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The Effects of Intracellular Monocarboxylates on the ATP-sensitive Potassium Channels in Rabbit Ventricular Myocytes

Nari Kim[1], Jin Han[1], Eui-Yong Kim[1], Won-Kyung Ho[2], and Yung E. Earm[2]

[1]Department of Physiology and Biophysics, College of Medicine, Inje University, Pusan 614–735, Korea; [2]Department of Physiology and Biophysics, College of Medicine, Seoul National University, Seoul 110–799, Korea

A regulating mechanism of the ATP-sensitive potassium channels (K_{ATP} channels) is yet to fully explained. This study was carried out to investigate the effects of intracellular application of monocarboxylates (acetate, formate, lactate, and pyruvate) on K_{ATP} channels in isolated rabbit ventricular myocytes. Single channel currents of K_{ATP} channels were recorded using the excised inside-out or permeabilized attached (open-cell) patch-clamp technique at room temperature. Intracellular application of acetate, formate and pyruvate led to an inhibition of channel activity, whereas intracellular application of lactate increased channel activity. These effects were reversible upon washout. Analysis of single channel kinetics showed that monocarboxylates did not affect open-time constant and close-time constant. These results suggest that monocarboxylates participate in modulating K_{ATP} channels activity in cardiac cells and that modulation of K_{ATP} channels activity may resolve the discrepancy between the low K_i in excised membrane patches and high levels of intracellular ATP concentration during myocardial ischemia or hypoxia.

Key Words: Rabbit ventricular myocytes, ATP-sensitive potassium channels, Monocarboxylates, Patch-clamp technique

INTRODUCTION

Since initial description by Noma (1983), the function of ATP-sensitive K^+ channels (K_{ATP} channels) in the cardiac cell membrane has been the subject of considerable speculation. The activation of the K_{ATP} channels brings about an increase in potassium efflux from myocardial cells and a shortening of action potential duration (APD) during cardiac ischemia, which reduces the risk of arrhythmia (Gasser et al, 1990). And extracellular K^+ accumulation is transmitted to the lumen of coronary vessels, it may serve to decrease coronary vascular resistance and counteract any original coronary vasospasm (Gasser & Dienstl, 1986). However, extracellular $[K^+]$ reaches to 18 mM/L within 10 minutes of ischemia due to reduction of vascular washout (Kleber, 1984). This causes a malignant arrhythmia by increasing the dispersion of electrophysiological properties in cardiac myocytes (Kantor, 1990). The main argument against the opening of the K_{ATP} channels, at least in the initial stage of ischemia, is that intracellular ATP concentration ($[ATP]_i$) has not decreased sufficiently, the channels would remain inhibited. The cytosolic [ATP] during early ischemia in intact heart is maintained typically millimolar levels (Weiss & Lamp, 1989), and the K_i (the concentration at the half-maximal inhibition level) for the K_{ATP} channel in isolated patches is micromolar levels (Noma, 1983). This problem has been discussed extensively with a variety of cell types where the channel exits, and no satisfactory solution has yet been provided. Acetate, major form of acetyl-CoA (active "acetate") in cell, is an essential material in the metabolic pathway of carbohydrates, fatty acids and a few of amino acids. During ischemia, acetate diminishes in cell. Pyruvate converts into acetate in aerobic condition and into lactate in anaerobic con-

Corresponding to: Eui-Yong Kim, Department of Physiology and Biophysics, College of Medicine, Inje University, Gaegeum-Dong, Pusanjin-Ku, Pusan 614-735, Korea (Tel) 82-51-890-6714 (Fax) 82-51-894-4500, E-mail: kimey308@chollian.dacom.co.kr

dition. During ischemia, pyruvate and lactate increase in cell. These have monocarboxyl group and relations with intracellular metabolic pathways. Based on these findings, we predicted that monocarboxylates would affect the K_{ATP} channel activity. To verify this theory, we have investigated the effects of intracellular monocarboxylates on K_{ATP} channel in rabbit ventricular myocytes.

METHODS

Preparation of single isolated cardiac myocytes

New Zealand white rabbits, weighing about 0.8~1.5 kg, were anesthetized with sodium pentobarbital (50 mg/kg i.v.). The heart was rapidly excised through a thoracotomy incision and mounted on a Langendoff perfusion apparatus. To dissociate single ventricular myocytes, we used a modified version of Mitra & Morad's method (1985). Initially, hearts were mounted on Langendorff apparatus and perfused with normal Tyrode solution until all signs of blood were removed with gentle squeezing of the heart. The hearts were then perfused with a normally Ca^{2+}-free Tyrode solution, and then perfused with a Ca^{2+}-free Tyrode solution containing 0.01% collagenase (5 mg/50 ml ,Yakult, Japan). After 15~20 min of collagenase treatment, Kraftbrühe (KB) solution was perfused. After 5 min of perfusion with KB solution, the heart was removed from the cannula, the atria were discarded, and the ventricular walls and septum were cut vertically into four to six pieces. These pieces were gently agitated in a small beaker with KB solution to obtain single cells. Isolated ventricular cells were stored in a KB solution at 4°C and used within 12 h. The rod shape of the cell and the clear striations of sarcomeres were important criteria used in selecting viable cells for study. Experiments were performed at room temperature.

Solutions

Tyrode solution was composed of (in mM): 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 16.6 glucose, 0.33 NaH_2PO_4, 5 N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES); the pH was adjusted to 7.4. In the patch-clamp experiment, the pipette solution contained (in mM): 140 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES; the pH was adjusted to 7.4. The solutions facing the inside of the cell membrane in the patch recordings contained (in mM): 127 KCl, 13 KOH, 1 $MgCl_2$, 10 HEPES, 5 ethylene glycol-bis (b-aminoethyl ether)-N, N, N',N'-tetraacetic acid (EGTA); the pH was adjusted to 7.4. ATP, glibenclamide, acetate, formate, pyruvate, and lactate were obtained from Sigma (St. Louis, MO, USA) and added to the intracellular solutions. The pH was readjusted to 7.4.

Electrophysiological recordings

Single channel activity was measured by using the inside-out patch (Hamill et al, 1981) and permeabilized attached patch technique configurations of the gigaohm seal patch-clamp technique. Channel activity was measured using a patch-clamp amplifier (Axopatch 200A, Axopatch instrument, Burlingarne, CA, USA). The permeabilized attached patch configuration was obtained as follows. After an on-cell patch was formed, β-Escin was perfused in bath solution. Pipettes of 15 to 20 MΩ resistance were pulled from borosilicate glass capillaries using a vertical puller. Their tips were coated with sylgard and fire polished. Membrane currents were stored in digitized format on digital audiotapes using a Biologic DTR-1200 recorder (Grenoble, France). For analysis of single channel activity, the data were transferred to a computer with pClamp software through a 12-bit Lab-master analogue-to-digital converter interface. The threshold for judging the open state was set at one-half of the single channel amplitude. The open-time histogram was formed from continuous recordings of >60 s. Open probability (P_o) was calculated using the formula (1) where t_j is the time spent at current levels corresponding to j=0, 1, 2, ..., N channels in the number of channels active in the patch. P_o was calculated over 60 s records. Data are presented in mean±S.E.M when appropriate.

$$P_o=\left(\sum_{j=1}^{N} t_j j\right)/(T_d N) \quad (1)$$

RESULTS

Properties of K_{ATP} channels in rabbit ventricular myocytes

Using a symmetrical transmembrane K^+ concentra-

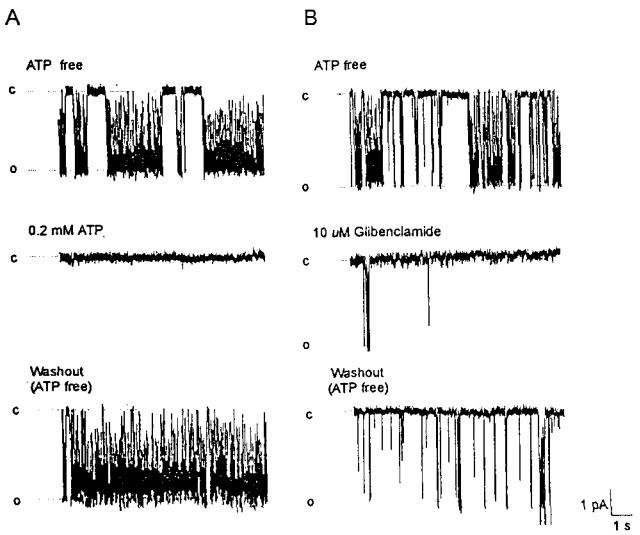

Fig. 1. ATP-sensitive K^+ (K_{ATP}) channels in rabbit ventricular myocytes. **A.** Current recordings were taken from a single patch of an inside-out configuration. The membrane holding potential was −40 mV. The electrode contained 140 mM K^+. During perfusion with ATP-free bathing solution, inward current through K_{ATP} channel increased. In addition of 0.2 mM ATP to the bath solution, it was closed. After washing out ATP, the channel was reactivated. **B.** Effect of 10 μM glibenclamide on K_{ATP} channel. Current recordings in open-cell patch configuration from rabbit ventricular myocytes. Currents flowing from the external to internal side are displayed downwards. K_{ATP} channel current was inhibited by application of 10 μM glibenclamide. This was reactivated partially by washing glibenclamide. "c" dashed line indicates zero current level. "o" dashed line indicates open current level. The data filtered at 1 kHz.

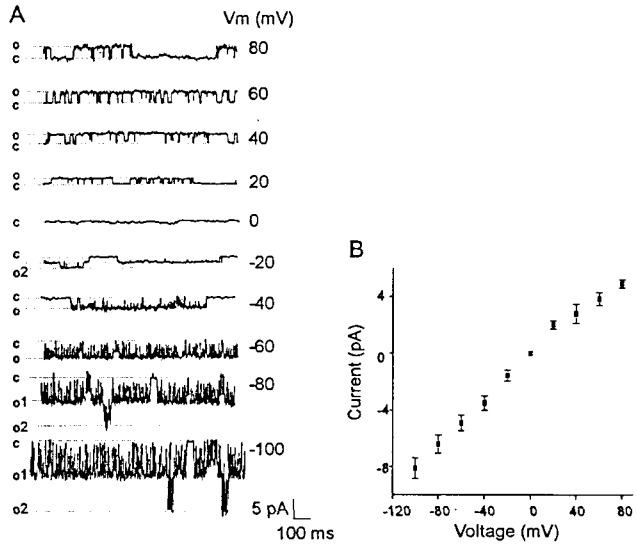

Fig. 2. The current-voltage relation of K_{ATP} channels. **A.** Single channel recordings were taken from an open-cell configuration. Outward currents (depolarization) were associated with long open times, whereas inward currents (hyperpolarization) showed fluctuation and burst kinetics. The zero current level is indicated by "c" dashed line. "o", "o_1" and "o_2" dashed lines show open current level. **B.** Current-voltage relationship of unitary single-channel current. The mean single-channel conductance was 80± 4.5 pS (n=5) and reversal potential was 0 mV. The K^+ concentration of pipette solution and bathing solution was 140 mM. The data filtered at 1 kHz.

tion (140 mM), on formation of the inside-out patches or permeabilized attached patches in ATP-free internal solution, a population of K^+ channels appeared. The channel was inhibited by 0.2 mM ATP or 10 μM glibenclamide, and activity showed recovery on the removal of ATP (Fig. 1A) or glibenclamide (Fig. 1B). Fig. 2A shows the current-voltage relationship of K_{ATP} channels. Outward currents (depolarization) were associated with long open times, whereas inward currents (hyperpolarization) showed fluctuation and burst kinetics. Fig. 2B shows the current-voltage relationship of unitary single-channel current from symmetrical 140 mM K^+ solutions. Reversal potential (E_k) is 0 mV, and hyperpolarizing holding potentials ranging from 0 mV to 100 mV negative to the reversal potential generated a linear I-V relationship with a slope conductance of 80±4.5 pS (n=5). During depolarizing holding potential (positive to E_k) of the same magnitude, the I-V curve displayed moderate inward rectification, with slope conductance becoming nonlinear at strong depolarizing holding potentials. To examine the gating kinetics of the channels, open- and close-time histograms were calculated at a membrane potential of 40 mV negative to E_k, which was analyzed from the current record filtered at a cutoff frequency of 1 kHz. The open-time histogram revealed a single-exponential distribution with a time constant of 4.386 ms in ATP-free solution and 4.084 ms in 0.1 mM ATP solution. The close-time histogram analysis using records filtered at a cutoff frequency of 1 kHz was fitted using a single-exponential function with time constant of 0.290 ms in ATP-free solution and 0.308 ms in 0.1 mM ATP solution. Fig. 3C shows all points amplitude histogram at 40 mV in ATP-free solution. The data filtered at 1 kHz. Bin width is 0.2 ms. The histogram illustrates that single channel current amplitude is 3.35 pA. Fig. 4 shows

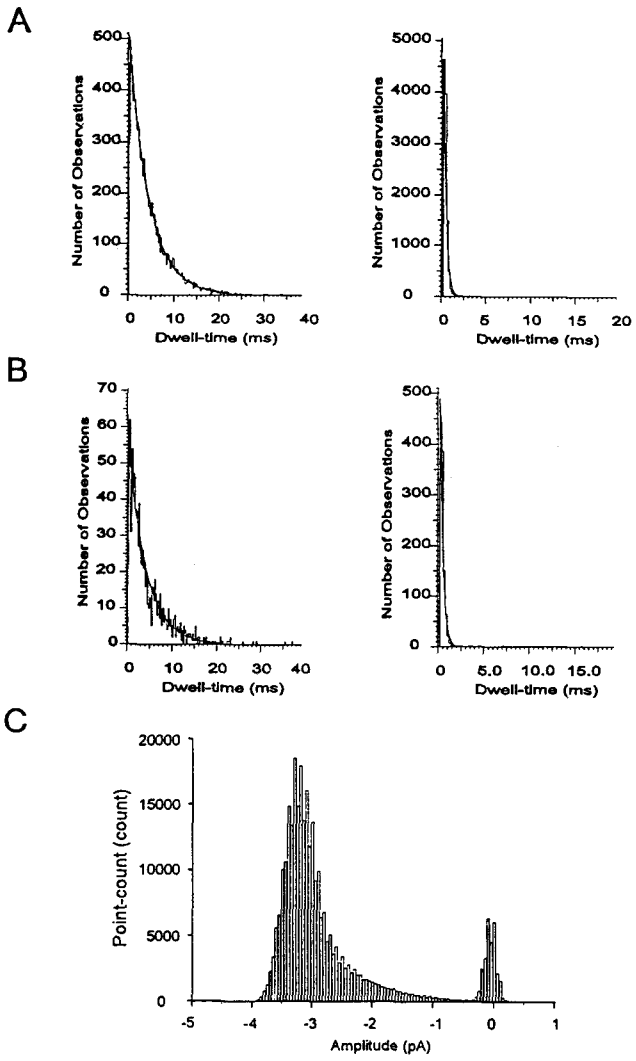

Fig. 3. A. Histograms of open- and close-time distribution in ATP-free solution. Single-channel currents recorded at −40 mV in inside-out patch. The data filtered at 1 kHz. The smooth curves were fitted by the least-squares algorithm to single exponential probability density function. Bin width is 0.2 ms. τ_o and τ_c are 4.386 ms and 0.290 ms. Left panel: open-time histogram, right panel: close-time histogram. **B.** Histograms of open- and close-time distribution at −40 mV in 0.1 mM ATP solution. τ_o and τ_c are 4.084 ms and 0.308 ms. Left panel: open-time histogram, right panel: close-time histogram. **C.** All points amplitude histogram at −40 mV in ATP-free solution. The data filtered at 1 kHz. Bin width is 0.2 ms. This histogram illustrates that single channel current amplitude is 3.35 pA.

apparent dose-response relationship for ATP inhibition of K_{ATP} channels in the inside-out patch configuration. In this and subsequent graphs, the ordinate

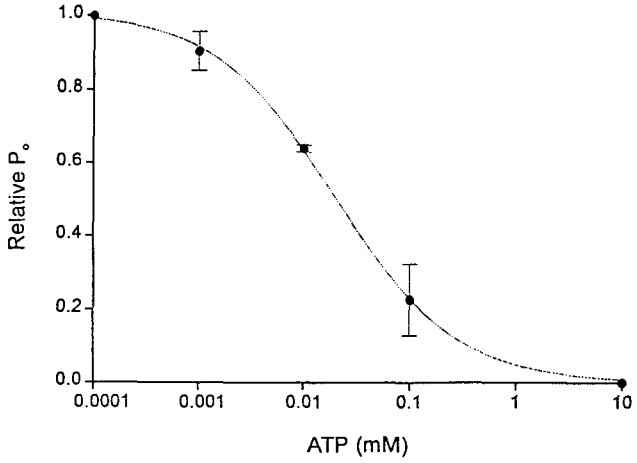

Fig. 4. The ATP dependence of K_{ATP} channel activity. Dose-response relationship for ATP inhibition of K_{ATP} channels. In this, the ordinate represents patch current relative to the current to zero ATP (I_{patch}/I_{max}), the points show means ± S.E.M., and n values are 4. The continuous line is fitted according to the general equation (2) in text. The ATP dependence of channel activity was well fitted by K_i=77 μM and H=1.19. K_i indicates ATP concentration causing half-maximal inhibition and H indicates the Hill coefficient.

represents patch current relative to the current to zero ATP (I_{patch}/I_{max}), the points show mean ± S.E.M., and n values are 4. The continuous line is a Least-squares fit to the general equation (2).

$$\text{Relative } P_o = 1/\{1+([ATP]_i/K_i)\}^H \quad (2)$$

where 'Relative P_o' is the patch P_o relative to the P_o in zero ATP, K_i=[ATP] causing half-maximal inhibition, and H=the Hill coefficient. In this, the ATP dependence of channel activity was well fitted by K_i=77 μM and H=1.19.

The effects of monocarboxylates on K_{ATP} channels

The effect of acetate on K_{ATP} channels: Fig. 5A shows a representative result obtained in the inside-out patch exposed to 0.2 mM acetate with 0.1 mM ATP in bath solution. 0.2 mM acetate inhibited the channel activity (0.0424→0.0055), which was reversible upon washout. Fig. 5B shows the current-voltage relationship. Application of 0.2 mM acetate increases slope conductance in hyperpolarizing holding potentials (70 pS→100 pS). The histograms of open- and close-time, which were analyzed from the current

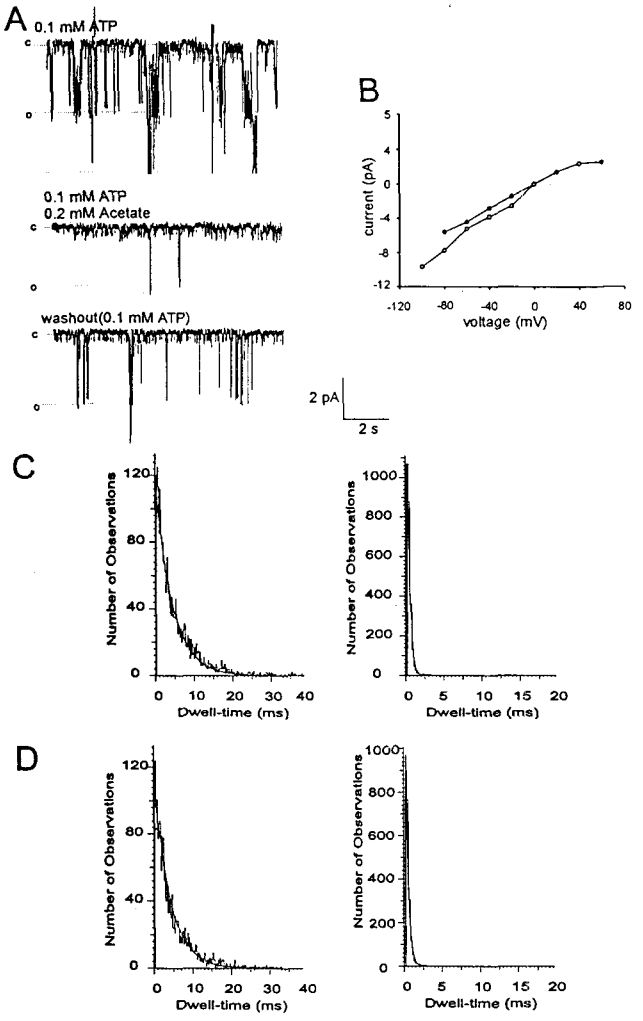

Fig. 5. Effects of acetate on K_{ATP} channel in inside-out patch voltage-clamped at −40 mV. **A.** Current recordings were taken from a single patch of an inside-out configuration. After recording the control current in the presence of 0.1 mM ATP, 0.2 mM acetate was added to the inner surface of the membrane. 0.2 mM acetate inhibited the channel activity, which was reversible upon washout. "c" dashed line indicates zero current level. "o" dashed line indicates open current level. **B.** Current-voltage relationship of unitary single-channel current. Application of acetate increases slope conductance in inward currents. Solid circle; control, open circle; application of acetate. **C.** Histograms of open- and close-time distribution in ATP-free solution. Single-channel currents recorded at −40 mV in inside-out patch. The data filtered at 1 kHz. The smooth curve was fitted by the least-squares algorithm to single exponential probability density function. Bin width is 0.2 ms. τ_o and τ_c are 4.391 ms and 0.309 ms. **D.** Histograms of open- and close-time distribution at −40 mV in the presence of 0.2 mM acetate. τ_o and τ_c are 4.070 ms and 0.325 ms. Left panel: open-time histogram, right panel: close-time histogram.

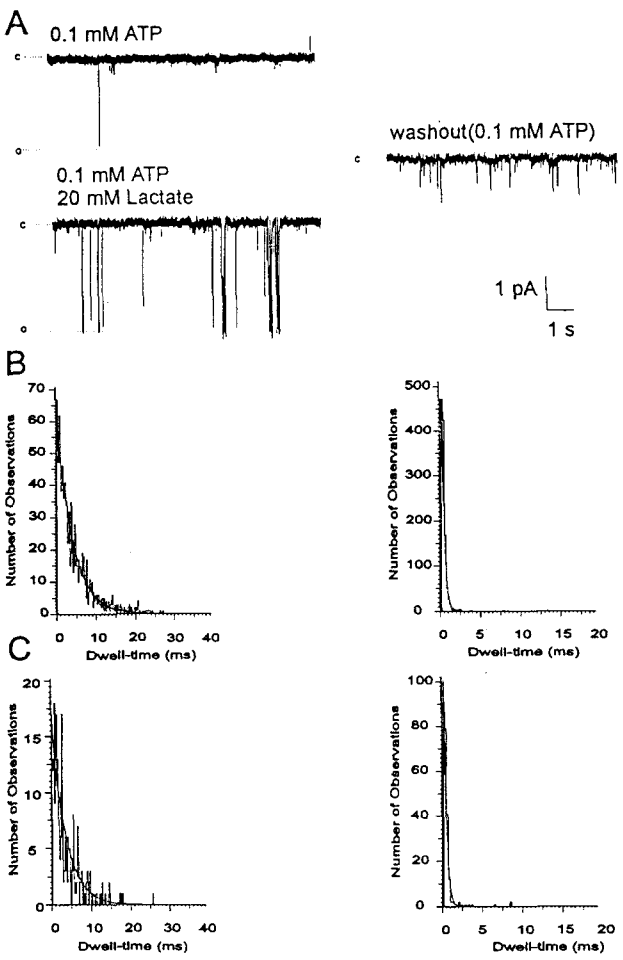

Fig. 6. Effects of lactate on K_{ATP} channel in open-cell patch voltage-clamped at −40 mV. **A.** Current recordings were taken from a single patch of an open-cell configuration. After recording the control current in the presence of 0.1 mM ATP solution in bath, 20 mM lactate was added to the inner surface of the membrane. 20 mM lactate activated the current. "c" dashed line indicates zero current level. "o" dashed line indicates open current level. **B.** Histograms of the open-time and close-time distribution in ATP-free solution. Bin width is 0.2 ms. τ_o and τ_c are 3.991 ms and 0.308 ms. **C.** Histograms of the open-time and close-time distribution in 20 mM lactate τ_o and τ_c are 3.559 ms and 0.310 ms. The data filtered at 1 kHz. Left panel: open-time histogram, right panel: close-time histogram.

record filtered at a cutoff frequency of 1 kHz, revealed a single-exponential distribution. In ATP-free solution, open-time constant (τ_o) was 4.391 ms, and close-time constant (τ_c) was 0.309 ms (Fig. 5C). In the presence of 0.2 mM acetate, τ_o was 4.070 ms, and τ_c was 0.325 ms (Fig. 5D).

The effect of lactate on K_{ATP} channels: Fig. 6A

shows a representative result obtained in the open-cell patch exposed to 20 mM lactate with 0.1 mM ATP in bath solution. 20 mM lactate increased the channel activity (0.001→0.1317), which was reversible upon washout. The histograms of open- and close-time, which was analyzed from the current record filtered at a cutoff frequency of 1 kHz, revealed a single-exponential distribution. In 0.1 mM ATP solution, τ_o was 3.991 ms, and τ_c was 0.308 ms (Fig. 6B). In the presence of 20 mM lactate, τ_o was 3.559 ms, and τ_c was 0.310 ms (Fig. 6C).

The effect of formate on K_{ATP} channels: Fig. 7A shows a representative result obtained in the inside-out patch exposed to 0.5 mM formate with 0.1 mM ATP in bath solution. 0.5 mM formate decreased the channel activity (0.6364→0.0017), which was reversible upon washout. The superposition of openings of the inward rectifier K channel (K_{ir} channel) and K_{ATP} channel was present in this patch. K_{ATP} channel was easily identified by larger conductance (80 pS) than that of K_{ir} channel (30 pS). The histograms of open- and close-time, which were analyzed from the current record filtered at a cutoff frequency of 1 kHz, revealed a single-exponential distribution. In ATP-free solution, τ_o was 2.310 ms, and τ_c was 0.298 ms (Fig. 7B). In the presence of 0.5 mM formate, τ_o was 2.728 ms, and τ_c was 0.242 ms (Fig. 7C).

The effect of pyruvate on K_{ATP} channels: Fig. 8A shows a representative result obtained in the open-cell patch exposed to 20 mM pyruvate with 0.1 mM ATP in bath solution. 20 mM pyruvate decreased the channel activity (0.2295→0.0182), which were

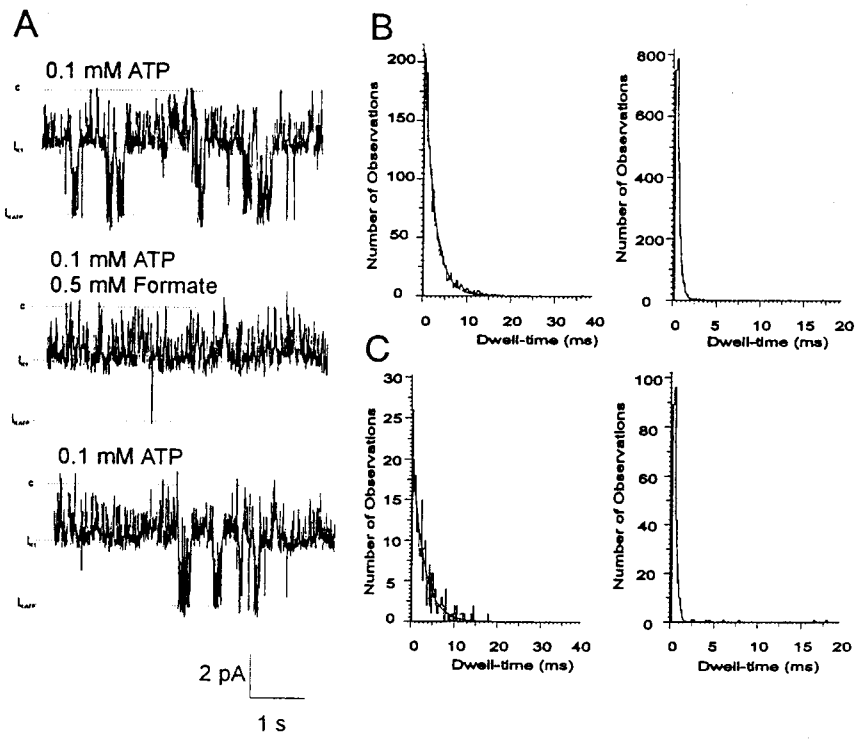

Fig. 7. Effects of formate on K_{ATP} channel in inside-out patch voltage-clamped at −40 mV. **A.** Current recordings were taken from a single patch of an inside-out configuration. After recording the control current with 0.1 mM ATP solution in bath, 0.5 mM formate was added to the inner surface of the membrane. 0.5 mM formate inhibited the current. "c" dashed line indicates zero current level. "I_{K1}" dashed line indicates inward rectifier K current-opening level. "I_{KATP}" dashed line indicates ATP-sensitive K current-opening level. **B.** Histograms of the open- and close-time distribution in ATP-free solution. τ_o and τ_c are 2.310 ms and 0.298 ms. **C.** Histograms of open- and close-time distribution in 0.5 mM formate. τ_o and τ_c are 2.728 ms and 0.242 ms. Bin width is 0.2 ms. The data filtered at 1 kHz. Left panel: open-time, right panel: close-time histogram.

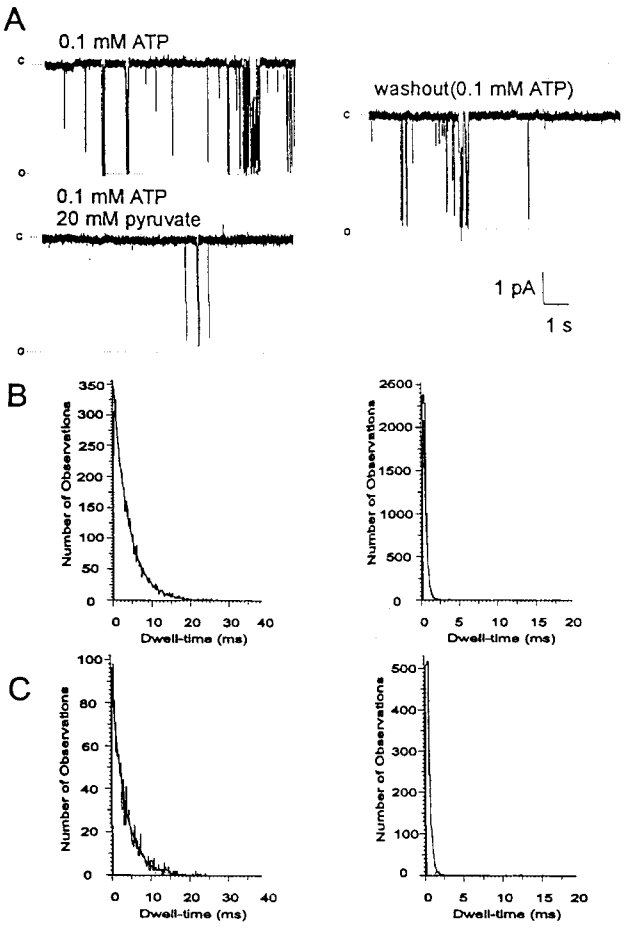

Fig. 8. Effects of pyruvate on K_{ATP} channel in open-cell patch voltage-clamped at −40 mV. **A.** Current recordings were taken from a single patch of an open-cell configuration. After recording the control current with 0.1 mM ATP solution in bath, 20 mM pyruvate was added to the inner surface of the membrane. 20 mM pyruvate inhibited the current. "c" dashed line indicates zero current level. "o" dashed line indicates open current level. **B.** Histograms of open- and close-time distribution in ATP-free solution. τ_o and τ_c are 3.755 ms and 0.317 ms. **C.** Histograms of open- and close-time distribution in 20 mM pyruvate. τ_o and τ_c are 3.22 ms and 0.261 ms. The data filtered at 1 kHz. Bin width is 0.2 ms. Left panel: open-time, right panel: close-time histogram.

reversible upon washout. The histograms of open- and close-time, which was analyzed from the current record filtered at a cutoff frequency of 1 kHz, revealed a single-exponential distribution. In ATP-free solution, τ_o was 3.755 ms, and τ_o was 0.317 ms (Fig. 8B). In the presence of 20 mM pyruvate, τ_o was 3.220 ms, and τ_c was 0.261 ms (Fig. 8C).

DISCUSSION

In cardiac muscle, K_{ATP} channels are inactive under physiological conditions, but open as the intracellular ATP level falls below a critical level. In this study, the inside-out patches or open-cell patches were used to activate K_{ATP} current. The unitary conductance of 80 ± 4.5 pS in 140 mM symmetrical K^+ we have measured in channels from rabbit ventricular myocytes is comparable to the reported values of 62 pS in 155 mM symmetrical K^+ in rat FDB muscle (McKillen et al, 1994) and 74 pS in 160 mM symmetrical K^+ in mouse skeletal muscle (Woll et al, 1989). The ATP concentration causing half-maximal inhibition (K_i) was 77 μM, which also appears similar in different muscle preparations. This concentration is very lower than intracellular ATP concentration in intact cells during early ischemia. The Hill coefficient of 1.19 suggests that the stochiometry for binding may be 1 : 1.

It has been implicated as a possible case of the marked increase in cellular K^+ efflux during ischemia and metabolic inhibition in heart (Noma, 1983). But, increased K^+ efflux can be detected within 30 s of inhibiting oxidative or glycolytic metabolism in heart at a time when total cellular ATP content is depressed only or even normal (Weiss & Hiltbrand, 1985).

Understanding the mechanism of the activity of K_{ATP} channel should provide important insights into discrepancy of ATP concentrations between excised patches and intact cells. Several possibilities have been suggested to account for the observed discrepancy. The estimated density of K_{ATP} channels in ventricular myocytes was estimated at ~5 channels per square micron of sarcolemma (Weiss et al, 1992a). Only a small increase in the open probability of K_{ATP} channel is required to shorten action potential duration during early ischemia or hypoxia (Weiss et al, 1992b). Another possibility is that the concentration of ATP binding sites to K_{ATP} channels may be maintained at a lower level than that of cytoplasm. A third possibility is that a number of intrinsic substances modulate the sensitivity of K_{ATP} channels to ATP (Han et al, 1995; Mubagwa et al, 1996; Kim et al, 1997). It is not the level of ATP itself that regulates the compensatory pathway but the formation of ATP breakdown products (adenosine monophosphate, inorganic phosphate, and adenosine) that acts as the regulator. A number of exogenous factors (ADP, G-pro-

tein, pH) also modify the sensitivity of the channels to ATP (Kurachi, 1982; Davies, 1990; Terzic et al, 1994). In this study, we observed the activation of K_{ATP} channel by intracellular monocarboxylates. During myocardial ischemia, lactate increases from 5 to 20~60 mM due to an inhibition of oxidative glycolysis (Elliot et al, 1989; Murry et al, 1990). In this study, intracellular applications of lactate increased channel activities. Coetzee (1992) demonstrated that the intracellular dialysis of cells with D-lactate (10 mM) led to a more rapid onset of K_{ATP} channel in the same preparations. Han et al (1993) found the activation of K_{ATP} channel by intracellular lactate in inside-out membrane patches isolated from rabbit ventricular myocytes. The exact mechanism underlying the effect of lactate on K_{ATP} channel still remains controversial. Intracellular application of pyruvate, which increases less than lactate during ischemia, decreases the channel activities. Coetzee (1992) demonstrated extracellular pyruvate exerted the inhibitory effect on K_{ATP} channel. Acetate and formate decreased the channel activities, which were reversible upon washout. In this study, ATP concentration is higher than that of half-maximal inhibition to K_{ATP} channel activity. Our results suggest that K_{ATP} channels in the heart may be modulated by intracellular monocarboxylates rather than ATP. Various anions (pyruvate, lactate, acetate, and gluconate) are most likely to act by stabilizing the structure of the channel protein (McKillen, 1994). Variability in negative surface charge density near the ATP-binding site(s) of K_{ATP} channels may in part explain the large differences in the ATP sensitivity of K_{ATP} channels in various tissues (Nicholas et al, 1994). The covalent attachment of a phosphate group affects ion channel function through conformation change. The function also can be affected by a direction electrostatic interaction between the negatively charged phosphate and charged substrates or ligands (Green & Andersen, 1991). Therefore negative charge, carboxyl group of monocarboxylates maybe affect K_{ATP} channel function through conformation change. Further studies are needed to estimate three dimensional structures, determine the relationship of monocarboxylates and K_{ATP} channel and to investigate the effect of monocarboxyl group on surface charge near the ATP-binding site(s) of K_{ATP} channels. Analysis of single channel kinetics showed that monocarboxylates did not affect open- and close-time constants. This suggests that monocarboxylates have no effect on gating mechanism of K_{ATP} channels.

In conclusion, monocarboxylates modulate K_{ATP} channel activity in cardiac cells and may play an important role in activation of K_{ATP} channel even at moderately high levels of cytosolic ATP concentrations during ischemia and hypoxia by reducing the sensitivity of K_{ATP} channel to ATP.

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