Inhibitory Action of Mibefradil on Calcium Signaling and Aldosterone Synthesis in Bovine Adrenal Glomerulosa Cells

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Accepted for publication June 23, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Mibefradil is a new cardiovascular drug with peculiar Ca\(^{2+}\) antagonistic properties. The most remarkable feature of mibefradil is its unique relative selectivity for T type calcium channels, a property that has been proposed to explain in part the beneficial pharmacological and clinical profiles of this drug. In adrenal glomerulosa cells, aldosterone biosynthesis and secretion in response to angiotensin II or extracellular potassium is dependent on a sustained influx of Ca\(^{2+}\) through T type Ca\(^{2+}\) channels. The effect of mibefradil on the steroidogenic function of glomerulosa cells was therefore investigated. Using the patch clamp technique, we found that mibefradil inhibits selectively and in a concentration-dependent manner (IC\(_{50}\) = 3 \(\mu\)M) Ba\(^{2+}\) T type currents in bovine glomerulosa cells. In addition to this tonic (voltage independent) inhibition, the drug also induced a shift of the steady-state inactivation curve of these channels toward hyperpolarized voltages, contributing to its efficacy to prevent Ca\(^{2+}\) influx into the cell through T type channels. Concomitantly, mibefradil reduced the cytosolic calcium responses to potassium and angiotensin II (as assessed with fluorescent probes), without affecting the capacitative Ca\(^{2+}\) influx, and inhibited pregnenolone and aldosterone formation. This inhibition of steroidogenesis was not exclusively due to mibefradil action on voltage-operated Ca\(^{2+}\) channels, because this agent also partially reduced steroid synthesis induced by adrenocorticotropic hormone or forskolin, two activators of the cyclic AMP pathway. In conclusion, mibefradil is highly effective in adrenal glomerulosa cells in reducing T type channel activity and aldosterone biosynthesis, two actions that should contribute to the beneficial effect of the drug in the treatment of hypertension.

Mibefradil, a benzimidazolyl-substituted tetraline derivative, is a recently developed calcium antagonist with unique chemical structure and promising cardiovascular profile, as compared to other drugs currently available (Clozel et al., 1991). The pharmacological properties and therapeutic advantages of mibefradil have been recently reviewed (Erkel and Clozel, 1997) and include: 1) a potent antihypertensive action with an efficacy similar to that of verapamil and dihydropyridines for reducing blood pressure (Hefti et al., 1990; Bernink et al., 1996), for relaxing aorta and coronary arteries (Boulanger et al., 1994a; Karila-Cohen et al., 1996; Boulanger et al., 1994b), for increasing coronary blood flow (Karila-Cohen et al., 1996) and for preventing blood pressure-related arterial hypertrophy (Li and Schiffrin, 1997, 1996); 2) a complete lack of negative inotropic effect at therapeutic doses (Clozel et al., 1989, 1990; Véniant et al., 1991; Cremer et al., 1997; Mulder et al., 1997), which is a major problem associated with the use of classical calcium antagonists for treating hypertension, particularly in patients with chronic heart failure; 3) a significant heart rate lowering activity (Clozel et al., 1991); 4) a marked selectivity for vascular (specifically coronary) smooth muscle over cardiac or visceral tissues (Osterrieder and Holck, 1989); and finally, 5) a high bioavailability and a long half-life (Hefti et al., 1990).

In the micromolar concentration range, mibefradil has been shown to bind and inhibit in a voltage-dependent manner various types of calcium channels (including L-, N-, P/Q- and R-types) expressed in Xenopus oocytes (Bezprozvanny and Tsien, 1995). The binding of the drug to L-type channels has been extensively characterized (Rutledge and Triggle, 1995; Schuster et al., 1996; Ratner et al., 1996) and appears to involve a site on the \(\alpha_1\) subunit of the channel, located close to the IVS6 segment, distinct from the binding site of dihydropyridines and partially overlapping that of verapamil.

Interestingly, whereas the efficacy of mibefradil on the cardiac L type and T type channels is highly dependent on membrane potential (Liang-min and Osterrieder, 1991; Laci-nova et al., 1995; Mangoni et al., 1997), suggesting a higher affinity for the inactivated state of the channel, mibefradil affects other T type (Mehrke et al., 1994) and vascular smooth muscle L type channels (Mishra and Hermanneyer,

ABBRIVIATIONS: AngII, angiotensin II; ACTH, adrenocorticotropic hormone; [Ca\(^{2+}\)]\(_{o}\), cytosolic free calcium concentration; Nic, nicardipine.

Received for publication January 30, 1998.

\(^1\) This work was supported by Grants 32-49297.96 and 31-42178.94 of the Swiss National Science Foundation and by the Helm Horten Foundation. M.P.R. is a recipient of a grant from the Prof. Max Cloetta Foundation.
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1994a; Bian and Hermansmyer, 1993) equally during both resting and depolarized conditions. This tissue-specific mode of action of mibefradil could be related to the differential expression of α1C (L type) splice variants in heart and smooth muscle (Hofmann et al., 1994) and may contribute to the relative sparing action on cardiac function of this novel calcium antagonist. Moreover, coexpression of particular β subunits has also been shown to influence the interaction of mibefradil with L type channels (Welling et al., 1995).

The most remarkable pharmacological feature of mibefradil, however, is certainly its mild selectivity for T type calcium channels (Értel and Clozel, 1997; Mishra and Hermansmyer, 1994b). This property, unique among Ca2+ antagonists, partially accounts for the peculiar pharmacological profile of this drug. In fact, although the weak negative inotropic effect of mibefradil is probably related to its weak potency in inhibiting Ca2+ current through L type channels in polarized cardiac tissue (Liang-min and Osterrieder, 1991), the selectivity of the drug for T type channels may also contribute to the beneficial cardiac actions of mibefradil (Roux et al., 1996). Moreover, the ability of mibefradil to inhibit smooth muscle cell proliferation and neointima formation after vascular injury has been proposed to be due to its effect on T type channel activity (Schmitt et al., 1995). In any case, the precise role of T type channels in cardiac or vascular physiology remains uncertain and further work is needed to confirm the mechanisms of the various actions of mibefradil (Értel and Ertel, 1997).

In contrast, the essential and specific function of T type channels in adrenal glomerulosa cells on stimulation of steroidogenesis by AngII or extracellular potassium (K+) has been clearly demonstrated (Barrett et al., 1991, 1995; Rossier et al., 1993, 1996) and reviewed elsewhere (Capponi and Rossier, 1996). Because of the demonstrated selectivity of mibefradil for T type channels in various tissues, and because of the possible involvement of mineralocorticoids in the onset of some forms of hypertension, the action of this novel calcium antagonist on bovine glomerulosa cell function and on aldosterone formation has been characterized in detail in our work.

Materials and Methods

Mibefradil (dihydrochloride) was kindly provided by Hoffmann-La Roche, Basel, Switzerland. Percoll was obtained from Pharmacia (Piscataway, NJ) and Cell-Tak from Inotech (Dottikon, Switzerland). Tetrodotoxin, sodium ATP, sodium GTP, nicardipine, nifedipine and pimozone were purchased from Sigma Chemical Co. (St. Louis, MO), and fura-2 acetoxymethyl ester from Molecular Probes (Eugene, OR). Thapsigargin was obtained from Anawa (Zurich, Switzerland), and [IIe6]AngII from Bachem AG (Bubendorf, Switzerland). WIN 19758 (cyanoketone) was kindly donated by Sterling-Winthrop (Renselaer, NY).

Adrenal glomerulosa cell isolation and culture. Bovine adrenal glands were obtained from a local slaughterhouse and glomerulosa cells were prepared by enzymatic dispersion, purified on a Percoll density gradient and maintained in culture for 2–4 days, as described in detail elsewhere (Rossier et al., 1993).

Patch-clamp measurements. The activity of voltage-operated Ca2+ channels in bovine adrenal glomerulosa cells was recorded under voltage clamp, in the whole cell configuration of the patch clamp technique, as previously described (Rossier et al., 1996). The reference electrode was placed in a KCl solution linked to the bath with an Agar bridge; the resulting liquid junction potential was smaller than 2 mV and has been neglected. The cell was voltage-clamped (Axopatch 1D, Axon Instruments Inc., Foster City, CA) at a holding potential of −90 mV and depolarized as indicated. The currents were filtered at 1 to 2 kHz and sampled at 6.2 kHz. Leak was subtracted either digitally after the experiment or automatically by a P/4 protocol (pclamp 6, Axon Instruments Inc.).

\[ \text{[Ca}^{2+}]_{i} \text{, [Ca}^{2+}]_{o} \text{, was determined with fura-2 in populations of cells, freshly isolated and purified on a Percoll density gradient. Fura-2 fluorescence (excitation at 340/380 nm and emission at 505 nm) was recorded with a Jasco CAF-110 fluorescence spectrometer (Hachioji City, Japan) and [Ca}^{2+}]_{o} \text{, was calibrated as previously described (Rossier et al., 1995; Grynkiewicz et al., 1985), using a } K_{d} \text{ value of 224 nM.} \]

Determination of aldosterone and pregnenolone formation. Glomerulosa cells, cultured for 3 days, were incubated, as described in detail elsewhere (Burnay et al., 1994), for 1 hr at 37°C in a modified Krebs-Ringer medium containing various agonists and increasing concentrations of mibefradil or other pharmacological inhibitors of Ca2+ channels. At the end of the incubation period, the aldosterone content of the medium was determined by direct radioimmunoassay, using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX). Cellular protein was measured in each dish using the Coomassie blue method of Bradford (1976).

For the assessment of pregnenolone production, WIN 19758 (5 μM) was included in the incubation medium to prevent conversion of pregnenolone into progesterone and the concentration of pregnenolone was determined as described elsewhere (Python et al., 1995), using (3H)pregnenolone and an antibody kindly donated by Professor P. Vescei (University of Heidelberg, Germany). The presence of mibefradil up to a concentration of 100 μM did not interfere with steroid or protein assays.

Results

Selectivity and mode of action of mibefradil on T type channels in bovine glomerulosa cells. To determine the selectivity of mibefradil for T type over L type Ca2+ channels in bovine adrenal glomerulosa cells, two different protocols have been used (fig. 1). Upon a sustained cell depolarization to 0 mV, both T and L type channels activated rapidly and the inward Ba2+ current reached a maximal amplitude approximately 20 msec after depolarization (fig. 1A). The current then decreased to a lower plateau, which was sustained for at least 500 msec. As discussed elsewhere (Rossier et al., 1993), the rapid decay of the current can be attributed to the fast inactivation of T type channels. The inactivation time constant of T type channels in bovine glomerulosa cells has been estimated to be 27 msec at 0 mV (not shown), and one is therefore entitled to consider that the current detected after a 450-msec depolarization is exclusively due to the slowly inactivating L type channels. The difference between the peak current value and the mean current measured between 100 and 150 msec after depolarization was used to estimate the activity of the T type channels. As shown in figure 1A, treatment of the cell with 10 μM mibefradil reduced both the peak and the plateau values, reflecting a 62% inhibition of T channel activity and a 45% inhibition of L channel activity in this particular cell.

Because of their lower threshold of activation and faster kinetics of inactivation, T type channels activate first upon a gradual (ramp) depolarization, and already inactivate when the voltage reached −20 mV; however, extensive overlap with L type current occurred. For this reason, the T and L compo-
nents of the total current were modeled by fitting current traces to two empirically defined functions (described in the legend of fig. 1). As indicated elsewhere (Rossier et al., 1996), when measured in the same cell, the amplitude of the T component determined by ramp depolarization was closely correlated with the amplitude of the slowly deactivating currents (exclusively attributed to T channels (Rossier et al., 1995)), demonstrating that the voltage ramp protocol allows one to discriminate between T and L type currents. By comparing the amplitudes of each current component in these traces, recorded before and after the addition of mibefradil, we found that, at 20 μM, the drug was able to reduce T channel activity by 79% and L channel activity by 49%.

The time course of mibefradil action was also investigated by repeatedly performing the same ramp voltage protocol every 30 sec, during a 5-min control period and after the addition of two concentrations of this agent (fig. 1C). The current elicited upon each depolarization was analyzed, T and L components determined and their amplitude plotted as a function of time. In this particular cell, T type current decreased by approximately 65% upon addition of 20 μM mibefradil, after a lag period of 3 min. To determine whether a use-dependence mechanism could be responsible for the long time scale of the response to mibefradil, we have compared the time elapsing between the addition of 10 μM of the drug and the half inhibition of the T current, in experiments in which the time intervals between the depolarizing steps were of different duration. Whereas the mean duration was estimated to be 5.3 ± 0.3 min (n = 3) when the cell was depolarized every 30 sec, this delay was reduced to 2.9 ± 0.7 min (n = 7) and to 1.8 ± 1.1 min (n = 3) when the cell was depolarized every 20 and every 10 sec, respectively. It therefore appears that the inhibition of T channels by mibefradil is characterized by a strong use dependence.

Under the same conditions, L type current was only reduced by approximately 40%. A second addition of mibefradil...
(100 µM) completely abolished T and substantially reduced L-type currents. Similar experiments, as illustrated in figure 1A and B, performed with various concentrations of the agent allowed us to establish the concentration dependence of mibefradil inhibition for each type of voltage-operated Ca⁺⁺ channel (fig. 1D). Mibefradil inhibited in a concentration-dependent manner T-type and L-type currents with IC₅₀ values of 3.2 and 27.4 µM, respectively. Inhibition of T-type channels was significantly more pronounced than that of L-type channels above 1 µM mibefradil. Because of possible cytotoxic effects (see below), concentrations of mibefradil of more than 100 µM could not be tested.

As previously reported for the cardiac and smooth muscle L-type channels (Lacinova et al., 1995; Bian and Hermsmeyer, 1993), mibefradil induced a shift of the steady-state inactivation curve of the adrenal glomerulosa cell T-type channels toward hyperpolarized membrane potentials (fig. 2). Indeed, 10 µM mibefradil induced a 20 mV shift of the inactivation curve V₁/₂ toward negative voltage values, without affecting activation properties of the channel. This resulted in a marked reduction of the size of the permissive voltage window (Rossier et al., 1995) and therefore of the amplitude of the steady-state current through T-type channels. This effect of mibefradil occurred in addition to a 83% decrease of the maximal steady-state current (I₀), which has been normalized in figure 2. A similar action of mibefradil (1–10 µM) was observed in each of six tested cells, in which the shift of activation V₁/₂ was on average −2.8 ± 1.4 mV (±S.E.M.) and the shift of steady-state inactivation V₁/₂ = −13.0 ± 4.4 mV. The action of mibefradil on the T-channel inactivation curve appeared to be concentration-dependent (not shown).

**Effect of mibefradil on cytosolic calcium signaling and steroidogenesis.** As shown in figure 3, mibefradil, in a concentration-dependent fashion, reduced the sustained [Ca⁺⁺], response induced by extracellular K⁺, with an IC₅₀ in the low micromolar range. Nifedipine, a dihydropyridine completely blocking both L and T type Ca⁺⁺ channels at 10 µM (Rossier et al., 1996), was systematically used to determine maximal inhibition. When used at concentrations of 100 µM or higher, mibefradil induced a slow and large [Ca⁺⁺], increase (50–350 nM), which was not prevented by the presence of dihydropyridines (data not shown), suggesting some cytotoxic effect of the drug at these high concentrations.

When the synthesis of pregnenolone, the first intermediate compound produced upon stimulation of steroidogenesis, or that of aldosterone, the final product, were assessed, an almost complete inhibition of the responses to K⁺ was observed at 10 µM mibefradil (fig. 4). This inhibition by mibefradil was clearly concentration dependent within the same range of concentrations required for inhibition of T-type channels, a finding that reflects the close relationship existing between the activity of this class of Ca⁺⁺ channels and the activation of steroidogenesis by extracellular K⁺ (Rossier et al., 1996). Interestingly, whereas a strong correlation (r = 0.949, P < .0001) was observed between pregnenolone and aldosterone production up to 10 µM mibefradil, a clear dissociation appeared in the presence of high, cytotoxic concentrations (100 µM) of the drug (not shown).

In contrast, the steroidogenic response to AngII appeared less sensitive to mibefradil and was affected only at concentrations of the drug of more than 3 µM. The action of AngII involving additional Ca⁺⁺ pathways, we further investigated the specificity of mibefradil on calcium signaling and steroidogenesis in adrenal glomerulosa cells.

**Lack of effect of mibefradil on the capacitative calcium influx.** Because AngII activates a capacitative calcium influx in addition to the voltage-operated Ca⁺⁺ channels (Burnay et al., 1994), the effect of mibefradil on this pathway was also investigated. As previously demonstrated, the activation of T- and L-type Ca⁺⁺ channels by adding 9 mM K⁺ (leading to a 12 mM final concentration in the medium) resulted in a sustained elevation of [Ca⁺⁺], which was rapidly reversed upon addition of 10 µM mibefradil (fig. 5A). In contrast, after complete inhibition of basal voltage-operated Ca⁺⁺ channel activity with 2 µM nicardipine (Burnay et al., 1994), the capacitative Ca⁺⁺ influx elicited by 500 nM thapsigargin, an inhibitor of the microsomal Ca⁺⁺ pumps, was insensitive to the addition of the drug (fig. 5B). Similarly, the sustained [Ca⁺⁺], response to AngII, reflecting Ca⁺⁺ influx occurring after completion of the Ca⁺⁺ release phase, remained unaffected by mibefradil when cells were preexposed to nicardipine (fig. 5D), but rapidly decreased when T- and L-type channels were allowed to open in response to the hormone (fig. 5C). From these results, we conclude that mibefradil, at 10 µM, selectively blocks voltage-operated Ca⁺⁺ channels without affecting the capacitative Ca⁺⁺ pathway.

**Specificity of the inhibition of steroidogenesis by various calcium antagonists.** The inhibitory action of mibefradil on aldosterone formation was compared to that of two other Ca⁺⁺ antagonists, nicardipine, a dihydropyridine blocking efficiently both T- and L-type channels, as previously demonstrated in glomerulosa cells (Burnay et al., 1994), and pimozide, a diphenylpiperidine derivative generally used as neuroleptic but also displaying Ca⁺⁺ antagonistic properties in bovine glomerulosa cells (Rossier MF, unpublished data).
As shown in figure 6, the steroidogenic response to extracellular K\(^+\) (12 mM), an agonist mobilizing Ca\(^{++}\) exclusively through voltage-operated channels, was almost completely abolished by micromolar concentrations of each of the three agents tested. In contrast, mibefradil was by far the most efficient antagonist when aldosterone synthesis was stimulated with AngII instead of K\(^+\). Indeed, whereas no or poor inhibition of aldosterone production was observed in the presence of pimozide or nicardipine, mibefradil reduced steroidogenesis by 52% (P < .005). Pimozide, at micromolar concentrations, was particularly efficient in discriminating between aldosterone stimulated by AngII and aldosterone stimulated by KCl (fig. 6, inset). This finding cannot be explained by a high voltage-dependence of pimozide action, because pimozide inhibited aldosterone formation induced by various concentrations of potassium (6–25 mM) with a similar efficacy (not shown). The action of pimozide provides a further demonstration that both AngII and K\(^+\) control steroidogenesis through distinct mechanisms.

When aldosterone biosynthesis was induced by agents acting through the cyclic AMP pathway, such as ACTH or forskolin, mibefradil was again the most powerful inhibitor (fig. 6). The aldosterone responses to both agonists were inhibited by mibefradil in a concentration-dependent manner, with IC\(_{50}\) values of 6.1 and 8.1 \(\mu\)M for the responses to ACTH and forskolin, respectively (not shown). Moreover, pregnenolone formation stimulated by forskolin was also reduced (by 55%) in the presence of 10 \(\mu\)M mibefradil. Altogether, these data suggest that mibefradil also affects steroidogenesis by additional mechanisms distinct from the blockade of Ca\(^{++}\) channels. In contrast, the conversion of 25-hydroxycholesterol into pregnenolone or corticosterone was only slightly affected (less than 10% inhibition in three independent experiments) by 10 \(\mu\)M mibefradil. This hydrophilic derivative of cholesterol bypasses the hormone-regulated transport of steroid precursors into the mitochondria, a limiting step normally controlled by cytosolic Ca\(^{++}\) or cyclic AMP (Python et al., 1995), and this observation therefore excludes the possibility of a cytotoxic action of mibefradil at this concentration.

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**Fig. 3.** Inhibition of the KCl-induced [Ca\(^{++}\)] response by mibefradil. Fura-2-loaded cells were sequentially exposed to 9 mM KCl (12 mM final concentration), to increasing concentrations of mibefradil, and finally to 10 \(\mu\)M nifedipine. Variations of [Ca\(^{++}\)] were recorded, quantified as described in "Materials and Methods," and expressed as percentage of the control levels, measured before the addition of the drug. Minimal [Ca\(^{++}\)] was systematically determined after completely blocking voltage-operated Ca\(^{++}\) channels with 10 \(\mu\)M nifedipine. Data (mean ± S.E.M. from five to nine experiments) were fitted to a four (two fixed) parameter logistic function to determine the IC\(_{50}\) (1.56 \(\mu\)M) and slope (0.60) values. Inset, A representative trace used to establish the inhibition curve. Mean basal [Ca\(^{++}\)] was estimated to be approximately 190 nM.

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**Fig. 4.** Effect of mibefradil on steroidogenesis. The inhibitory action of increasing concentrations of mibefradil on steroidogenesis was determined by measuring, as described in “Materials and Methods,” pregnenolone (A) and aldosterone (B) formation induced in cultured glomerulosa cells by 12 mM KCl (□) or 100 nM AngII (○). Data (mean value ± S.E.M. from three independent experiments performed in triplicate) were fitted to a 4-parameter (1 fixed) logistic function to determine the IC\(_{50}\) values (indicated in figure). Basal (unstimulated) pregnenolone and aldosterone productions were on average 1080 and 3.02 pmol/mg prot/hr, respectively.
The treatment of hypertension and cardiovascular diseases with classical Ca\(^{2+}\) antagonists (mostly acting on L type channels) has been hampered for a long time by the negative inotropic action exerted by these drugs on cardiac function and the risk associated with this type of medication has been the object of a recent controversy (Furberg et al., 1995; Cohen, 1996; Schulz et al., 1996; Zanchetti, 1996). A search for Ca\(^{2+}\) channel blockers with different specificity has logically been launched to decrease undesirable side effects and has led to the development of second and third generation Ca\(^{2+}\) antagonists (Palma-Gamiz, 1997; Toyo-Oka and Nayler, 1996; van Zwieten et al., 1996). Among these recently characterized Ca\(^{2+}\) antagonists, mibefradil appeared as a particularly promising therapeutic agent and some of its clinical advantages have been attributed to its specificity in inhibiting preferentially T type Ca\(^{2+}\) channels in various cell types (Ertel and Clozel, 1997). In our study, a similar selectivity for T channels, with an IC\(_{50}\) in the low micromolar range, was demonstrated in bovine adrenal glomerulosa cells (fig. 1).

Although an important effect of the drug on L type channels was also observed, confirming the lack of selectivity of mibeferadil for one single type of Ca\(^{2+}\) channel, this drug is unique because the other Ca\(^{2+}\) antagonists currently used for treating hypertension display a marked preference for L type channels. For instance, in bovine glomerulosa cells, nifedipine inhibits L type currents at concentrations at least two orders of magnitude lower than those required for affecting T type currents (Rossier et al., 1996).

In addition to the tonic (voltage-independent) inhibition of T type channels exerted by mibefradil, a shift of the inactivation curve of the channels toward more negative values of voltage was also induced by this agent, further reducing the amplitude of the steady-state influx of Ca\(^{2+}\) predicted to occur through these channels upon a sustained cell depolarization (Rossier et al., 1995). Thus, in the presence of mibefradil, not only are fewer T type channels available but the remaining channels are more easily inactivated. This combination of effects makes mibefradil particularly efficient to prevent Ca\(^{2+}\) entry through T type channels, a privileged pathway in the activation of aldosterone biosynthesis (Barrett et al., 1991; Capponi and Rossier, 1996). More surprising was the high efficacy of mibefradil in inhibiting the cytosolic calcium response to extracellular K\(^+\) (fig. 3), a parameter directly linked to the activity of L type Ca\(^{2+}\) channels (Rossier et al., 1996). A possible explanation could be that, as it is the case for T type channels, mibefradil also exerts a voltage-dependent inhibition on L type channels and that a Ca\(^{2+}\) channel blockers with different specificity has logically been launched to decrease undesirable side effects and has led to the development of second and third generation Ca\(^{2+}\) antagonists (Palma-Gamiz, 1997; Toyo-Oka and Nayler, 1996; van Zwieten et al., 1996). Among these recently characterized Ca\(^{2+}\) antagonists, mibefradil appeared as a particularly promising therapeutic agent and some of its clinical advantages have been attributed to its specificity in inhibiting preferentially T type Ca\(^{2+}\) channels in various cell types (Ertel and Clozel, 1997). In our study, a similar selectivity for T channels, with an IC\(_{50}\) in the low micromolar range, was demonstrated in bovine adrenal glomerulosa cells (fig. 1).

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In addition to the tonic (voltage-independent) inhibition of T type channels exerted by mibefradil, a shift of the inactivation curve of the channels toward more negative values of voltage was also induced by this agent, further reducing the amplitude of the steady-state influx of Ca\(^{2+}\) predicted to occur through these channels upon a sustained cell depolarization (Rossier et al., 1995). Thus, in the presence of mibefradil, not only are fewer T type channels available but the remaining channels are more easily inactivated. This combination of effects makes mibefradil particularly efficient to prevent Ca\(^{2+}\) entry through T type channels, a privileged pathway in the activation of aldosterone biosynthesis (Barrett et al., 1991; Capponi and Rossier, 1996). More surprising was the high efficacy of mibefradil in inhibiting the cytosolic calcium response to extracellular K\(^+\) (fig. 3), a parameter directly linked to the activity of L type Ca\(^{2+}\) channels (Rossier et al., 1996). A possible explanation could be that, as it is the case for T type channels, mibefradil also exerts a voltage-dependent inhibition on L type channels and that a
sustained influx of Ca\textsuperscript{2+} through L channels becomes particularly sensitive to the drug at the potential (about −60 mV) maintained by 12 mM extracellular potassium. Further investigation will be required to confirm this hypothesis.

Because mineralocorticoid excess is a major cause of hypertension, inhibition of aldosterone secretion from glomerulosa cells, in addition to vascular relaxation, by the same therapeutic agent is certainly beneficial in the treatment of hypertensive patients. However, most classical Ca\textsuperscript{2+} antagonists, probably because they are targeted against L-type channels, are inefficient in preventing aldosterone secretion. For example, no effect or even an increase of aldosterone production upon treatment with antagonist dihydropyridines have been reported both in \textit{vivo} (Hrnciar et al., 1997; Landmark et al., 1995; Levy et al., 1994; Shibasaki et al., 1994) and \textit{in vitro} (Barrett et al., 1995; Rossi et al., 1996). In contrast, mibefradil appears as a potent inhibitor of K\textsuperscript{+}-induced steroidogenesis in glomerulosa cells (fig. 4). In this regard, mibefradil action can be compared to that of tetrandrine, an alkaloid extracted from a Chinese medicinal herb and antagonizing Ca\textsuperscript{2+} channels with a slight selectivity for T type channels (Rossier et al., 1993). Tetrandrine is traditionally used in the treatment of hypertension and efficiently reduces aldosterone secretion in bovine glomerulosa cells.

Interestingly, mibefradil similarly affected steroidogenesis induced by AngII, although with a slightly higher IC\textsubscript{50}. This result was surprising because AngII stimulates aldosterone synthesis via additional pathways, including a capacitative Ca\textsuperscript{2+} influx (Barnard et al., 1994; Rohacs et al., 1994). These properties of the hormone may explain why some Ca\textsuperscript{2+} antagonists such as nicardipine (Barnard et al., 1994) or pimozide (fig. 6, inset) affect much more efficiently the response to KCl than the response to AngII. We therefore tested whether mibefradil could prevent the capacitative Ca\textsuperscript{2+} influx induced by AngII or thapsigargin. The drug had no effect on the Ca\textsuperscript{2+} signal induced by either thapsigargin or AngII when voltage-operated-Ca\textsuperscript{2+} channels were previously blocked by nicardipine. In contrast, mibefradil partially reduced the \([\text{Ca}^{2+}]\), response to AngII when it was mediated by both the voltage-operated-Ca\textsuperscript{2+} channels and the capacitative influx. We therefore conclude that mibefradil does not affect the capacitative Ca\textsuperscript{2+} influx in adrenal glomerulosa cells, a finding apparently in disagreement with the blockade of receptor-operated channels by mibefradil reported in human platelets (Hahn et al., 1995) or in rabbit vascular smooth muscle cells (Cheglakov et al., 1997). However, it is noteworthy that, in the latter studies, mibefradil action was not tested in the presence of dihydropyridines, a condition that can lead to artifactual results (Barnard et al., 1994); moreover, different types of capacitative Ca\textsuperscript{2+} influx with different properties have been described and the possibility of a distinct sensitivity toward mibefradil has also to be considered.

Because the potency of mibefradil in inhibiting the aldosterone response to AngII cannot be attributed to an action on the capacitative Ca\textsuperscript{2+} influx, its effect on the response to other agonists, mobilizing distinct messenger systems, was also investigated. We found that mibefradil significantly reduced the steroidogenic response to both ACTH and forskolin (fig. 6), two agonists of steroidogenesis acting through an elevation of cyclic AMP, suggesting once again that the mibefradil-induced inhibition of aldosterone secretion cannot be attributed only to the effect of the drug on T and L type channels.

In conclusion, it appears that the inhibitory action of mibefradil on the steroidogenic function of glomerulosa cells cannot be entirely accounted for by its marked and specific effect on the T type voltage-operated Ca\textsuperscript{2+} channels. Nevertheless, the best therapeutic agents are not necessarily the most selective drugs but rather drugs having the most appropriate combination of effects. In this regard, mibefradil appears particularly well suited for treating hypertension and the efficacy with which it inhibits aldosterone production may contribute to its recognized beneficial clinical action.

Acknowledgments

The authors are particularly grateful to Gisèle Dorenter, Liliane Bockhorn and Walda Dimeck for their technical assistance.

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