Full Paper

Hypotonic Stress Increases Efficacy of Rilmakalim, but Not Pinacidil, to Activate ATP-Sensitive K^+ Current in Guinea Pig Ventricular Myocytes

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Abstract. The aim of this study was to investigate the influence of hypotonic challenge on the effects of potassium channel openers (PCO), rilmakalim and pinacidil, on activation of the ATP-sensitive K^+ current. The whole cell configuration of the patch-clamp technique was applied to guinea pig ventricular myocytes exposed to isotonic and hypotonic solutions. Difference in osmolarity was about 100 mOsm due to different mannitol concentrations. Rilmakalim, a second generation PCO [(3*S*,4*R*)-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-6-phenylsulfonyl-chromanhemihydrate], activated time-independent K^+ current in the isotonic solution with pD2 ($-\log EC_{50}$) = 6.42 ± 0.12 and $E_{max} = 19.74 \pm 2.16$ pA/pF, $n = 7$, at 0 mV. The effects of the cyanoguanidine compound pinacidil were similar to those of rilmakalim, but the action appeared slower and with about 600-fold less potency than the former. Efficacy of rilmakalim, but not pinacidil, was enhanced in hypotonic solution, with $E_{max} = 30.87 \pm 5.40$ pA/pF ($P < 0.05$, $n = 7$), and the current was completely inhibited by glibenclamide. Additionally, rilmakalim concentration-effects correlation coefficient (R) decreased from 0.96 to 0.86 and Hill's coefficient increased from 1.21 to 1.45. Pretreatment with phalloidin (20 μ M), a cytoskeleton stabilizer, prevented an intensification of the effects of rilmakalim in hypotonic solution and returned R and Hill's coefficients to the control values. We conclude that osmotic stress increases efficacy of rilmakalim to activate K_{ATP} channels in guinea pig ventricular myocytes due to the specific interaction with actin filaments.

Keywords: hypotonic swelling, ATP-sensitive K^+ channel, patch clamp, cytoskeleton, rilmakalim

Introduction

ATP-sensitive K^+ channels (K_{ATP}) in the heart and other tissues can be activated by metabolic changes such as ischemic insults or by application of potassium channel openers (PCO) (1, 2). Recently it has been shown that depletion of intracellular ATP is not sufficient to explain the mechanism of K_{ATP} channel activation (3). For instance, lactate dehydrogenase, creatine phosphate, and phosphatidylinositol biphosphate (PIP_2) are shown to be involved in regulation of K_{ATP} channels (4–7). On the other hand, ischemia causes accumula-

tion of metabolic products in cardiac cells, thereby increasing cellular osmolarity. This hypotonic stress under such a condition is related to increased intracellular osmolarity. Hence, water enters the cells, increasing their volume, and alters the function of ion channels (8–10). There is an indication that cardiac cell swelling could activate K_{ATP} channels (11), albeit in the presence of low concentration of ATP in the pipette solution (12). We have recently shown that hypotonic stress itself cannot activate K_{ATP} channels in guinea pig ventricular myocytes in the presence of 5 mM Mg-ATP in the pipette solution (13). These results suggest that there are additional factors to regulating the channel openings during hypotonic cell swelling. Organization of the actin cytoskeleton has been shown to be involved

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in regulation of K_{ATP} channels (14–16). Nevertheless, there is no data about the effects of hypotonic stress on the PCOs action in the heart. In order to explore further interaction between hypotonic stress and K_{ATP} channels, we studied effects of PCO, pinacidil, and rilmakalim on whole cell currents in guinea pig ventricular myocytes under isotonic and hypotonic conditions, and explored a possible contribution of actin cytoskeleton to modulation in their action.

Materials and Methods

All experimental procedures were approved by Tokyo Medical and Dental University Ethic Committee for Animal Experiments and are in accordance with ethical standards published by the USA National Institutes of Health No. 85-23, revised in 1996.

Cell isolation

We used guinea pigs of both sexes weighing 300–400 g, kept under standard laboratory conditions. The animals were anesthetized with pentobarbital- Na^+ (30 mg/kg, i.p.), heparinized (5000 i.u., i.v.), and artificially ventilated during isolation of the heart. The ventricular myocytes were isolated enzymatically using an established procedure presented elsewhere (13).

Solutions and drugs

Normal Tyrode's solution contained 145.0 mM NaCl, 4.0 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5.0 mM HEPES; the pH of Tyrode's solution was adjusted to 7.3 by NaOH. Isotonic solution was made by omitting 85 mM NaCl and adding mannitol to achieve osmolarity 280 ± 8 mOsm as measured by the Vapor Pressure Osmometer (5520; Wescor, Logan, UT, USA). The hypotonic solution contained all components of isotonic solution, except mannitol was added in a small quantity allowing us to adjust the solution to about 100 mOsm lower osmolarity than isotonic solution (180 ± 12 mOsm). Pipette solution contained 20.0 mM KCl, 0.02 mM $CaCl_2$, 5.0 mM HEPES, 100.0 mM K^+ -aspartate, 5.0 mM Mg^{2+} -ATP, 5.0 mM K^+ -creatine phosphate, and 0.05 mM EGTA. All drugs except rilmakalim (formerly HOE 234) and chromanol (kindly donated from Hoechst-Roussel, Frankfurt, Germany) were purchased from Sigma, St. Louis, MO, USA. Rilmakalim [(3*S*,4*R*)-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-6-phenyl sulfonyl chromanhemihydrate], pinacidil, phalloidin, and glibenclamide were dissolved in dimethyl sulfoxide (DMSO) in the stock solution of 0.1 mM (rilmakalim), 10 mM (pinacidil), 1 mM (phalloidin), and 0.1 mM (glibenclamide). The final concentration of DMSO in

all experimental solutions was less than 0.3% and did not affect the effects of rilmakalim and pinacidil. All solutions were freshly prepared before each experiment.

Electrophysiology

The whole-cell configuration of the patch clamp technique was employed to record membrane currents using a patch clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA, USA) at a temperature of $35 \pm 1^\circ C$. Recorded signals were filtered at 2 kHz, monitored on an oscilloscope (DCS 7040; Kenwood, Tokyo), digitized (Digidata 1200, Axon Instruments), and stored in a personal computer. Data analysis were performed by pCLAMP software (version 8.0) and curve fittings were done by Origin software. Suction electrodes were pulled to have resistances between 1.0 and 2.0 M Ω after filling with a pipette solution. Junction potential was minimized before approaching the preparations and checked again after the end of the experiments. If the values differed by more than 2 mV, data were discarded from the analysis. Series resistance compensation was achieved to 50–70%. A descending ramp was applied from +60 to –110 mV for 4.5 s (holding potential, –40 mV).

Experimental protocols

The effects of PCOs, rilmakalim and pinacidil, were estimated by adding incremental concentrations of the drug first in isotonic solution. In the case of rilmakalim, a steady-state effect developed after about 3 min, and therefore, the concentration of the drug was changed every 3 min. In the case of pinacidil, equilibration of the effect with a certain concentration appeared after about 5 min, and hence, the concentration was changed every 5 min. To study effects of PCOs in hypotonic solution, we examined the currents in hypotonic solution in the absence of PCOs during the period of 15–25 min needed for application of 4–5 different concentrations of the tested drugs. All experiments were performed in the presence of 20 μM chromanol, a blocker of slow component of delayed rectifier K^+ current (I_{Ks}) (17), and 1 mM anthracene-9-carboxylic acid to block volume-sensitive chloride current ($I_{Clswell}$) (13). During 25 min in hypotonic solution, in the absence of PCOs, we did not observe any time-independent K^+ currents. It is noteworthy that we observed depolarization of resting potentials and positive shifting of reversal potentials of whole cell currents in all examined cells started after about 3 min to reach steady-state after about 5 min in hypotonic solution, which is in accordance with our previous data (13).

To study the possible interaction between PCOs and cytoskeleton in hypotonic solutions, we had to use

some actin filament stabilizers. Based on our previous experiments with different actin filament stabilizers (14) and pilot study, we have chosen phalloidin at 20 μ M as optimal compound that does not interfere with K_{ATP} channel activity in isotonic solution. For instance, PIP₂, another actin filament stabilizer, can activate K_{ATP} channels (5). Therefore, all experiments with PCOs rilmakalim and pinacidil in hypotonic solutions have been repeated in the presence of 20 μ M phalloidin.

Data analyses

We compared normalized current values obtained at 0 mV, which was close to a minimum value in the control. For construction and analysis of concentration-response relation of examined PCO, we used Pharmacological Calculation System/PCS version 4 software (18). Efficacy of each PCO referred to the maximum current density obtained at 0 mV, while potency was calculated as the concentration needed to produce 50% of the maximum observed effect (EC₅₀). The Hill's coefficients were calculated using the computer program written by Barlow (19). Data are presented as means $\pm$ S.E.M. Two-way ANOVA with a multiple comparison test (data with normal distribution) and the Kruskal-Wallis non-parametric test (data without normal distribution) were used for analysis of statistical significance. $P < 0.05$ was considered to indicate a significant difference.

Results

Changes of reversal potentials of whole cell currents in hypotonic solution

As we have shown previously, hypotonic challenge of guinea pig ventricular myocytes besides other changes of membrane potentials induced positive shifting of whole cell current-reversal potentials (13). We have found that those changes are not related to activation or inhibition of specific ion currents, but probably attributable to the dilution of ion concentration in some subcellular spaces due to water entering the cells during hypotonic challenge (13). In this study, positive shifting of reversal potential of whole cell current (elicited by descending ramp protocol from +60 to -110 mV for 4.5 s) occurred in all examined cells exposed to hypotonic solution in the presence of 1 mM 9-AC to block I_{Clswell} (13) and 20 μ M chromanol to block I_{Ks} (13) and it was 4.9 ± 1.2 mV, $n = 15$.

Effects of rilmakalim

Figure 1A shows responses of a cardiomyocyte to different concentrations of rilmakalim in isotonic solution elicited by descending ramp from +60 to -110 mV.

Fig. 1. Original traces obtained from one typical cell in isotonic solution (A) and another cell in hypotonic solution (B) in the presence of different concentrations of rilmakalim elicited by descending ramp protocol from +60 to -110 mV for 4.5 s. a: control; b: rilmakalim, 0.3 μ M; c: rilmakalim, 1.0 μ M; d: rilmakalim, 3.0 μ M; e: rilmakalim, 10.0 μ M; f: rilmakalim, 10.0 μ M + glibenclamide, 1.0 μ M. Similar results were obtained from 7 cells.

When the concentration after change produced the same or a smaller response as the previous concentration of the drug, we assumed the response reached was the maximal effect and applied 1 μ M glibenclamide to assure that activated current represented I_{K(ATP)}. The same procedure was done in hypotonic solutions (Fig. 1B), but tested on a different cardiomyocyte. The maximal effect of rilmakalim was observed in hypotonic solution at 10 μ M; therefore, the values were taken as 100% for calculating potency and efficacy of rilmakalim (Fig. 2). The rilmakalim-activated currents in isotonic and hypotonic solutions were reversibly inhibited by glibenclamide identifying the current as I_{K(ATP)}. To find out whether the actin cytoskeleton contributes to enhanced maximum effect of rilmakalim in hypotonic solution, we repeated the experiments in the presence

Fig. 2. Average effects of rilmakalim presented as % of the maximal effect (30.87 pA/pF) in isotonic, hypotonic, and hypotonic + phalloidin solutions. $**P < 0.01$, significant difference between effects of rilmakalim in hypotonic and isotonic solutions; $\#P < 0.05$, significant differences between the values in hypotonic solution in the absence and presence of phalloidin. Mean $\pm$ S.E.M. from 7 experiments.

of 20 μ M of phalloidin, an actin filaments stabilizer (see Materials and Methods). As Fig. 2 shows, phalloidin attenuated the maximum effect induced by rilmakalim in hypotonic solution.

Effects of pinacidil

We used the same experimental procedure as for rilmakalim to study the effects of pinacidil. Figure 3A shows concentration-dependent effects of pinacidil on whole cell current elicited by ramp protocol in a typical example of cardiomyocytes in isotonic solution. The action of pinacidil to elicit outward current developed slower than that of rilmakalim; therefore, the concentration of pinacidil was changed after 5 min. The procedure was repeated in hypotonic solution (Fig. 3B). The maximal effect of pinacidil was not significantly different from the effects in isotonic solution (Fig. 4). The pinacidil-activated current was also blocked by glibenclamide in isotonic and hypotonic solutions. Comparing the efficacy and potency of rilmakalim and pinacidil, we found about a 600-fold higher potency of rilmakalim, but the efficacies were similar in the isotonic solution (Fig. 5) (Table 1). The correlation and Hill's coefficients are also presented in Table 1.

Discussion

In this paper, we present results indicating that hypotonic challenge significantly enhances efficacy of rilmakalim on activation of K_{ATP} channel currents, but not of pinacidil in guinea pig ventricular myocytes.

Fig. 3. Original traces obtained from one typical cell in isotonic solution (A) and another cell in hypotonic solution (B) in the presence of different concentrations of pinacidil (induced by the same ramp protocol as in Fig. 1). a: control; b: pinacidil, 30 μ M; c: pinacidil, 100 μ M; d: pinacidil, 300 μ M; e: pinacidil, 1000 μ M; f: pinacidil, 1000 μ M + glibenclamide, 1.0 μ M. Similar results were obtained from 6 cells.

Moreover, the above-mentioned effect of rilmakalim was significantly attenuated in the presence of phalloidin, a stabilizer of actin cytoskeleton. Phalloidin itself at a concentration of 20 μ M did not affect whole cell current either in isotonic or hypotonic solutions, and exposure of the ventricular myocytes to hypotonic solution for as long as 25 min did not elicit time-independent K^+ current (data not shown). Therefore, it seems that actin filaments interacted specifically with rilmakalim to increase its efficacy to activate glibenclamide-sensitive current, $I_{K(ATP)}$.

There are two points in this study requiring critical evaluations. Firstly, it is necessary to prove that obtaining maximal effects of rilmakalim and pinacidil are reliable, and secondly, that enhanced efficacy of

Fig. 4. Average data expressed as percentage of the maximal effect of pinacidil observed in isotonic and hypotonic solutions (21.9 pA/pF). Mean $\pm$ S.E.M. from 6 experiments.

Fig. 5. Comparison of the maximal effects of rilmakalim and pinacidil in isotonic and hypotonic solutions. White bar: isotonic solution, Gray bar: hypotonic solution, Black bar: hypotonic solution + phalloidin. ** P <0.01, significant difference between rilmakalim effects in hypotonic and isotonic solutions; # P <0.05, significant difference between the maximal effects of rilmakalim and pinacidil; & P <0.05, significant difference between the effects of rilmakalim in the absence and presence of phalloidin in hypotonic solution.

rilmakalim in hypotonic solution is due to the interaction with the cytoskeleton.

We used rilmakalim and pinacidil in a wide concentration range and carefully observed concentration-response relations in the isotonic solution in order to find out the precise time needed for steady-state effects for both examined compounds. According to our assumption, lack of significant increase of the effect of examined compound despite of increase of its concentration means that drug reached its maximal effect (Fig. 2B and Fig. 4B). Although, a positive shifting of reversal potential of whole cell currents observed in hypotonic solutions suggested participation of some other hypotonic-sensitive currents in the effects of rilmakalim and pinacidil, especially $I_{Clswell}$, we obtained the mentioned results in the presence of 9-AC and chromanol, blockers of $I_{Clswell}$ and I_{Ks} . Our previous data indicate that 9-AC at 1 mM completely prevented activation of $I_{Clswell}$. Moreover, we have shown previously (13) and also in this study that positive shifting of reversal potential of whole cell current in hypotonic solution appearing in all guinea pig ventricular myocytes has a non-specific character.

On the other hand, hypotonic challenge of cardiomyocytes is associated with cell swelling and deformation or disruption of actin filaments might be expected under such a condition. An involvement of the actin cytoskeleton in the regulation of K_{ATP} channel gating mechanisms was previously reported. Furukawa et al. (14) showed that the actin cytoskeleton regulated the processes of rundown and reactivation of K_{ATP} channels in guinea pig ventricular myocytes. The principal finding of that study was that actin filament depolymerizing agents accelerated K_{ATP} channels rundown while a stabilizer phalloidin abolished rundown of fully activated K_{ATP} channels and reactivated channels already partially rundown. It has also been noted that disruption of actin filaments can increase K_{ATP} channels activity (15, 20). One possible explanation for this discrepancy are different channel conditions for stimulating (before rundown) and inhibiting effects of actin filament

Table 1. Comparison of the effects of rilmakalim and pinacidil exposed to isotonic and hypotonic solutions in guinea pig ventricular myocytes

	Rilmakalim		Pinacidil	
	Isotonic	Hypotonic	Isotonic	Hypotonic
pD ₂ (−log EC ₅₀)	6.42 $\pm$ 0.12	6.10 $\pm$ 0.18	3.80 $\pm$ 0.11	3.7 $\pm$ 0.10
EC ₅₀ (μ M)	0.37 $\pm$ 0.02	0.74 $\pm$ 0.03	151.0 $\pm$ 24.1	195.0 $\pm$ 24.1
R	0.96 $\pm$ 0.03	0.86 $\pm$ 0.02**	0.96 $\pm$ 0.02	0.97 $\pm$ 0.02
Hill coefficient	1.21 $\pm$ 0.02	1.45 $\pm$ 0.021 [#]	0.90 $\pm$ 0.018	0.92 $\pm$ 0.02

Mean $\pm$ S.E.M. from 6–8 experiments; ** P <0.01, in comparison with corresponding values obtained in isotonic solution; [#] P <0.05, value different from unity.

network on K_{ATP} channels activity (15). Effects of cytoskeleton modulating agents on PCO action in isotonic and hypotonic solutions were not studied. Our results may imply that hypotonic stress destabilizes the actin cytoskeleton and enhances stimulatory action of rilmakalim on K_{ATP} channels activity. We used phalloidin at 20 μ M as an optimal cytoskeleton stabilizer based on our previous experiments (14). Interestingly, effects of pinacidil were not augmented by exposure to hypotonic solution. This apparent difference between rilmakalim (21) and pinacidil is probably related to different chemical structure and affinity to SUR2A receptors as well as sensitivity to actin filaments organization. We found that rilmakalim was about 600-fold more potent than pinacidil either in isotonic or in hypotonic solutions. Moreover, rilmakalim effects developed faster than that of pinacidil. The increase in efficacy of rilmakalim appeared at higher concentration and was associated with significantly lower R-coefficient (related to correlation between concentrations and effects) and higher Hill coefficient, suggesting an additional mechanism of action apart from simple stimulation of SUR2A receptors. Hence, in our opinion, hypotonic stress-induced disorganization/disruption of actin filaments could delay rundown of the channels, thereby causing opening of more channels in the presence of high rilmakalim concentration. By this means, rilmakalim effectiveness could be increased, similar to an earlier explanation of synchronization of channel opening (22). Another possibility comes from a study with diadenosine-tetraphosphate (Ap4A) and K_{ATP} channel gating (23). The authors showed that Ap4A, recently discovered as very important regulatory compound in the ischemic heart, inhibited K_{ATP} channel gating only in the presence of an intact cytoskeleton, and thus, disruption of cytoskeleton increased activity of K_{ATP} channels. It is in accordance with our data related to rilmakalim, but does not explain lack of similar action of pinacidil. There is evidence that actin cytoskeleton disruption can decrease inhibitory effects of ATP and glibenclamide on K_{ATP} channels in cardiomyocytes and smooth muscle cells (24, 25). We used 1 μ M glibenclamide, enough to block completely rilmakalim and pinacidil-induced currents in isotonic solutions and to abolish currents elicited in hypotonic solutions. Although, effects of glibenclamide in hypotonic solution seem to be slightly smaller than in isotonic solution, the observed differences were not significant (data not shown). This apparent discrepancy is probably due to the facts that we did not use any pharmacological tools to disrupt the actin cytoskeleton, but swelling of the cells in hypotonic solution. Also, whole cell configuration of the patch clamp technique, which is

mandatory in a such condition (to study effects of hypotonic challenge), may account for the above-mentioned differences, as the authors of the cited papers used inside-out configured patches.

Taken all together we can conclude that hypotonic challenge of guinea pig ventricular myocytes increases the efficacy of rilmakalim by a mechanism involving the actin cytoskeleton. This interaction is probably due to the better synchronization of K_{ATP} channel opening in the presence of high concentrations of fast acting rilmakalim. Although, further work is required to clarify the precise mechanism involved in this interaction.

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