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Reduction of Muscarinic K^+ Channel Activity by Transferrin in Ischemic Rat Atrial Myocytes

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It has been demonstrated that an unidentified cytosolic factor(s) reduces K_{ACh} channel function. Therefore, this study attempted to elucidate the cytosolic factor. Fresh cytosol isolated from normal heart (FC) depressed the K_{ACh} channel activity, but cytosol isolated from the ischemic hearts (IC) did not modulate the channel function. Electrophorectic analysis revealed that a protein of ~80 kDa was markedly reduced or even lost in IC. By using peptide sequencing analysis and Western blot, this 80 kDa protein was identified as transferrin (receptor-mediated Fe^{3+} transporter, 76 kDa). Direct application of transferrin (100 nM) to the cytoplasmic side of inside-out patches decreased the open probability (P_o, 12.7±6.4%, n=4) without change in mean open time (τ_o, 98.5±1.3%, n=4). However, the equimolar apotransferrin, which is free of Fe^{3+}, had no effect on the channel activity (N^*P_o, 129.1±13.5%, n=3). Directly applied Fe^{3+} (100 nM) showed results similar to those of transferrin (N^*P_o: 21.1±3.9%, n=5). However Fe^{2+} failed to reduce the channel function (N^*P_o, 106.3±26.8%, n=5). Interestingly, trivalent cation La^{3+} inhibited N^*P_o of the channel (6.1±3.0%, n=3). Taken together, these results suggest that Fe^{3+} bound to transferrin can modulate the K_{ACh} channel function by its electrical property as a polyvalent cation.

Key Words: Muscarinic K^+ channel, Cardiac ischemia, Transferrin, Ferric iron, Cytosolic factor

INTRODUCTION

Ischemic attack causes rapid disturbances in electrical and mechanical function of heart. Extensive studies indicate that interruption of cardiac electrical excitation results from increases in K^+ conductances (gK^+) and intracellular Na^+, and from altered Ca^{2+} homeostasis (Vleugels et al, 1980; Kusuoka & Marbon, 1992; Boyett et al, 1996; Arita & Shigematsu, 1999) . In elevating gK^+ upon ischemia, many kinds of K^+ channels such as ATP-sensitive K^+ (K_{ATP}) channel, Na-activated K^+ channel, and K^+ channels activated by fatty acids or phospholipids, have been suggested to be related (Noma, 1983; Mitani & Shattock, 1992; Boyett et al, 1996). When these channels are highly activated, net K^+ efflux becomes dominant enough to cease the excitability and to make action potential duration short due to an accelerated repolarization process, eventually leading to arrhythmia (Isenberg & Benndorf, 1990). These ischemia-induced complications have been observed commonly in ventricular myocytes.

Cardiac inward rectifier K^+ channels play a critical role in determining resting potential and in setting electrical rhythm (Nichols et al, 1995; Barry & Nerbonne, 1996). Unlikely to ventricular myocytes, ACh-activated K^+ (K_{ACh}) channels are densely present only in atrial myocytes (Breitwieser & Szabo, 1985; Pfaffinger et al, 1985; Kurachi et al, 1986; Krapivinski et al, 1995). K_{ACh} channels determine the resting potential and the firing rate of atria, and more importantly regulate the electrical rhythm in response to the vagal stimulation (Kurachi et al, 1986; Kurachi, 1995). If ischemic or hypoxic stress deteriorates the electrical function of atria, the activation of K_{ACh} channels could be a possible underlying mechanism.

Recent study has shown that cytosolic factor(s) modulated the K_{ACh} channel function. The factor(s)reduced the K_{ACh} channel activity as if channels were in a fully desensitized state (Hong et al, 1996). Unfortunately, this component remained still unidentified (Kim, 1991; Hong et al, 1996). Thus, this study was aimed to examine what kind of cytosolic component(s) was/were responsible for the modulation of the K_{ACh} channel function. To find this factor(s), we first tried to test the cytosolic proteins as candidates responsible for cytosolic factor(s). This was based on the earlier study that both boiled or trypsinized cytosol was ineffective to reverse ATP-induced change in K_{ACh} channel function (Hong et al, 1996). We further hypothesized that the property of the factor(s) could be altered or reduced under ischemic stress.

We found that cytosolic proteins with 68 kDa and 80

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ABBREVIATIONS: FC, fresh cytosol; IC, ischemic cytosol; K_{ACh} channel, ACh-activated K^+ channel; AIKA, ATP-induced increased in K_{ACh} channel activity; CURL, compartment of uncoupling of receptor and ligand; UFAs, unsaturated fatty acids; GPCR, G protein-coupled receptor.

kDa mass were significantly decreased or even lost in ischemic cytosol, compared to normal. Peptide sequencing analysis revealed that the proteins of 68 kDa and 80 kDa were albumin and transferrin, respectively. Of these two, transferrin dramatically inhibited the K_{ACh} channel activity, suggesting that it could be, at least in part, a cytosolic factor responsible for modulating the K_{ACh} channel function under the ischemic condition.

METHODS

Isolation of single atrial myocytes

Atrial myocytes from adult rats (Sprague Dawley, supplied from Yuhan Central Research Institute, Korea) more than 8 weeks old were used.

Whole heart was excised from chest of the rat anesthetized with ketamin HCl and rompum (5 mg/kg, respectively) and then transferred to the Langendorff column. Initially, the isolated heart was perfused hydrostatically (100 cmH_2O) with the bicarbonate/ phosphate- buffered Krebs solution containing 1.8 mM Ca^{2+}, ensuring beats to recover and be kept steadily. After 5 min, the Ca^{2+}-free Krebs solution was followed, which causes the heart to dilate and to stop its beating. Finally, perfusion was switched to a Ca^{2+}-free solution containing 0.4 mg/ml collagenase (Type II, Worthington, USA) for 30 min. All solutions were kept at 37°C during perfusion in a column.

Following the enzyme perfusion, both atria were cut out and into small pieces (2×5 mm). The smaller atrial tissues were gently agitated to make a cell suspension in the experimental 140 mM KCl solution. This cell suspension was mounted on 12 mm round cover slips placed in plastic 35 mm Petri dishes. Cells were kept at 4°C before use.

Preparation of cytosols

Cytosolic proteins were obtained from whole rat hearts. The quickly excised heart was perfused for ~5 min with O_2-saturated Krebs-Henseleit (K-H) solution for the fresh cytosol (FC), and perfused for 2 hrs with N_2-saturated solution for the ischemic cytosol (IC) in a Langendorff column. After perfusion, hearts were weighed and then cut into small pieces in a HEPES buffer and homogenized. Tissues were then centrifugated at 3,300 xg for 15 min, and the supernatant was diluted with concentrated cytosolic extraction buffer (0.3 M HEPES, 1.4 mM KCl, 30 mM $MgCl_2$). Well-mixed supernatant with CE buffer was again centrifugated at 100,000 xg for 1 hr, and the supernatant was collected as cytosolic fractions. For FC, all procedures were performed at 4°C.

SDS-PAGE and N-terminal sequencing analysis

Alteration in cytosolic protein fractions isolated from ischemic hearts was analyzed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE with 15 μg/ml protein per lane was run at 150 V for 4 hrs. The gel was stained with Coomassie brilliant blue R250 (Sigma, MO, USA) diluted to 0.1% with 50% methanol. For peptide analysis, the proteins were electrotransferred onto PVDF membrane after SDS-PAGE, fixed with 10% acetic acid for 15 min and stained with Ponceau S solution (0.5% in 7.5% trichloroacetic acid in water, Gelman Scinences, MI, USA) for 40 min. The membranes were destained with water, and excised bands were then subjected to N-terminal sequence analysis using the protein sequencer (PE 491A, CA, USA) at the Korea Basic Science Institute (Taedok, Korea).

Immunoblot analysis

To identify the protein band of 80 kDa, cytosolic proteins were analyzed by anti-transferrin antibody using Western blot analysis. Cytosolic fraction of whole cardiac muscle was separated by 8% SDS-PAGE and then transferred onto a nitrocellulose membrane. The transferred membrane was incubated with monoclonal antibody (Fitzerald, MA, USA) diluted at a ratio of 1,000-fold and subsequently labeled with alkaline phosphatase-conjugated antimouse IgG (Promega, USA). The bands were visualized by reaction with the substrate solution consisted of nitroblue tetrazolium and 5-Br-4-chloro-3-indolyl phosphate.

Chemicals and experimental solutions

Acetylcholine (ACh) and transferrin were purchased from Sigma Chemical (USA), collagenase (Type II) from the Worthington Chemicals (NJ, USA), ATP and GTP from Boeringer Mannheim (Germany), alkaline phosphatase from Amresco (Ohio, USA), and glibenclamide was from Research Biochemicals International (RBI, USA).

Experimental solution contained; 118.5 mM KCl, 2.0 mM $MgCl_2$, 5 mM KOH/EGTA, and 10 mM KOH/HEPES, and pH adjusted to 7.2 with HCl.

Concentration of ATP and GTP were 1 mM and 100 μ M throughout the study, unless otherwise addressed. ACh concentration in the pipette was steadily kept at 10 μ M. To block K_{ATP} channel, glibenclamide dissolved in dimethyl sulfoxide (DMSO) was used. When chemicals dissolved in DMSO were applied into bath, they were diluted more than a thousand times not to exceed 0.1% of DMSO concentration. Cytoplasmic sides of excised patches were perfused with desired solutions through plastic tubing at a rate of ~2 ml/min.

Transferrin concentration was determined to 100 nM, similar to the plasma level of the transferrin. Ferric iron (Fe^{3+}), ferrous iron (Fe^{2+}), and La^{3+} were kept at 100 nM throughout this study.

Electrophysiology

Gigaseals were formed with Sylgard (Sylgard elastomer 184, Dow Corning, USA) coated pipette of 5 MΩ resistances, and channel currents were recorded using the method described by Hamill et al. (1981). Membrane potential was held at −60 to −80 mV. Channel activities were recorded with a patch clamp amplifier (EPC-7, List, FRG), low-pass filtered at 2 kHz using an eight-pole Bessel filter (AI 2040, Axon Instruments, USA), and stored on magnetic tape for video cassette recorder (Samsung, SV-606) via a pulse code modulator (PCM-2, Medical Systems, USA). Later, digitized data were transferred directly into an IBM-clone personal computer and analyzed to obtain histograms for duration, amplitudes and probabilities as well as channel activity (averaged N^*P_o), using analysis program (pClamp 6.02, Axon Instrument, USA).

Single-channel openings in expanded scale presented in Figures were filtered at 1 kHz, and channel tracings

obtained from pen recorder (Grass 7,400, Astro-Med Inc, USA) were filtered at 100 Hz. All experiments were performed at 22~24°C, and data were presented as means ±SE (standard error). *Student's t-test* was used to check significant differences ($p<0.05$).

Because of multiple channel openings in most patches, accurate measurements of mean open times (τ_o) were not possible. However since alterations in open time duration occurred in these experiments, mean open times were determined by selecting patches having only up to two open channels for general comparison purposes. In this study, patches containing only K_{ACh} channels were used.

RESULTS

Inhibitory action of ATP-induced change in K_{ACh} channel function is lost in ischemic cytosol

During long exposure to ACh, K_{ACh} channels were rapidly desensitized within a minute (Kim, 1991). This desensitization could not be observed in an excised inside-out patch configuration. When adding ATP (1 mM) to bathing solution, the K_{ACh} channel activity increased more than two folds, and Further addition of cytosol reversed the ATP-induced increased in K_{ACh} channel activity (AIKA) by to a level before the addition of ATP (Hong et al, 1996). These results suggest that cytosolic function is quite contrast to that of ATP.

To examine whether ischemic cytosol (IC) lost factor(s) that reversed the ATP effect on the K_{ACh} channel, we compared the effect of normal cytosol (FC) and ischemic cytosol (IC) on the ATP-induced change in a same excised membrane patch (Fig. 1). With the inside-out (I/O) patches, K_{ACh} channel activity was remarkably increased by applying ATP to the cytoplasmic side. As expected, τ_o was increased 2~4 folds during tens of seconds after adding 1 mM ATP (from 1.86±0.20 ms to 3.42±0.81 ms, n=19), and N^*P_o was also doubled (180%). The increased channel activity was maintained after washout of ATP. However, IC (1 mg/ml) applied to the cytoplasmic side failed to reverse AIKA (Fig. 1A and B).

With treatment of IC for 3 min, τ_o was hardly decreased (from 3.04±1.09 to 2.43±0.80, n=5), comparing to that of FC (1.46±0.83, n=3, left in Fig. 1C). Relative N^*P_o was little changed by IC (105.1±82.3%, n=5), but strongly by FC (40.7±9.6%, n=3). These results clearly show that FC (1 mg/ml) recovers ATP-induced changes in τ_o and N^*P_o, but IC did not.

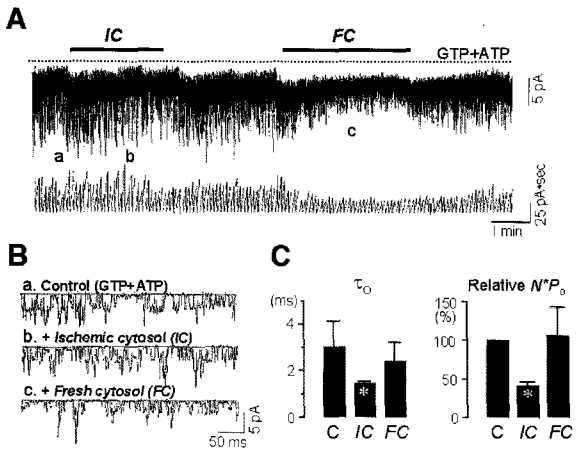

Fig. 1. Effect of ischemic cytosol (*IC*) and fresh cytosol (*FC*) on the K_{ACh} channel activity. A, Representative changes in single channel activity in the presence of *IC* or *FC* at inside-out patch configuration. Ischemic cytosol and fresh cytosol were perfused at least for 2~3 min. Between applications of *IC* or *FC*, ATP was perfused to recover channel function. Relative channel activities at every 5 sec are shown as ramp spikes below the channel current tracing. B, Expanded time scaled channel currents in the presence of IC or FC. Each trace was adopted from tracing A before applying cytosol (a), in the presence of *IC* (b) or *FC* (c). C, Summarized data of the mean open time (left) and relative channel activity (right) as bar plots. Each bar is mean±S.E. from 3~5 patches. (*, $p<0.05$).

The alteration in cytosolic protein of the ischemic heart

Failure of IC suggested that ischemic stress might have altered a factor in the cytosolic fraction, which modulated the K_{ACh} channel function in a normal state. To find the factor(s), we compared the differences in cytosolic proteins profile between IC and FC. SDS-PAGE analysis revealed that 68 kDa and 80 kDa proteins were remarkably decreased or lost in IC (Fig. 2, second and third lanes). Thus, we further challenged the proteins on two bands. To specify whether these proteins have an action to suppress AIKA, we divided whole FC into three fractions, >100 kDa, 100~50 kDa and <50 kDa (left column of Fig. 3). As shown in Fig. 3, 100~50 kDa fraction containing both 68 kDa and 80 kDa proteins was more potent in reducing N^*P_o (78.8%) than the other two fractions (40% for the fraction >100 kDa and 37.4% for the fraction <50 kDa). Interestingly, the 100~50 kDa fraction decreased N^*P_o mainly due to the reduction in open probability (P_o, 21.2%; from 0.033 to 0.007), rather than modulation of τ_o (74.2% from 3.14 ms to 1.93 ms) (Fig. 3C). These results suggest that 68 and 80 kDa proteins might be the cytosolic factor that recover

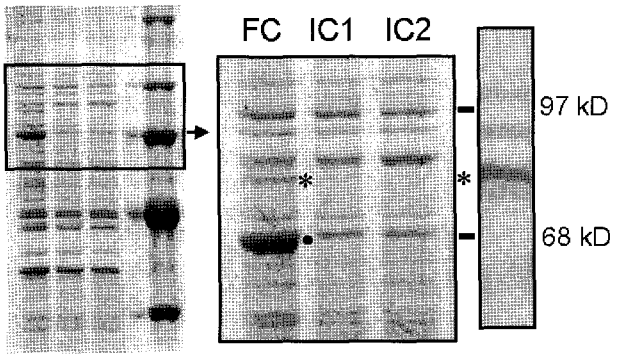

Fig. 2. Ischemia-induced change in cytosolic protein. Fresh cytosols (FC) and two ischemic cytosols, labelled as IC1 and IC2, were loaded from the left. Two band proteins clearly decreased or lost in lanes loaded with ICs are indicated by asterisk (* , ca. 80 kDa) and closed ciricle (• , ca. 68 kDa) in the magnified three lanes of inset in the left. Right lane represents the 80 kDa protein blotted with anti-transferin monoclonal antibody indicated by asterisks (*) with size markers of 97 kDa and 68 kDa indicated by bars. IC1 and IC2 were independently isolated from heart tissue of two rats.

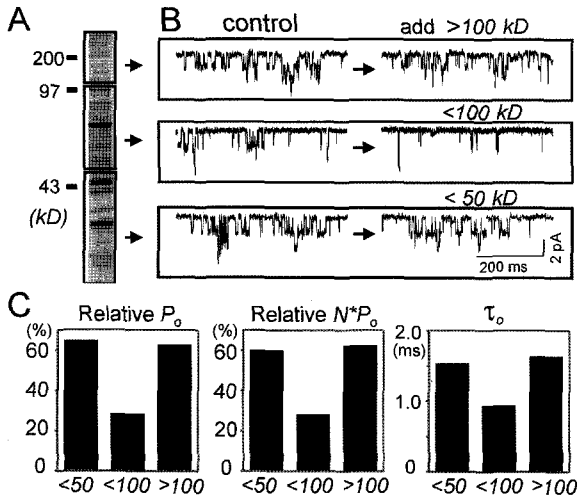

Fig. 3. Effects of the fractionated cytosol on the K_{ACh} channel activity. A, Fractionation of the whole cytosol by the molecular size. Boxes on the single lane of PAGE indicate three fractions, above 100 kDa (>100), 50~100 kDa (<100) and below 50 kDa (<50). B, Changes in the K_{ACh} channel activities by adding each cytosolic fraction are indicated by arrow. Channel currents before and after adding cytosolic fractions (1 mg/ml) are shown in each box. These single channel currents were observed from the different patches. C. Summarized data shown as bar charts. Each data were obtained from B. Data in percent were normalized to those of channel currents recorded before adding the fraction as control.

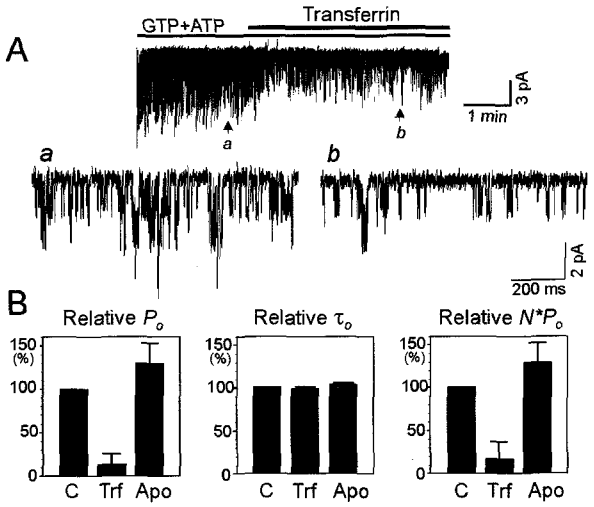

Fig. 4. Modulation of the K_{ACh} channel activity by transferrin. A, Reduced channel activity in the presence of transferrin (100 nM). Expanded channel current tracing recorded before (*a*) and after applying transferrin (*b*) are shown. B, Summarized results shown as bar charts. Relative data were normalized to control (C) recorded in the absence of transferrin (Trf) or apotransferrin (Apo).

AIKA.

Because the 100~50 kD fraction was effective to revert AIKA, we sequenced 11 amino acids (VPDKTVK*XX*AV, where *X* means amino acid unreliable) for 80 kDa and 12 amino acids (EAHKSEIAHRFK) for 68 kDa. From the BLAST Network Service protein database, our search with the amino acid sequence and molecular sizes estimated on PAGE provided that 68 kDa and 80 kDa band proteins matched with albumin (609 amino acids, MW 69,399) and transferrin (698 amino acids, MW 76,393), respectively. Western blot with the monoclonal antibody of transferrin confirmed that the protein of 80 kDa was transferrin (data not shown).

Transferrin suppresses the K_{ACh} channel activity

If albumin and transferrin were decisive factors, these proteins should reverse AIKA. We focused on transferrin as a candidate responsible for the cytosolic function in this experiment, because it has been known that albumin could modulate the K_{ACh} channel function by bound substances such as phospholipids or fatty acid, rather than by the albumin itself (Elliott et al, 1989; Duprat et al, 1995). Furthermore, albumin-bound phospholipids increase the K_{ACh} channel function, whereas albumin-containing fatty acids can suppress the channel function (Kim & Plueum-samran, 2000).

As shown in Fig. 4, transferrin (100 nM) added to the bathing solution (cytoplasmic side) strongly suppressed the channel activity. Remarkable decrease in N^*P_o by transferrin was consistently reproduced (17.1±10.0%, n=4). This was entirely due to the marked reduction in P_o (12.7±6.4%, n=4) rather than in τ_o (98.5±1.3%, n=4), clearly suggesting

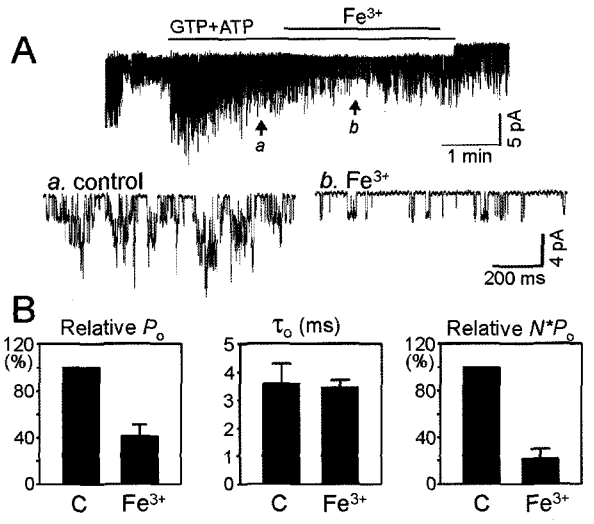

Fig. 5. Effect of ferric iron (Fe^{3+}) on the K_{ACh} channel activity. A. Changes in single K_{ACh} channel currents in the presence of Fe^{3+} (100 nM). B, Summarized bar plots of Fe^{3+}-induced changes in open probability (P_o), mean open time (τ_o), and channel activity (N^*P_o). Relative data normalized to those without application of Fe^{3+} were compared as percentage (%).

that transferrin inhibits the channel openings.

Transferrin has been known to be recycled via receptor-mediated endocytosis and the intrernal CURL, compartment of uncoupling of receptor and ligand (Harding et al, 1983; Dautry-Varsat, 1986). It contains two ferric irons (Fe^{3+}) and releases them to store ferritin in cytoplasm. During its cycling, therefore, released Fe^{3+} may participate in modulating K_{ACh} channel function. To confirm if the effect of transferrin was due to release of Fe^{3+} from transferrin, we examined the effect of apotransferrin, which is free of

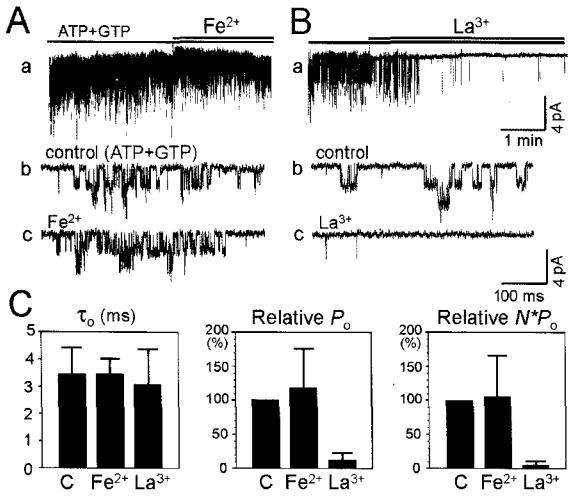

Fig. 6. Reduced K_{ACh} channel activity by trivalent ions. A, Effect of the ferrous iron (Fe^{2+}) on the K_{ACh} channel current. B, Effect of La^{3+} on the K_{ACh} channel current. Representative traces of K_{ACh} channel currents before and after adding 100 nM Fe^{2+} and equimolar La^{3+} are shown at the left and the right, respectively. Both scale bars have same scale of time (200 ms) and current size (2 pA). C, Analyzed data for the effect of Fe^{2+} and La^{3+} on the K_{ACh} channel activity.

Fe^{3+}. As summarized in Fig. 4B, apotransferrin did not affect τ_o (104.1±1.0%, n=3) and N^*P_o (129.1±13.5%, n=3).

Effect of Fe^{3+} on the K_{ACh} channel function

Since apotransferrin was ineffective, free iron (Fe^{3+}) might be a candidate. To examine this assumption, we studied the direct effect of Fe^{3+} on AIKA (Fig. 5). When 100 nM Fe^{3+} was added, ATP-induced increase in K_{ACh} channel activity was quickly reduced (N^*P_o: 21.1±3.9%, n=5). Similar to transferrin, Fe^{3+} dramatically decreased P_o (40.7±4.5%, n=5) without any significant change of τ_o (from 3.58±0.33 ms to 3.45±0.12 ms, n=5).

Next, we attempted to discriminate whether the inhibitory effect of Fe^{3+} was due to its electrical trivalent properties or a specific property of iron independent of the electrical charge. To examine this, we compared ATP-induced changes in K_{ACh} channel function in response to ferrous iron (Fe^{2+}) or trivalent La^{3+} (Fig. 6). Equimolar Fe^{2+} (100 nM) was ineffective, and it rather slightly increased N^*P_o (106.3±26.8%, n=5) without changing the open time (3.45±0.44 vs 3.46±0.25 ms, n=5). In contrast to Fe^{2+}, La^{3+} (100 nM) completely reduced the channel activity (6.1± 3.0%, n=3, Fig. 6B), preferentially due to the decrease in P_o (12.2±5.9%, n=3, Fig. 6C). After washout of La^{3+}, the channel activity was not recovered. These results strongly indicate that the K_{ACh} channel was suppressed not by transferrin, but by trivalent electrical charge of iron bound to transferrin.

DISCUSSION

This study first shows that ferric iron (Fe^{3+}) bound in transferrin directly regulates the K_{ACh} channel function in atrial tissue. Our data also provided that ischemic cytosol (IC) failed to modulate channel function due to loss or reduction of transferrin during cardiac ischemia. This finding was clearly demonstrated by results that either Fe^{3+} or transferrin potently inhibited the K_{ACh} channel activity but apotransferrin did not.

We assumed that K_{ACh} channels should be involved in elevating atrial gK^+ at early ischemia in atrial tissue, since these channels densely present in atria are critical in keeping atrial resting potential and regulating cardiac rhythm (20, 21). In the preliminary examination under an ischemic condition, K_{ACh} channel activity was dramatically increased about four-folds by application of KCN (unpublished data), thus supporting our assumption.

Dramatic change in the K_{ACh} channel activity could be observed in desensitization process (Kim, 1991). During the rapid decline phase (~30 s) in desensitization, the channel activity (N^*P_o) was rapidly decayed due to decrease in mean open time (τ_o). Previous studies indicated that cytosolic protein(s) was responsible for this decaying process (Hong et al, 1996). If this decay did not occur under ischemic condition, cytosolic factor(s) might have been denatured or suppressed. In the experiment to assess the responsible protein, transferrin (79 kDa) was found to be lost in the ischemic cytosol (IC) (see Fig. 2).

Transferrin transports two Fe^{3+} into the intracellular compartment by receptor-mediated endocytosis. Complex of transferrin bound to its specific receptor forms clathrin-coated pits and subsequently so-called CURL (compartment of uncoupling of receptor and ligand) via formation of an endosome. With ATP-driven proton pump, acidified endosome and CURL release Fe^{3+} from transferrin, since acidity leads to the dissociation of protein-receptor complexes and lowers the affinity of transferrin for Fe^{3+}. Finally Fe^{3+}-free transporter, apotransferrin leaves the cell interior and recovers affinity for Fe^{3+} in plasma of pH 7.4. It takes sixteen minutes in HepG2 cells for one cycle (Stryer, 1995). During this recycling, ATP is required in both processes of the internalization of transferrin-receptor complex and releasing Fe^{3+} from endosome and CURL. In an ischemic or hypoxic state, both intracellular level of Fe^{3+} and transferrin might be decreased due to lowered level of ATP. Since the lower level of transferrin or Fe^{3+} could not play its potent inhibitory action any more, it could not decrease the K_{ACh} channel activity. Therefore, it is likely that the K_{ACh} channel maintains an elevated activity due to the loss of an inhibitory action of transferrin or Fe^{3+} under the ischemic condition.

Mechanism of action of transferrin on the K_{ACh} channel function

The present study implicated two possibilities for the transferrin-blockade of the K_{ACh} channel. One is due to an electrical charge of Fe^{3+} bound in transferrin. This is based on observations that Fe^{3+} or La^{3+} could reduce N^*P_o of the K_{ACh} channel while ferrous ion (Fe^{2+}) could not (Fig. 5 and Fig. 6). It has been well established that polyvalent molecule such as spermine (IC_{50}=~10 nM) binds to a specific site of the cytoplasmic side of the channel, thereby pluging the pore to block the inward rectifiers (Lopatin et al, 1995; Yamada & Kurachi, 1995). For the K_{ATP} channel, negatively charged phosphoinositol 4,5-biphosphate (PIP_2) in the membrane interacts with several positively charged residues in the COOH terminus, thereby shifting confor-

mation of the channel to the active state and resulting in the increased N^*P_o (Fan & Makielski, 1999). Similar to the property of sperimine, Fe^{3+} is likely to play an inhibitory action on the K_{ACh} channel as a member of the GIRK channel family.

The other is that Fe^{3+} can be considered as a redox molecule. Free radicals increased the current of inward rectifier K^+ channels (Duprat et al, 1995; Jeglitsch et al, 1999), and reactive oxygen species (ROS) generated from hypoxanthine/xanthine oxidase system increased the K_{ACh} current without involvement of the G protein or even in the absence of ACh (Jeglitsch et al, 1999). Xanthine oxidase catalyses the formation of uric acid from either xanthine or hypoxanthine. During this process, Fe^{3+} generates ROS such as the hydroxyl radical (OH*) (Halliwell & Gutteridge, 1999). Therefore, the ROS generated might affect the channel conformation and/or the channel regulator to alter the K_{ACh} channel activity under ischemia. Under low O_2 condition and lower pH, Fe^{3+} can be reduced to Fe^{2+}, and reduced Fe^{2+} is ineffective on the K_{ACh} channel activity (Fig. 6). Further study is required to clarify the effect of Fe^{3+} as a redox molecule on the K_{ACh} channel activity.

Can transferrin be a cytosolic factor for modulating the K_{ACh} channel function?

To be a responsible factor, transferrin should counteract the enhancement of the K_{ACh} channel function. During KCN-induced ischemia, striking increase (~4 times) in a mean open time (τ_o) gave rise to increased channel activity (N^*P_o) of the K_{ACh} channel (unpublished data). However, transferrin inhibited N^*P_o by severely decreased P_o without a significant change in τ_o (Fig. 4). Rather than transferrin, ferric iron (Fe^{3+}) directly decreased the channel activity. Therefore, it is unlikely that transferrin is a correct responsible factor.

Recently, unsaturated fatty acids (UFAs) have been emerged as an alternative candidate for the cytosolic factor, since UFAs inhibit N^*P_o by reducing τ_o rather than P_o (Kim & Plueumsamran, 2000). Furthermore free fatty acid (FFA) concentration was increased to alter the K_{ACh} channel function, leading to loss of the cardiac excitability under pathological state such as hypoxia and ischemia. Study on isolated ischemic rat hearts indicated that fatty acid did not accumulate earlier than 20~45 min after the onset of ischemia (Van Bilsen et al, 1989), although long-chain polyunsaturated linoleic and arachidonic fatty acids were highly accumulated 120 min after the onset of ischemia (Van Der Vusse et al, 1982). Considering that acute ischemic attack or KCN rapidly inhibited the K_{ACh} channel function within ~1 min, an increase in N^*P_o could not be entirely attributed by accumulation of UFAs at the onset of ischemia.

Internalization or endocytosis of the related receptor such as the G protein-coupled receptor (GPCR) should also be considered, because the K_{ACh} channel gated by $G\beta\gamma$ is activated by typical GPCR, muscarinic m2 receptors (m2AChR). Desensitization of m2AChR is commonly regarded as the internalization of GPCR (Bunemann & Hosey, 1999; Shui et al, 2001). If ischemia-induced increase in the K_{ACh} channel function were considered to be due to an impairement of the rapid desensitization phase, then ischemic stress might interrupt cytosolic component(s) involved in both internalization or endocytosis of GPCRs and in pathway linked with the K_{ACh} channel protein. This implication was made, because ischemia interrupted the receptor-mediated endocytosis of transferrin (unpublished data). However it remains unclear whether the effect of ischemia on the endocytosis of m2AChR is linked to desensitization of the K_{ACh} channel.

In summary, the present study showed for the first time that the atrial K_{ACh} channel activity was rapidly increased at the early stage of ischemia, at least partly, by decreased cytosolic transferrin and bound Fe^{3+}.

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REFERENCES

Arita M, Shigematsu S. Role of ATP-sensitive potassium channels in ischemia/referfusion-induced ventricular arrythmias. In: Kurachi Y, Jan LL, Lazdunski M, (eds) *Potassium Ion Channels molecular structure, Function, and Disease, Current topics in membranes*, Vol 46 (Chap 22) Academic Press, p 417−434, 1999

Barry DM, Nerbonne. Myocardial potassium channels: Electrophysiological and molecular diversity. *Annu Rev Physiol* 58: 363−394, 1996

Boyett MR, Harrison SM, White E. Potassium channels and cardiac contraction. *In K channels in Cardiovascular Medicine, From basic science to clinical practice* (Escande, D. and Standen, N., editors). Springer-Verlag, p 69−85, 1993

Breitwieser G, Szabo G. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* 317: 538−540, 1985

Bunemann M, Hosey MM. G-protein coupled receptor kinases as modulators of G-protein signalling. *J Physiol* 517: 5−23, 1999

Dautry-Varsat A. Receptor-mediated endocytosis: the intracellular journey of transferrin and its receptor. *Biochimie* 68(3): 375−381, 1986

Duprat F, Guillemare E, Romey G, Fink M, Lesage F, Lazdunski M, Honore E. Susceptibility of cloned K^+ channels to reactive oxygen species. *Proc Natl Acad Sci USA* 92(25): 11796−11800, 1995

Elliott AC, Smith GL, Allen DG. Simultaneous measurements of action potential duration and intracellular ATP in isolated ferret hearts exposed to cyanide. *Circ Res* 64(3): 583−591, 1989

Fan Z, Makielski JC. Phosphoinositides decrease ATP sensitivity of the cardiac ATP-sensitive K^+ channel. A molecular probe for the mechanism of ATP-sensitive inhibition. *J Biol Chem* 272: 5388−5395, 1999

Halliwell B, Gutteridge JMC. Free radicals, other reactive species and disease (Chap 9). *In Free radicals in Biology and Medicine. 3rd ed.*, Oxford University Press, New York, p 645−655, 1999

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391(2): 85−100, 1981

Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* 97(2): 329−339, 1983

Hong SG, Pleumsamran A, Kim D. Regulation of atrial muscarinic K^+ channel activity by a cytosolic protein via G protein-independent pathway. *Am J Physiol* 270: H526−H537, 1996

Isenberg G, Benndorf. Action potential and membrane currents during metabolic inhibition. In: J. Vereecke, van Bogaert PP and Verdonck F, (*eds*) *Ionic currents and ischemia*. Leuven University Press, Krakenstraat, Belgium, p 77−97, 1990

Jeglitsch G, Ramos P, Encabo A, Tritthart HA, Esterbauer H, Groschner K, Schreibmayer W. The cardiac acetylcholine-activated, inwardly rectifying K^+-channel subunit GIRK1 gives

rise to an inward current induced by free oxygen radicals. *Free Radic Biol Med* 26(3−4): 253−259, 1999

Kim D. Modulation of acetylcholine-activated K^+ channel function in rat atrial cells by phosphorylation. *J Physiol* 473: 133−155, 1991

Kim D, Plueumsamran A. Cytoplasmic unsaturated free fatty acid inhibit ATP-dependent gating of the G protein-gated K^+ channel. *J Gen Physiol* 115: 287−304, 2000

Krapivinski G, Gordon EA, Wickman K, Velimiroviy B, Krapivinski L, Clapham DE. The G-protein-gated atrial K^+ channel $I_{K,Ach}$ is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature* 374: 135−141, 1995

Kurachi Y. G protein regulation of cardiac muscarinic potassium channel. *Am J Physiol* 269: C821−C830, 1995

Kurachi Y, Nakajima T, Sugimoto T. Acetylcholine activation of K^+ channels in cell-free membrane of atrial cells. *Am J Physiol* 251: H681−H684, 1986

Kusuoka H, Marbon E. Cellular mechanisms of myocardial stunning. *Annu Rev Physiol* 54: 234−256, 1992

Lopatin, AN, Makhina EN and Nichols CG. The mechanism of inward rectification of potassium channels: "long-pore plugging" by cytoplasmic polyamines. *J Gen Physiol* 106(5): 923−955, 1995

Mitani A, Shattock MJ. Role of Na-activated K channel, Na-K-Cl cotransport, and Na-K pump in $[K]_e$ changes during ischemia in rat heart. *Am J Physiol* 263: H333−340, 1992

Nichols, CG, Makhina EN, Pearson WL, Sha Q, Lopatin AN. Inward rectification and implications for cardiac excitability. *Circ Res* 78: 1−7, 1995

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

Pfaffinger PJ, Martin JM, Hunter DD, Nathanson NM, Hille B. GTP-binding proteins couple cardiac muscarinic receptors to a K^+ channel. *Nature* 317: 536−538, 1985

Shui Z, Yamanushi TT, Boyett MR. Evidence of involvement of GIRK1/GIRK4 in long-term desensitization of cardiac muscarinic K^+ channels. *Am J Physiol* 280: H2554−H2562, 2001

Van Bilsen M, Van Der Vusse GJ, Willemsen PHM, Coumans WA, Roemen THM, Roneman RS. Lipid alterations in isolated, working rat hearts guring ischemia and reperfusion: its relation to myocardial gamage. *Circ Res* 64: 304−314, 1989

Van Der Vusse GJ, Roeman THM, Prinzen FW, Coumans WA, Reneman RS. Uptake and tissue content of fatty acids in dog myocardium under normoxic and ischemic conditions. *Circ Res* 50: 538−546, 1982

Vleugels A, Vereecke J, Carmeliet EE. Ionic current during hypoxia in voltage clamped cat ventricular muscle. *Circ Res* 47: 501−508, 1980

Yamada, M. and Kurachi, Y. Spermine gates inward-rectifying muscarinic but not ATP-sensitive K^+ channels in rabbit atrial myocytes. Intracellular substance-mediated mechanism of inward rectification. *J Biol Chem* 270(16): 9289−9294, 1995