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Effects of Ca^{2+} channel blockers on Ca^{2+} loading induced by metabolic inhibition and hyperkalemia in cardiomyocytes

Tianlai Tang [1], Rose Duffield, Andrew K.S. Ho *

Division of Clinical Pharmacology, Department of Biomedical and Therapeutic Sciences, University of Illinois College of Medicine at Peoria, One Illini Dr., Peoria, IL 61605, USA

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Abstract

The effects of the L-type (nifedipine and verapamil) and the T-type (mibefradil) Ca^{2+} channel blockers on the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by NaCN metabolic inhibition and hyperkalemia were examined in chicken cardiomyocytes using fluorescence imaging with Fura-2. NaCN induced a slow and sustained rise in $[Ca^{2+}]_i$, which was not affected by pretreating the cells for 5 min with nifedipine, verapamil, or mibefradil at 100 nM or 10 μM. Pretreatment of the cells with 10 μM nifedipine, verapamil, or mibefradil for 5 min remarkably inhibited the K^+-induced increase in $[Ca^{2+}]_i$. These inhibitory effects diminished after 48-h pretreatment with nifedipine or verapamil but not with mibefradil. Ryanodine also induces an increase in $[Ca^{2+}]_i$, and this effect was enhanced by 48-h pretreatment of the cells with 10 μM verapamil but not with 10 μM mibefradil. We conclude that the NaCN-induced increase in $[Ca^{2+}]_i$ is independent of the Ca^{2+} influx though the L-type or T-type Ca^{2+} channels. Chronic inhibition of the L-type Ca^{2+} channels but not T-type channels may enhance the ryanodine receptor-mediated Ca^{2+} release, which may be responsible for the development of tolerance to their inhibitory effects on K^+-induced increase in $[Ca^{2+}]_i$.

Keywords: Ca^{2+} channel blocker; Ca^{2+}, intracellular; Ryanodine; Cyanide; Hyperkalemia; Cardiomyocyte

1. Introduction

In order to achieve normal function of myocardium, the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in cardiomyocytes is well regulated, and it must be able to undergo rapid and precise increase and return to the original low resting level. Under certain pathological conditions, such as ischemia, hypoxia, or hyperkalemia, there is often a pronounced and sustained elevation of $[Ca^{2+}]_i$ which may result in various forms of cardiac injury. For example, in the early phase of ischemia, the rise of $[Ca^{2+}]_i$ may trigger ventricular arrhythmia. When the ischemic myocardium is reperfused during the reversible phase of ischemic injury, the rise of $[Ca^{2+}]_i$ may interfere with postischemic contractile function. Furthermore, a prolonged rise in $[Ca^{2+}]_i$ can trigger a cascade of intracellular enzyme activities which ultimately result in cell death (Katz and Reuter, 1979; Naylor, 1981). In addition, it has been proposed that the regional increase in the extracellular potassium concentration $[K^+]_o$ may also contribute to the increase in $[Ca^{2+}]_i$ in ischemic cardiac tissues. High $[K^+]_o$ state may occur in other clinical settings such as hyperkalemia and cardioplegia with high potassium solution used in cardiac surgery. The major risk of high $[K^+]_o$ is to trigger arrhythmias and ventricular dysfunction which is possibly related the increase in $[Ca^{2+}]_i$. Among the commonly used anti-ischemic agents, the L-type calcium channel blockers have been widely prescribed for their efficacy and paucity of side effects. Previous studies in several in vivo and in vitro models of myocardial ischemia have supported the notion that calcium channel blockers exert their cardioprotective effects (Chouariri et al., 1995; Hensley et al., 1997) through inhibition of Ca^{2+} influx by blocking the sarcolemmic calcium channels (Hashimoto et al., 1993; Tsukube et al., 1996; Tenthorey et al., 1998). However, little evidence is available to show that the calcium channel blockers inhibit the increase in $[Ca^{2+}]_i$ in energy-compromised cardiomyocytes. Furthermore, their anti-ischemic efficacy and safety have been questioned, especially since recent studies showed that the short-acting calcium channel blockers may actually lead to a higher risk of myocardial infarction and

* Corresponding author. Tel.: +1-309-671-8533; Fax: +1-309-671-8403

[1] Present address: Department of Psychiatry, Vanderbilt University, Nashville, TN, USA.

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mortality (Psaty et al., 1995; Furberg et al., 1995). Although this opinion is still under debate, medical practitioners tend to be more cautious in prescribing these drugs. Therefore, there is a need to find new anti-ischemic agents. Recently, a new category of calcium channel blockers, the T-type calcium channel blocker, has received much attention because of its potential anti-ischemic effect. A recently developed T-type calcium antagonist, mibefradil (Ro40-5967), has undergone several clinical trials and has been suggested to be more effective than the L-type calcium channel blockers in improving exercise tolerance and reducing ischemic episodes (Richard et al., 1995; Massie, 1997). To the best of our knowledge, the effect of mibefradil on the regulation in $[Ca^{2+}]_i$ in cardiomyocytes has not been studied. It is the objective of this study to examine the protective effects of mibefradil versus the conventional L-type calcium channel blockers against the increase in $[Ca^{2+}]_i$ induced by metabolic inhibition and hyperkalemia.

2. Materials and methods

2.1. Culture of chicken cardiomyocytes

Fertile white Leghorn eggs (supplied by SPAFAS, Roanoke, IL) were incubated at high humidity at 38.5°C. On day 14 of incubation, the eggs were opened, the embryos sacrificed and the hearts immediately removed and placed in warm Medium 199. The ventricular tissues were dissected, washed free of blood and clots, then placed in 2 ml of Medium 199 containing 0.125% trypsin. The cleansed ventricular tissues were minced in this medium into pieces of 1–2 mm diameter. The pieces were incubated in trypsin solution for 20 min at 37°C. Cells were separated by agitation, the chunks allowed to settle out of suspension, and the cardiomyocyte rich supernate removed for plating. One to 2×10^5 cells per ml were plated onto collagen-coated, glass bottomed, 33 mm culture dishes at a volume of 1 ml per dish. Cells were cultured in Medium 199 containing 5% fetal calf serum at 37.5°C with 5% CO_2. After 3 days of culture, the cardiomyocytes were easily identified by their characteristic spindle shape, and in some cases they exhibited spontaneous beating. Cells were maintained in culture for 3–6 days before performing Ca^{2+} determinations.

2.2. Measurement of intracellular calcium

$[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fura-2-AM (Molecular Probes, Eugene, OR). Cells grown in 33 mm diameter, glass bottom culture dishes were washed 4 times with clear Medium 199, loaded with 5 μM Fura-2 in clear medium 199 for 1 h after which the free fura-2 was then removed. The cells were washed 4 times to remove any free fura-2, and returned to the incubator for 30 min to 1 h. During the experiment, cells were treated gently by adding each agent in 10 μl aliquot to the dish. The fluorescent signal from preselected individual cells adherent to the glass bottom of the temperature regulated (37.5°C) culture dish was measured with a microscope-based spectrofluorometer (Intracellular Imaging, Cincinnati, OH) with dual excitation at 340 and 380 nm. The calcium concentration was calibrated before each experiment with an intracellular calcium imaging calibration kit (Molecular Probes, Eugene, OR).

2.3. Chemicals

Mibefradil was a gift from Hoffman LaRoche Bioscience. Nifedipine, verapamil, ryanodine, medium 199, and trypsin were obtained from Sigma. Fura-2-AM and intracellular calcium imaging calibration kit were obtained from Molecular Probes (Eugene, OR).

2.4. Statistical analysis

All of the data are represented as mean ± S.E.M. The statistical method was Student's *t* test to compare the increase in $[Ca^{2+}]_i$ in the presence of nifedipine, verapamil, and mibefradil to the control group.

3. Results

3.1. Effects of calcium channel blockers on cyanide-induced increases in $[Ca^{2+}]_i$

The results obtained showed that the resting $[Ca^{2+}]_i$ in cultured chicken cardiomyocytes was 82 ± 4 nM (total $n = 167$, from 24 separate experiments). To produce a state of metabolic hypoxia, we added 4 mM NaCN to the cell culture to block ATP synthesis from oxidative phosphorylation. We also added 20 mM 2-deoxyglucose to block anaerobic glycolysis. After adding CN, $[Ca^{2+}]_i$ began to increase after about 10 min and gradually reached the peak level of 248 ± 5 nM within 40 min and maintained around that level until the end of recording. To test the effect of calcium channel blockers on the CN-induced increase in $[Ca^{2+}]_i$, we added 100 nM and 10 μM mibefradil, nifedipine, or verapamil to the cell culture at 5 min before adding CN. Fig. 1 shows the examples of averaged tracings for the time course of the CN-induced increase in $[Ca^{2+}]_i$ in presence of 10 μM of mibefradil, nifedipine, or verapamil versus control (in absence of calcium channel blocker). It appears that 10 μM of mibefradil, nifedipine, or verapamil failed to alter the time course of CN-induced increase in $[Ca^{2+}]_i$, as indicated by no apparent shift of the time–response curves. In control and the calcium channel blocker-treated cells, we measured the peak of the CN-induced increase in $[Ca^{2+}]_i$, as

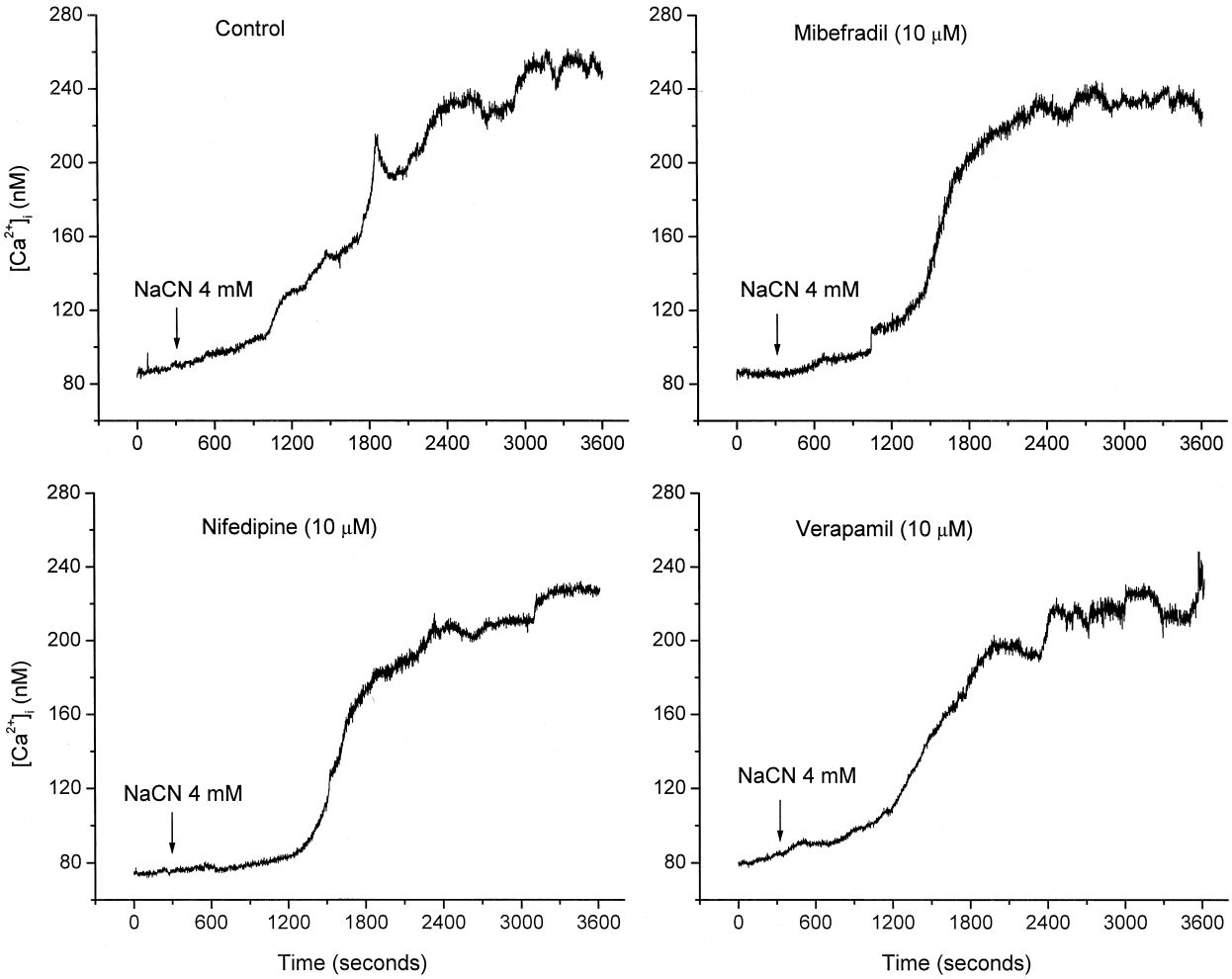

Fig. 1. Tracing of CN-induced increase in $[Ca^{2+}]_i$ in absence of calcium channel blocker (control) or in presence of 10 μM mibefradil, nifedipine, or verapamil. Each calcium channel blocker was added into the culture dish at the beginning of each recording. 4 mM NaCN was then added into the dish at 5 min later as shown by the arrow. The change of $[Ca^{2+}]_i$ was recorded for up to one h. The tracing represents the mean from four to six independent experiments. For each experiment, 8 to 15 cells were selected.

well as the time to reach the half of the magnitude of $[Ca^{2+}]_i$ ($t_{1/2}$) as an estimate of the rate of $[Ca^{2+}]_i$ increase. The results were shown in Table 1. At concentration of 100 nM or 10 μM, nifedipine, verapamil, and mibefradil failed to result in significant alterations of the peak level of the CN-induced increase in $[Ca^{2+}]_i$, as well as the $t_{1/2}$ value. These findings indicate that those calcium channel blockers have little effects on the CN-induced increase in $[Ca^{2+}]_i$.

3.2. Effects of calcium channel blockers on KCl-induced increase in $[Ca^{2+}]_i$

In normal cardiomyocytes, high K^+ is known to depolarize the cell membrane which is accompanied by Ca^{2+} influx through voltage sensitive Ca^{2+} channels, leading to an increase in $[Ca^{2+}]_i$. To examine the effects of the calcium channel blockers on the K^+-induced increase in $[Ca^{2+}]_i$, we tested the actions of acute (5 min) versus

Table 1
Effects of mibefradil, nifedipien, and verapamil (100 nM and 10 μM) on CN-induced increase in $[Ca^{2+}]_i$

Ca^{2+} channel blockers	Basal $[Ca^{2+}]_i$ (nM)	Peak $[Ca^{2+}]_i$ (nM)	$T_{1/2}$ (Min.)
Control	82±4	246±7	29±1
Nifedipine 100 nM	87±7	240±21	29±4
10 μM	79±5	236±12	27±2
Verapamil 100 nM	81±6	230±17	30±3
10 μM	80±5	233±12	28±3
Mibefradil 100 nM	90±8	211±18	28±4
10 μM	85±4	240±16	26±2

Each agent was added in two concentrations (100 nM and 10 μM) into the culture dish at one min after the beginning of each recording. Four millimolar NaCN was then added into the dish at 4 min thereafter. In control group, no calcium channel blocker was added. For each experiment, change in $[Ca^{2+}]_i$ was recorded for up to 1 h. Basal $[Ca^{2+}]_i$ was measured before adding each calcium channel blocker. Peak $[Ca^{2+}]_i$ was measured at the highest level of $[Ca^{2+}]_i$ after adding CN. $T_{1/2}$ represents the time to reach the half of the magnitude of $[Ca^{2+}]_i$ increase (Peak $[Ca^{2+}]_i$–Basal $[Ca^{2+}]_i$). Each value of $[Ca^{2+}]_i$ and $T_{1/2}$ represents the Mean±S.E.M. from four to six independent experiments.

chronic (48 h) treatment of cardiomyocytes with 10 μM nifedipine, verapamil, or mibefradil. KCl induces an abrupt increase in $[Ca^{2+}]_i$ in a dose-dependent manner. As shown in Fig. 2, acute treatment with 10 μM nifedipine had pronounced inhibition of the K^+-induced increase in $[Ca^{2+}]_i$ except at 85 mM of added K^+. Chronic treatment with 10 μM nifedipine resulted in diminished rise in $[Ca^{2+}]_i$ stimulated by 45 mM and 65 mM added K^+. The effect of verapamil is shown in Fig. 3. Acute treatment with 10 μM verapamil produced strong inhibition of the K^+-induced increase in $[Ca^{2+}]_i$ for all KCl concentrations tested. These inhibitions were significantly reduced at all concentrations of added K^+ in the cells pretreated with verapamil for 48 h. Interestingly, the action of mibefradil was different from nifedipine and verapamil. As shown in Fig. 4, both acute and chronic treatment with 10 μM mibefradil exerted remarkably similar inhibition of the K^+-induced increase in $[Ca^{2+}]_i$ at all concentrations, indicating a lack of desensitization for mibefradil following chronic treatment.

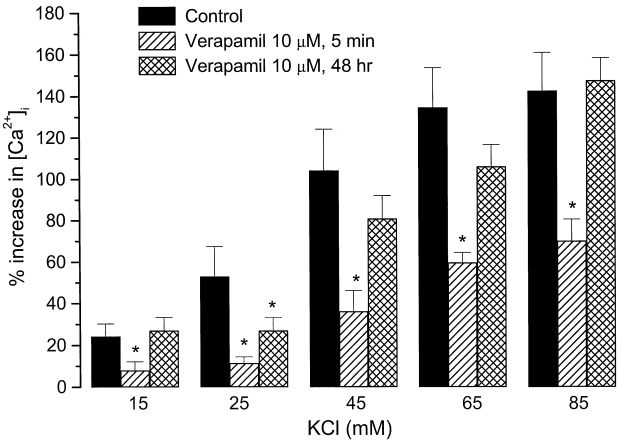

Fig. 3. Increases in $[Ca^{2+}]_i$ induced by 15, 25, 45, 65, and 85 mM KCl in untreated cells (control) or cells treated with 10 μM verapamil for 5 min or 48 h. For acute treatment, 10 μM verapamil was added into cell culture at the beginning of each recording and followed by adding KCl at about 5 min subsequently. For chronic treatment, the cells were continuously incubated with 10 μM verapamil for 48 h before exposure to KCl. Bars represent the means ± S.E.M. of four to six independent experiments. * Significant differences from control (in absence of nifedipine) $[Ca^{2+}]_i$ (* $P < 0.05$).

3.3. Chronic effects of calcium channel blockers on ryanodine-induced increase in $[Ca^{2+}]_i$

Nifedipine and verapamil have been thought to exert their effects through the dihydropyridine receptor which may have direct interaction with the sarcoplasmic reticulum ryanodine receptor in regulation of intracellular Ca^{2+} release (Rios and Pizarro, 1991; Rios et al., 1993). In order to explore the potential compensation pathway that might be involved in the desensitization of the inhibitory actions of nifedipine and verapamil after chronic treatment, we tested the effects of verapamil and mibefradil on the ryanodine-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 5, ryanodine at concentrations of 1 μM, 10 μM, or 100 μM increased $[Ca^{2+}]_i$ by 2%, 7%, and 30%, respectively. Acute treatment with 10 μM verapamil shows 67% inhibition of the 100 μM ryanodine-induced increase in $[Ca^{2+}]_i$, whereas chronic treatment with 10 μM verapamil en-

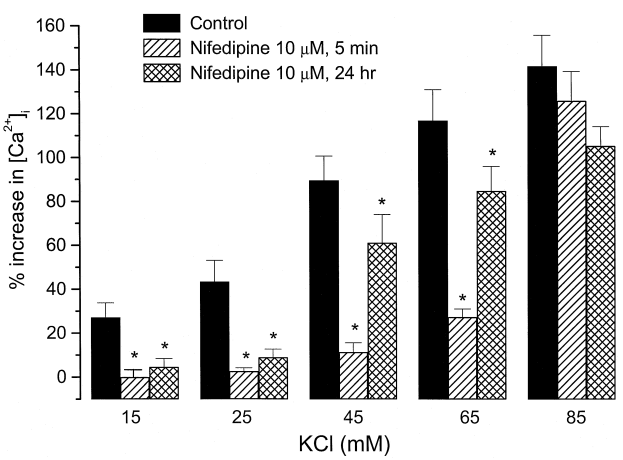

Fig. 2. Increases in $[Ca^{2+}]_i$ induced by 15, 25, 45, 65, and 85 mM KCl in untreated cells (control) or cells treated with 10 μM nifedipine for 5 min or 48 h. For acute treatment, 10 μM nifedipine was added into cell culture at the beginning of each recording and followed by adding KCl at about 5 min subsequently. For chronic treatment, the cells were continuously incubated with 10 μM nifedipine for 48 h before exposure to KCl. Bars represent the means ± S.E.M. of four to six independent experiments. * Significant differences from control (in absence of nifedipine) $[Ca^{2+}]_i$ (* $P < 0.05$).

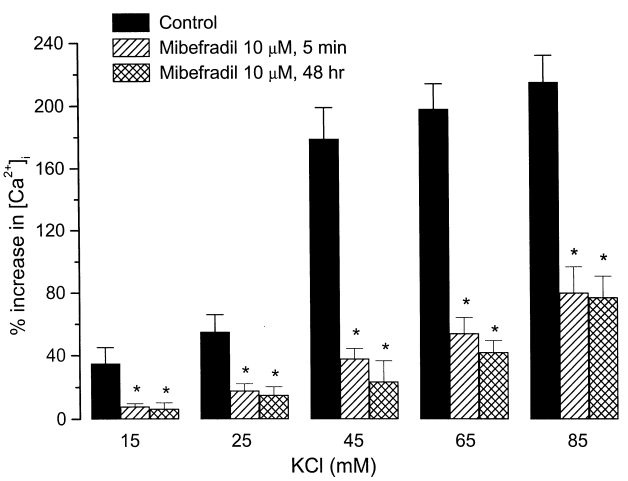

Fig. 4. Increases in $[Ca^{2+}]_i$ induced by 15, 25,45, 65, and 85 nM KCI in untreated cells (control) or cells treated with 10 μM mibefradil for 5 min or 48 h. For acute treatment, 10 μM mibefradil was added into cell culture at the beginning of each recording and followed by adding KCI at about 5 min subsequently. For chronic treatment, the cells were continuously incubated with 10 μM mibefradil for 48 h before exposure to KCI. Bars represent the means ± S.E.M. of four to six independent experiments. * Significant differences from control (in absence of nifedipine) $[Ca^{2+}]_i$ (* $P < 0.05$).

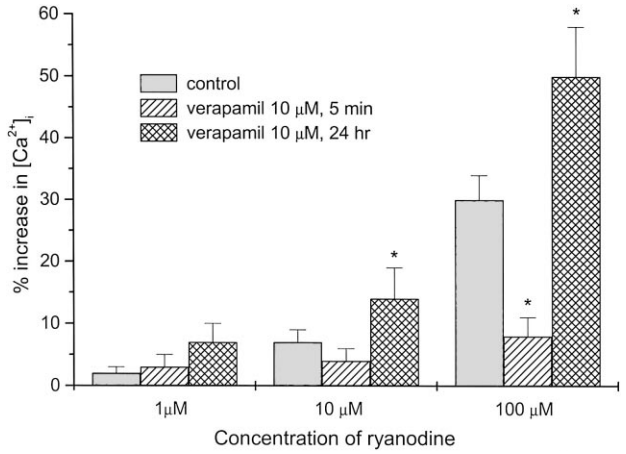

Fig. 5. Increases in $[Ca^{2+}]_i$ induced by 1, 10, 100 μM ryanodine in untreated cells (control) or cells treated with 10 μM verapamil for 5 min or 48 h. For acute treatment, 10 μM verapamil was added into cell culture at the beginning of each recording and followed by adding ryanodine at about 5 min subsequently. For chronic treatment, the cells were continuously incubated with 10 μM verapamil for 48 h before exposure to ryanodine. Bars represent the means ± S.E.M. of four to six independent experiments. * Significant differences from control (in absence of verapamil) $[Ca^{2+}]_i$ ($* P < 0.05$).

hanced the actions of 10 μM and 100 μM ryanodine, in which the $[Ca^{2+}]_i$ were increased by 14% and 50%, respectively. In contrast, acute treatment with 10 μM mibefradil showed no significant effect on ryanodine-induced increase in $[Ca^{2+}]_i$, whereas chronic treatment with 10 μM mibefradil inhibited the 100 μM ryanodine-induced increase in $[Ca^{2+}]_i$ by 28% (Fig. 6). These findings suggest that the ryanodine-induced calcium release is inhibited by acute action of verapamil and enhanced by chronic action of verapamil. However, ryanodine-induced calcium release is unaffected by acute treatment with mibefradil but inhibited by chronic treatment with mibefradil.

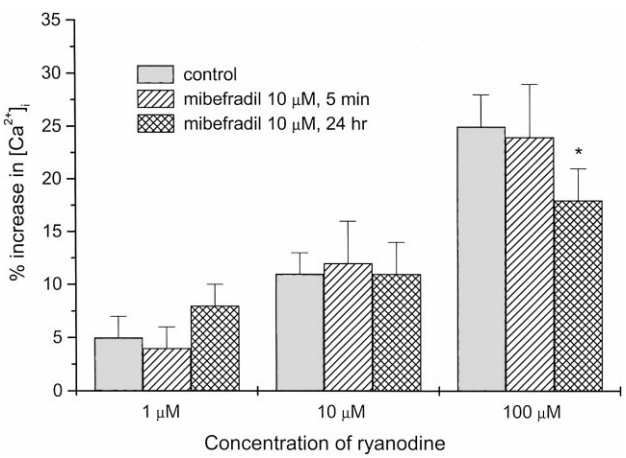

Fig. 6. Increases in $[Ca^{2+}]_i$ induced by 1, 10, 100 μM ryanodine in untreated cells (control) or cells treated with 10 μM mibefradil for 5 min or 48 h. For acute treatment, 10 μM mibefradil was added into cell culture at the beginning of each recording and followed by adding ryanodine at about 4 min subsequently. For chronic treatment, the cells were continuously incubated with 10 μM mibefradil for 48 h before exposure to ryanodine. Bars represent the means ± S.E.M. of four to six independent experiments. * Significant differences from control (in absence of mibeframil) $[Ca^{2+}]_i$ ($* P < 0.05$).

4. Discussion

It has been shown that under energy distress, several mechanisms might contribute to Ca^{2+} overloading. These include the perturbations of multiple Ca^{2+} regulators, such as the sarcolemmal Ca^{2+} channels (Shattock and Bers, 1989), the Na^+–Ca^{2+} exchanger (Kiang and Smallridge, 1994; Jiang et al., 1994), the ryanodine receptor-coupled Ca^{2+} channels in sarcoplasmic Ca^{2+} stores (Feher et al., 1989; Tani et al., 1996; Zucchi and Ronca-Testoni, 1997), and the sarcoplasmic reticulum (SR) Ca^{2+} pumps (Tani, 1990; Steenbergen et al., 1997). In our study, CN induced a slow and sustained increase in $[Ca^{2+}]_i$ which was similar to that observed with the SR Ca^{2+} pump inhibitor, thapsigargin (Vigne et al., 1992; Metz et al., 1994). It is likely that the inhibition of the SR Ca^{2+} pump due to insufficient intracellular ATP is a major mechanism responsible for the CN-induced increase in $[Ca^{2+}]_i$. Our results clearly demonstrate that nifedipine, verapamil, and mibefradil failed to influence the CN-induced increase in $[Ca^{2+}]_i$, indicating that the Ca^{2+} influx through the L-type or T-type calcium channels may not primarily contribute to the CN-induced increase in $[Ca^{2+}]_i$. Furthermore, our results do not support the commonly held notion that the calcium channel blockers exert their cardioprotective effect by inhibiting the Ca^{2+} influx through L-type or T-type calcium channels.

Another possible mechanism responsible for the increase in $[Ca^{2+}]_i$ during ischemia and metabolic inhibition may involve the elevation of the $[K^+]_o$, which may result from the accumulation of K^+ in the extracellular space due to cellular K^+ loss or impaired microcirculation (Wilde et al., 1990). There is evidence that high K^+ concentration present in cardioplegic solution induces an increase in $[Ca^{2+}]_i$ (Cyran et al., 1992; Lopez et al., 1996). Although the underlying mechanism is not totally understood, it has been proposed that high K^+ can depolarize the cell membrane which is accompanied by Ca^{2+} influx through voltage-sensitive calcium channels, leading to an overall increase in $[Ca^{2+}]_i$ (Cyran et al., 1992; Lopez et al., 1996). As the intracellular Ca^{2+} plays a pivotal role in the cardiac excitation–contraction coupling, abnormal increase in $[Ca^{2+}]_i$ may cause arrhythmia and ventricle dysfunction. In this regard, calcium channel blockers should have potential protective effect against high $[K^+]_o$-induced myocardial dysfunction. Paradoxically, the traditional L-type calcium channel blockers have little therapeutic value for cardioprotection in hyperkalemia or hyperkalemic cardio-

plegia. Our study demonstrates that acute treatment of the cells with both nifedipine and verapamil produced a pronounced inhibition of the K^+-induced increase in $[Ca^{2+}]_i$. However, these inhibitory effects were remarkably desensitized when the cells were exposed to nifedipine and verapamil for 48 h. We speculate that an intracellular compensation pathway, such as enhancing the Ca^{2+} release from Ca^{2+} stores, may contribute to the desensitization following chronic treatment. There is ample evidence supporting the concept of direct interaction between the dihydropyridine receptor and the ryanodine receptors. In particular, sarcolemmal depolarization is thought to produce a conformational change in the dihydropyridine receptor that is transmitted to the ryanodine receptor and induces the release of Ca^{2+} from the SR (Rios and Pizarro, 1991; Rios et al., 1993; Schneider, 1994). Our results suggest that acute blocking of the dihydropyridine receptor by verapamil may suppress the ryanodine-induced Ca^{2+} release, whereas chronic blocking of the dihydropyridine receptor may enhance the ryanodine receptor function, possibly by up-regulation of the ryanodine receptor or increasing its sensitivity. It is possible that the development of tolerance to nifedipine and verapamil is due to activation of the compensation pathway such as promoting Ca^{2+} release through ryanodine receptor-coupled Ca^{2+} channels. It would be interesting to know weather this tolerance is a generalized phenomenon or just limited to the inhibitory effect on K^+-induced increase in $[Ca^{2+}]_i$. Further study has been planned to test this desensitization in other cellular injury models. In contrast to nifedipine and verapamil, the T-type calcium channel blocker, mibefradil, had different action following chronic treatment. Although acute treatment with mibefradil also inhibited K^+-induced increase in $[Ca^{2+}]_i$, desensitization of this action was not observed after chronic treatment for 48 h. In addition, chronic treatment with mibefradil did not enhance the action of ryanodine. Instead, it inhibited the 100 μM ryanodine-induced increase in $[Ca^{2+}]_i$. In summary, the results obtained suggest that chronic inhibition of the L-type calcium channel but not T-type channels may enhance the ryanodine receptor-mediated Ca^{2+} release, which may be responsible for the development of tolerance to the inhibitory effects on K^+-induced increase in $[Ca^{2+}]_i$ by nifedipine and verapamil. The observation that mibefradil failed to develop tolerance suggests that it may have potential therapeutic value in protecting myocardium against hyperkalemic injury.

Acknowledgements

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