



Mibefradil (Ro 40-5967) inhibits several Ca^{2+} and K^{+} currents in human fusion-competent myoblasts

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1 The effect of mibefradil (Ro 40-5967), an inhibitor of T-type Ca^{2+} current ($I_{\text{Ca(T)}}$), on myoblast fusion and on several voltage-gated currents expressed by fusion-competent myoblasts was examined.

2 At a concentration of 5 μM , mibefradil decreases myoblast fusion by 57%. At this concentration, the peak amplitudes of $I_{\text{Ca(T)}}$ and L-type Ca^{2+} current ($I_{\text{Ca(L)}}$) measured in fusion-competent myoblasts are reduced by 95 and 80%, respectively. The IC_{50} of mibefradil for $I_{\text{Ca(T)}}$ and $I_{\text{Ca(L)}}$ are 0.7 and 2 μM , respectively.

3 At low concentrations, mibefradil increased the amplitude of $I_{\text{Ca(L)}}$ with respect to control.

4 Mibefradil blocked three voltage-gated K^{+} currents expressed by human fusion-competent myoblasts: a delayed rectifier K^{+} current, an *ether-à-go-go* K^{+} current, and an inward rectifier K^{+} current, with a respective IC_{50} of 0.3, 0.7 and 5.6 μM .

5 It is concluded that mibefradil can interfere with myoblast fusion, a mechanism fundamental to muscle growth and repair, and that the interpretation of the effect of mibefradil in a given system should take into account the action of this drug on ionic currents other than Ca^{2+} currents.

Keywords: Myoblast; potassium current; mibefradil; myoblast fusion

Abbreviations: $I_{\text{Ca(L)}}$, L-type Ca^{2+} current; $I_{\text{Ca(T)}}$, T-type Ca^{2+} current; $I_{\text{h-eag}}$, Human *ether-à-go-go* K^{+} current; $I_{\text{K(DR)}}$, Delayed rectifier K^{+} current; $I_{\text{K(IR)}}$, Inward rectifier K^{+} current; $I_{\text{K(NI)}}$, Non-inactivating K^{+} current

Introduction

Mibefradil is a pharmacological agent accepted as a rather selective inhibitor of Ca_{T} channels (Ertel & Ertel, 1997). This agent has recently been used in the therapy of some cardiovascular diseases (Clozel *et al.*, 1997) before being withdrawn from the market in June 1998 due to its interactions with other drugs (Po & Zhang, 1998).

Recently, we have proposed that a low-threshold transient Ca^{2+} current ($I_{\text{Ca(T)}}$) of fusion-competent myoblasts might contribute to an inward flux of Ca^{2+} ions (Liu *et al.*, 1998) and thereby play a role in myoblast fusion, a process essential to skeletal muscle development and repair. For this reason, we examined the effect of mibefradil on myoblast fusion, expecting it to be reduced if $I_{\text{Ca(T)}}$ was involved. We found that mibefradil markedly reduces myoblast fusion. However, we also found that, in addition to inhibiting $I_{\text{Ca(T)}}$, mibefradil very efficiently blocks several types of voltage-gated K^{+} channels, as shall be described here.

Methods

Dissociation and cell cultures

Samples of human skeletal muscle were obtained during corrective orthopaedic surgery of young patients (9 months to 17 years old) without any known neuromuscular disease in accordance with the guidelines of the ethical committee of the University Hospital of Geneva (written informed consent was

obtained from patients or their legal guardians). Myoblasts (Baroffio *et al.*, 1993) and fusion-competent myoblasts (Krause *et al.*, 1995) were prepared as described.

Electrophysiology

Whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used as in Bernheim *et al.* (1996). Leak current subtraction procedures are discussed in the Figure legends. (i) Potassium currents. Extracellular solution (mM): NMG-Cl (100), KCl (5), MgCl_2 (3), HEPES (5), NaOH (50), acetic acid (50), and glucose (8). The pH was adjusted to 7.3 with NMG. Intracellular (pipette) solution (mM): KCl (110), NaCl (5), MgCl_2 (1), HEPES (5), BAPTA (20), and glucose (5). The pH was adjusted to 7.3 with KOH. (ii) Currents through calcium channels. Extracellular solution (mM): BaCl_2 (10), TEA-Cl (90), tetrodotoxin (10 μM), NMG-Cl (50), KCl (5), MgCl_2 (2), HEPES (5), and glucose (8). The pH was adjusted to 7.3 with NMG. Intracellular (pipette) solution (mM): KCl (145), MgCl_2 (2), HEPES (10), BAPTA (1), Mg-ATP (3), and glucose (5). The pH was adjusted to 7.3 with KOH.

Fusion index

The fusion index is defined as the number of nuclei in myotubes divided by the total number of nuclei counted. Cultures were fixed 5 min with ice-cold 100% methanol, and stained with haematoxylin. Nuclei were counted in 20 randomly chosen microscope fields in two separate cultures. One microscope field usually contains between 100 and 150 nuclei. In *t*-tests, *n* refers to the number of microscope fields counted.

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Statistics

All data are expressed as means \pm s.e.mean.

Results

Effect of mibefradil on myoblast fusion

Human myoblasts can be maintained in an undifferentiated state for several weeks, as long as they are exposed to a growth medium (Baroffio *et al.*, 1993; Ham *et al.*, 1989). Alternatively, myoblast fusion can be rapidly induced when the proliferation medium is replaced with differentiation medium (StClair *et al.*, 1992), and maximum fusion is usually reached within 48 h.

In preliminary experiments, we examined the effect on fusion of a concentration of mibefradil known to block a large fraction of $I_{\text{Ca(T)}}$ ($5 \mu\text{M}$; Clozel *et al.*, 1997). Figure 1 illustrates the dramatic effect $5 \mu\text{M}$ mibefradil has on myoblast fusion. In Figure 1A, it can be seen that after 30 h of exposure to the differentiation medium containing the drug, the fusion index was reduced by 43% with respect to control. Eight hours later, when fusion was nearly maximal in the control cultures (Baroffio *et al.*, 1996), the fusion index in sister cultures exposed to mibefradil was still at the same low level and was reduced by 57% with respect to control. The photomicrographs in Figure 1B illustrate the difference between a control culture (top) and its sister culture exposed to mibefradil (bottom). In the presence of the drug, although the cells look perfectly healthy, there are very few multinucleated myotubes, with the vast majority of the cells remaining as mononucleated myoblasts.

Effect of mibefradil on Ca^{2+} currents

The sensitivity of voltage-gated Ca^{2+} channels to mibefradil was examined in fusion-competent myoblasts. Fusion-competent

myoblasts are obtained by plating myoblasts at a very low density in differentiation medium. Under these conditions, the cells are induced to differentiate and thus begin to express Ca^{2+} and K^+ currents (Bernheim *et al.*, 1996; Liu *et al.*, 1998), but they are prevented from fusing due to lack of neighbouring cells (Krause *et al.*, 1995). In the differentiation medium, not all myoblasts necessarily express the same currents at a given time. This is due to the fact that (1) only about 70% of the myoblasts are going to fuse (Baroffio *et al.*, 1993), (2) cells are not strictly synchronized, and (3) the currents appear in a sequential order with some currents being probably expressed just before fusion. Regarding Ca^{2+} currents, Figure 2A (inset) shows the inward current activated by a depolarization in a myoblast expressing exclusively $I_{\text{Ca(T)}}$. This current was recorded in the presence of 10 mM Ba^{2+} in the extracellular solution. Also, in order to eliminate any contribution of a voltage-gated sodium current (Hamann *et al.*, 1994), tetrodotoxin ($10 \mu\text{M}$) was added to the bath solution which contained no sodium ions. It can be seen that the inward current activates rapidly and then quickly inactivates within a few tens of ms. The addition of mibefradil ($2 \mu\text{M}$) markedly decreased the inward current (the effect was reversible; data not shown). Figure 2A shows the current-voltage relationship of $I_{\text{Ca(T)}}$, together with that recorded in the presence of $2 \mu\text{M}$ mibefradil. The current suppressed by mibefradil activates at potentials more depolarized than -60 mV . This current is also blocked efficiently by amiloride and Ni^{2+} (data not shown). These data all consistently indicate that the inward current is $I_{\text{Ca(T)}}$.

In Figure 2B, similar data are shown for a myoblast expressing $I_{\text{Ca(L)}}$ in addition to $I_{\text{Ca(T)}}$. Inset, it can be seen that the same concentration of mibefradil ($2 \mu\text{M}$) was less efficient in blocking $I_{\text{Ca(L)}}$ than $I_{\text{Ca(T)}}$. The addition of $3 \mu\text{M}$ nifedipine, a blocker of $I_{\text{Ca(L)}}$, markedly reduced the current remaining in the presence of mibefradil, indicating that this steady inward current was indeed mainly $I_{\text{Ca(L)}}$. Comparison of the current-voltage relationship of Figure 2B in the absence and in the

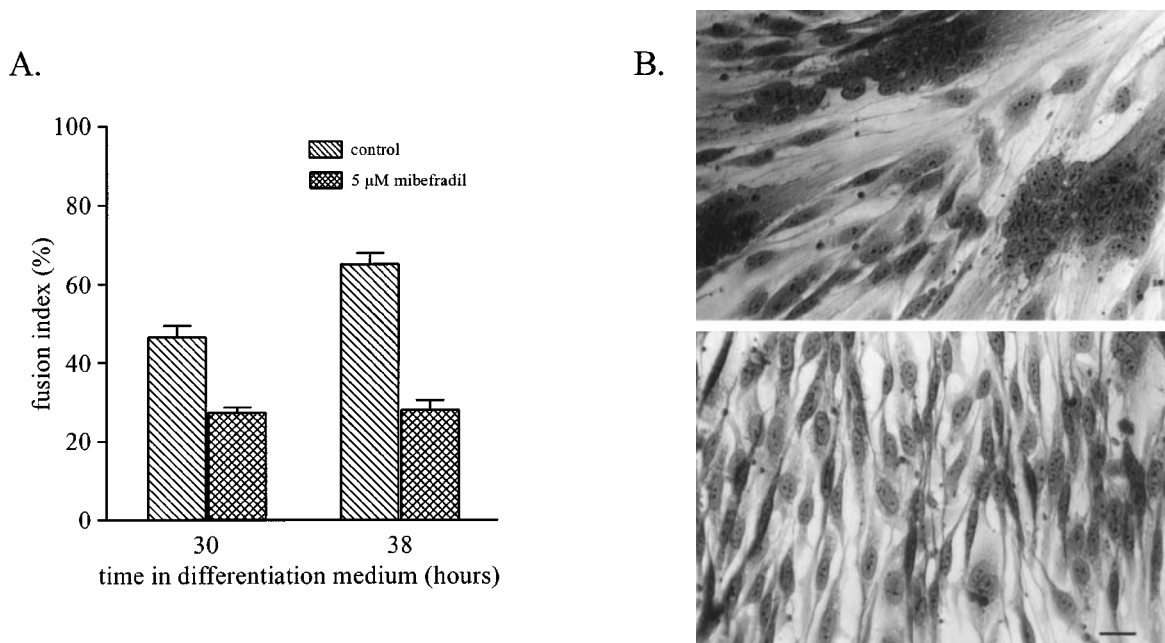


Figure 1 Mibefradil reduces myoblast fusion in primary culture. (A) Myoblast fusion was induced by exposing myoblasts to differentiation medium and the fusion index was evaluated after 30 and 38 h, in control conditions and in sister cultures exposed to $5 \mu\text{M}$ mibefradil. Mibefradil decreased myoblast fusion significantly ($P < 0.0001$) at the two time points evaluated. (B) Upper photograph: culture fixed after 38 h in differentiation medium (control condition) and stained with haematoxylin. Lower photograph: sister culture after 38 h in differentiation medium containing $5 \mu\text{M}$ mibefradil. Scale bar is $25 \mu\text{m}$.

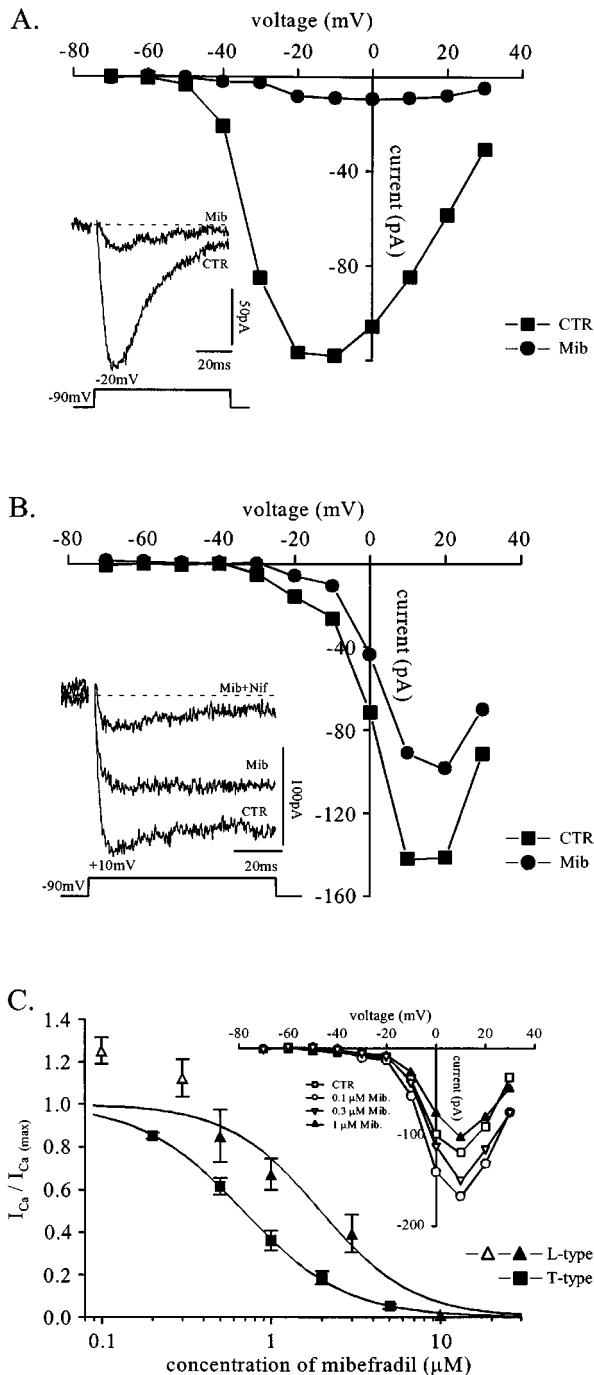


Figure 2 Effect of mibefradil on Ca^{2+} currents of fusion-competent myoblasts. (A) Recording from a fusion-competent myoblast (membrane capacitance 42 pF) exposed 3 days to differentiation medium. The cell, superfused with a solution containing 10 mM Ba^{2+} (see Methods), was held at -90 mV and stepped for 80 ms to a series of potentials between -70 and $+30$ mV. Leak current was estimated by adding 1 mM cadmium at the end of the experiment and was subtracted. Peak Ba^{2+} currents (current through T-type Ca^{2+} channels) were plotted as a function of the voltage during a step in the absence and in the presence of $2 \mu\text{M}$ mibefradil. Inset: Ba^{2+} current recorded during a step to -20 mV with and without $2 \mu\text{M}$ mibefradil. (B) Fusion-competent myoblast (membrane capacitance 10 pF) exposed 3 days to differentiation medium. Steady-state Ba^{2+} currents (current through mainly L-type Ca^{2+} channels) measured at the end of a 80 ms step and plotted as a function of the voltage during the step, in the absence and in the presence of $2 \mu\text{M}$ mibefradil. Leak current was estimated as in (A) and subtracted. Inset: Ba^{2+} current recorded during a step to $+10$ mV in control, in $2 \mu\text{M}$ mibefradil, and $3 \mu\text{M}$ nifedipine in addition to mibefradil. (C) Peak Ba^{2+} currents (current through T-type Ca^{2+} channels) recorded during a step to -20 mV and normalized to their amplitude

presence of mibefradil with that of Figure 2A confirms that $I_{\text{Ca(L)}}$ is less efficiently inhibited by mibefradil than $I_{\text{Ca(T)}}$ at all voltages.

Figure 2C illustrates the degree of inhibition of $I_{\text{Ca(T)}}$ and $I_{\text{Ca(L)}}$ at different mibefradil concentrations. The IC_{50} of mibefradil for $I_{\text{Ca(T)}}$ is $0.7 \mu\text{M}$. For $I_{\text{Ca(L)}}$, as few fusion-competent myoblasts express this current, the inhibition by mibefradil was tested both on fusion-competent myoblasts ($n=3$) and in freshly formed myotubes ($n=5$). The two sets of data were pooled as there was no difference between them. Interestingly, the effect of mibefradil on $I_{\text{Ca(L)}}$ of fusion-competent myoblasts and freshly-formed myotubes is bimodal. At $0.1 \mu\text{M}$, there is an increase in the amplitude of the inward current by 25% with respect to the control level. At higher concentrations, however, inhibition takes place. The IC_{50} for $I_{\text{Ca(L)}}$, taking into account exclusively the filled triangles, is near $2 \mu\text{M}$.

Figure 2C indicates that the concentration of mibefradil ($5 \mu\text{M}$) used to inhibit fusion in Figure 1 would have blocked 95% of $I_{\text{Ca(T)}}$ and 80% of $I_{\text{Ca(L)}}$. As we observed previously that $I_{\text{Ca(L)}}$ is not required for fusion of primary cultures of human myoblasts (Bernheim *et al.*, 1996), the effect of mibefradil on myoblast fusion would suggest that a functional $I_{\text{Ca(T)}}$ is important in the fusion process. This, however, only applies if the effect of mibefradil is restricted to Ca^{2+} currents. Indeed, we recently demonstrated that a block of an inward rectifier K^+ current ($I_{\text{K(IR)}}$), which contributes to the final hyperpolarization of myoblasts to -65 mV, could nearly totally inhibit myoblast fusion (Liu *et al.*, 1998). Therefore, it was important to demonstrate that mibefradil is not affecting $I_{\text{K(IR)}}$, or any of the other K^+ currents, which also contribute to the hyperpolarization of myoblasts, i.e. $I_{\text{K(NI)}}$, a non-inactivating K^+ current (Bernheim *et al.*, 1996), which we know now is the human *ether-à-go-go* K^+ channel ($I_{\text{h-eag}}$, Bijlenga *et al.*, 1998; Occhiodoro *et al.*, 1998), and $I_{\text{K(DR)}}$, a delayed rectifier K^+ current (Widmer *et al.*, 1995).

Effect of mibefradil on K^+ currents

Figure 3A illustrates the effect of mibefradil on $I_{\text{K(DR)}}$. The cell represented had no $I_{\text{h-eag}}$, which occurs in only a small fraction of fusion-competent myoblasts (Bernheim *et al.*, 1996), so that $I_{\text{K(DR)}}$ could be studied in isolation. The inset shows the marked reduction of the outward current when the myoblast was exposed to $3 \mu\text{M}$ mibefradil. The blocking effect occurred at all voltages, as attested by the current-voltage relationship in Figure 3A.

In Figure 3B, it can be seen that mibefradil is also very efficient at inhibiting another outward K^+ current, $I_{\text{h-eag}}$. As $I_{\text{h-eag}}$ does not inactivate with time, the inset illustrates the steady current recorded at $+40$ mV in the absence and in the

measured in control solution are plotted as a function of mibefradil concentration. The line is a Hill equation ($\text{IC}_{50}=0.68 \mu\text{M}$, Hill coefficient=1.4, $n=5$). All recordings were from fusion-competent myoblasts cultured 3 days in differentiation medium. A similar experiment was performed with the current through L-type calcium channels. Normalized steady-state Ba^{2+} currents measured at the end of the 80 ms voltage step to $+10$ mV were plotted as a function of mibefradil concentration and fitted with a Hill equation ($\text{IC}_{50}=2 \mu\text{M}$, Hill coefficient=1.6, $n=4$ or more); the open triangles represent Ba^{2+} currents potentiated by low concentrations of mibefradil ($n=8$). Inset: Effect of low concentrations of mibefradil on current through L-type calcium channels. Steady state Ba^{2+} currents (leak subtracted) were measured at the end of the 80 ms step and plotted as a function of the voltage during the step for different mibefradil concentrations.

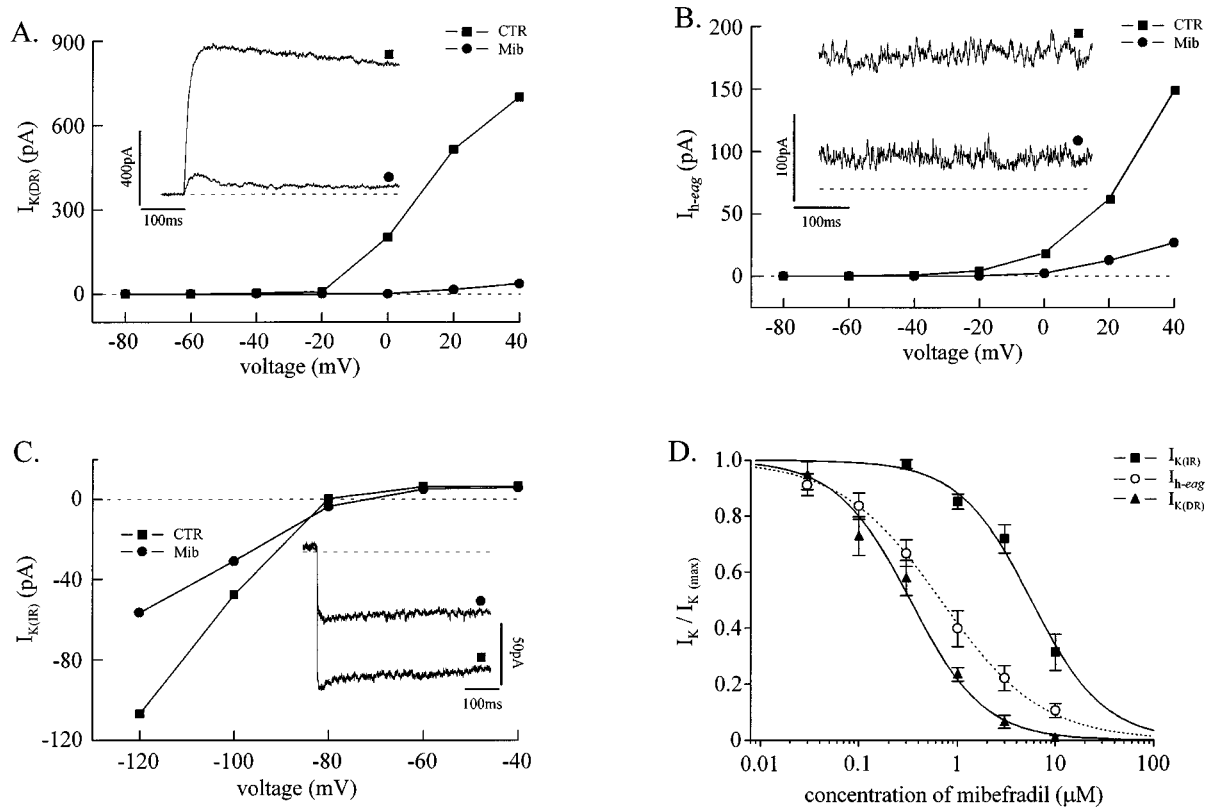


Figure 3 Mibefradil inhibits different types of K^+ currents of fusion-competent myoblasts. All experiments were done with fusion-competent myoblasts exposed to differentiation medium for 3 days. (A) A myoblast (membrane capacitance 18 pF) was held steadily at -80 mV and stepped for 500 ms to a series of voltages between -80 and $+40$ mV. The outward current obtained at the end of the step was plotted as a function of the voltage during a step in the absence and in the presence of $3 \mu\text{M}$ mibefradil. Leak current was obtained by linear extrapolation from the current-voltage relationship between -80 and -40 mV and subtracted. This cell had no I_{h-eag} , so that the outward current represents exclusively $I_{K(DR)}$. Currents recorded from the same cell (during a step to $+40$ mV) without and with $3 \mu\text{M}$ mibefradil are shown. (B) Another fusion-competent myoblast (membrane capacitance 17 pF) was held steadily at $+40$ mV for 3 min, and stepped for 500 ms to a series of voltages between -80 and $+40$ mV. Symbols represent the amplitude of I_{h-eag} recorded in the absence and in the presence of $3 \mu\text{M}$ mibefradil. Leak current was obtained as in (A) and subtracted. Steady-state currents at $+40$ mV in the absence and in the presence of $3 \mu\text{M}$ mibefradil are shown (same cell). The dotted line indicates the leak at $+40$ mV. (C) A fusion-competent myoblast (membrane capacitance 26 pF) expressing $I_{K(IR)}$ was held steadily at -60 mV and stepped to a series of voltages between -120 and -40 mV for 500 ms. Leak subtracted $I_{K(IR)}$ currents measured in the absence and in the presence of $10 \mu\text{M}$ mibefradil were plotted against the voltage during the step. Leak current was obtained by adding $500 \mu\text{M}$ Ba^{2+} to the bath solution. Inward K^+ currents recorded from the same cell at -120 mV in the absence and in the presence of $10 \mu\text{M}$ mibefradil are shown. (D) $I_{K(DR)}$, I_{h-eag} , and $I_{K(IR)}$ normalized to their respective control values are plotted as a function of mibefradil concentration. The three data sets were fitted with a Hill equation.

presence of $3 \mu\text{M}$ mibefradil. As for $I_{K(DR)}$, the block of I_{h-eag} by mibefradil occurred at all voltages.

At a higher concentration range, mibefradil can also block $I_{K(IR)}$. Figure 3C shows that $I_{K(IR)}$ is reduced by 50% by mibefradil at a concentration of $10 \mu\text{M}$. The complete dose-response curve for five cells is illustrated in Figure 3D. The IC_{50} of mibefradil for $I_{K(IR)}$ is $5.6 \mu\text{M}$.

Figure 3D also illustrates the effect of various mibefradil concentrations on $I_{K(DR)}$ and I_{h-eag} . The IC_{50} of mibefradil for $I_{K(DR)}$ and I_{h-eag} are 0.3 and $0.7 \mu\text{M}$, respectively, i.e. very similar to that of $I_{\text{Ca}(T)}$ in human fusion-competent myoblasts.

Discussion

This study on the effect of mibefradil on human myoblasts was initiated in order to evaluate the role of $I_{\text{Ca}(T)}$ in myoblast fusion. Unfortunately, despite its strong inhibitory effect on fusion, mibefradil did not allow us to assess the importance of $I_{\text{Ca}(T)}$ in the fusion process. However, an interesting observation emerged from this study: mibefradil inhibits several types of voltage-gated K^+ currents including an inward rectifier, and

is as efficient an inhibitor of the outward K^+ currents as of $I_{\text{Ca}(T)}$.

Selectivity of mibefradil on T -type Ca^{2+} current

In venous smooth muscle myocytes and heart cells, mibefradil has a selectivity for $I_{\text{Ca}(T)}$ over $I_{\text{Ca}(L)}$ in an excess of 10 (Ertel & Ertel, 1997; Mishra & Hermsmeyer, 1994). In neurons, however, the situation is different. In embryonic rat spinal cord motoneurons, all inward Ca^{2+} currents were blocked in the same concentration range with an IC_{50} of $1.4 \mu\text{M}$ (Viana *et al.*, 1997). In myoblasts, the IC_{50} of mibefradil for $I_{\text{Ca}(L)}$ is in the same range ($2 \mu\text{M}$) as the IC_{50} for rat motoneurons, although, the IC_{50} for $I_{\text{Ca}(T)}$ is three times smaller ($0.7 \mu\text{M}$). Hence, in skeletal muscle myoblasts, the selectivity of mibefradil for $I_{\text{Ca}(T)}$ is halfway between that of neurons and smooth muscle cells. It is possible that the differences in results on various cell types with respect to selectivity for $I_{\text{Ca}(T)}$ reflects the existence of different types of Ca_v channels in these cells. A Ca_v channel has recently been cloned ($\alpha 1\text{G}$; Perez-Reyes *et al.*, 1998), and it appears that there exists more than one type of Ca_v channel ($\alpha 1\text{H}$; Cribbs *et al.*, 1998). In any case, the

difference in mibefradil IC₅₀ for low- and high-voltage activated Ca²⁺ channels in skeletal muscle myoblasts is not sufficiently large to allow a very specific inhibition of I_{Ca(T)} versus I_{Ca(L)}.

Enhancement of I_{Ca(L)} by low concentrations of mibefradil

Low concentrations of mibefradil significantly and very reproducibly enhanced I_{Ca(L)} in human fusion-competent myoblasts. An increase of I_{Ca(L)} by low concentrations of mibefradil has also been described in Chinese hamster ovary (CHO) cells co-transfected with the smooth muscle L-type $\alpha 1_{Cb}$ subunit and an L-type β subunit. This enhancement of I_{Ca(L)} by mibefradil was, however, only observed in a subpopulation of transfected CHO cells, and was not present when CHO cells were transfected with the $\alpha 1_{Cb}$ subunit alone (Welling *et al.*, 1995). At higher concentrations, an inhibitory effect of mibefradil was, on the other hand, always observed. It has been hypothesized that this dual action could be caused by the existence of two separate binding sites for mibefradil (Welling *et al.*, 1995). We did not further explore the underlying mechanism but if this should occur in cell types that are the pharmacological target of this type of agent, an increase in Ca²⁺ influx through depolarized membranes containing Ca_v(L) channels could occur at certain mibefradil concentrations.

Effect of mibefradil on K⁺ currents

There are several indications that mibefradil may act on channels other than Ca²⁺ channels. For example, mibefradil inhibits Ca²⁺-activated Cl⁻ currents and volume-sensitive Cl⁻ currents in endothelial cells with an IC₅₀ in the range of 5 μ M (Nilius *et al.*, 1997). However, in these cells, mibefradil at concentrations up to 30 μ M did not affect an inwardly rectifying K⁺ channel (Kir1.0 or Kir4.0 families; Voets *et al.*, 1996; Nilius *et al.*, 1997), and we have not noticed any report of an effect of this drug on outward K⁺ currents. Therefore, it was unexpected to observe that, when compared to I_{Ca(T)},

mibefradil could block outward K⁺ currents with equal efficiency (I_{h-eag}) or even more efficiently (I_{K(DR)}). Interestingly, these two outward currents belong to different superfamilies, as I_{K(DR)} is a member of the *Shaker* superfamily, and I_{h-eag} is the human *ether-à-go-go* K⁺ channel (Bijlenga *et al.*, 1998; Occhiodoro *et al.*, 1998). If this effect of mibefradil on K⁺ channels occurs in other cell types, effects on action potential duration, resting membrane potential or even rhythmic activity should be expected. Regarding I_{K(IR)} in human myoblasts (Kir2.0 family; Liu *et al.*, 1998), the sensitivity to mibefradil is clearly present but is more comparable to that of I_{Ca(L)}, than to that of I_{Ca(T)} or of the outward K⁺ currents of the same preparation.

Effect of mibefradil on fusion

There is a marked effect of mibefradil on myoblast fusion. However, at the concentration used in the experiment illustrated in Figure 1, more than 90% of I_{Ca(T)}, I_{K(DR)} and I_{h-eag} and approximately 50% of I_{K(IR)} would have been blocked. Our experiences with caesium ions on myoblast fusion indicated that blocking 50% of I_{K(IR)} reduced fusion by approximately 25%. Therefore, the block of I_{K(IR)} by mibefradil cannot alone explain the 57% inhibition of fusion seen with 5 μ M mibefradil. At present, we cannot distinguish whether there is an isolated or combined contribution of I_{Ca(T)}, I_{K(DR)} or I_{h-eag} to the residual 32% inhibition in fusion by mibefradil. It is worth noting however that a high concentration of this drug interferes with a mechanism essential to muscle growth and repair.

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