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Protection of cardiomyocytes by pinacidil during metabolic inhibition and hyperkalemia

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Abstract

The objective of this study is to understand the mechanism underlying the cardioprotective effects of pinacidil, an ATP-sensitive K^+ channel (K_{ATP}) opener. We examined the effects of 10 μM pinacidil in cultured chicken cardiomyocytes. Pinacidil caused a concentration-dependent delay in metabolic inhibition-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and creatine phosphokinase release, and this action was antagonized by glyburide, a K_{ATP} blocker. Neither verapamil, an L-type Ca^{2+} channel blocker, nor bepridil, a Na^+–Ca^{2+} exchange inhibitor, affected the time course of increase in $[Ca^{2+}]_i$ induced by metabolic inhibition. Pinacidil did not have an effect on the amplitude of K^+-induced increase in $[Ca^{2+}]_i$, but accelerated the rate of decline following peak stimulation. In contrast, glyburide reduced the amplitude of K^+-induced increase in $[Ca^{2+}]_i$ and prolonged the rate of decline. These results provide direct evidence that pinacidil protects cardiomyocytes from metabolic inhibition-induced injury by cyanide (CN) through a delay in the onset of increase in $[Ca^{2+}]_i$, rather than by inhibition of the L-type Ca^{2+}-channels or by alteration of Na^+–Ca^{2+} exchange.

Keywords: Cardioprotection; Pinacidil; $[Ca^{2+}]_i$ transient; K_{ATP} channel opener; Cyanide; Hyperkalemia; Cardiomyocyte

1. Introduction

The ATP-sensitive K^+ (K_{ATP}) channels play an important role in the regulation of cellular response to myocardial distress such as ischemia and cardioplegic arrest. Under normal conditions, the physiologic level of ATP inhibits K_{ATP} channels. The probability of channel opening is increased when ATP levels fall below a critical concentration. Thus, activation of these channels in the heart may provide endogenous cardioprotection under conditions in which cardiac metabolism is compromised, such as myocardial ischemia (Beuckelmann et al., 1993; Wickenden et al., 1998). The selective K_{ATP} channel openers have been the focus of much recent attention due to their potential cardioprotective effects of improved postischemic recovery of contractile function, delayed onset of ischemic contraction, and reduced infarct size in several cardiac ischemia models in vitro (Galinanes et al., 1992; Cavaro et al., 1995; Hearse, 1995: Lawton et al., 1996). However, much less is known about the mechanism underlying these effects. It was initially suggested that a direct cardioprotective effect for K_{ATP} openers is due to the shortening of the action potential duration and subsequent reduction in Ca^{2+} entry and cardiac work (Cole et al., 1991); yet later evidence showed that the protective effects of K_{ATP} are independent of action potential duration shortening (Grover et al., 1995a,b). It is generally agreed that the abnormal rise in $[Ca^{2+}]_i$ induced by oxygen deprivation and by the local rise in extracellular K^+ is a common pathological process during ischemia and cardioplegic arrest (Cheung et al., 1986; Opie and Du Tit, 1992). Recently, we have reported that the metabolic inhibition-induced increase in $[Ca^{2+}]_i$ is independent of Ca^{2+} influx through the L-type or T-type Ca^{2+} channels as evidenced by the fact that verapamil and nifedipine (L-type Ca^{2+} channel blockers) and mibefradil (T-type Ca^{2+} channel blocker) failed to affect $[Ca^{2+}]_i$ transient in the cardiomyocytes. However, the K^+-induced $[Ca^{2+}]_i$ transient was significantly reduced by both L- and T-type Ca^{2+}-channel blockers (Tang et al., 1998). Previous studies have shown that K_{ATP} channel

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openers prevented the intracellular Ca^{2+} loading induced by ischemia (Behling and Malone, 1995) and by hyperkalemic challenge (Lopez et al., 1996a,b). However, it was not shown whether prevention of the increase in $[Ca^{2+}]_i$ would result in protection of cellular injury in those models. In order to answer this question, we tested the effects of pinacidil, a recently developed K_{ATP} channel opening drug (Arena and Kass, 1989), on the time course of the metabolic inhibition-induced increase in $[Ca^{2+}]_i$ and creatine phosphokinase release simultaneously. We also examined the effect of pinacidil on K^+-induced increase on $[Ca^{2+}]_i$ and discussed the potential action of pinacidil to facilitate Ca^{2+} homeostasis.

2. Materials and methods

2.1. Materials

Glyburide, verapamil, bepridil, creatine phosphokinase assay kit, culture media and supplements, trypsin were obtained from Sigma (St. Louis, MO, USA). Pinacidil was a gift from Eli Lilly (Indianapolis, IN, USA). Fura-2, AM, the product name for (5-Oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-(acetyloxy)methyl ester) and Fura-2, AM calibration kit were obtained from Molecular Probes (Eugene, OR, USA). 33-mm glass culture plates with heating electrodes were obtained from Bioptechs (Butler, PA, USA).

2.2. 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 (Sigma). The ventricular tissues were dissected, washed free of blood and clots and placed in 2 ml of Medium 199 containing 0.125% trypsin. The cleansed ventricular tissues were minced into pieces of 1–2 mm diameter and incubated in the 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. Using densities in the range of 1 to 2×10^5 cells per ml, the cells were plated onto collagen-coated, glass bottom, 33-mm culture dishes at a volume of 1 ml per dish. Cells were cultured in Medium 199 supplemented with 5% fetal calf serum at 37°C with 5% CO_2. After 3 days in culture, the cardiomyocytes were easily identified by their characteristic spindle shape and in some cases exhibited spontaneous beating. Cells were maintained in culture for 3–6 days before performing $[Ca^{2+}]_i$ determinations.

2.3. Measurement of intracellular Ca^{2+}

The $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fura-2, AM as described by Tang et al. (1998). Cells grown in 33-mm glass bottom culture dishes were washed four times with clear Medium 199 (Sigma), loaded with 5 μM Fura-2, AM in clear Medium 199 for 1 h. The cells were washed four times with clear Medium 199 to remove any free Fura-2, AM, and returned to the incubator for 30 min to 1 h. During the experiments, cells were maintained at 37.5°C by a thermostat controlled heated stage (Bioptechs). 10 μl aliquots of each compound to be tested were added to the dish at appropriate intervals during the experiment. The fluorescent signal from individual cells adherent to the glass bottom of the culture dish was measured with a microscope-based spectrofluorometer (Intracellular Imaging, Cincinnati, OH) with dual excitation at 340 and 380 nm. The Ca^{2+} concentration was calibrated before each experiment with an intracellular Ca^{2+} imaging calibration kit (Molecular Probes).

2.4. Metabolic inhibition by cyanide

4 mM NaCN was added to the cell culture to block ATP synthesis from oxidative phosphorylation and produce metabolic hypoxia. 20 mM 2-deoxyglucose was added to block anaerobic glycolysis that might interfere with the experiments. Continuous tracings of $[Ca^{2+}]_i$ for individual cells in culture were made over a period of 60 min after the addition of NaCN. $T_{1/2}$ was calculated as the time, after addition of NaCN, for the $[Ca^{2+}]_i$ to reach 50% of the maximum level achieved in response to added NaCN.

2.5. Creatine phosphokinase assay

The extent of metabolic inhibition-induced injury to ventricular myocytes was quantitatively determined by the amount of creatine phosphokinase released into the medium. Samples were collected at time 0, 20, 40, and 60 min following addition of 4 mM NaCN by taking 10 μl aliquots from the total 1 ml in the culture dish. Creatine phosphokinase activity was measured by using an assay kit (Sigma) and a UV-spectrophotometer with a wavelength of 340 nM. According to the Sigma protocol, the following formula was used to determine creatine phosphokinase activity: creatine phosphokinase activity (Units/ml) = $\Delta A/\text{min} \times \text{TV}/6.22 \times \text{LP} \times \text{SV}$, where $\Delta A/\text{min}$ is change in absorbance per minute at 340 nm; TV is total volume (ml); SV is sample volume (ml); 6.22 is millimolar absorptivity of NADH at 340; LP is light path (1 cm).

2.6. Statistical analysis

All of the data are represented as mean ± S.E.M. Student's *t*-test was used to compare the increase in $[Ca^{2+}]_i$

and creatine phosphokinase release in the presence of pinacidil or glyburide to the control groups.

3. Results

3.1. Effects of pinacidil and glyburide on metabolic inhibition-induced increases in $[Ca^{2+}]_i$ and creatine phosphokinase release

The basal, unstimulated level of $[Ca^{2+}]_i$ in cultured chicken cardiomyocytes was determined to be 84 ± 3 nM ($n = 286$ cells, 36 experiments). Following CN treatment, $[Ca^{2+}]_i$ began to increase after about 10 min and gradually reached a plateau level of 240–250 nM within 40 min, which was sustained until the end of recording. At the end of the experiments, when $[Ca^{2+}]_i$ had reached the plateau level, average mean $[Ca^{2+}]_i$ was 246 ± 7 nM, 234 ± 13 nM, 238 ± 12 nM, and 211 ± 15 nM, respectively, for control and 0.1 μM, 1.0 μM and 10 μM pinacidil. $T_{1/2}$, half the time required for $[Ca^{2+}]_i$ to reach a CN stimulated plateau, was determined for control and pinacidil-treated cardiomyocytes. Pinacidil alone showed no effect on the resting $[Ca^{2+}]_i$. However, pinacidil treatment produced a concentration-dependent delay in the metabolic inhibition-induced increase in $[Ca^{2+}]_i$ as evidenced by the findings that at the low concentration of pinacidil (0.1 μM), the rate of onset and maximal response were similar to the controls, whereas at higher concentration the rate of onset and rapid rise in $[Ca^{2+}]_i$ were significantly ($P < 0.01$) delayed. The $T_{1/2}$ for 10 μM pinacidil was almost twice as long (55 min) as for controls (29 min). To elucidate whether the delay of the $[Ca^{2+}]_i$ increase is associated with the activation of K_{ATP} channels, glyburide, a selective K_{ATP} channel blocker (Brady et al., 1996), was added to the incubation mixture to evaluate its blocking effect on pinacidil. Glyburide (10 μM) alone had no significant effect on the resting $[Ca^{2+}]_i$, but it shortened the time of onset and rapid rise in $[Ca^{2+}]_i$ after NaCN to a $T_{1/2}$ of 22 min (Fig. 1). Adding glyburide (10 μM) along with pinacidil (10 μM) reversed the pinacidil action to a $T_{1/2}$ of 32 min. These results suggest that the activation of the

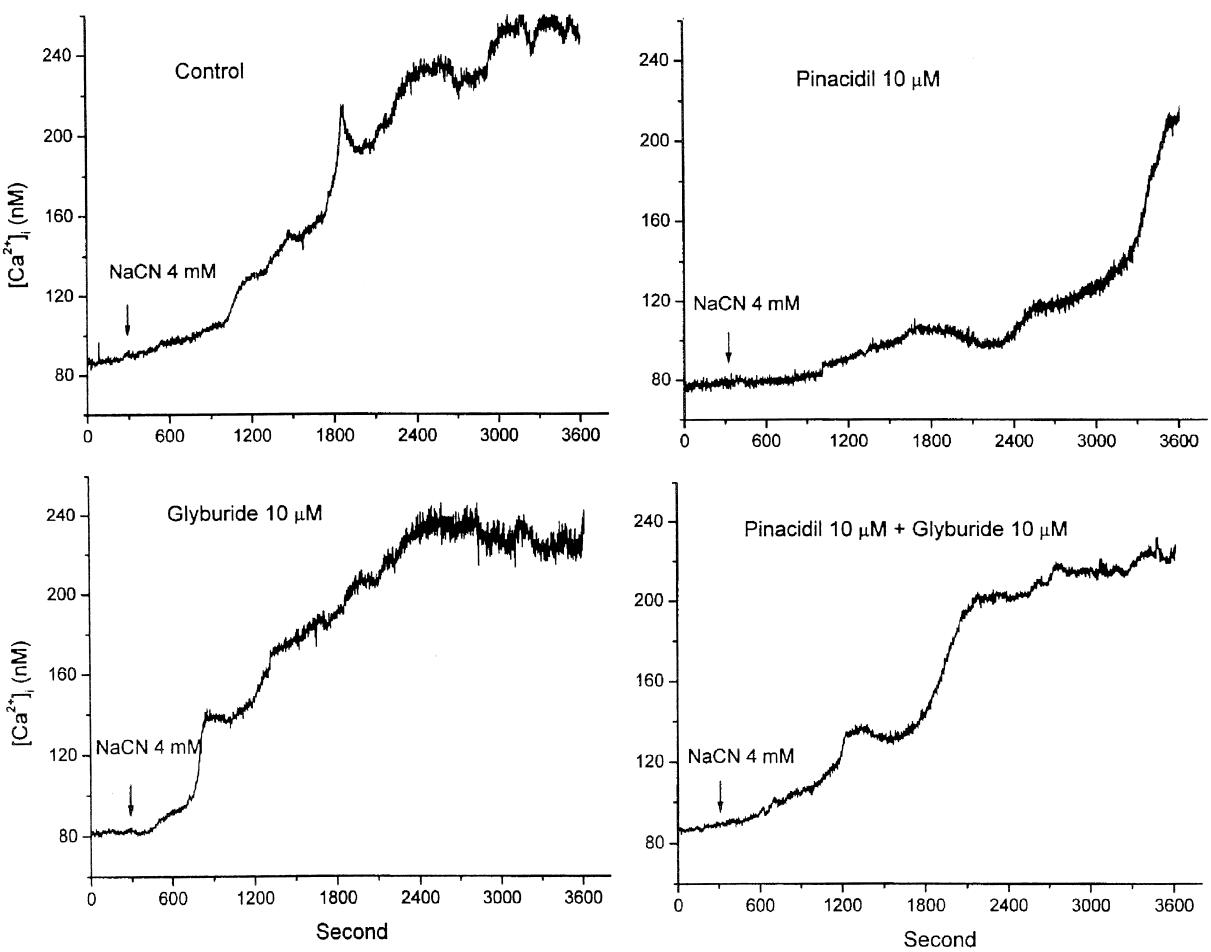

Fig. 1. Glyburide (10 μM) antagonism of pinacidil (10 μM) action on the time course of metabolic inhibition-induced increase in $[Ca^{2+}]_i$. Pinacidil, glyburide, or a combination was added into the culture dish at 1 min after the beginning of each recording. 4 mM NaCN was added 4 min later, as shown by the arrow. The experiments were recorded for up to 1 h. Each $[Ca^{2+}]_i$ tracing represents the mean from four to six independent experiments.

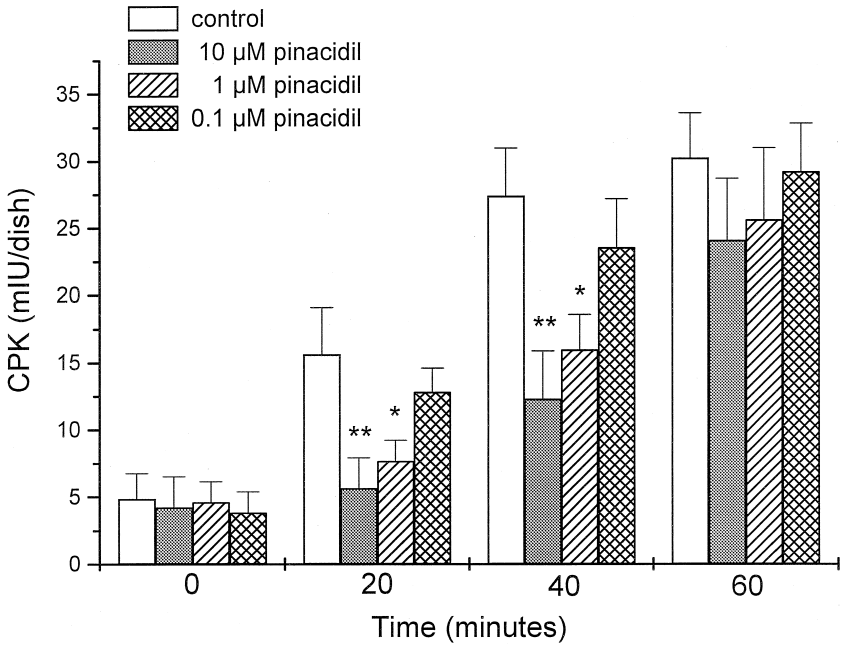

Fig. 2. Effects of pinacidil (0.1, 1.0, and 10 μM) on metabolic inhibition-induced increase in creatine phosphokinase release at 0, 20, 40, and 60 min after adding 4 mM NaCN. Pinacidil was added at 1 min after the beginning of recording, followed by NaCN 4 min later. Values are ± S.E.M. for four to six independent experiments. * Significant differences from control (in absence of pinacidil) level of creatine phosphokinase (* $P < 0.05$; ** $P < 0.01$).

K_{ATP} channel is responsible for the pinacidil effect of decreasing the rate of increase in $[Ca^{2+}]_i$ induced by metabolic inhibition.

To further evaluate the protective effect of pinacidil against cell injury induced by metabolic inhibition, we tested the level of creatine phosphokinase released into the culture medium during NaCN exposure. Pinacidil (1 μM and 10 μM) significantly reduced the creatine phosphokinase release at 20 and 40 min following CN treatment (Fig. 2). However, pinacidil at all concentrations failed to influence the creatine phosphokinase release at the end of the experiment. This pattern of pinacidil action on creatine

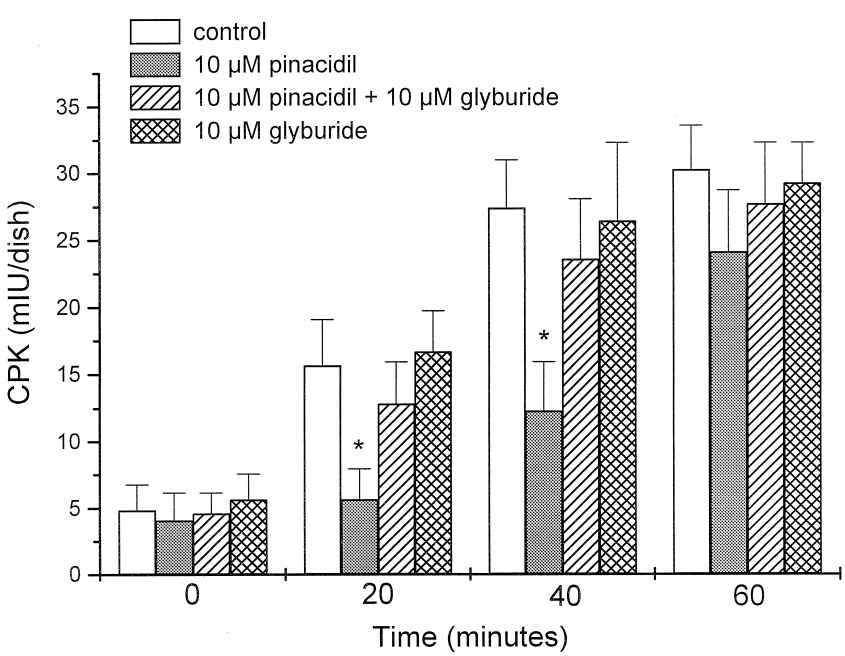

Fig. 3. Glyburide (10 μM) antagonism of pinacidil (10 μM) action on metabolic inhibition-induced increase in creatine phosphokinase release measured at 0, 20, 40, and 60 min after addition of 4 mM NaCN. Pinacidil, glyburide, or a combination was added at 1 min, and NaCN was added 4 min later. * Significant ($P < 0.05$) difference from control creatine phosphokinase.

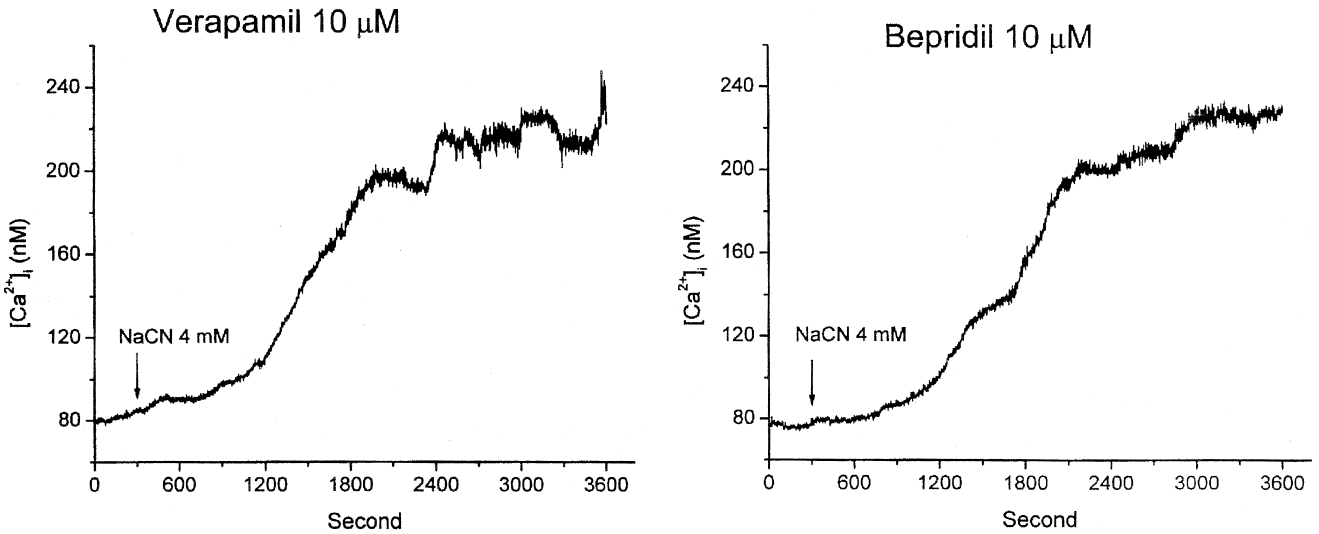

Fig. 4. Effects of verapamil and bepridil on metabolic inhibition-induced increase in $[Ca^{2+}]_i$. Verapamil (10 μM) or bepridil (10 μM) was added into the culture dish at 1 min after the beginning of each recording. 4 mM NaCN was added 4 min later, as shown by the arrow. Each tracing represents the mean from four to six independent experiments.

phosphokinase release appears to be in parallel with the time course of its effect on the increase in $[Ca^{2+}]_i$ induced by metabolic inhibition. Furthermore, glyburide (10 μM) alone had no effect on the metabolic inhibition-induced increase in creatine phosphokinase release, but reversed the inhibitory effect of pinacidil (Fig. 3).

To test whether the L-type Ca^{2+} channels and the Na^+–Ca^{2+} exchange system are involved in Ca^{2+} overload during metabolic inhibition by NaCN, we examined the effects of the L-type Ca^{2+} channel blocker, verapamil, and the Na^+–Ca^{2+} exchange inhibitor, bepridil (Labrid et al., 1979; Schwartz et al., 1985) on the increase in $[Ca^{2+}]_i$ induced by metabolic inhibition. Neither verapamil (10 μM) nor bepridil (10 μM) effectively alters the time course for metabolic inhibition-induced increase in $[Ca^{2+}]_i$, with $T_{1/2}$ of 28 min and 31 min, respectively (Fig. 4). Preliminary data also indicated that verapamil treatment had no effect on the course of creatine phosphokinase release (data not shown). These results suggest that neither L-type Ca^{2+} channels nor Na^+–Ca^{2+} exchange are involved in the increase in $[Ca^{2+}]_i$ induced by metabolic inhibition.

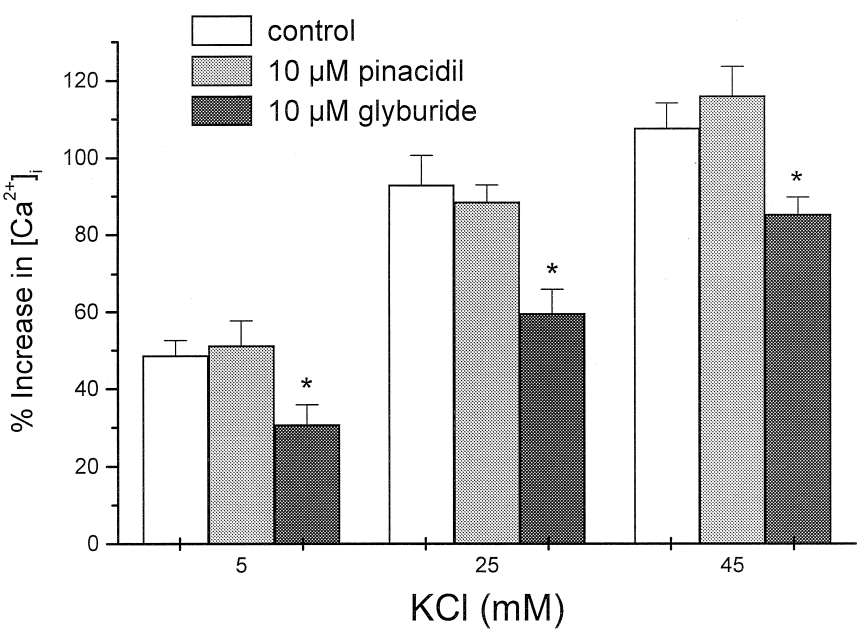

Fig. 5. Increases in $[Ca^{2+}]_i$ induced by 15, 25, and 45 mM KCl in untreated (control) cells or cells treated with 10 μM pinacidil or glyburide. Pinacidil or glyburide was added at 1.5 min and KCl was added 5 min later. Bars represent the means ± S.E.M. of four to six independent experiments. Significant differences from control ($^*P < 0.05$).

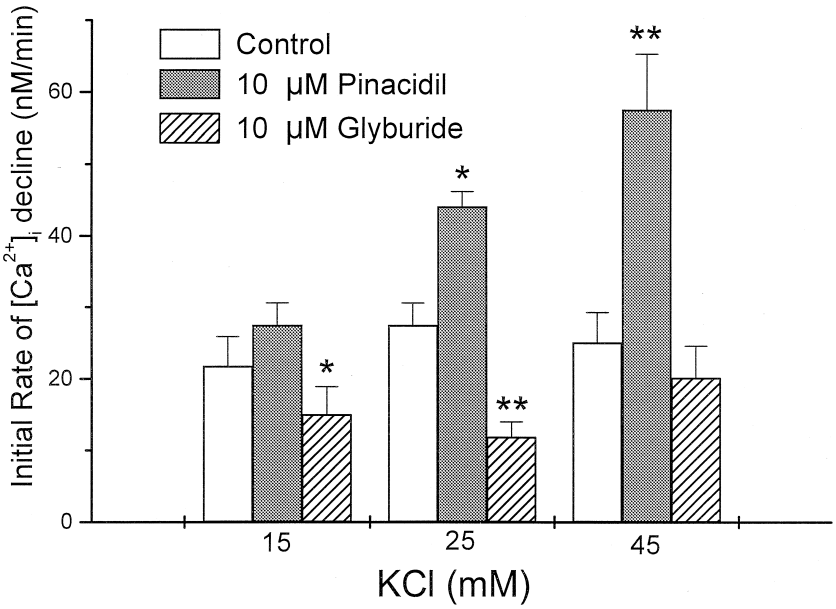

Fig. 6. Effects of 10 μM pinacidil and glyburide on the initial rate of decline (in the first 1–3 min) after peak $[Ca^{2+}]_i$ stimulation by 15, 25, and 45 mM KCl. Pinacidil or glyburide was added at 1.5 min and KCl was added 5 min later. * Significant differences from control (* $P < 0.05$; ** $P < 0.01$).

3.2. Effects of pinacidil and glyburide on KCl-induced increase in $[Ca^{2+}]_i$

The effects of pinacidil and its antagonist, glyburide, on the KCl-induced increase in $[Ca^{2+}]_i$ were examined. Pinacidil (10 μM) did not significantly affect the peak level of $[Ca^{2+}]_i$ induced by 15, 25, and 45 mM KCl, while glyburide (10 μM) suppressed the peak level of KCl-induced increase in $[Ca^{2+}]_i$ (Fig. 5). Pinacidil and glyburide also had different influences on the rate of decline of $[Ca^{2+}]_i$ following the initial peak level. In control cells, KCl elicits an instant surge in $[Ca^{2+}]_i$ followed by a gradual recovery to a level slightly higher than the initial resting $[Ca^{2+}]_i$. Both pinacidil and glyburide (10 μM) failed to alter the rate of the initial rise in $[Ca^{2+}]_i$ but exerted different effects during the recovery phase. We estimated the relative rates of decline by measuring the change in $[Ca^{2+}]_i$ induced by varying concentrations of KCl from the time point of peak $[Ca^{2+}]_i$ and the slope of the decline curve at their respective steady state levels over a fixed period (1–3 min). As shown in Fig. 6, pinacidil accelerated the rate of $[Ca^{2+}]_i$ decline at 25 and 45 mM KCl. The effect was especially prominent in response to 45 mM KCl. In contrast, glyburide significantly decreased the rate of $[Ca^{2+}]_i$ decline in response to 15 and 25 mM KCl.

4. Discussion

Myocardial functions are highly dependent upon mitochondrial oxidative phosphorylation to generate ATP which regulates the opening of K_{ATP} channels. Under physiologic conditions, K_{ATP} channels are closed in the presence of adequate levels of ATP and opened when ATP falls. Therefore, impairment of the mechanism of ATP production, which occurs not only with CN treatment, but also during ischemia, can result in metabolic cellular injury, and this process is thought to involve $[Ca^{2+}]_i$. The finding that CN, an inhibitor of oxidative phosphorylation, induces opening of ATP-regulated channels in single mammalian heart cells was first reported by Noma (1983), and this model has since been used to simulate an ischemic condition in cardiomyocytes (Lopez et al., 1996a; Koyama et al., 1996). It has been suggested that Ca^{2+} loading during myocardial injury can be the consequence of either perturbation of the sarcolemmal Ca^{2+} transport systems (e.g., Ca^{2+} channels and Na^+–Ca^{2+} exchanger) leading to an increase in Ca^{2+} influx or uncontrolled release and sequestration of Ca^{2+} by the sarcoplasmic reticulum and mitochondrial Ca^{2+} pools (Tani, 1990; Steenbergen et al., 1997). In our study, metabolic inhibition induced a slow and sustained increase in $[Ca^{2+}]_i$, which is evidenced by the loss of the ability of the cardiomyocytes to regulate Ca^{2+} homeostasis, i.e., to maintain the low free $[Ca^{2+}]_i$ of 10^{-7} M against the millimolar extracellular Ca^{2+}. It has been suggested that ATP generated by sarcoplasmic reticulum-associated glycolytic enzymes may be involved in the support of cellular Ca^{2+} homeostasis by driving the Ca^{2+} pump (Xu et al., 1995). The use of 2-deoxyglucose to inhibit glycolysis may play a role in inhibiting the sarcoplasmic reticulum and sarcolemmal Ca^{2+} pumps which may explain, at least in part, the prolongation of $[Ca^{2+}]_i$ by pinacidil. A similar pattern of increase in $[Ca^{2+}]_i$ was also observed in rat atrial cells treated with the sarcoplasmic reticulum Ca^{2+} pump inhibitor, thapsigargin (Vigne et al., 1992; Metz et al., 1994). Therefore, it is

likely that the inhibition of the sarcoplasmic reticulum Ca^{2+} pump due to insufficient intracellular ATP is a major mechanism responsible for the metabolic inhibition-induced increase in $[Ca^{2+}]_i$. Under our experimental conditions, the metabolic inhibition-induced increase in $[Ca^{2+}]_i$ reached a peak level of approximately 250 nM which may seem modest in view of the previous observation that an increase in $[Ca^{2+}]_i$ of 1 μM or greater may be required to cause fatal injury of the cells in other animal species (Fletcher et al., 1992). Nevertheless, data obtained showed that the time course of increase in $[Ca^{2+}]_i$ paralleled well with the time course of cellular injury as estimated by creatine phosphokinase release. These results suggest that in chicken cardiomyocytes, a sustained increase in $[Ca^{2+}]_i$ may lead to cellular injury even at 25% of the reported lethal concentration in other species.

Cardiac ischemia is known to be accompanied by a rapid accumulation of extracellular K^+ ($[K^+]_e$), a major cause of cardiac arrhythmias (Weiss and Shine, 1982; Watanabe et al., 1987). It is well known that K^+-induced membrane depolarization is followed by an influx of Ca^{2+}. To test whether a K_{ATP} opener has cardioprotective effect, we used pinacidil, a drug proven effective as an anti-hypertensive in several clinical trials (Friedel and Brodgen, 1990; Vigne et al., 1992). Pinacidil has also been shown to protect the heart against experimental ischemia in terms of improved coronary blood flow upon reperfusion and cell survival after ischemia (Lawton et al., 1996; Critz et al., 1997). It is generally assumed that cardioprotection by pinacidil during acute ischemia is attributable to K^+ efflux via the ATP-sensitive K^+ channel. Glyburide and tolbutamide have been shown to reduce the rise of $[K^+]_e$ (Kantor et al., 1990; Wilde et al., 1990; Venkatesh et al., 1991). In our study, pinacidil was shown to be effective in delaying the onset of the metabolic inhibition-induced increases in $[Ca^{2+}]_i$ and creatine phosphokinase release. During the earlier period of exposure to NaCN (before 40 min), pinacidil also exhibited inhibition of the increases in $[Ca^{2+}]_i$ and creatine phosphokinase release, but these inhibitory effects were abolished at 60 min of NaCN exposure. The delay in the onset of the increase in $[Ca^{2+}]_i$ by pinacidil was reversed by glyburide which by itself also shortened $[Ca^{2+}]_i$ increase after metabolic inhibition. The fact that glyburide alone showed no effect on creatine phosphokinase release indicates that cellular damage was not directly induced by glyburide, but rather as the result of metabolic inhibition. It appears that the cardioprotection by pinacidil is a time-dependent action which may account for its effectiveness only during the early stage of cellular energy stress. The fact that verapamil and bepridil failed to inhibit or delay the increase in $[Ca^{2+}]_i$ induced by metabolic inhibition suggests that the L-type Ca^{2+} channel and the Na^+–Ca^{2+} exchange system may not be involved in the cardioprotective effects of pinacidil. These findings are in general agreement with our previous report that both L-type and T-type Ca^{2+} channel blockers failed to exert cardioprotection from Ca^{2+} loading induced by NaCN in cultured cardiomyocytes (Tang et al., 1998). Such characteristics of pinacidil appear to be different from other K_{ATP} openers, such as cromakalim and the cyanoguanidine derivative, BMS-180448, which have been shown to inhibit the increase in $[Ca^{2+}]_i$ during ischemia through a mechanism similar to that observed in rat heart treated with Ca^{2+} channel blockers (Grover et al., 1995b).

The pinacidil effect obtained on KCl-induced $[Ca^{2+}]_i$ transient is consistent with the earlier study of $[Ca^{2+}]_i$ in ventricular myocytes by using glycine, *N*-(2-((8-(bis(carboxymethyl) amino) -6-methoxy-2-quinolinyl) methoxy) -4-methylphenyl)-*N*-(carboxymethyl)-(QUIN2) as a Ca^{2+} indicator, showing that hyperkalemia elicited a $[Ca^{2+}]_i$ transient followed by gradual decline (Powell et al., 1984). It has been postulated that K^+ depolarization elicits an initial Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Influx of Ca^{2+} subsequently triggers the release of intracellular Ca^{2+} from various stores (Feher et al., 1989). Weir (1990) summarized the biochemical evidence in mammalian ventricle and supported the concept that the Ca^{2+}-ATPase of the sarcoplasmic reticulum may play a dominant role in determining the decline of the $[Ca^{2+}]_i$ once the release of Ca^{2+} is over. In addition, the overloaded $[Ca^{2+}]_i$ can be extruded out of the cells via membrane Ca^{2+} pump or Na^+–Ca^{2+} exchange mechanism. It should be noted that the added K^+ was maintained throughout the experiment. At the high concentration of 45 mM K^+, the K^+ equilibrium potential, as predicted by the Nernst equation, is expected to approach that of the membrane potential. Our data showed that pinacidil did not alter the amplitude of the $[Ca^{2+}]_i$ transient induced by KCl, since the K^+ channel opener is expected to have no net effects on the net K^+ flux. Nevertheless, the accelerated decline of $[Ca^{2+}]_i$ produced by pinacidil suggests that activation of K_{ATP} channels may facilitate Ca^{2+} homeostasis. Our data showed a concentration dependent effect by pinacidil on the accelerated rate of $[Ca^{2+}]_i$ decline after KCl stimulation. In the presence of pinacidil and 45 mM KCl, the duration of K_{ATP} channel opening might be prolonged during the recovery phase, thus delaying membrane repolarization which requires Na^+/K^+ exchange. We speculate that $[Ca^{2+}]_i$ efflux could be indirectly enhanced as a result. If this assumption is correct, it may also explain why glyburide significantly decreased the rate of $[Ca^{2+}]_i$ decline and prolonged the Ca^{2+} recovery phase after $[K^+]_e$ stimulation, since glyburide may exert its action by partially blocking the K^+ channels and thus reduce the effect of K^+-induced depolarization and the net K^+ flux during repolarization. The reduced amplitude of K^+-induced increase in $[Ca^{2+}]_i$ following glyburide is unexpected, since glyburide has not been reported to affect $[Ca^{2+}]_i$ in hyperkalemic challenged cells (Lopez et al., 1996b; Jovanovic et al., 1997), nor to influence the $[Ca^{2+}]_i$ in cells exposed to hypoxia or metabolic inhibition (Russ et al., 1996). It should be noted that other K_{ATP} openers

may have different actions during hyperkalemic treatment. For example, aprikalim and nicorandil have been shown to prevent 16 mM K^+, but not 32 mM K^+, from inducing an increase in $[Ca^{2+}]_i$ in cardiomyocytes (Lopez et al., 1996a), and this action has been used to explain myocardial protection by K_{ATP} openers in hyperkalemic cardioplegia (Lawton et al., 1996, Lopez et al., 1996a).

In summary, our findings provide direct evidence of the cardioprotective effect of pinacidil at the cellular level by showing that pinacidil delayed the onset of cellular injury induced by metabolic inhibition as indicated by Ca^{2+} overloading and creatine phosphokinase release. Facilitation of Ca^{2+} homeostasis as observed during hyperkalemic challenge may explain the cardioprotective effects of the pinacidil type of K_{ATP} openers. This action of pinacidil has potentially therapeutic benefit in terms of delaying the onset of cardiac injury during ischemia and thus prolonging the time for other therapeutic interventions to be made available. Regulation of $[Ca^{2+}]_i$ by K^+ appears to be basic and the effects may be similar across various animal species.

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References

Arena, J.P., Kass, R.S., 1989. Enhancement of potassium-sensitive current in heart cells by pinacidil: evidence for modulation of the ATP-sensitive potassium channel. Circ. Res. 65, 436–445.

Behling, R.W., Malone, H.J., 1995. K_{ATP} channel openers protect against increased cytosolic calcium during ischemia and reperfusion. J. Mol. Cell. Cardiol. 27 (9), 1809–1817.

Beuckelmann, D.J., Nabauer, M., Erdmann, E., 1993. Alterations of K^+ currents in isolated human ventricular myocytes from patients with terminal heart failure. Circ. Res. 75, 379–385.

Brady, P.A., Zhang, S., Lopez, J.R., Javanovic, A., Alekseev, A.E., Terzic, A., 1996. Dual effect of glyburide, an antagonist of K_{ATP} channels, on metabolic inhibition-induced Ca^{2+} loading in cardiomyocytes. Eur. J. Pharmacol. 308, 343–349.

Cavaro, I., Djellas, Y., Guillon, J.M., 1995. Ischemic myocardial cell protection conferred by the opening of ATP-sensitive potassium channels. Cardiovasc. Drugs Ther. 9 (suppl. 2), 245–255.

Cheung, J.V., Bonventre, J.V., Malis, C.D., Leaf, A., 1986. Calcium and ischemic injury. N. Engl. J. Med. 314, 1670–1676.

Cole, W.C., McPherson, C.D., Sontag, D., 1991. ATP-regulated K^+ channels protect the myocardium against ischemia/reperfusion damage. Circ. Res. 69 (3), 571–581.

Critz, S.D., Liu, G.S., Chujo, M., Downey, J.M., 1997. Pinacidil but not nicorandil opens ATP-sensitive K^+ channels and protects against simulated ischemia in rabbit myocytes. J. Mol. Cell. Cardiol. 29 (4), 1123–1130.

Feher, J.J., LeBolt, W.R., Manson, N.H., 1989. Differential effect of global ischemia on the ryanodine-sensitive and ryanodine-insensitive calcium uptake of cardiac sarcoplasmic reticulum. Circ. Res. 65, 1400–1408.

Fletcher, A.E., Battersby, C., Adnitt, P., Underwood, N., Jurgensen, H.J., Bulpitt, C.J., 1992. Quality of life on antihypertensive therapy: a double-blind trial comparing quality of life on pinacidil and nifedipine in combination with thiazide diuretic. J. Cardiovasc. Pharmacol. 20, 108–114.

Friedel, H.A., Brodgen, R.N., 1990. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in the treatment of hypertension. Drugs 39, 929–967.

Galinanes, M., Shatock, M.M., Hearse, D.J., 1992. Effects of potassium channel modulation during global ischemia in isolated rat heart with and without cardioplegia. Cardiovasc. Res. 26, 1063–1068.

Grover, G.J., D'Alonza, A.J., Parham, C.S., Barbensio, R.B., 1995a. Cardioprotection with the K_{ATP} opener cromakalim is not correlated with ischemic myocardial action potential duration. J. Cardiovasc. Pharmacol. 26, 145–152.

Grover, G.J., D'Alonza, A.J., Hess, T., Sleph, P.G., Darbensiio, R.B., 1995b. Glyburide-reversible cardioprotective effect of BMS-180448 is independent of action potential shortening. Cardiovasc. Res. 30, 731–738.

Hearse, D.J., 1995. Activation of ATP-sensitive potassium channels: a novel pharmacological approach to myocardial protection?. Cardiovasc. Res. 30, 1–17.

Jovanovic, A., Alekseev, A.E., Lopez, J.R., Shen, W.K., Terzic, A., 1997. Adenosine prevents hyperkalemia-induced calcium loading in cardiac cells: relevance for cardioplegia. Ann. Thorac. Surg. 63, 153–161.

Kantor, P.F., Coetzee, W.A., Carmeliet, E.E., Dennis, S.C., Opie, L.H., 1990. Reduction of ischemic K^+ loss and arrhythmias in rat hearts: effect of glibenclamide, a sulfonylurea. Circ. Res. 66, 478–485.

Koyama, T., Boston, D., Ikenouchi, H., Barry, W.H., 1996. Survival of metabolically inhibited ventricular myocytes is enhanced by inhibition of rigor and sarcoplasmic reticulum Ca^{2+} cycling. Am. J. Physiol. 271, H643–H650.

Labrid, C., Grossel, A., Dureng, G., Mironneau, J., Duchene-Marullaz, P., 1979. Some membrane interactions with bepridil, a new antianginal agent. J. Pharmacol. Exp. Ther. 211, 546–554.

Lawton, J.S., Harrington, G.C., Allen, C.T., Hsia, P.W., Damiano, R.J. Jr., 1996. Myocardial protection with pinacidil cardioplegia in the blood-perfused heart. Ann. Thorac. Surg. 61 (6), 1680.

Lopez, J.R., Ghanbari, R.A., Terzic, A., 1996a. A K_{ATP} channel opener protects cardiomyocytes from Ca^{2+} waves: a laser confocal microscopy study. Am. J. Physiol. 39, H1384–H1389.

Lopez, J.R., Jahangir, R., Jahangir, A., Shen, W.K., Terzic, A., 1996b. Potassium channel openers prevent potassium-induced calcium loading of cardiac cells: possible implications in cardioplegia. J. Thorac. Cardiovasc. Surg. 112 (3), 820–831.

Metz, D.C., Pradhan, T.K., Mrozinski, J.E. Jr., Jensen, R.T., Turner, R.J., Gardner, J.D., 1994. Effect of inhibition of microsomal Ca^{2+}-ATPase on cytoplasmic calcium and enzyme secretion in pancreatic acini. Biochim. Biophys. Acta 13 (1220(2)), 199–208.

Noma, A., 1983. ATP-regulated K channels in cardiac muscle. Nature 305, 147–148.

Opie, L.D., Du Tit, E.F., 1992. Postischemic stunning: the two-phase model for the role of calcium as pathogen. J. Cardiovasc. Pharmacol. 20, S1–S4.

Powell, T., Tatham, P.E.R., Twist, V.W., 1984. Cytoplasmic free calcium measured by QUIN2 fluorescence in isolated ventricular myocytes at rest and during potassium depolarization. Biochem. Biophys. Res. Commun. 122, 1012–1020.

Russ, U., Englert, H., Scholkens, B.A., Gogelein, H., 1996. Simultaneous recording of ATP-sensitive K^+ current and intracellular Ca^{2+} in anoxic rat ventricular myocytes. Effects of glibenclamide. Pflug. Arch. 432 (1), 75–80.

Schwartz, A., Matlib, M.A., Balwierczak, J., Lathrop, D.A., 1985. Pharmacology of calcium antagonists. Am. J. Cardiol. 55, 3C–7C.

Steenbergen, C., Murphy, E., Levy, L., London, R.E., 1997. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. Circ. Res. 60, 700–707.

Tang, T., Duffield, R., Ho, A.K., 1998. Effects of Ca^{2+} channel blockers on Ca^{2+} loading induced by metabolic inhibition and hyperkalemia in cardiomyocytes. Eur. J. Pharmacol. 360, 205–211.

Tani, M., 1990. Mechanism of Ca^{2+} overload in reperfused ischemic myocardium. Annu. Rev. Physiol. 52, 543–559.

Venkatesh, N., Lamp, S.T., Weiss, J.N., 1991. Sulfonylureas, ATP-sensitive K^+ channels, and cellular K^+ loss during hypoxia, ischemia and metabolic inhibition in mammalian ventricles. Circ. Res. 69, 623–637.

Vigne, P., Breittmayer, J.P., Frelin, C., 1992. Thapsigargin, a new inotropic agent, antagonizes action of endothelin-1 in rat atrial cells. Am. J. Physiol. 263 (6), H1689–H1694, pt. 2.

Watanabe, I., Johnson, T.A., Buchanan, J., Engle, C.L., Gettes, C., 1987. Effect of graded coronary flow reduction on ionic, electrical, and mechanical indexes of ischemia in the pig. Circulation 76, 1127–1134.

Weir, W.G., 1990. Cytoplasmic $[Ca^{2+}]_i$ in mammalian ventricle: dynamic control by cellular processes. Annu. Rev. Physiol. 52, 467–485.

Weiss, J.N., Shine, K.I., 1982. $[K^+]_o$ accumulation and electrophysiological alterations during early myocardial ischemia. Am. J. Physiol. 243, H318–H327.

Wickenden, A.D., Kaprielian, R., Kassiri, Z., Tsoporis, J.N., Tsushima, R., Fishman, G.I., Backx, P.H., 1998. The role of action potential prolongation and altered intracellular calcium handling in the pathogenesis of heart failure. Cardiovasc. Res. 37, 312–323.

Wilde, A.A.M., Escande, D., Schumacher, C.A., Thuringer, D., Meestre, M., Fiolet, J.W.T., Jance, M.J., 1990. Potassium accumulation in the globally ischemic mammalian heart: a role for the ATP-sensitive potassium channel. Circ. Res. 67, 835–843.

Xu, K.Y., Sweier, J.L., Becker, L.C., 1995. Functional coupling between glycolysis and sarcoplasmic reticulum Ca^{2+} transport. Circ. Res. 77, 88–97.