心不全と心房細動におけるミトコンドリア ATP 感受性 K^+ チャネルのリモデリング

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研究代表者　佐藤俊明

（千葉大学大学院医学研究院助教授）

はしがき

近年，ミトコンドリア膜には様々なイオンチャネルが存在し，細胞の生死に深く関与していることが明らかとなった。われわれは，ミトコンドリア ATP 感受性 K^+（$mitoK_{ATP}$）チャネルが虚血プレコンディショニング（可逆性虚血によって心筋が虚血耐性を獲得する内因性心筋防御機構）に重要な役割を担っていることを報告した。心筋細胞表面膜のイオンチャネルは，心不全・心肥大あるいは心房細動によってリモデリングが起こることが知られている。しかしながら，心筋細胞ミトコンドリアイオンチャネルのリモデリングに関する知見はこれまで全く得られていない。本研究は，$mitoK_{ATP}$ チャネルのリモデリングの病態を明らかにし，心不全におけるプレコンディショニングの修飾，あるいは心房細動の発生・慢性化との関連を解明することを目的に行なわれた。

実験は，BIO 14.6 ハムスターを心不全モデルとして用いて行なった。われわれの研究室は，この BIO 14.6 ハムスターでは心筋細胞表面膜イオンチャネルのリモデリングが起こり，L 型 Ca^{2+} チャネル電流（I_{Ca}）や一過性外向き電流（I_{to}）などが減少して活動電位持続時間が延長していることを報告した。そこで，$mitoK_{ATP}$ チャネル活性化の指標としてフラボプロテイン自家蛍光を測定し，$mitoK_{ATP}$ チャネル開口薬であるジアゾキシドの効果を検討した。その結果，BIO 14.6 ハムスター心室筋細胞では $mitoK_{ATP}$ チャネルの密度は減少していないと考えられる。しかしながら，フラボプロテイン酸化反応の latency が延長しているので，$mitoK_{ATP}$ チャネル活性化の細胞内情報伝達系とくに PKC に対する応答に異常があるのではないかと推測される。

今回の実験結果は，$mitoK_{ATP}$ チャネルにリモデリングが起こっている可能性を示唆するものである。最近，$mitoK_{ATP}$ チャネルの分子構造は，ミトコンドリア内膜にある ABC 蛋白のひとつである MABC1 と succinate dehydrogenase がその構

成分子であることが示唆されている。しかしながら，チャネル孔を構成する分子の同定には至っていない。今後，mitoK$_{ATP}$チャネルの分子構造が解明されれば，チャネルのリモデリングに関する新たな知見も得られることが期待できる。また，心房細動におけるmitoK$_{ATP}$チャネルのリモデリングやmitoK$_{ATP}$チャネル以外のミトコンドリアイオンチャネル（Ca^{2+}活性化K^{+}チャネルなど）のリモデリングなど解明すべき課題が多く残されている。本研究がその一助となれば幸いである。

研究組織

研究代表者　佐藤俊明（千葉大学・大学院医学研究院・助教授）

研究分担者　中谷晴昭（千葉大学・大学院医学研究院・教授）

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研究成果

心不全ハムスター（BIO 14.6）におけるミトコンドリア ATP 感受性 K^+チャネルのリモデリング

佐藤俊明，中谷晴昭

千葉大学大学院医学研究院薬理学

Ⅰ　はじめに

心筋細胞のイオンチャネルは，心不全・心肥大あるいは心房細動によってリモデリングが起こることが知られている[1]。心筋細胞ミトコンドリア内膜にある ATP 感受性 K^+（mitoK$_{ATP}$）チャネルは，虚血プレコンディショニング（可逆性虚血によって心筋が虚血耐性を獲得する現象）に重要な役割を担っている[2]。リモデリング心筋ではプレコンディヨニングによる心筋保護効果が減弱することが報告されているが[3]，これが mitoK$_{ATP}$チャネルのリモデリングに起因するかどうかは全く不明である。よって本研究の目的は，mitoK$_{ATP}$チャネルのリモデリングの病態を明らかにし，心不全におけるプレコンディショニングの修飾，あるいは心房細動の発生・慢性化との関連を解明することである。

Ⅱ　方法

心不全モデルとして BIO 14.6 ハムスター[4]，対照群として F1β ハムスターを用いて実験を行なった。

(1) BIO 14.6 と F1β ハムスターの心室筋細胞をそれぞれコラゲナーゼ処理により単離し実験に供した。ミトコンドリアの酸化還元状態を安定させるため，単

離した心室筋細胞を培養液（5%牛血清を含むDMEM）に少なくとも3時間以上保存した。

(2) 心室筋細胞を蛍光顕微鏡ステージに置いたチャンバーに入れ，グルコースを含まないTyrode液で灌流した。Tyrode液の組成は，NaCl 140, KCl 5.4, $CaCl_2$ 1.8, NaH_2PO_4 0.33, $MgCl_2$ 0.5, HEPES 5 (pH=7.4)である。

(3) $MitoK_{ATP}$チャネル活性化の指標としてフラボプロテイン自家蛍光を測定した。$MitoK_{ATP}$チャネルの開口によりK^+が細胞質からマトリックスへ流入すると，呼吸鎖におけるH^+の排出が促進されるためフラボプロテイン酸化反応が亢進して蛍光が増強する。フラボプロテイン自家蛍光は励起波長480 nm，測光波長520 nmにて測定した[5]。

(4) 蛍光強度は脱共役薬である2,4-dinitrophenol (DNP) を実験終了時に添加して，最大酸化反応を起こしたときのフラボプロテイン自家蛍光を100%とした相対値で表示した。

(5) データは平均値±標準誤差で表示してある。有意差の検定はANOVA (Fisher's post hoc test) を用いて，$p<0.05$を有意差ありとみなした。

Ⅲ 結果

心筋細胞にmitoK$_{ATP}$チャネルの選択的開口薬であるdiazoxideを添加するとフラボプロテイン自家蛍光の強度が増加する[5]。図1AとBIO 14.6とF1βハムスターの心筋細胞にdiazoxide (100 μM) を添加したときのフラボプロテイン自家蛍光の経時的変化の典型例をそれぞれ示している。BIO 14.6の心筋細胞は，diazoxideを添加してからフラボプロテイン自家化蛍光が増加しはじめるまでの時間 (latency) が約15分あり，そのあと蛍光は徐々に増加してDNPの蛍光強度の約40%まで達した。一方，対照群であるF1βの心筋細胞では，latencyが

BIO 14.6 より短く（約 8 分間），そのあとフラボプロテイン自家蛍光は DNP の蛍光強度の約 40%まで増加した。図 1C は diazoxide（100 μM）によるフラボプロテイン酸化反応を多数例でまとめたものである。BIO 14.6 は 48.1±5.7%，F1β は 45.1±5.6%までフラボプロテイン自家蛍光がそれぞれ増加し，両者に差はみられなかった。しかしながら，図 1D に示すごとく，BIO 14.6 の latency は 15.2±0.7 分，F1β は 8.0±1.2 分であり，BIO 14.6 のほうが有意に延長していた。

われわれは，mitoK$_{ATP}$ チャネルが protein kinase C（PKC）により活性化されると，latency が短縮することを報告した [5,6]。そこで，PKC を活性化する bradykinin の BIO 14.6 心筋細胞 mitoK$_{ATP}$ チャネルに対する作用を検討した。図 2A にフラボプロテイン自家蛍光の経時的変化の典型例を示す。BIO 14.6 の心筋細胞は，diazoxide（100 μM）を添加すると約 15 分の latency があり，そのあと DNP の蛍光強度の約 50%まで増加した。Diazoxide を washout したあと，bradykinin（1 μM）を単独で添加してもフラボプロテイン自家蛍光は変化しなかった。しかしながら，bradykinin 存在下に diazoxide を添加すると，非存在下と比較して，latency は約 7 分へ短縮し，フラボプロテイン自家蛍光も DNP の蛍光強度の約 70%まで増加した。図 2B に示すように，diazoxide 単独では 43.8±6.8%であったフラボプロテイン自家蛍光が，bradykinin 添加により 63.7±3.6%に有意に増加した（$p<0.05$）。また，図 2C に示すように，bradykinin 存在下では latency が 16.2±0.7 分から 8.4±0.7 分へ有意に短縮した（$p<0.05$）。すなわち，bradykinin により diazoxide のフラボプロテイン酸化反応が増強した。

Ⅳ　考察

本研究では心筋症ハムスターBIO 14.6 を用いて mitoK$_{ATP}$ チャネルの機能解析

を行なった。このハムスターでは心筋細胞表面膜のイオンチャネルのリモデリングが起こり，L型Ca^{2+}チャネル電流（I_{Ca}）や一過性外向き電流（I_{to}）が減少して活動電位持続時間が延長している[7]。今回の実験では，BIO 14.6 ハムスター心筋細胞は，対照群であるF1βと比較して，mitoK_{ATP}チャネル活性化の指標であるフラボプロテイン酸化反応の latency は延長していた。しかしながら，フラボプロテイン自家蛍光の増加の程度には差がなかった。両群で同程度の酸化反応が起こったことから，mitoK_{ATP}チャネルの密度は BIO 14.6 心筋細胞で減少していないと推察される。BIO 14.6 心筋細胞の latency が bradykinin により短縮し，かつ酸化反応も増強した。この効果は，adenosine が PKC を介して mitoK_{ATP}チャネルを活性化し latency を短縮させることと一致している[6]。したがって，BIO 14.6 心筋細胞は，細胞内情報伝達系とくに PKC の活性化に異常があるのではないかと推測される。実際，BIO 14.6 では PKC の活性化が低下しているとの報告もあり[8]，この点はさらに検討をする必要がある。

今回は心室筋細胞を用いて検討したが，心房筋細胞の mitoK_{ATP}チャネル機能や心房細動におけるリモデリングについても今後検討する予定である。最近，mitoK_{ATP}チャネルの分子構造は，ミトコンドリア内膜にある ABC 蛋白のひとつである MABC1 と succinate dehydrogenase がその構成分子であることが示唆されている[9]。残念ながら，チャネル孔を構成する分子の同定にはまだ至っていない。今後，mitoK_{ATP}チャネルの分子構造が解明されれば，チャネルのリモデリングに関する新たな知見も得られるであろう。最近，心筋細胞のミトコンドリア内膜にはCa^{2+}活性化K^+（mitoK_{Ca}）チャネルが存在することが報告された[10]。われわれは，mitoK_{Ca}チャネルが protein kinase A によって活性化され，mitoK_{ATP}チャネルと同様にミトコンドリア内 Ca^{2+}過負荷を抑制して心筋保護的に働くことを報告した[11]。この mitoK_{Ca}チャネルのリモデリングについても，非常に興味のあ

る重要な研究課題であり，今後検討したいと考えている。

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図1

BIO 14.6とF1βハムスター心筋細胞におけるdiazoxideのフラボプロテイン酸化反応。DIAZ: diazoxide(100 μM), DNP: 2.4-dinitrophenol(100 μM)。＊p<0.05 vs. F1β。

図2

BIO 14.6ハムスター心筋細胞におけるdiazoxideとbradykininのフラボプロテイン酸化反応。DIAZ: diazoxide (100 μM), BK: bradykinin (1 μM), DNP: 2.4-dinitrophenol (100 μM)。＊p<0.05 vs. DIAZ。

Figure 1, Sato *et al*

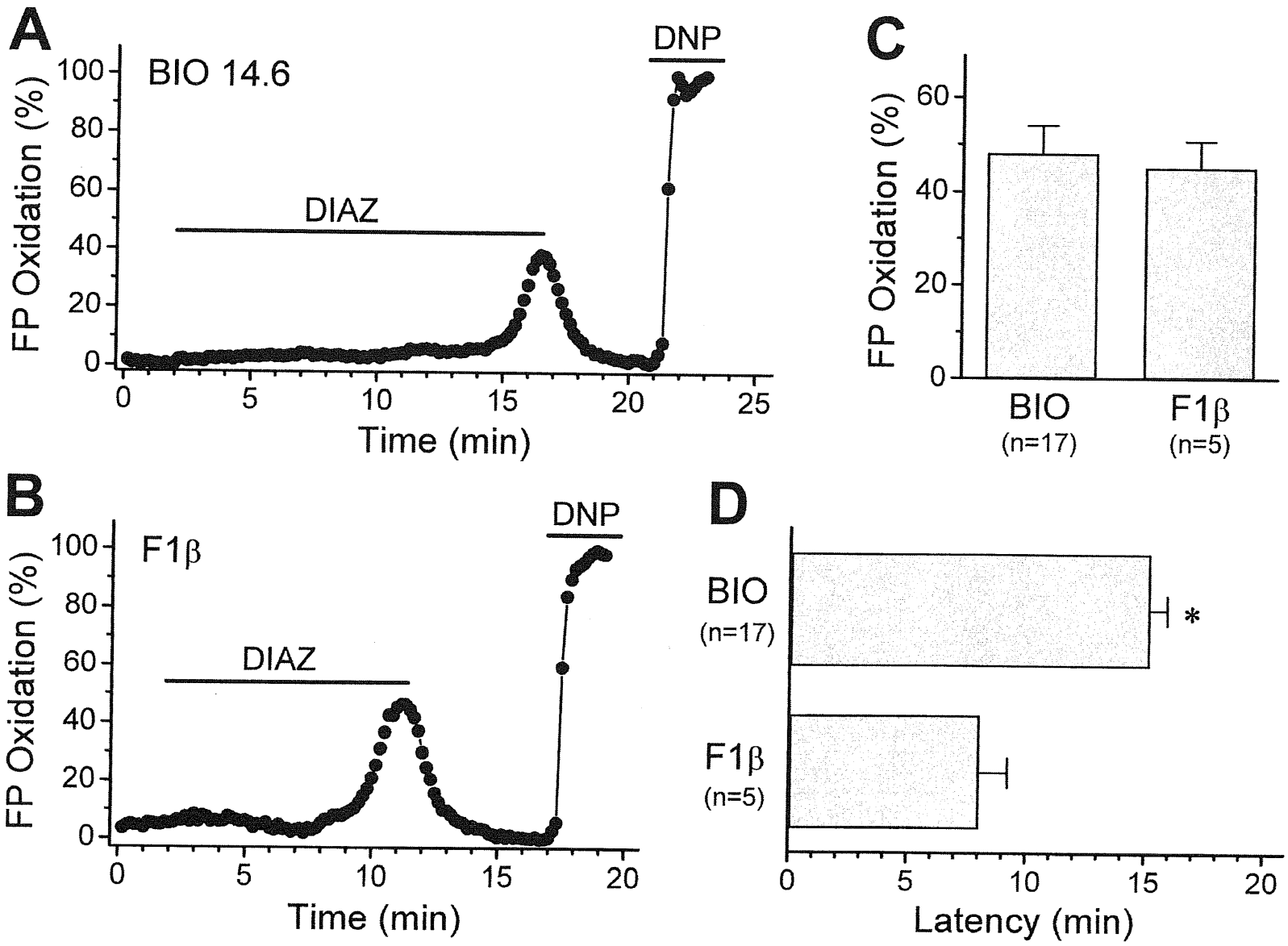
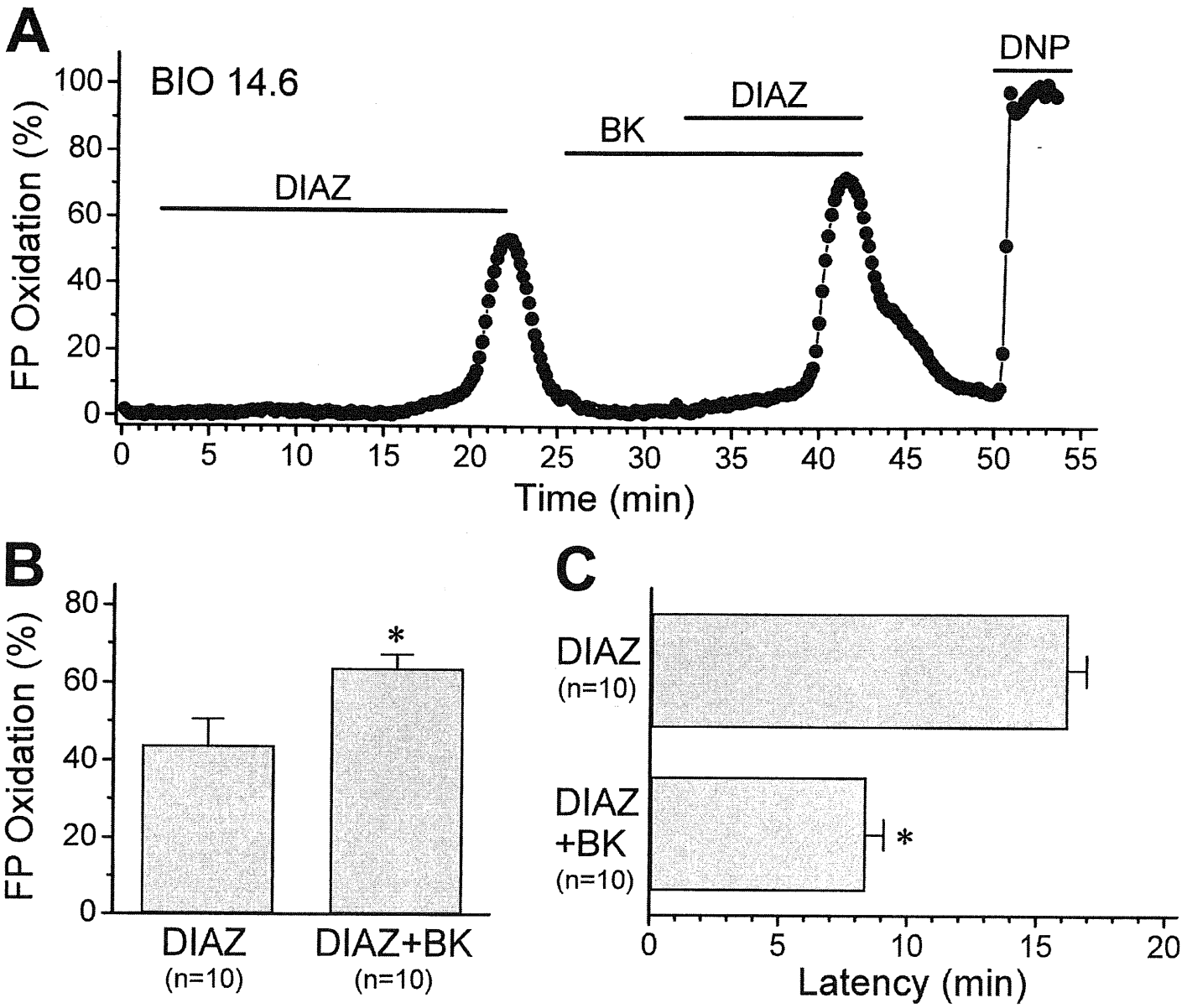


Figure 2, Sato *et al*



Kir6.2-deficient mice are susceptible to stimulated ANP secretion: K_{ATP} channel acts as a negative feedback mechanism?

Noriko Saegusa [a,b], Toshiaki Sato [a], Tomoaki Saito [a], Masaji Tamagawa [a], Issei Komuro [b], Haruaki Nakaya [a]

[a] *Department of Pharmacology, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan*

[b] *Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan*

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Corresponding author:

Haruaki Nakaya, MD, PhD

Department of Pharmacology

Chiba University Graduate School of Medicine

1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Tel: +81-43-226-2051; Fax: +81-43-226-2052

E-mail: nakaya@faculty.chiba-u.jp

Abstract

Objective: While atrial natriuretic peptide (ANP) has been shown to be released mainly from cardiac muscle cells in response to atrial distension, the regulatory mechanisms of ANP secretion are still not fully understood. We sought to determine whether ATP-sensitive K^+ (K_{ATP}) channel modulates the secretion of ANP, using mice with homozygous knockout of the Kir6.2 (a pore-forming subunit of cardiac K_{ATP} channel) gene. **Methods:** K_{ATP} channel currents were recorded from isolated mouse atrial cells with patch clamp techniques. Plasma ANP concentration in anesthetized mice, ANP content and secretion in isolated atrial preparations were determined by radioimmunoassay. Action potentials were recorded from the isolated atria. **Results:** Exposure to 2,4-dinitrophenol (100 μM) evoked a glibenclamide-sensitive K_{ATP} channel current in atrial cells from wild-type (WT) but not Kir6.2 knockout (Kir6.2 KO) mice. Although there were no significant differences in the basal plasma ANP level between WT and Kir6.2 KO mice, volume expansion caused a significant elevation of plasma ANP concentration in Kir6.2 KO but not WT mice with accompanying hypotension. When isolated left atria were stretched, ANP secreted into the bath from Kir6.2 KO atria was significantly higher than that from WT atria. Furthermore, stretching the atria from WT but not Kir6.2 KO mice significantly shortened the action potential duration. A hypotonic stretch of the membrane induced the glibenclamide-sensitive K_{ATP} channel current in atrial cells from WT but not Kir6.2 KO mice. **Conclusions:** Kir6.2 is essential for the function of K_{ATP} channel in mouse atrial cells. Given that Kir6.2 KO mice are susceptible to stretch-induced secretion of ANP, our results suggest that K_{ATP} channel may act as a negative feedback mechanism for the control of ANP secretion.

Keywords: natriuretic peptide; K-ATP channel; myocytes

1. Introduction

ATP-sensitive K^+ (K_{ATP}) channels, originally discovered in cardiac muscle [1], are present in many tissues and play an important role in various cellular responses [2]. The molecular identity of sarcolemmal K_{ATP} channels is now known to be a hetero-octameric complex of four pore-forming subunit (Kir6.x) and four sulfonylurea receptor regulatory subunit (SUR) [2]. Previous studies have shown that mutant mice lacking the Kir6.2 subunit of K_{ATP} channels (Kir6.2 KO mice) display obvious impairment of the insulin response to glucose [3] and are susceptible to generalized seizures after brief hypoxia [4]. Our recent studies using the Kir6.2 KO mice have provided direct evidence that Kir6.2 forms the pore region of ventricular K_{ATP} channels [5] and the activation of K_{ATP} channels plays an important role in cardioprotection [6]. More recently, Kir6.2 KO mouse models highlight the importance of K_{ATP} channels in the adaptation to stress beyond their role in cytoprotection [7].

Although considerable advances have been made in recent years towards understanding the nature of ventricular K_{ATP} channels, the molecular identity and functional role of atrial K_{ATP} channels are poorly understood. Atrial distention causes release into the circulation of atrial natriuretic peptide (ANP), a hormone that plays a role in the regulation of cardiovascular homeostasis [8]. Since the gating of the atrial K_{ATP} channel is mechanosensitive [9], the relationship to the process of ANP secretion is a subject of considerable interest. Kim et al. [10] reported that the K_{ATP} channel blocker glibenclamide suppressed the stretch-stimulated ANP secretion from rat atria. On the other hand, conflicting observation was reported that the K_{ATP} channel blocker tolbutamide increased the release of ANP in neonatal rat atrial myocytes [11]. Moreover, Xu et al. [12] reported that the stretch-induced ANP secretion was inhibited by K_{ATP} channel openers. Thus, the results so far obtained by using K_{ATP} channel blockers and

openers are not conclusive. In this study, we sought to determine whether atrial K_{ATP} channel regulates the secretion of ANP, using Kir6.2-deficient mice. The results show that Kir6.2 is essential for the function of atrial K_{ATP} channel and Kir6.2-deficient mice are susceptible to the stretch-induced secretion of ANP.

2. Methods

2.1. Kir6.2-deficient mice

All procedures were performed in conformity with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996), and were approved by the Institutional Animal Care and Use Committee of Chiba University. A mouse line deficient in the K_{ATP} channels was generated by targeted disruption of the gene coding for Kir6.2, as described previously [3]. C57BL/6 mice were used as control because they had been back crossed to a C57BL/6 strain for five generations. Twelve- to fourteen-weeks old Kir6.2-deficient mice or C57BL/6 control mice were used in this study.

2.2. Electrophysiology

Single atrial cells were enzymatically isolated by the method of Suzuki et al. [5] with some modifications. Single-channel and whole-cell membrane currents were recorded by the patch-clamp method as previously described [5,6]. Whole-cell current recordings were performed at 36°C with nystatin in the pipette solution. Single atrial cells were superfused with the HEPES-Tyrode's solution (in mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5, and HEPES-NaOH buffer 10 (pH = 7.4). The pipette solution contained (in mM): K-aspartate 110, KCl 20, $MgCl_2$ 1.0, $CaCl_2$ 1.0, EGTA 0.1, and HEPES-KOH buffer 5 (pH = 7.4), with 250 μg/ml nystatin. A ramp-pulse protocol (–100 to +50 mV over 2.5 seconds, repeated at 30-second intervals) was used to record the quasi-steady-state membrane current. For the experiments of hypotonic stretch of the membrane (Fig. 6), the isolated atrial cells were exposed to the low-chloride isotonic or hypotonic solution in order to minimize swelling-induced chloride current contamination [13]. Isotonic solution contained (in mM): Na-aspartate

80, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5, mannitol 119, and HEPES-NaOH buffer 10 (pH = 7.4, ≈36°C) with the osmolarity of 300 mosmol/kg H_2O. Hypotonic solution was prepared by simply omitting mannitol from the isotonic solution with the osmolality of 180 mosmol/kg H_2O. For single-channel recordings from the cell-attached patches, symmetrical high K^+ external and internal solutions (≈22°C) were used. The external solution contained (in mM): KCl 140, EGTA 5, Na_2-ATP 0.1, and HEPES-KOH buffer 5 (pH = 7.4), and the pipette solution contained (in mM): KCl 140, $MgCl_2$ 1.8, $CaCl_2$ 1.8, and HEPES-KOH buffer 5 (pH = 7.4). The current signals were digitized at 2 kHz for data analysis with pClamp software (Axon Instruments, Foster City, CA).

2.3. Volume expansion

The mice were anesthetized with urethane (1.5 g/kg, i.p.) and placed on a heating pad to maintain rectal temperatures at 37°C. The left external jugular vein was cannulated with small polyethylene catheters for intravenous infusion. Volume expansion was performed by a modification of the method of Kishimoto et al. [14]. The lactated Ringer's solution containing 4.5% bovine serum albumin was infused at a rate of 200 μl/h/g (body weight) for 30 min. The rate of infusion was then lowered to 4.3 μl/h/g for another 30 min. In some experiments the initial infusion rate was increased from 200 to 400 μl/h/g for 30min, after which the infusion was continued at a rate of 4.3 μl/h/g for 30 min. These infusions were carried out with a microinfusion pump (Harvard Apparatus, MA). At the end of the volume expansion, blood (700 μl) was drawn from the right carotid artery, collected into tube containing EDTA and aprotinin, and centrifuged for 20 min at 4°C. Plasma samples were stored at –80°C until analyzed by ANP radioimmunoassay.

2.4. Hemodynamic measurements

Arterial blood pressure was measured continuously in mice anesthetized with urethane. The right carotid artery was cannulated with small polyethylene catheters for the measurement of blood pressure via a pressure transducer. Heart rate was derived from the arterial blood pressure signal.

2.5. Isolated atrial preparations

Tissue bath preparations of left atria were prepared by a modification of the method described by Bilder et al. [15]. Briefly, left atria from WT and Kir6.2 KO mice were dissected free, and placed in a 3-ml water jacketed tissue bath containing HEPES-Tyrode's solution (pH = 7.4) gassed with 100% O_2 (37°C). The edge of preparation was pinned to the rubber base of the tissue bath and electrically paced at a frequency of 5 Hz. After 10 min equilibration, the bath solution was discarded and 1 ml of fresh HEPES-Tyrode's solution was added to the bath. Atrial preparations were incubated in this solution with or without stretch stimulus. The atria connected to a string were stretched with a fixed resting tension of 0.5 g. After 10 min of incubation, the bath solution was collected into a tube containing EDTA and aprotinin, and stored at –80°C until analyzed by ANP radioimmunoassay.

2.6. ANP content of the atria

The hearts were rapidly removed from the anesthetized mice and the left atria were dissected out and immediately frozen in liquid nitrogen. The lysates were obtained from three preparations by homogenization in ice-cold buffer (0.1 mM acetic acid). The lysates were incubated for 8 min at 100°C, kept on ice for 15 min and cleared by centrifugation at 66,000 × g for 20 min at 4°C. Protein concentration was determined by the BCA protein assay kit (PIERCE, Rockford, IL).

2.7. ANP radioimmunoassay

The concentration of immunoreactive ANP was measured by commercially available radioimmunoassay, as described previously [16]. The threshold for detection of ANP was 1.5 pg/ml.

2.8. Action potential recordings

The preparations of left atria from WT and Kir6.2 KO mice were mounted in a recording chamber and perfused at a constant flow (5 ml/min) with Tyrode's solution (in mM): NaCl 125, KCl 4.0, $CaCl_2$ 2.7, $MgCl_2$ 0.5, NaH_2PO_4 1.8, glucose 5.5, and $NaHCO_3$ 25 and gassed with 95% O_2/5% CO_2 (37°C). Action potentials were evoked by electrical field stimulation at 5 Hz (2-ms rectangular pulses at 2× threshold intensity) and recorded by use of a 3 M KCl-filled microelectrode (tip resistance 10–20 MΩ). The edge of left atrial preparation was stretched with a fixed resting tension of 0.5 g. Transmembrane potential was recorded by a direct current preamplifier (MEZ-7200, Nihon Kohden, Tokyo, Japan) and digitized (PowerLab 2/20, ADInstruments, Castle Hill, Australia).

2.9. Drugs

The following drugs were used: nystatin (Wako Pure Chemicals, Osaka, Japan); glibenclamide (Sigma-Aldrich Japan, Tokyo, Japan). Nystatin was dissolved in methanol at a concentration of 10 mg/ml and added to the pipette solution at a concentration of 250 μg/ml just before experiments. Glibenclamide was dissolved in dimethyl sulfoxide as a stock solution of 10 mM, and final concentration of dimethyl sulfoxide was less than 0.1%.

2.10. Statistics

All data are presented as mean ± SEM. Statistical comparisons were made with the use of Student's *t* test or ANOVA combined with Fisher post hoc test, as appropriate. A p value of less than 0.05 was considered significant.

3. Results

3.1. K_{ATP} channel function in atrial cells

We first evaluated K_{ATP} channel function in atrial cells isolated from wild-type (WT) and Kir6.2-deficient (Kir6.2 KO) mice. Whole-cell membrane currents were recorded using a ramp-pulse protocol (Figs. 1A and 1B). There were no significant differences in the density of the outward current at 0 mV between atrial cells isolated from WT (1.4 ± 0.1 pA/pF, n = 6) and Kir6.2 KO mice (1.6 ± 0.1 pA/pF, n = 6) in the control condition. In WT cells, metabolic inhibition with a glucose-free, 100 μM DNP-containing solution induced an outward current (9.1 ± 0.1 pA/pF at 0 mV, n = 6), which, by virtue of its blockade by 1 μM glibenclamide (1.3 ± 0.1 pA/pF, n = 6), was confirmed to be the K_{ATP} current. However, such a membrane current change was not observed in Kir6.2 KO cells (1.6 ± 0.1 pA/pF after metabolic inhibition, n = 6). When the atrial cells were exposed to a solution containing DNP (100 μM), single K_{ATP} channel activity could be recorded from 25 of 25 cell-attached patches of 9 WT mice (Fig. 1C). The channel openings were inhibited by addition of 1 μM glibenclamide to the solution (data not shown). The linear slope conductance, obtained from the current-voltage relationship from –100 mV to –40 mV for the single channel current of WT cells, was 71.6 ± 1.7 pS (n = 7). In contrast, opening of K_{ATP} channels could not be recorded from any cell-attached patches of Kir6.2 KO cells (n = 18). These results indicate that Kir6.2 is essential for the function of K_{ATP} channel in atrial cells.

3.2. Effect of volume expansion on plasma ANP level

In anesthetized mice, there were no significant differences in the basal plasma ANP concentrations between WT (28.7 ± 3.0 pg/ml, n = 7) and Kir6.2 KO mice (36.6 ± 4.9 pg/ml, n = 6). After volume expansion (200 μl/h/g for 30 min and 4.3 μl/h/g for

another 30 min), the plasma ANP level was increased to 31.8 ± 1.6 pg/ml (n = 7) in WT mice, but this change was not statistically significant (p = 0.385 vs. baseline). On the other hand, Kir6.2 KO mice reacted to volume expansion and the plasma ANP concentration significantly increased to 73.5 ± 13.4 pg/ml (n = 5, p = 0.021 vs. baseline). The elevation of plasma ANP levels observed in Kir6.2 KO mice was notably higher than that observed in WT mice (p = 0.004) (Fig. 2). When the initial infusion rate was increased from 200 to 400 μl/h/g for 30min (then the rate of infusion was lowered to 4.3 μl/h/g for 30 min) to give a severe volume expansion, the plasma ANP concentration in WT mice significantly increased to 49.8 ± 8.2 pg/ml (n = 4, p = 0.024 vs. baseline). This value was not significantly different from that in Kir6.2 KO mice (53.9 ± 4.9 pg/ml, n = 4). These results indicate that Kir6.2 KO mice are susceptible to the ANP secretion by volume expansion.

3.3. Hemodynamic effects of volume expansion

There were no significant differences in the basal values of mean arterial pressure (MAP) between WT and Kir6.2 KO mice (Fig. 3). The values of MAP at 30 min of volume expansion were similarly increased in both WT (112.0 ± 4.1% of baseline, n = 4) and Kir6.2 KO mice (111.3 ± 2.3% of baseline, n = 4). In WT mice, MAP returned to baseline levels after 60 min of volume expansion (96.7 ± 6.2% of baseline, n = 4). In contrast, 60 min of volume expansion in the Kir6.2 KO mice produced significant decrease in MAP (81.0 ± 4.7% of baseline, n = 4, $p < 0.05$). There were no significant differences in the basal values of heart rate between WT (654 ± 31 bpm, n = 7) and Kir6.2 KO mice (642 ± 50 bpm, n = 5). Volume expansion did not alter the heart rate in both WT (689 ± 25 bpm, n = 7) and Kir6.2 KO mice (644 ± 50 bpm, n = 5).

3.4. ANP secretion from isolated atria

Isolated mouse atrial preparations were used to study the direct effect of mechanical stretch on ANP release. As shown in Fig. 4, in most cases the ANP concentration did not exceed the threshold for detection (≥1.5 pg/ml). We therefore repeated the experiment until the ANP secretion was detected in 4 or 5 of the preparations in each group. In control (non-stretch) conditions, ANP secretion was detected in 4 of 17 atria from WT and in 5 of 13 atria from Kir6.2 KO, respectively. The mean ANP concentration in each of 4 or 5 preparations (WT; 2.2 ± 0.2 pg/ml, Kir6.2 KO; 2.8 ± 0.4 pg/ml) was not statistically significant. After mechanical stretch for 10 min, ANP secretion was detected in 4 of 15 atria from WT. In Kir6.2 KO atria, ANP secretion was detected in 4 of 8 preparations, and the mean ANP concentration (4.9 ± 0.7 pg/ml) was notably higher that that observed in WT atria (2.4 ± 0.3 pg/ml) ($p = 0.046$).

ANP content of atria obtained from WT and Kir6.2 KO mice was 0.64 ± 0.30 ng/mg protein and 0.74 ± 0.23 ng/mg protein, respectively; these values did not reach statistical significance ($n = 3$, $p = 0.696$).

3.5. Action potentials in isolated atria.

Representative recordings of action potentials before and after 5 min of stretch are shown in Figs. 5A and 5B. The action potential duration (APD) in the WT atrium was shortened at 5 min of stretch, while the APD remained unaltered in the Kir6.2 KO atrium. As summarized in Fig. 5C, stretching the WT atria significantly shortened the APD measured at 90% repolarization (APD_{90}) to 71.8 ± 3.4 % of control ($n = 4$, $p = 0.004$). Stretch-induced shortening of APD_{90} was not observed in Kir6.2 KO atria (98.6 ± 0.7 %, $n = 4$).

3.6. Hypotonic stretch-induced K_{ATP} current

To determine whether K_{ATP} channel currents could be mechanically induced, the nystatin-perforated patch was used to record whole-cell membrane currents, and atrial cells were perfused with either isotonic or hypotonic solutions. Fig. 6A shows the representative current traces recorded in response to voltage ramps from –100 to +50 mV. In an atrial cell of WT, a hypotonic stretch of the membrane for 10 min evoked an outward current that could be reduced by the subsequent application of glibenclamide (1 μM). The reversal potential of the glibenclamide-sensitive current, which isolated by digital subtraction of the current trace in the presence of glibenclamide from that under hypotonic condition, was close to the K^+ equilibrium potential (~–80 mV, data not shown). On the other hand, a hypotonic stretch of the membrane slightly increased the outward current in Kir6.2 KO cells, but this current was not blocked by glibenclamide. As summarized in Fig. 6B, hypotonic stretch of WT cells significantly increased the glibenclamide-sensitive outward current at 0 mV from 0.23 ± 0.03 to 1.73 ± 0.36 pA/pF ($n = 4$, $p < 0.05$). However, in Kir6.2 KO cells, a glibenclamide-sensitive outward current was not evoked under hypotonic stretch (from 0.13 ± 0.03 to 0.16 ± 0.07 pA/pF, $n = 4$, $p = 0.66$). These results indicate that K_{ATP} channel current is activated by hypotonic stretch of the membrane in atrial cells from WT but not Kir6.2 KO mice.

4. Discussion

4.1. Kir6.2 forms the pore of atrial K_{ATP} channels

A previous study using primary cultured neonatal rat atrial cells suggested that molecular composition of atrial K_{ATP} channels may be different from that of ventricular K_{ATP} channels based on the functional and pharmacological profiles [17]. In the present study, however, a glibenclamide-sensitive K_{ATP} channel current could be observed during metabolic inhibition in atrial cells of WT but not Kir6.2 KO mice (Figs. 1A and 1B). In addition, we found that the unitary conductance of single K_{ATP} channel current in WT mouse atrial cells (71.6 ± 1.7 pS, n = 7, Fig. 1C) was close to that of ventricular cells (75.9 ± 1.3 pS, n = 7, data not shown). Therefore, it can be concluded that Kir6.2 is essential for the function of mouse atrial K_{ATP} channels and Kir6.2 KO mice are potentially useful to examine whether K_{ATP} channel modulates the secretion of ANP.

4.2. K_{ATP} channel modulates ANP secretion in vivo and in vitro

It is acknowledged that ANP is stored as pro-ANP within the granules of cardiomyocytes and released in response to atrial stretch [18,19]. Pro-ANP is cleaved during the release process by a cardiac protease, corin, to form the biologically active C-terminal ANP [20]. Here we examined, in vivo and in vitro, whether K_{ATP} channel is crucial for the regulation of ANP secretion. Plasma volume expansion was used as a means of producing release of the cardiac ANP granules in vivo. There were no significant differences in the basal plasma ANP levels (Fig. 2) between WT and Kir6.2 KO mice. After the initial volume expansion (200 μl/h/g for 30min), MAP in WT and Kir6.2 KO mice increased to similar extents (Fig. 3), suggesting that the volume expansion seemed to be equally effective in both WT and Kir6.2 KO mice. In WT mice, however, volume expansion was insufficient to cause a significant increase in plasma

ANP concentration. Despite a rather mild volume expansion, a significant increase of plasma ANP concentration was observed in Kir6.2 KO mice (Fig. 2). ANP promotes diuresis/natriuresis and reduces vascular tone [8]. Consequently, hypotension might occur in Kir6.2 KO but not WT mice (Fig. 3), accompanied by an excessive secretion of ANP.

ANP secreted into the bath from atrial tissue in vitro was small and ≤30% of preparations exceeded the threshold for detection in control conditions. However, 4 of 8 preparations (50%) from Kir6.2 KO mice exceeded the threshold for detection after mechanical stretch and the ANP concentration was greater than that of WT atria (Fig. 4). Thus, in vitro study using isolated atria was consistent with the findings in vivo. Since ANP content of WT atria was not statistically different from Kir6.2 KO atria (Fig. 5), in vivo and in vitro studies suggest that Kir6.2 KO mice are susceptible to stretch-induced secretion of ANP. Furthermore, given the fact that an excess of ANP produced hypotension in Kir6.2 KO mice, it is reasonable to suppose that activation of K_{ATP} channels is a mechanism for feedback inhibition of stimulated ANP release.

4.3. Activation of atrial K_{ATP} channels by mechanical stretch

Based on pharmacological experiments, it has been proposed that K_{ATP} channels regulate ANP secretion [10,11]. In the present study, we found that mechanical stretch shortened the action potential duration in WT atria, but not in Kir6.2 KO atria (Fig. 5). Van Wagoner [9] reported that K_{ATP} channels in rat atrial cells were mechanosensitive and activated by a hypotonic swelling. Later on, Baron et al. [17] also demonstrated a hypotonic-induced activation of the atrial appendage K_{ATP} channel currents. In agreement with previous reports, we could record the glibenclamide-sensitive K_{ATP} channel current in response to a hypotonic stretch in atrial cells of WT but not Kir6.2 KO mice (Fig. 6). Although we did not investigate the hypotonic stretch-induced ANP

secretion, Jiao et al. [11] have demonstrated that a hypotonic swelling increases ANP secretion in rat atrial myocyte culture. Together, these findings suggest that mechanical stimulus of atrial myocytes activates K_{ATP} channel in association with ANP secretion.

4.4. Possible mechanisms underlying K_{ATP} channel-mediated regulation of ANP secretion

Our observations raise the question of how stretch-induced opening of K_{ATP} channels prevents excessive release of ANP. The diagram of Fig. 7 shows the possible mechanism underlying K_{ATP} channel-mediated regulation of ANP secretion. Mechanical stretch in isolated atrial tissues was reported to increase the intracellular Ca^{2+} transients and the late duration of the action potentials, which was ascribed to the stretch-induced channel activation and resultant augmentation of the Na^{+}/ Ca^{2+}-exchanger inward current [21,22]. It has also been proposed that intracellular Ca^{2+} plays a critical role in ANP secretion from atrial cells, and changes of cytosolic Ca^{2+} concentration affects ANP secretion [8,23,24]. Activation of atrial K_{ATP} channels by mechanical stretch may decrease the cytosolic Ca^{2+} concentration by abbreviating the action potentials, which is expected reduce the time for Ca^{2+} influx via L-type Ca^{2+} channels and to increase the time for Ca^{2+} extrusion through the Na^{+}/ Ca^{2+}-exchange system. Whatever the mechanism involved, the present study clearly demonstrates that K_{ATP} channels negatively regulate ANP secretion from atrial tissues.

4.5. Conclusions

ANP is known to produce a variety of physiological actions such as diuretic, vasodilative [25,26], anti-ischemic [27] and antihypertrophic actions [28]. In the present study we have provided evidence that Kir6.2 is essential for the function of K_{ATP} channel in atrial myocytes, and that K_{ATP} channel-dependent mechanism contributes to

the regulation of ANP secretion. Recently, a functional study using Kir6.2 KO mice has demonstrated that disruption of K_{ATP} channel function leads to impaired Ca^{2+} handling, cardiac dysfunction and lethal arrhythmias under vigorous sympathetic stimulation, suggesting requirement of K_{ATP} channels for adaptation to physiological stress [7]. Furthermore, it has been reported that in failing hearts the metabolic dysregulation of K_{ATP} channels can occur and resultant loss of protective mechanisms expands the risk of disease progression [29]. Therefore, ANP secretion induced by impaired K_{ATP} channel function may play a compensatory role by protecting the heart under pathophysiological states. It is likely, however, that under physiological conditions K_{ATP} channel may act as a negative feedback mechanism for the control of ANP secretion.

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Figure Legends

Fig. 1. (A) Effect of metabolic inhibition with a glucose-free, DNP-containing (100 μM) solution and coapplication of glibenclamide (GLB, 1 μM) on the nystatin-perforated whole-cell membrane currents recorded from atrial cells of WT (left) and Kir6.2 KO mice (right). (B) Current densities at 0 mV from control (n = 6 of WT, n = 6 of Kir6.2 KO, black bar), DNP-treated (n = 6 of WT, n = 6 of Kir6.2 KO, grey bar) and DNP+GLB (n = 6 of WT, dark grey bar) mice of atrial cells are summarized. Values are mean ± SEM. * $p < 0.01$ vs. control. (C) Single-channel current recordings in the cell-attached mode by addition of DNP (100 μM) from atrial cells of WT (left) and Kir6.2 KO mice (right). The test potential is indicated on the left.

Fig. 2. Effect of volume expansion on plasma ANP levels of WT and Kir6.2 KO mice. Top panel shows the experimental protocol. Bottom panel shows summarized data for plasma ANP concentration. Blood samples were drawn at baseline (n = 7 of WT, n = 6 of Kir6.2 KO, grey bar), and after volume expansion (n = 7 of WT, n = 5 of Kir6.2 KO, black bar). Values are mean ± SEM.

Fig. 3. Effect of volume expansion on mean arterial pressure (MAP) in anesthetized mice. (A) Changes of MAP before (baseline) and after 30 and 60 min of volume expansion. (B) MAPs are given as a percentage of the value measured at baseline. Data represent mean ± SEM of 4 experiments for each group. * $p < 0.05$ vs. baseline, # $p < 0.05$ vs. 30 min.

Fig. 4. Effects of mechanical stretch on ANP secretion in isolated atria from WT and Kir6.2 KO mice. The threshold for detection of ANP (1.5 pg/ml) is represented as dotted

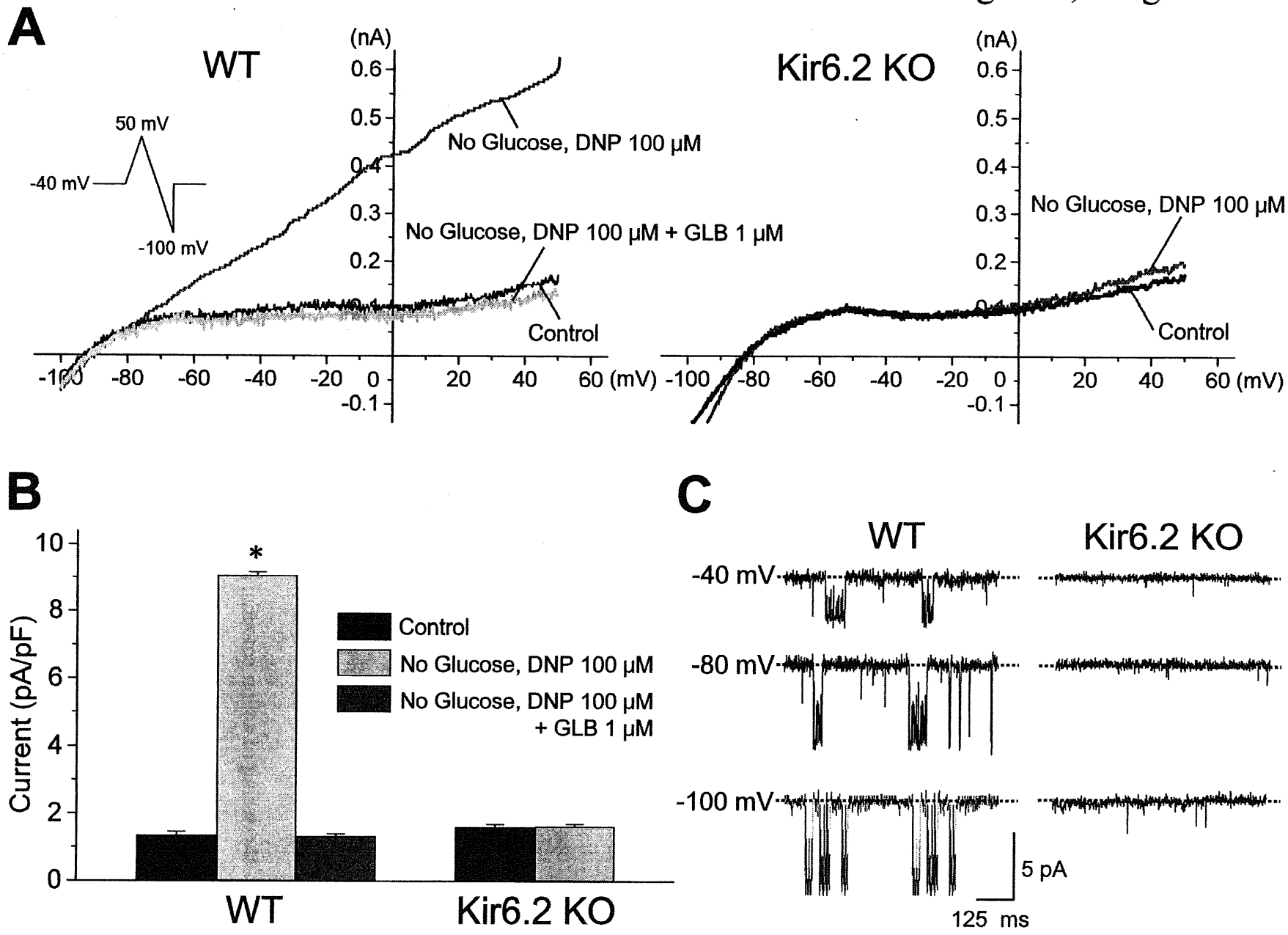
line. Each symbol represents data from individual preparations. Closed squares indicate the mean and the vertical bars indicate SEM.

Fig. 5. Representative recording of action potentials before (Control, black) and 5 min after stretch (Stretch, grey) in the atrium of WT (A) and Kir6.2 KO (B) mice. (C) Summarized effects of stretch on the action potential duration. The action potential duration at 90% repolarization (APD_{90}) was measured 5 min after stretch and given as a percentage of the control value. Data represent mean ± SEM of 4 experiments for each group. * $p < 0.05$ vs. control.

Fig. 6. Activation of the atrial K_{ATP} current by hypotonic stretch. (A) Original whole-cell voltage-ramp currents traces of atrial cells from WT (left) and Kir6.2 KO mice (right). (B) Glibenclamide-sensitive current densities at 0 mV in WT ($n = 4$) and Kir6.2 KO ($n = 4$) atrial cells are summarized. Values are mean ± SEM. * $p < 0.05$ vs. control.

Fig. 7. Hypothetical representation of mechanism by which opening of K_{ATP} channels acts as a negative modulator of ANP secretion. Mechanical stretch in atrial tissue increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and thereby stimulates the release of ANP. On the other hand, K_{ATP} channel opens when stretched and decreases $[Ca^{2+}]_i$ by shortening of action potential duration (APD), which in turn inhibits the release of ANP. (+): stimulation, (–): inhibition. (See text for details.)

Figure 1, Saegusa *et al*



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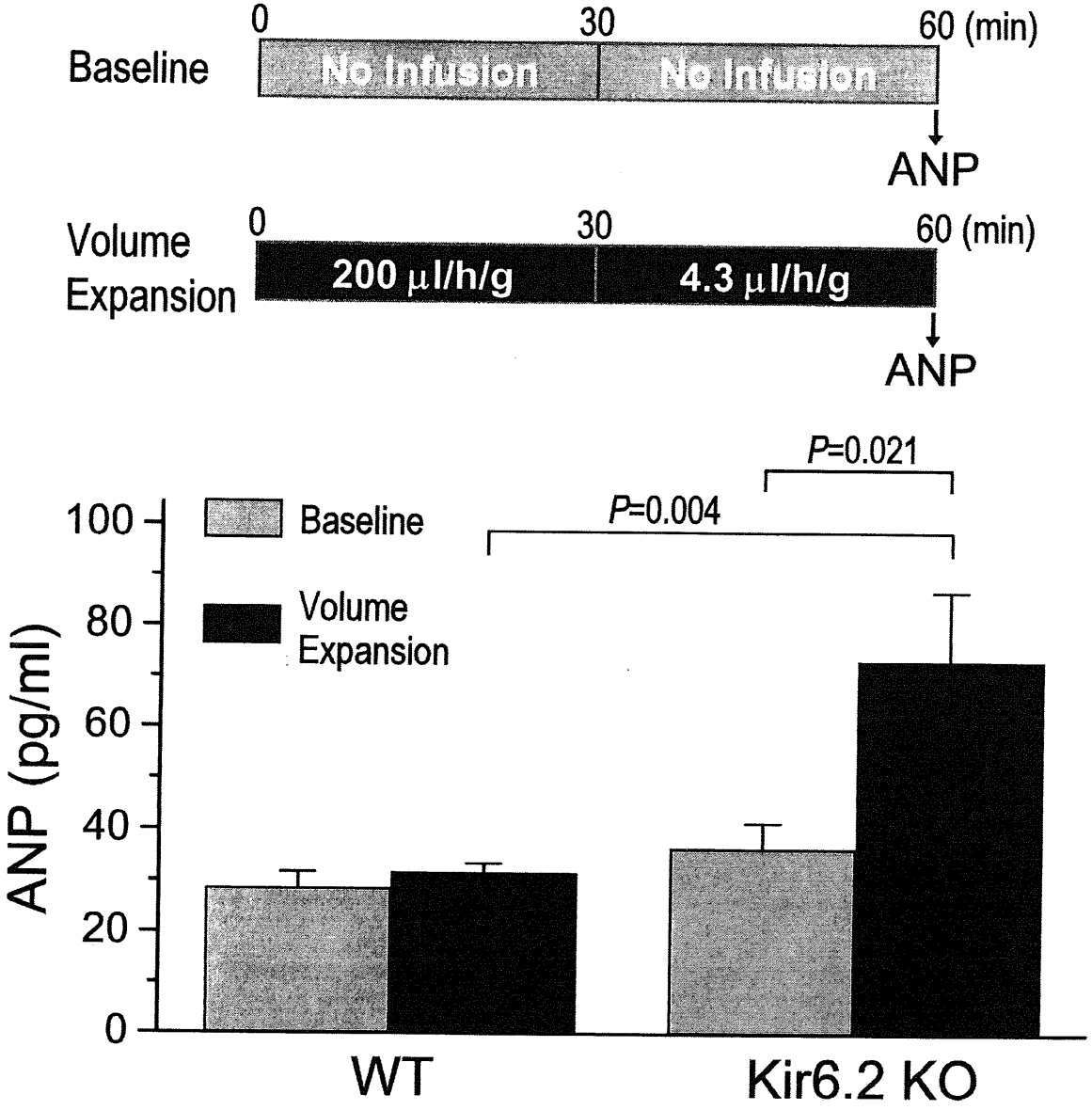

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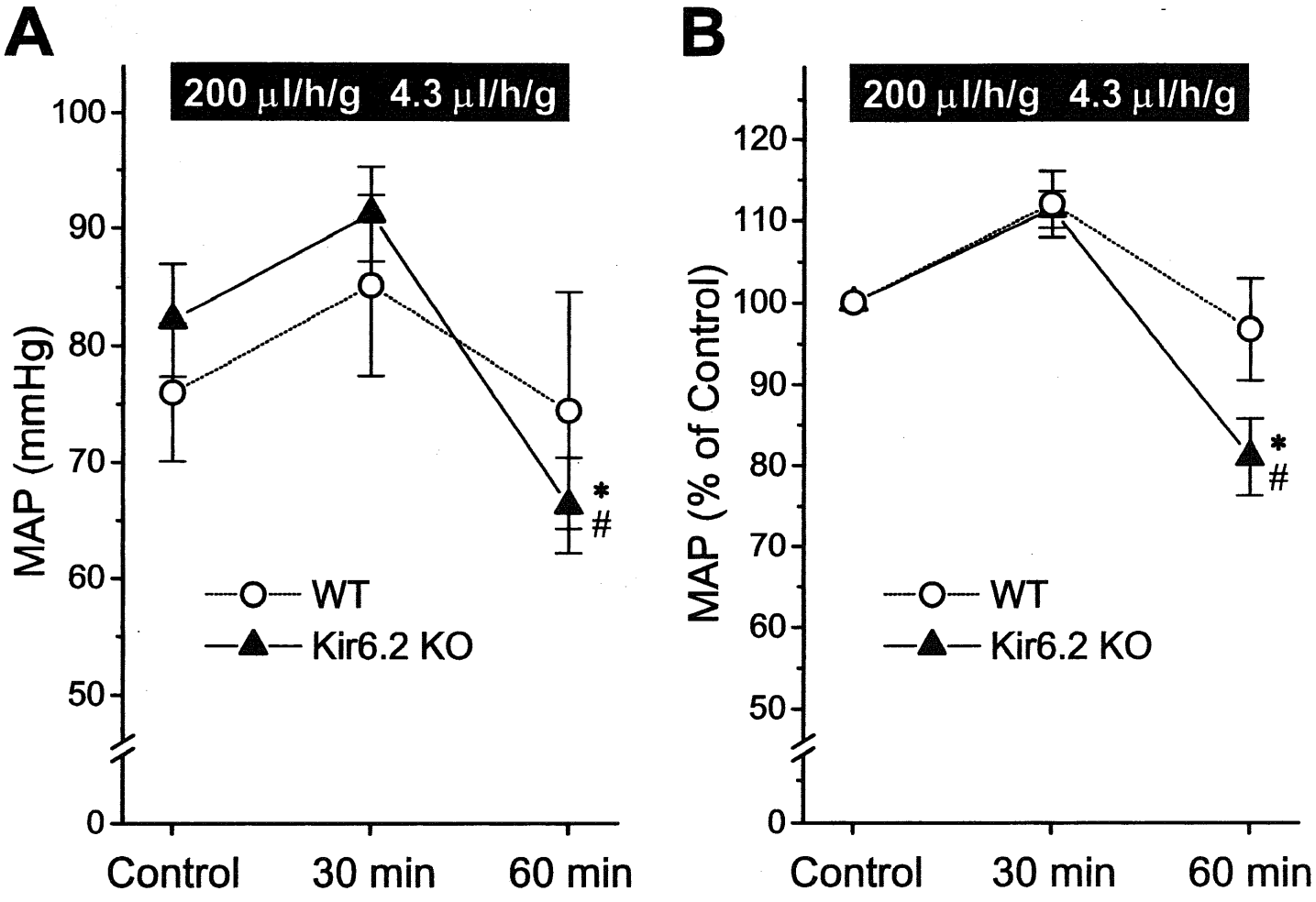

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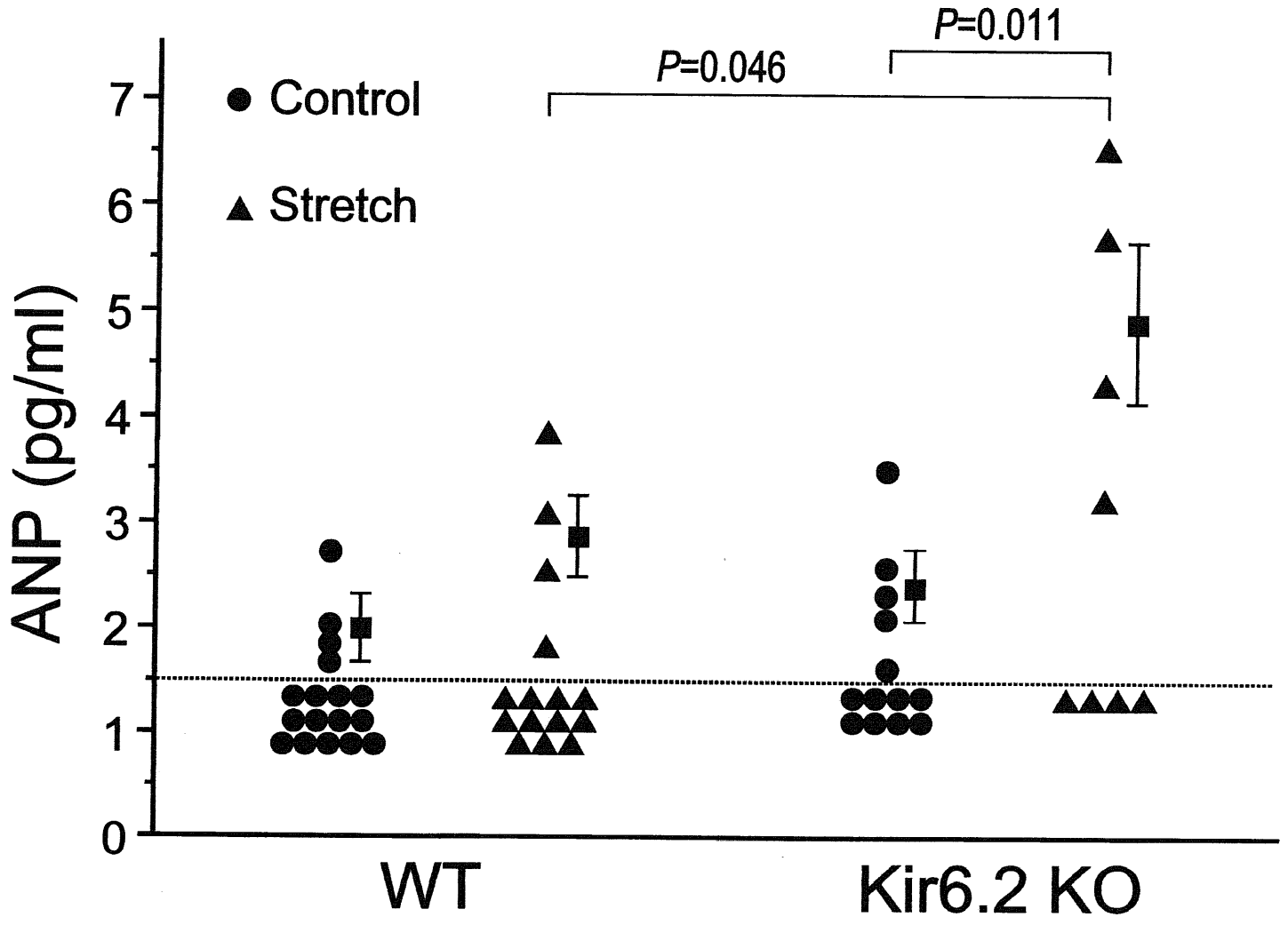

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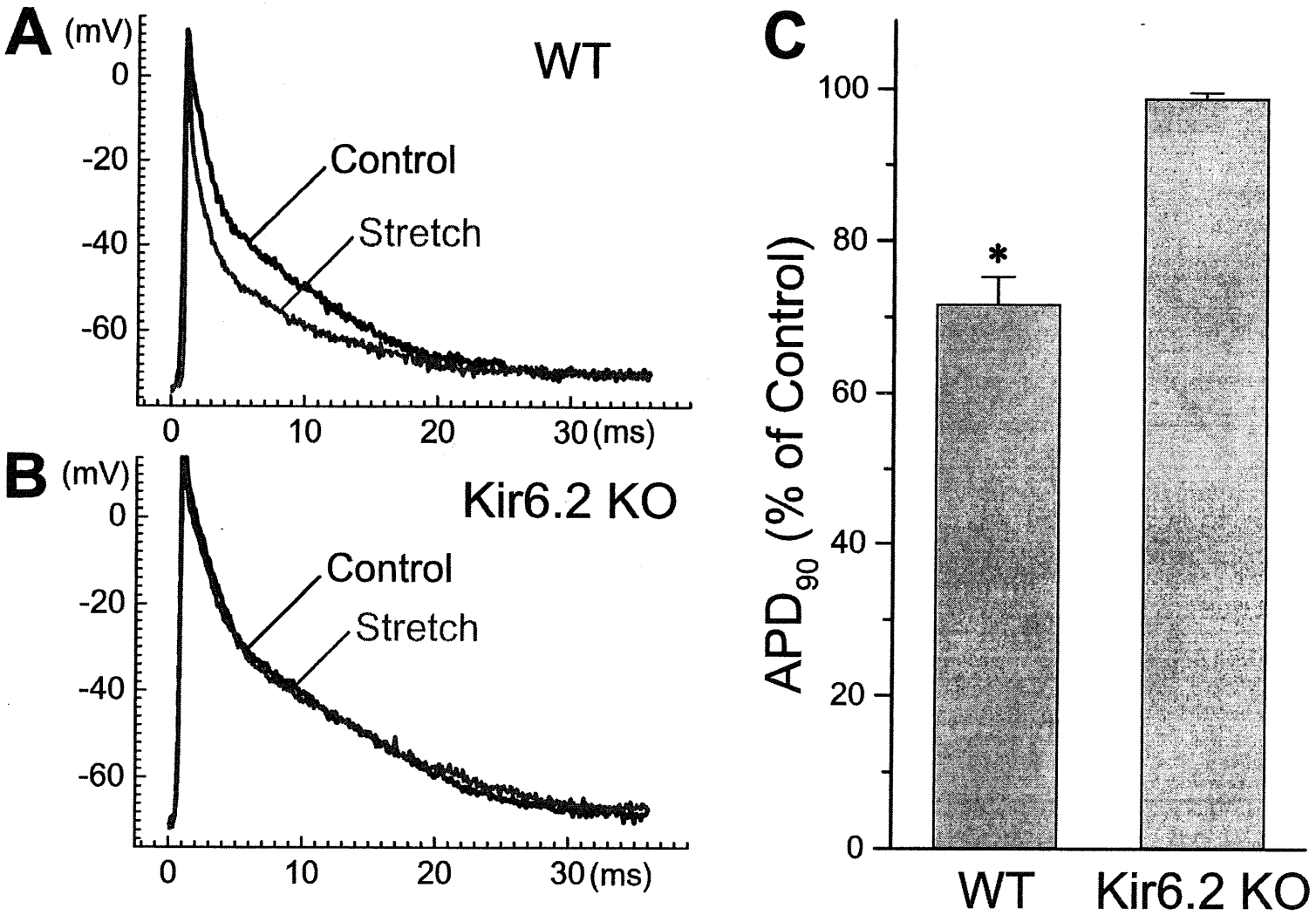

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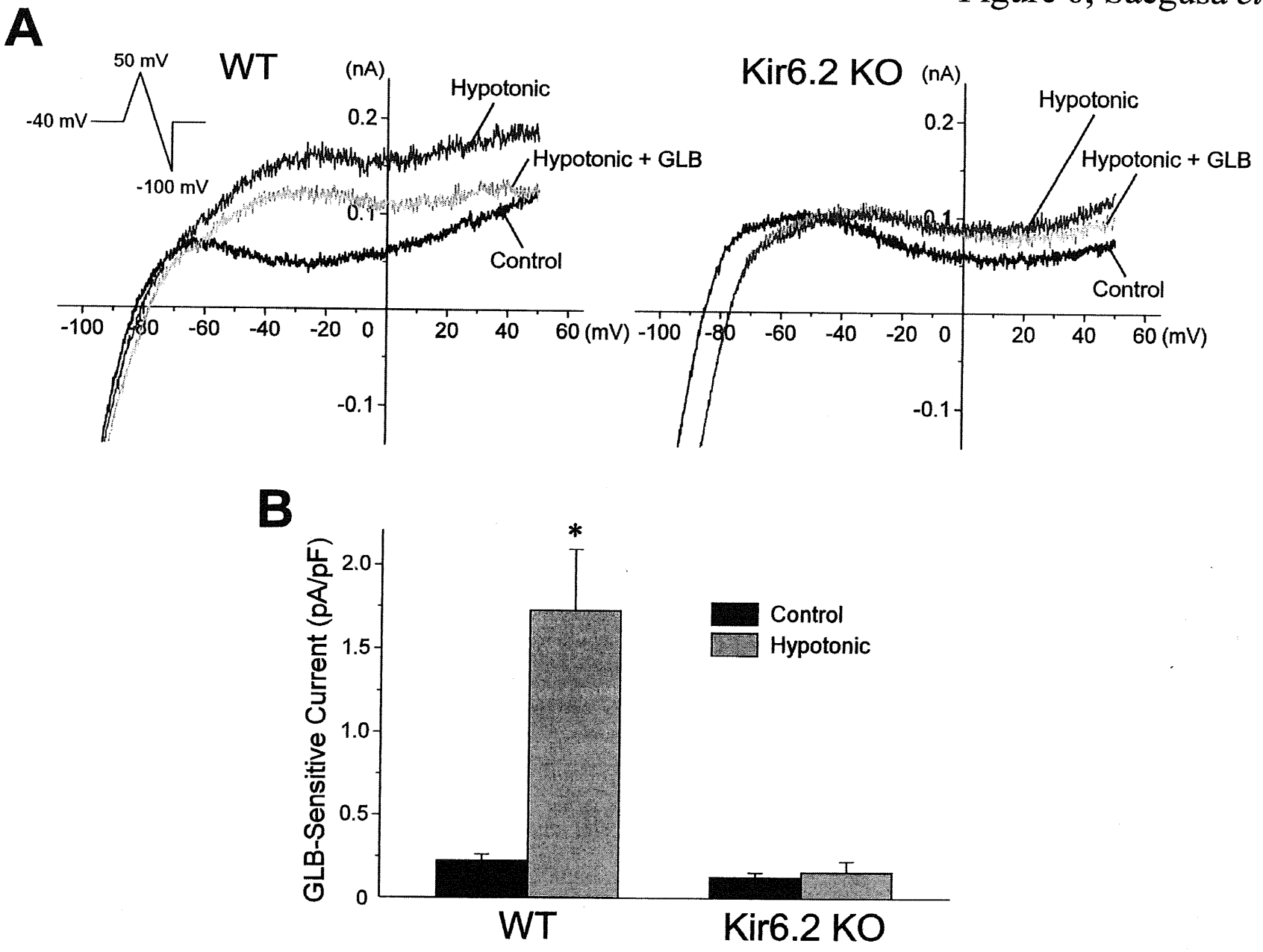

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