency defines a limitation to its specificity as an ion channel blocker and suggests that interaction with K^+ channels may be involved in its therapeutic or toxic effects.

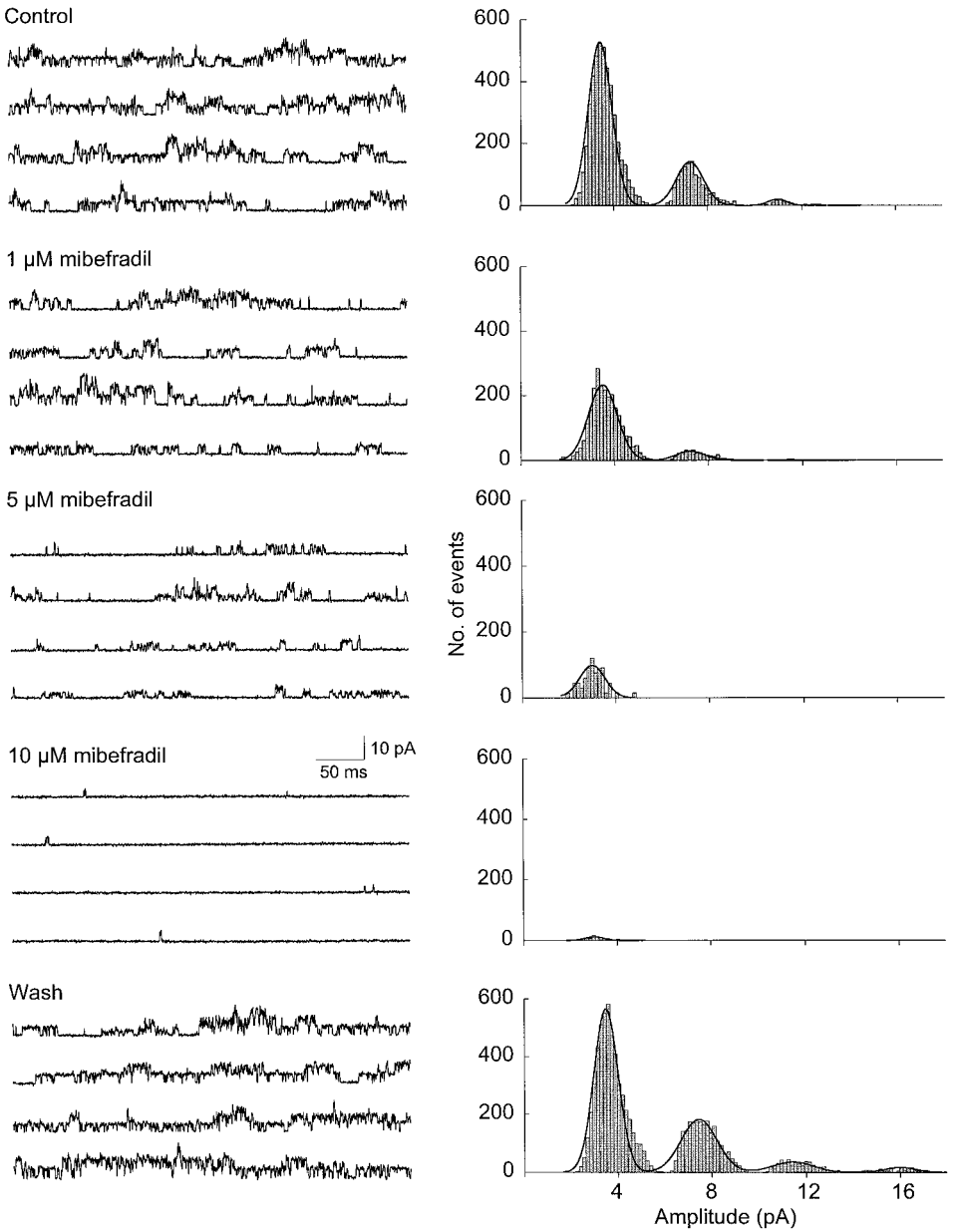

Fig. 3. Effect of mibefradil on unitary I_{AC} K^+ currents. Unitary I_{AC} currents were recorded in the outside-out configuration using pipettes containing standard solution supplemented with 2 mM MgATP. Voltage steps to +30 mV were applied at 10-s intervals from a holding potential of −40 mV. Each amplitude histogram was constructed from idealized channel openings obtained from unitary currents recorded in response to 80 to 96 separate voltage steps of 400-ms duration applied at 0.25 Hz. Unitary current amplitudes were distributed into bins of 0.15-pA width. Traces and corresponding amplitude histograms in control saline, in the presence of 1, 5, and 10 μM mibefradil and 7 min after return to control saline (wash) as indicated. The continuous line in the histograms represents the fit of Gaussian distributions to the data. Currents were filtered at a cutoff frequency of 2 kHz and sampled at 5 kHz. Similar results were obtained in each of eight outside-out patches.

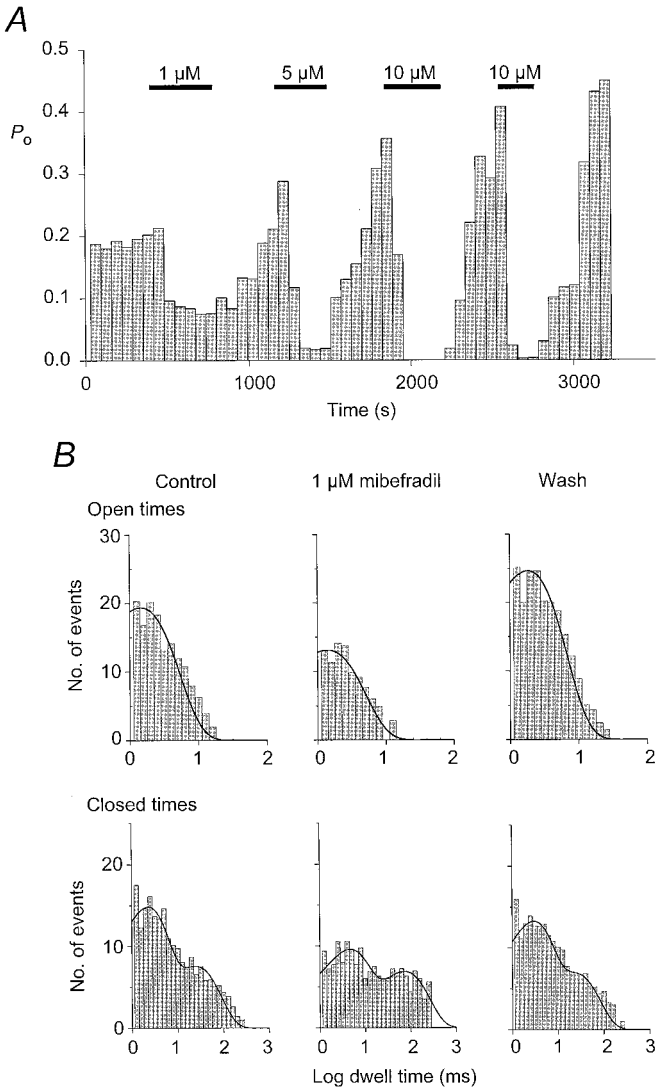

Fig. 4. Effect of mibefradil on I_{AC} channel P_o and dwell times. The effect of mibefradil on I_{AC} K^+ channel P_o and mean open (τ_o) and closed (τ_{c1} and τ_{c2}) time constants was determined from experiments such as those in Fig. 3. A, P_o was calculated by integrating the area of the current traces and dividing this value by the product $N \cdot \hat{i} \cdot T$, where N is the number of functional channels in the patch, $\hat{i}$ is the unitary current, and T is total recording time. Each P_o value was calculated from channel openings recorded in response to 16 voltage steps to +30 mV. B, dwell time histograms with logarithmic time axes and square-root ordinates. Open and closed events were accumulated in bins whose width was proportional to the logarithm of the event duration. Open time histograms were fit (continuous line) with single exponentials (τ_o), and the closed time histograms were fit with two exponentials (τ_{c1} and τ_{c2}). τ_o values for control, mibefradil, and wash were 1.73, 1.51, and 1.63 ms. Respective τ_{c1} and τ_{c2} values were 1.56 and 17.88 ms (control), 4.09 and 70.60 ms (mibefradil), and 2.40 and 23.52 ms (wash). Events of <0.5 ms in duration were disregarded.

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