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Blockade of Adenosine Triphosphate-sensitive Potassium Channels by Thiamylal in Rat Ventricular Myocytes

Yasuo Tsutsumi, M.D.,* Shuzo Oshita, M.D.,† Hiroshi Kitahata, M.D.,‡ Yasuhiro Kuroda, M.D.,§ Takashi Kawano, M.D.,* Yutaka Nakaya, M.D.∥

Background: The adenosine triphosphate (ATP)—sensitive potassium (K_{ATP}) channels protect myocytes during ischemia and reperfusion. This study investigated the effects of thiamylal on the activities of K_{ATP} channels in isolated rat ventricular myocytes during simulated ischemia.

Methods: Male Wistar rats were anesthetized with ether. Single, quiescent ventricular myocytes were dispersed enzymatically. Membrane currents were recorded using patch-clamp techniques. In the cell-attached configuration, K_{ATP} channel currents were assessed before and during activation of these channels by 2,4-dinitrophenol and after administration of 25, 50, and 100 mg/l thiamylal. The open probability was determined from current–amplitude histograms. In the inside-out configuration, the current–voltage relation was obtained before and after the application of thiamylal (50 mg/l).

Results: In the cell-attached configuration, 2,4-dinitrophenol caused frequent channel opening. 2,4-Dinitrophenol-induced channel activities were reduced significantly by glibenclamide, suggesting that the channels studied were K_{ATP} channels. Open probability of K_{ATP} channels was reduced by thiamylal in a concentration-dependent manner. K_{ATP} channels could be activated in the inside-out configuration because of the absence of ATP. Thiamylal inhibited K_{ATP} channel activity without changing the single-channel conductance.

Conclusions: The results obtained in this study indicate that thiamylal inhibits K_{ATP} channel activities in cell-attached and

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Address reprint requests to Dr. Tsutsumi: Department of Anesthesiology, Tokushima University School of Medicine, 3–18-15 Kuramoto, Tokushima 770-8503, Japan. Address electronic mail to: tsutsumi@clin.med.tokushima-u.ac.jp

inside-out patches, suggesting a direct action of this drug on these channels. (Key words: Anesthetic; K_{ATP} channel; patch-clamp configuration.)

ADENOSINE triphosphate (ATP)-sensitive potassium (K_{ATP}) channels are a family of potassium channels inhibited by intracellular ATP. 1-3 Because K_{ATP} channels are gated by intracellular ATP, they are thought to link cellular metabolism with membrane excitability.1 In heart cells, K_{ATP} channels are activated by depletion of intracellular ATP, hypoxia, or exposure to metabolic inhibitors^{1,4-6} and cause an increase in potassium ion outward flow. This activation of KATP channels may be an endogenous protective mechanism against cardiac damage during myocardial ischemia. In addition, it has been reported that K_{ATP} channels play an important role in ischemic preconditioning⁷⁻⁹: A brief period of ischemia can make the heart more resistant to subsequent, more severe episodes of ischemia. As a possible mechanism of cardioprotective action of KATP channels, the activation of sarcolemmal KATP channels has been thought to protect the ischemic myocardium by shortening the action potential duration. That is, the shortening of action potential duration decreases calcium influx through voltage-dependent calcium channels, 10 and causes a decrease in contractility, 11 which is a major source of ATP consumption, and decreases in calciuminduced cell toxicity, 12 thereby resulting in the protection of myocytes from further depletion of ATP and irreversible impairment of energy metabolism.¹³ It was suggested recently that mitochondrial rather than sarcolemnal KATP channels might be playing an important role in the cardioprotective action of K_{ATP} channels. $^{14-16}$

Studies of the effects of anesthetics on the K_{ATP} channel activities in the heart have been performed. *In vivo* experiments revealed that volatile anesthetics, including halothane, isoflurane, and enflurane, activate K_{ATP} chan-

^{*} Postgraduate Student, Department of Anesthesiology.

[†] Professor and Chairman, Department of Anesthesiology.

[‡] Associate Professor, Department of Anesthesiology.

 $[\]$ Assistant Professor, Division of Intensive Care Medicine.

^{||} Professor and Chairman, Department of Nutrition.

nels and have cardioprotective effects. 17-23 In contrast, using a patch-clamp technique, Ko et al. 12 found that ketamine inhibits the KATP channel activities in a concentration-dependent manner in rat ventricular myocytes. Kozlowski and Ashford²⁴ also revealed that in CRI-G1 insulin-secreting cells thiopental inhibits K_{ATP} channel activities if applied to cell-attached patches or excised inside-out patches. These findings suggest that ketamine and thiobarbiturates may attenuate the cardioprotective effects of K_{ATP} channels during ischemia and reperfusion in the myocardium. Because thiobarbiturates are used a great deal in all types of anesthesia in patients who have coronary artery disease and are undergoing a variety of surgical procedures, it is important to evaluate the direct effects of these drugs on the KATP channel activities in the ventricular myocardium during ischemia. Therefore, we evaluated the effects of thiamylal on the K_{ATP} channel activities in isolated rat ventricular myocytes during simulated ischemia.

Materials and Methods

Cell Isolation

This study was approved by the Animal Investigation Committee of Tokushima University and followed the guidelines of the American Physiological Society (Bethesda, Maryland) for the humane use of animals in research. Fifty-one male Wistar rats (weight, 250-300 g) were anesthetized with ether, and 1.0 IU/g heparin was injected intraperitoneally 30 min before surgery. The chest was opened, and the beating heart was dissected as soon as the response to tail clamp was lost. The heart was perfused in a retrograde manner via the aorta using a Langendorff apparatus with standard Tyrode's solution prewarmed to 37°C and saturated with a 95% oxygen and 5% carbon dioxide gas mixture. The composition of standard Tyrode's solution (pH 7.4) was as follows: NaCl: 125 mm; NaHCO₃: 24 mm; KCl: 5.4 mm; NaH₂PO₄: 0.47 mm; MgCl₂: 1.05 mm; dextrose: 5.5 mm; and CaCl₂: 1.8 mm. The heart was perfused with Ca²⁺-free Tyrode's solution for approximately 5 min until the heart stopped beating and then was digested with Ca²⁺-free Tyrode's solution containing collagenase (0.2 mg/ml) and pronase (0.05 mg/ml) for 15 min. After enzymatic digestion, the heart was perfused with low-Ca2+ Tyrode's solution (0.1 mm CaCl₂) for several minutes to wash away the enzymes. The ventricle was cut into pieces in low-Ca²⁺ Tyrode's solution, and the myocytes were filtered. The cells were centrifuged, and the supernatant was removed. All cells used in these experiments were rod shaped and striated.

Electrophysiologic Measurements

Membrane currents were recorded in the cell-attached and inside-out configurations using a patch-clamp amplifier as described by Hamill et al. 25 The composition of bath solution for cell-attached mode was as follows: KCl: 140 mm; HEPES: 10 mm; dextrose: 5.5 mm; and ethyleneglycoltetracetic acid (EGTA): 0.5 mm. The pipette solution for cell-attached mode contained 140 mm KCl, 10 mm HEPES, and 5.5 mm dextrose. The composition of bath solution for inside-out mode was as follows: KCl: 140 mm; HEPES: 10 mm; dextrose: 5.5 mm; MgCl₂: 1 mm; and ethyleneglycoltetracetic acid: 0.5 mm. The composition of pipette solution for the inside-out mode was the same as that for the cell-attached mode. Soft-glass pipettes prepared in an electrode puller (PP-830; Narishige, Tokyo, Japan) were used after being coated with Sylgard (Dow Corning Co., Midland, MI). The electrical resistance of the patch pipette was 5 to 7 M Ω for singlechannel recording. Experiments were conducted with solution temperatures of 35-37°C. pClamp version 6.0 software (Axon Instruments, Foster, CA) was used to analyze the data for single-channel currents. The open probability (NPo) was determined from current amplitude histograms and was calculated using the following equation:

$$NP_{O} = \sum_{n=0}^{N} {n \cdot P_{n}}$$

where N is the number of channels in the patch and Pn is the integrated channel opening. Open probability of K_{ATP} channels was determined from recordings longer than 60 s in duration.

Drugs

The thiamylal sodium (Yoshitomi Chemical, Osaka, Japan) ampule was opened before use. 2,4-Dinitrophenol (DNP; Sigma Chemical, St. Louis, MO) and glibenclamide (Sigma) were prepared as stock solutions. All other solutions were made daily. Collagenase (Yakult, Tokyo, Japan) and pronase (Sigma) were used for enzymatic dissociation.

Statistical Analysis

Data are expressed as the mean \pm SD. Differences among data sets were evaluated by the Student t test. A P value < 0.05 was considered statistically significant.

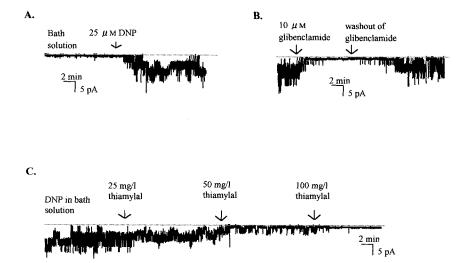


Fig. 1. Effects of thiamylal on the adenosine triphosphate-sensitive potassium (KATP) channel activities in the cell-attached configuration. Membrane potentials were clamped at -50 mV. The dashed line is the zero current level. (A) No significant current was observed before 2,4-dinitrophenol (DNP) treatment. DNP in the bath solution activated KATP channels. (B) DNP-induced KATP channel activity was inhibited by glibenclamide. This blockade was reversible, and the channel activities were restored by washing out glibenclamide. (C) Dose-dependent effects of thiamylal on KATP channels. The channels show a decreased open probability as thiamylal concentration is increased.

Results

Effects of Thiamylal on K_{ATP} Channels in the Cellattached Configuration

To investigate whether thiamylal affects the K_{ATP} channel activities in intact ventricular myocytes, we studied single-channel KATP currents using the cell-attached configuration. As shown in figure 1A, we did not observe any channel openings in the standard bath solution. If 25-µM DNP, an inhibitor of mitochondrial ATP synthesis, was administered, frequent channel openings were observed (fig. 1A). DNP at a concentration of 25 μ m was sufficient to identify K_{ATP} channels; at higher concentrations, there were so many opened channels during the burst that we could not determine the number of channels. DNP-induced KATP channel activities were inhibited by 10-µm glibenclamide, a specific inhibitor of K_{ATP} channels (fig. 1B). This blockade by glibenclamide was reversible (fig. 1B). Figure 1C shows the blockade of K_{ATP} channels by thiamylal. Figure 2 shows the relation between relative K_{ATP} channel activities and the concentration of thiamylal. In 22 patches, the open probabilities were suppressed by increase the concentration of thiamylal. The relative channel activities after administration of thiamylal were 0.70 ± 0.05 (25 mg/l thiamylal; significantly different from DNP solution; P < 0.05), 0.41 \pm 0.04 (50 mg/l thiamylal; P < 0.05), and 0.01 \pm 0.01 (100 mg/l thiamylal; P < 0.05). These results indicate that inhibition of K_{ATP} channels by thiamylal occurs in a dose-dependent manner. A decreased open probability of K_{ATP} channel activities induced by thiamylal returned toward baseline values almost completely after thiamylal washout.

Effects of Thiamylal on K_{ATP} Channels in the Inside-out Configuration

We also studied whether thiamylal could block K_{ATP} channels directly from the cytosolic side of ventricular myocytes. Because K_{ATP} channels are inhibited by intracellular ATP, we studied the activities of these channels in the absence of ATP. In this series of experiments, bath application of thiamylal also inhibited the K_{ATP} channel

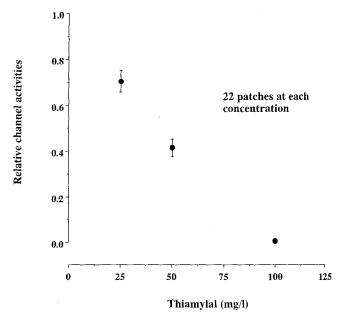
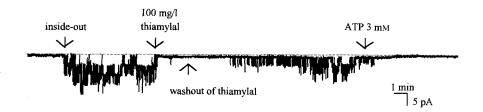


Fig. 2. The concentration-dependent decrease in the relative channel activities of adenosine triphosphate-sensitive potassium channels at three concentrations (25, 50, and 100 mg/l) of thiamylal. In general, 90-120 s of data were recorded for each thiamylal concentration. Each data point (vertical bars) is presented as the mean \pm SD.

Fig. 3. In the inside-out configuration, application of thiamylal inhibits the adenosine triphosphate (ATP)-sensitive potassium ($K_{\rm ATP}$) channel activities. In ATP-free solution, the $K_{\rm ATP}$ channel activities decreased gradually with time. This is called "run-down." This figure also shows that washout of thiamylal restores channel activities and that inhibition of channel activities is caused not only by rundown, but also by thiamylal.



activities in the inside-out configuration, and washout of thiamylal reactivated the channel (fig. 3). The average percentage recovery of the K_{ATP} channel activity after thiamylal washout was 89.3 \pm 6.2% of open probability obtained before application of thiamylal (n = 26).

The current-voltage relation obtained from 19 patches before and after the application of thiamylal (50 mg/l) is shown in figure 4. The current-voltage curves before and after the application of thiamylal were linear in the negative potential range, with single-channel conductances of 73.4 ± 1.7 picosiemens (pS) and 75.1 ± 2.2 pS before and after 50 mg/l thiamylal, respectively. There were no statistical differences between control and thiamylal-treated series. These results suggest that thiamylal does not change the K_{ATP} channel conductances in the inside-out configuration.

Discussion

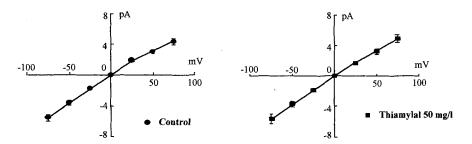
The principal findings of this study are that thiamylal inhibits the K_{ATP} channel activities without changing the single-channel conductance in the inside-out patches and in the cell-attached patches. Although we cannot discriminate whether thiamylal acts directly on the K_{ATP} channel protein or indirectly via alteration of the physical characteristics of the membrane lipids, thiamylal inhibits the K_{ATP} channel activities in a membrane-delimited manner rather than through a cytosolic second messenger, which is absent in the excised inside-out patch.

Adenosine triphosphate-sensitive potassium channels

are important for regulation of cellular energy metabolism associated with membrane excitability. During ischemia and reperfusion, KATP channels open to induce several protective responses in the heart, K_{ATP} channels are responsible for an increase in potassium permeability and reduction of the action potential duration during hypoxia and ischemia. 12 Thus, opening of K_{ATP} channels benefits the heart, possibly by reducing calcium influx through voltage-dependent calcium channels, slowing ATP depletion, and decreasing calcium-induced toxicity. 12 In addition, with reperfusion or reoxygenation, oxygen free radicals are generated, which could trigger a chain of damaging chemical reactions resulting in "reperfusion injury." Free radical-induced injury can be attenuated by potassium channel openers that act on K_{ATP} channels.²⁶

Although Han *et al.*²⁷ reported that, in rabbit ventricular myocytes, isoflurane inhibits the K_{ATP} channel activities in the inside-out patch-clamp configurations, there are many *in vivo* or isolated whole-heart experiments showing that volatile anesthetics, including halothane, isoflurane, and enflurane, activate K_{ATP} channels and have cardioprotective effects.^{17–23} Kanaya and Fujita²⁸ and Kersten *et al.*^{19,20} reported that isoflurane improves regional myocardial contraction and preserves high-energy phosphate concentrations in a canine model of myocardial stunning. Boutros *et al.*²¹ found in isolated rat hearts that ischemic preconditioning, halothane, and isoflurane provide significant protection against ischemia. In addition, Kersten *et al.*²⁹ demonstrated that pretreatment with isoflurane, similar to ischemic pre-

Fig. 4. The current-voltage relation for adenosine triphosphate-sensitive potassium channels during control conditions and after the application of thiamylal (50 mg/l). The curve is linear in the negative membrane potential range but shows rectification with depolarization beyond zero. Each data point (vertical bars) is presented as the mean \pm SD.



conditioning, produces functional recovery of stunned myocardium in anesthetized dogs. Because the recovery of regional contractile function enhanced by isoflurane is abolished by pretreatment with glibenclamide, 19,20,29 it has been thought that cardiac KATP channels would be activated by volatile anesthetics. In contrast, we found that thiamylal directly inhibits KATP channels in cardiac myocytes. These effects of thiamylal on the K_{ATP} channel activities are similar to those of ketamine. Ko et al. 12 studied the effects of ketamine on single rat ventricular myocytes and revealed that ketamine inhibits the KATP channel activities in a concentration-dependent manner in both the inside-out and the cell-attached patch configurations. The results of the current study and findings reported by Ko et al. 12 suggest that thiamylal and ketamine may increase the extent of myocardial damage during anesthesia.

Our study had several limitations. First, in our cellattached configurations, we used DNP to simulate ischemia. Because DNP inhibits mitochondrial ATP synthesis, we could observe marked opening of K_{ATP} channels. The lag period between DNP exposure and induction of K_{ATP} channel current was a few minutes. This latency is considered to reflect the time for the depletion of endogenous energy sources before intracellular ATP levels decreased. However, the activation of K_{ATP} channels by DNP may be different from that by ischemia or hypoxia. 12 Second, for clinical uses in humans, the peak plasma concentration of thiamylal during induction of general anesthesia is approximately 100-150 mg/l, with an intravenous dose of 4-5 mg/kg.³⁰ In the current investigation, 100 mg/l thiamylal completely inhibited the KATP channel activities. This concentration may be sufficient to inhibit the K_{ATP} channel activities and potentially reverse the antiischemic effects mediated by this channel. However, it should be noted that free drug concentration at the myocyte in situ may be much lower than that stated previously, because its affinity for plasma proteins is very high. Third, high extracellular potassium concentration (140 mm) and a resultant membrane depolarization might have altered the behavior of the channel and the sensitivity of thiamylal. 12 Therefore, we should be careful in extending the current results to the human heart.

In summary, thiamylal inhibits the K_{ATP} channel activities without affecting the channel conductances during simulated ischemia in the cell-attached and inside-out patch-clamp configurations of single rat myocytes. These results suggest that thiamylal inhibits the K_{ATP} channel

activities in a membrane-delimited manner rather than through cytosolic second messengers.

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