Syntaxin-1A Actions on Sulfonylurea Receptor 2A Can Block Acidic pH-induced Cardiac $K_{\text{ATP}}$ Channel Activation*

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During cardiac ischemia, ATP stores are depleted, and cardiomyocyte intracellular pH lowers to <7.0. The acidic pH acts on the Kir6.2 subunit of $K_{\text{ATP}}$ channels to reduce its sensitivity to ATP, causing channel opening. We recently reported that syntaxin-1A (Syn-1A) binds nucleotide binding folds (NBF)-1 and NBF2 of sulfonylurea receptor 2A (SUR2A) to inhibit channel activity (Kang, Y., Leung, Y. M., Manning-Fox, J. E., Xia, F., Xie, H., Sheu, L., Tsushima, R. G., Light, P. E., and Gaisano, H. Y. (2004) *J. Biol. Chem.* 279, 47125–47131). Here, we examined Syn-1A actions on SUR2A to influence the pH regulation of cardiac $K_{\text{ATP}}$ channels. $K_{\text{ATP}}$ channel currents from inside-out patches excised from Kir6.2/SUR2A expressing HEK293 cells and freshly isolated cardiac myocytes were increased by reducing intracellular pH from 7.4 to 6.8, which would be blocked by increasing concentrations of Syn-1A added to the cytoplasmic surface. Syn-1A had no effect on C-terminal truncated Kir6.2 (Kir6.2–ΔC26) channels expressed in TSA cells without the SUR subunit. In vitro binding and co-immunoprecipitation studies show that Syn-1A binding to SUR2A or its NBF-1 and NBF-2 domain proteins increased progressively as pH was reduced from 7.4 to 6.0. The enhancement of Syn-1A binding to SUR2A by acidic pH was further regulated by Mg$^{2+}$ and ATP. Therefore, pH regulates Kir6.2/SUR2A channels not only by its direct actions on the Kir6.2 subunit but also by modulation of Syn-1A binding to SUR2A. The increased Syn-1A binding to the SUR2A at acidic pH would assert some inhibition of the $K_{\text{ATP}}$ channels, which may serve as a “brake” to temper the fluctuation of low pH-induced $K_{\text{ATP}}$ channel opening that could induce fatal re-entrant arrhythmias.

Myocardial ATP-sensitive potassium channels ($K_{\text{ATP}}$), composed of four pore-forming Kir6.2 (inwardly rectifying potassium) channel subunits and four regulatory sulfonylurea receptor 2A (SUR2A) subunits (1–3), are closed during normoxia (4) because the intracellular ATP levels are high enough to maintain the channels in their closed state. During periods of severe cardiac stress, such as ischemia, cardiomyocyte ATP stores become depleted, and intracellular pH (pHi) lowers to <7.0 (5–7), which decreases the channel sensitivity to ATP and increases the channel open state probability. The resulting Kir6.2/SUR2A channel activation leads to shortening of the action potential, which is believed to be cytoprotective but can render the heart susceptible to life-threatening arrhythmias.

With accumulating evidence that has indicated that protons are an important $K_{\text{ATP}}$ channel regulator in addition to ATP, ADP, and phospholipids (4, 8–10), the precise mechanism by which pH modulates $K_{\text{ATP}}$ activity is now becoming clearer. Using C-terminal truncated Kir6.2 (Kir6.2ΔC26 and Kir6.2Δ36), which allows the channel pore subunit to be expressed in a functional form in the plasma membrane without the SUR subunit (11, 12), a study by Xu et al. (12) showed that these truncated Kir6.2 channels could be activated by hypercapnic and intracellular acidosis. These effects of acidic pH on the truncated Kir6.2 channels were very similar to those effects of acidic pH previously observed in cardiac and skeletal muscle cells (10, 13–15). Further elegant and detailed site-directed mutation studies (16–20) demonstrated that full pH sensitivity requires multiple and specific residues and domains within the Kir6.2 subunit, including Thr-71 in the N terminus, Cys-166 in the M2 region, and His-175 in the C terminus. His-175 was found to be the only potentially titratable residue and is, therefore, postulated to be the protonation site in Kir6.2. ATP reduces the ability of acidic pH to activate the cloned Kir6.2 channel, and *vice versa*, acidic pH reduces the channel sensitivity to ATP (19). This allosteric modulation of the Kir6.2 channel by protons and ATP depends on His-175 and Cys-185, respectively, and is significantly enhanced by the SUR subunit (19). Similar results were observed with frog skeletal muscle $K_{\text{ATP}}$ channels, the ATP and pH sensitivity of which are reciprocally modulated by each other (21). Intriguingly, a most

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¶ The abbreviations used are: $K_{\text{ATP}}$, channel, ATP-sensitive potassium channel; SUR, sulfonylurea receptor; NBF, nucleotide binding fold; Syn-1A, syntaxin-1A; GST, glutathione S-transferase; HEK, human embryonic kidney; pHi, intracellular pH; MES, 4-morpholineethanesulfonic acid.
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recent report by Wang et al. (22) revealed that mutations of Thr-71 and Cys-166 could reverse the Kur channel gating by pH and ATP, whereby ATP became the activator and pH became the inhibitor of the channel.

We had reported that syntaxin-1A (Syn-1A), a soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein originally shown to mediate exocytic membrane fusion (23), also serves as a platform to modulate not only Ca\(^{2+}\) channels (24) but also voltage-gated K\(^{+}\) channels to orchestrate the events leading to secretion. We further showed that Syn-1A is also present in non-secretory cells, particularly cardiac myocytes, in which Syn-1A directly binds (25, 26) to nucleotide binding folds (NBF)-1 and NBF2 of SUR2A to inhibit cardiac K\(_{ATP}\) channels (27). These findings prompted us to investigate whether Syn-1A action on SUR2A is able to influence pH regulation of cardiac K\(_{ATP}\) channels.

**EXPERIMENTAL PROCEDURES**

**Constructs and Recombinant GST Fusion Proteins**—Gifts include plasmid pGEX-4T-1-Syn-1A from W. Trimble (Hospital for Sick Children, Toronto, ON, Canada), PEC-Ekir6.2 from S. Seino (Chiba University, Chiba, Japan), and pcDNA3-Kir6.2ΔC26 from F. M. Ashcroft (Oxford University, UK). pGEX-4T-1-SUR2A-NBF1 (encoding amino acids 684–872) and pGEX-4T-1-SUR2A-NBF2 (amino acids 1321–1499) were generated as described previously (27). For generating the construct pERIS-DSRed2/SUR2A-3×FLAG, the 3×FLAG epitope (MDYKDHDG-DYKDHDIDYKDDDDK) was introduced into the third extracellular loop of rat SUR2A (following residue 332). The position of this loop was deduced by comparison with the transmembrane topology derived for SUR1 by Conti et al. (28). A fragment of SUR2A cDNA encoding this region was replaced by a modified fragment, produced by overlap PCR. All constructs were verified by sequencing. GST fusion protein expression, purification, and thrombin cleavage were performed following the manufacturer’s instructions (Amersham Biosciences).

**Cell Culture and Transfection**—A HEK293 cell line stably expressing Kir6.2/SUR2A has previously been described by Giblin et al. (29). These HEK293 cells were cultured at 37 °C in 5% CO\(_2\) in minimum essential medium (Invitrogen) containing 2 g/liter glucose and supplemented with 10% fetal bovine serum. Cells were trypsinized and plated on glass coverslips overnight prior to voltage clamp experiments. TSA cells were cultured under the same conditions as HEK293 cells but in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 4.5 g/liter glucose supplemented with 10% fetal bovine serum. TSA cells were transiently co-transfected with green fluorescent protein and pcDNA3-Kir6.2ΔC26 using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. After 18–24 h, cells were trypsinized, plated on glass coverslips, and cultured overnight before voltage clamp experiments. For binding or co-immunoprecipitation studies, the HEK293 cells were transiently co-transfected with pIREs2-DsRed2/SUR2A-3×FLAG plus pECE-Kir6.2 or pcDNA3-Syn-1A plus Kir6.2 or transfected with pIREs2-DsRed2/SUR2A-3×FLAG or PEC-E-Kir6.2 alone using Lipofectamine 2000 (Invitrogen) as described above.

**Isolation of Rat Cardiac Myocytes**—Male Sprague-Dawley rats (200–225 g) were injected with heparin (3000 units/kg intraperitoneal). After 10 min, animals were sacrificed under anesthesia with phenobarbital (Somnotol, 75 mg/kg intraperitoneal). Hearts were rapidly excised and immersed in cold Ca\(^{2+}\)-free Tyrode’s solution (in mM, 140 NaCl, 4 KCl, 1 MgCl\(_2\), 10 glucose, and 5 HEPES; pH 7.4). The aorta was cannulated, and the hearts were perfused in a retrograde fashion on a Langendorff apparatus with Tyrode’s solution containing 1 mM CaCl\(_2\) for 3 min (9.6 ml/min, 37 °C) followed by perfusion with Ca\(^{2+}\)-free Tyrode’s solution for 5 min. The hearts were then perfused for 8.5 min with Ca\(^{2+}\)-free Tyrode’s solution containing 0.15 mg/ml collagenase (500 units/mg; Yakult Pharmaceutical Inc. Co., Ltd., Tokyo, Japan) and 0.04 mg/ml protease (Type XIV, 5.4 units/mg; Sigma) followed by another 10-min perfusion with Ca\(^{2+}\)-free Tyrode’s solution. All solutions were bubbled with oxygen for 10 min prior to and during perfusion. The right free wall of the right ventricle was dissected from the heart into 10 ml of Kraft-Brühe solution (in mM, 100 potassium glutamate, 10 potassium aspartate, 25 KCl, 20 glucose, 10 KH\(_2\)PO\(_4\), 2 MgSO\(_4\), 20 taurine, 5 creatine, 0.5 EGTA, and 5 HEPES; pH 7.2) and cut into 8–10 pieces. The solution was then passed through a nylon mesh and stored at room temperature (22–24 °C) until used. Single cardiac myocytes used were calcium-tolerant, quiescent, and had sharp cross-striations without membrane blebs.

**Electrophysiology**—Current recordings were performed using standard patch clamp techniques in the inside-out configuration, in a cell line stably expressing Kir6.2/SUR2A and in freshly isolated rat cardiac myocytes, under symmetrical K\(^{+}\) conditions (in mM, 140 KCl, 1 MgCl\(_2\), 1 EGTA, 10 HEPES, MgATP and pH as indicated). Recording pipettes were pulled from 1.5-mm borosilicate glass capillary tubes using a programmable micropipette puller (Sutter Instrument Co., Novato, CA) and fire-polished. The typical tip resistance when filled with solution was 1.0–1.5 megohms for cell line experiments and 0.5–0.8 megohms for rat cardiac myocytes. Syn-1A or GST recombinant proteins were added to bath solutions as indicated. To examine pH sensitivity, currents were evoked by ramp command potentials from −110 to 100 mV in 450 ms from a holding potential of 0 mV for both cell line and cardiac myocytes. To study the effects of Syn-1A on pH 6.8-induced K\(_{ATP}\) channel opening, currents were evoked by 500-ms hyperpolarizations to −120 mV for cell line experiments and by 100-ms depolarizations to +60 mV for rat cardiac myocytes experiments at 10-s intervals from a holding potential of 0 mV. Patches were excised in 0 mm ATP bath solution and then quickly subjected to perfusion of 1 mm ATP solution to confirm ATP sensitivity of currents recorded. The difference in current magnitude between 0 and 1 mm ATP was defined as the maximal current for each patch. To prevent channel run-down and to partially inhibit channel activity, 100 μM ATP was applied to bath solutions. Results are reported as the difference in current level when pH was changed from 7.4 to 6.8, expressed as a percentage of the maximal current of each individual patch. Each membrane patch was subjected to two pH 7.4–6.8 changes, with the solution used for the subsequent pH change containing either GST or Syn-1A. The effect of GST or Syn-1A can then be com-
pared with its own control within the same patch. All electrophysiological experiments were performed at room temperature (22-24°C) using an EPC9 amplifier with Pulse8.6 acquisition software (HEKA Electronik, Lambrecht, Germany). Data were filtered at 2 KHz and sampled at 10 KHz.

In Vitro Binding Assay and Western Blotting—Two days after transfection, the HEK293 cells were washed with ice-cold saline-buffered solution (phosphate-buffered saline; pH 7.4), then harvested in binding buffer (15 mM MES plus 15 mM HEPES (pH 5.5-8.0), 100 mM KCl, 1.5% Triton X-100, 2 µM pepstatin A, 1 µg/ml leupeptin, and 10 µg/ml aprozin). The cells were lysed by sonication, and insoluble materials were removed by centrifugation at 55,000 × g at 4 °C for 30 min. The detergent-extract (0.3 ml, 1.2 µg/µl protein) of HEK293 cells was incubated with GST (as a negative control) or GST-Syn-1A (all bound to glutathione agarose beads, 500 pmol of protein each) at 4 °C for 2 h. The samples were then washed three times with binding buffer, separated on 10 or 12% SDS-PAGE, transferred to nitrocellulose membrane, and identified with mouse anti-FLAG monoclonal antibody (Sigma, 1:700) or goat anti Kir6.2 antibody (Santa Cruz Biotechnology, 1:500). For binding of Syn-1A to SUR2A domain proteins, thrombin-cleaved Syn-1A (500 pmol of protein) was incubated with GST-SUR2A-NBF1 or -NBF2 (all bound to beads, 500 pmol of protein each) in 250 µl of binding buffer (pH 6.0-7.4) as described above. The precipitated proteins were separated on 15% SDS-PAGE and probed with anti-Syn-1A monoclonal antibody (Sigma, 1:1000).

Immunoprecipitation—HEK293 cell lysate extract was prepared as described above in immunoprecipitation buffer (same as binding buffer but containing 2 mM EDTA). 0.4 ml of cell extract (1.1 µg of protein/µl) was precleared by incubation with 35 µl of protein A-Sepharose beads (50% slurry) (Sigma) at 4 °C for 2 h. After centrifugation, the precleared extract was incubated with 5 µg of primary antibody (rabbit anti-FLAG from Sigma or goat anti Kir6.2 from Santa Cruz Biotechnology), which was prelinked to protein A-Sepharose beads by incubation with the beads at 4 °C for 2 h and then washing with immunoprecipitation buffer three times. The preimmune rabbit serum was used as a negative control. The precipitated proteins were washed three times with immunoprecipitation buffer and identified by Western blotting.

Data Analysis—Data analysis and curve fitting were performed using Origin 6.0 (Microcal Software Inc., Northampton, MA). Data are presented as means ± S.E. Differences in means were compared using the paired Student’s t-test or repeated measures analysis of variance followed by Dunnett’s test, with p < 0.05 considered as statistically significant.

RESULTS

Biphasic Effect of Intracellular pH on Cardiac K_{ATP} Channel Activity—Previous reports have shown a biphasic effect of intracellular pH on K_{ATP} channels, including cardiac Kir6.2/SUR2A, vascular smooth muscle Kir6.1/SUR2B, and also brain/pancreatic islet Kir6.2/SUR1 isoforms. We confirmed the effect of intracellular pH on the cardiac Kir6.2/SUR2A K_{ATP} channel either expressed stably in HEK293 cell line (Fig. 1A) or in freshly isolated rat ventricular myocytes (Fig. 1B). We performed inside-out patch clamp recordings to allow us to expose the intracellular face of the plasma membrane of these cells to different pH solutions. The effect of intracellular pH was examined with 100 µM ATP in the bath solutions to partially inhibit current levels and to prevent channel rundown. Results are expressed as a percentage of the maximal current (Fig. 1C), which is defined as the difference between current amplitudes at 0 and 1 mM ATP.

In the HEK293 cell line stably expressing Kir6.2/SUR2A (Fig. 1A and C, filled squares), the current amplitude at pH 7.4 was

\[
\begin{array}{c|c|c|c|c|c|c|c|c|c|c}
\text{pH} & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 \\
\text{ATP} (mM) & 7.4 & 7.4 & 6.8 & 6.5 & 6.0 & 5.0 & 7.4 & 7.4 & 7.4 & 7.4 \\
\end{array}
\]

FIGURE 1. Biphasic effect of intracellular pH on cardiac K_{ATP} channels. Sensitivity to intracellular pH was studied by recording macroscopic currents in the inside-out mode under symmetrical K^+ (140 mM) conditions. Inward rectifying currents were evoked by ramp command potentials from −110 to 100 mV in 450 ms from a holding potential of 0 mV. Inhibition by 1 mM ATP confirms ATP sensitivity of currents recorded. Each panel represents a superposition of eight consecutive traces. Similar biphasic effect of pH was observed in a cell line stably expressing Kir6.2/SUR2A (A) and in freshly isolated rat cardiac myocytes (B). C, summary of results from cell line and cardiac myocyte experiments. Data are presented as the means ± S.E. (n = 6 in each group), and repeated measures analysis of variance followed by Dunnett’s test was employed for statistical analysis (*, p < 0.05 from pH 7.4).
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A

i

\[
\begin{align*}
\text{[ATP]} & \text{ (mM)} \\
0 & 0.1 & 1 & 0.1 & 1 & 0 \\
7.4 & 6.8 & 7.4 & 6.8 & 7.4 & \text{pH} \\
\end{align*}
\]

GST

\[
\Delta_1 \Delta_2
\]

Time (min)

0 5 10 15 20 25

\[
\begin{align*}
\text{[ATP]} & \text{ (mM)} \\
0 & 0.1 & 1 & 0.1 & 1 & 0 \\
7.4 & 6.8 & 7.4 & 6.8 & 7.4 & \text{pH} \\
\end{align*}
\]

Syn-1A 1nM

\[
\Delta_1 \Delta_2
\]

Time (min)

0 5 10 15 20 25 30

\[
\begin{align*}
\text{[ATP]} & \text{ (mM)} \\
0 & 0.1 & 1 & 0.1 & 1 & 0 \\
7.4 & 6.8 & 7.4 & 6.8 & 7.4 & \text{pH} \\
\end{align*}
\]

Syn-1A 10nM

\[
\Delta_1 \Delta_2
\]

Time (min)

0 5 10 15 20 25 30

\[
\begin{align*}
\text{[ATP]} & \text{ (mM)} \\
0 & 0.1 & 1 & 0.1 & 1 & 0 \\
7.4 & 6.8 & 7.4 & 6.8 & 7.4 & \text{pH} \\
\end{align*}
\]

Syn-1A 100nM

\[
\Delta_1 \Delta_2
\]

Time (min)

0 5 10 15 20 25 30 35

\[
\begin{align*}
\text{[ATP]} & \text{ (mM)} \\
0 & 0.1 & 1 & 0.1 & 1 & 0 \\
7.4 & 6.8 & 7.4 & 6.8 & 7.4 & \text{pH} \\
\end{align*}
\]

Syn-1A 300nM

\[
\Delta_1 \Delta_2
\]

Time (min)

0 5 10 15 20 25 30 35

B

\[
\begin{align*}
\text{pH 7.4} & \rightarrow \text{pH 6.8} (\Delta_1) \\
\text{pH 7.4} & \rightarrow \text{pH 6.8}^+ (\Delta_2) \\
^+ & = \text{GST/Syn-1A}
\end{align*}
\]

% Change

0 25 50 75

GST 1 10 100 300

[Syn-1A] (nM)

* * *
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Concentration-dependent inhibitory effect of Syn-1A on acid-induced K$_{ATP}$ channel activation in a cell line stably expressing Kir6.2/SUR2A.

Macroscopic currents were recorded in the inside-out mode under symmetrical K$^+$ conditions. Currents were evoked by 500-ms hyperpolarizations to $-120$ mV at 10-s intervals from a holding potential of 0 mV. A, the time course of the experiment protocol, with each membrane patch subjected to two pH changes. The subsequent pH change ($\Delta_{pH}$) containing either GST (panel i) or 1 nM Syn-1A (panel ii), 10 nM (panel iii), 100 nM (panel iv), or 300 nM (panel v) was compared, with the first pH change ($\Delta_1$) serving as a control within the same patch. B, summary of the results with $n = 5-8$ in each group. Changes in amplitude are expressed as the percentage of the maximal current of each patch. Data are presented as the means ± S.E., and the paired student’s t test was employed for statistical analysis ($^*, p < 0.05$, **, $p < 0.01$).

14.5 ± 4.5% of the maximal current ($n = 6$). Decreasing pH to 6.8, 6.5, and 6.0 progressively increased current levels to 46.1 ± 10.6%, 71.1 ± 8.4%, and 71.8 ± 7.9%, respectively. However, further decreasing pH to 5.0 caused a reduction in current amplitude to 49.6 ± 7.6%. Current amplitudes at pH 6.8, 6.5, 6.0, and 5.0 were significantly different from the control level at pH 7.4 ($n = 6$, $p < 0.05$).

A similar biphasic pattern was observed in freshly isolated rat cardiac myocytes (Fig. 1, B and C, open squares). Current amplitude was 14.0 ± 2.9% ($n = 6$) at pH 7.4 but increased to 35.6 ± 6.6, 39.8 ± 4.5, and 45.1 ± 2.9% when pH was decreased to 6.8, 6.5, and 6.0, respectively. As with the cell line results, further decreasing pH to 5.0 caused a reduction in current level to 31.4 ± 4.4%. Current amplitudes at pH 6.8, 6.5, 6.0, and 5.0 were significantly different from the control level at pH 7.4 ($n = 6$, $p < 0.05$). Acid-induced channel activation was more pronounced in the HEK293 cell line (no endogenous Syn-1A) when compared with cardiac myocytes, presumably due to the presence of intrinsic Syn-1A in the cardiac myocyte plasma membrane (27).

Effect of Syn-1A on Reversing Acidic pH-induced K$_{ATP}$ Channel Opening—Since mild decreases in pH increased K$_{ATP}$ channel activity, we determined whether the increased binding of Syn-1A to SUR2A at lower pHs could affect acid-induced K$_{ATP}$ channel opening in the physiological range of pH 7.4-6.8 in the cell line stably expressing Kir6.2/SUR2A (Fig. 2A) and in cardiac myocytes (Fig. 3A). Each cell membrane patch was subjected to two pH 7.4-6.8 changes with the solution used for the subsequent pH change containing either GST or Syn-1A. The effect of GST or Syn-1A can then be compared with its own control within the same patch. In Figs. 2A and 3A, the change in current amplitude from pH 7.4 to pH 6.8 in the absence of GST or Syn-1A is indicated as $\Delta_1$, and the effect of the subsequent pH change in the presence of GST or Syn-1A on current amplitude is indicated as $\Delta_2$. Results are summarized (Figs. 2B and 3B) as the difference in current level when pH was changed from 7.4 to 6.8, expressed as a percentage of the maximal current in each individual patch.

In the cell line stably expressing Kir6.2/SUR2A (Fig. 2), GST at 300 nM had no effect on pH 6.8-induced channel opening ($\Delta_1$, 38.5 ± 7.9% versus $\Delta_2$, 35.8 ± 8.0%, $n = 7$, Fig. 2A, panel i, and B). Similarly, no significant effect was found with Syn-1A at <10 nM (Fig. 2A, panels ii and iii, and B). However, Syn-1A was able to reverse pH 6.8-induced channel opening significantly at higher concentrations of 100 nM ($\Delta_1$, 35.7 ± 6.5% versus $\Delta_2$, 17.1 ± 5.8%, $n = 8$, $p < 0.05$, Fig. 2A, panel iv, and B) and almost completely at 300 nM ($\Delta_1$, 33.2 ± 3.6% versus $\Delta_2$, 4.9 ± 4.7%, $n = 8$, $p < 0.01$, Fig. 2A, panel v, and B).

To determine whether the observed results also occur in primary native cells, the experiment was repeated in freshly isolated rat cardiac myocytes (Fig. 3). Similarly, acid (pH 6.8)-induced K$_{ATP}$ channel opening was not affected by GST at 300 nM ($\Delta_1$, 16.1 ± 1.5% versus $\Delta_2$, 17.1 ± 1.7%, $n = 8$, Fig. 3A, panel i, and B) but was reversed by Syn-1A at 300 nM ($\Delta_1$, 17.1 ± 1.9% versus $\Delta_2$, 6.6 ± 2.4%, $n = 8$, $p < 0.01$, Fig. 3A, panel ii, and B).

Taken together, these recordings on K$_{ATP}$ channel activity in cardiac myocytes and HEK cells indicate that Syn-1A is capable of reversing acidic pH-induced K$_{ATP}$ channel opening.

Syn-1A Has No Effect on Functional Kir6.2/SUR2A Channels in Absence of SUR2A—To verify that Syn-1A effects on cardiac K$_{ATP}$ channel gating is due to its action on SUR2A and not the Kir6.2 subunit, the same electrophysiological study was conducted on TSA cells expressing only the pore-forming subunit Kir6.2/SUR2A. Truncation of the C-terminal region of Kir6.2 allows fully functional pore-forming subunits to be expressed on the cell surface without their SUR counterparts (11). Using this approach, Syn-1A, at a concentration that caused significant inhibition of acid-induced channel opening in Kir6.2/SUR2A channels, had no effect on Kir6.2/SUR2A channel activity (Fig. 4, A and B). As with Kir6.2/SUR2A channels expressed in HEK293 cells, lowering the pH from 7.4 to 6.8 caused a significant increase in current amplitude ($\Delta_1$, 36.3 ± 8.2%, $n = 4$). However, unlike the channels with their SUR subunits, Syn-1A at 300 nM failed to inhibit acid-induced activation of Kir6.2/SUR2A-mediated K$_{ATP}$ currents ($\Delta_1$, 38.1 ± 4.6%, $n = 4$, $p = 0.88$ when compared with $\Delta_1$). These results indicate that Syn-1A mediates its inhibitory effects on K$_{ATP}$ Channel specifically through its interaction with regulatory SUR2A but not pore-forming Kir6.2.

Effect of pH on Syn-1A Binding to SUR2A—To understand the mechanism by which Syn-1A could inhibit pH-induced K$_{ATP}$ channel activation, we investigated the possibility that pH changes might affect Syn-1A binding to SUR2A. Because of the lack of a high affinity SUR2A-specific antibody to examine endogenous SUR2A proteins, we used GST-Syn-1A bound to glutathione beads to pull down FLAG-tagged SUR2A overexpressed in HEK293 cells under different pH conditions (pH 8-5.5), and the precipitated SUR2A proteins were then probed with anti-FLAG antibody. This assay allowed us to examine how pH changes influence the direct interactions of Syn-1A with SUR2A without the confounding effects of undefined endogenous proteins in cardiac membranes that might also influence Syn-1A-SUR2A binding. As shown in Fig. 5A, Syn-1A binding to SUR2A was progressively enhanced as pH was reduced from 8.0 to 6.0 (half-maximal effective pH value (EV_50) = 6.54 ± 0.05, $n = 4$). However, Syn-1A binding to SUR2A was slightly reduced at pH 5.5 when compared with pH 6.0. As we had previously determined that NBF-1 and NBF-2 are the putative SUR2A binding domains for Syn-1A (27), we performed the reciprocal binding study. GST-SUR2A-NBF1 or -NBF2 domain proteins bound to glutathione agarose beads were used to pull down thrombin-cleaved Syn-1A at different
levels of pH, and then the precipitated Syn-1A proteins were identified with anti-Syn-1A antibody. Fig. 5B shows that as pH reduced within the physiologic to pathophysiologic (in cardiac ischemia) range of pH 7.4-6.0, Syn-1A binding to SUR2A-NBF1 and -NBF2 domains progressively increased in a manner that was remarkably similar to that observed with the wild type SUR2A protein (Fig. 5A), with EV50s of pH 6.66 ± 0.03 and pH 6.59 ± 0.02 (n = 3), respectively. GST, as a negative control, did not bind to SUR2A or to thrombin-cleaved Syn-1A at the full pH range tested (data not shown).

To confirm that Syn-1A-SUR2A interactions are between properly folded expressed proteins, we performed immunoprecipitation studies of HEK cells co-expressing SUR2A-FLAG and Syn-1A. Rabbit anti-FLAG antibody was used to co-precipitate SUR2A-FLAG and Syn-1A, and the results are expressed as the percentage of the values determined at pH 7.4. As shown in Fig. 6A, anti-FLAG antibody precipitated only 85.3 ± 5.5% of SUR2A-FLAG protein at pH 6.8 when compared with pH 7.4 (n = 3), suggesting that acidic pH can decrease the primary antibody binding to its antigen. However, acidic pH (pH 6.8) actually enhanced co-precipitated Syn-1A by 67.1% (167.1 ±
21.1) when compared with pH 7.4 ($n = 3, p < 0.05$) (Fig. 6B). Taken together, this study clearly indicates that SUR2A binds much more Syn-1A at pH 6.8 than at pH 7.4.

To determine whether Syn-1A is able to bind Kir6.2, goat anti-Kir6.2 antibody was used to co-precipitate Syn-1A from Kir6.2- and Syn-1A-expressing HEK293 cell lysate extract (Fig. 7A). As a second approach, GST-Syn-1A bound to agarose glutathione beads was used to pull down thrombin-cleaved Syn-1A at different pH conditions as indicated. The precipitated proteins were identified with Western blotting with antibody against FLAG. The top panel is a representative blot of four separate experiments. The bottom panel shows the quantitative densitometry scanning of the specific bands from four separate experiments. The values (mean ± S.E.) are expressed as a percentage of the maximal value at pH 6.0 performed in every experimental condition to control for inter-assay variation. B, GST-SUR2A-NBF1 (top panel) and -NBF2 (bottom panel), all bound to glutathione beads, were used to pull down thrombin-cleaved Syn-1A at different pH conditions as indicated. Shown are representative blots of two separate similar experiments. Molecular masses of marker proteins are shown on the left.

Effects of ATP and Mg$^{2+}$ on pH Regulation of Syn-1A Binding to SUR2A—In addition to a fall in intracellular pH (6, 7) during myocardial ischemia, other major cellular events in the cardiac myocyte can influence $K_{ATP}$ channel activity, including the rapid depletion of ATP to as low as 1 mM and elevation of free Mg$^{2+}$ levels to 5-10 mM (31). We therefore examined whether ATP and Mg$^{2+}$ would affect the pH regulation of Syn-1A binding to SUR2A. The results were normalized to the percentage of the maximal binding values determined at pH 6.0 in the absence of ATP and Mg$^{2+}$. As shown in Fig. 8A, when 1 mM ATP was added (1 mM ATP, 0 mM Mg$^{2+}$, second panel), Syn-1A binding to SUR2A was reduced in the entire pH range tested when compared with controls (0 mM ATP, 0 mM Mg$^{2+}$, first panel) performed in parallel, with significant differences at pH 7.4.

**FIGURE 5.** pH effects on Syn-1A binding to SUR2A, in vitro pull down study. A, the binding assay was performed using GST-Syn-1A bound to agarose glutathione beads to pull down FLAG-tagged SUR2A from Kir6.2/SUR2A-FLAG-expressing HEK293 cell lysate extract at different pH conditions as indicated. The precipitated proteins were identified by Western blotting with antibody against FLAG. The top panel is a representative blot of four separate experiments. The bottom panel shows the quantitative densitometry scanning of the specific bands from four separate experiments. The values (mean ± S.E.) are expressed as a percentage of the maximal value at pH 6.0 performed in every experimental condition to control for inter-assay variation. B, GST-SUR2A-NBF1 (top panel) and -NBF2 (bottom panel), all bound to glutathione beads, were used to pull down thrombin-cleaved Syn-1A at different pH conditions as indicated. Shown are representative blots of two separate similar experiments. Molecular masses of marker proteins are shown on the left.

**FIGURE 6.** pH effects on Syn-1A binding to SUR2A, co-immunoprecipitation (IP) study. A co-immunoprecipitation assay was used to detect the association of Syn-1A with SUR2A. Rabbit anti-FLAG antibody (Anti-FLAG Ab) co-precipitated SUR2A-FLAG and Syn-1A at pH 7.4 or pH 6.8, and then the precipitated proteins were identified with anti-FLAG (in A) or anti-Syn-1A (in B) antibodies. The top panel in A and B is a representative blot of three separate experiments. The bottom panel is a summary of three experiments. The results (mean ± S.E.) are expressed as a percentage of the value at pH 7.4. * indicates $p < 0.05$; WB, Western blot.
Kir6.2/SUR2A Channel Modulation by Syntaxin-1A and pH

**DISCUSSION**

The cardiac myocyte Kir6.2/SUR2A channel is regulated by multiple factors, including adenine nucleotides (ATP, ADP), Mg\(^{2+}\), phospholipids, and pH (1, 3, 4). The Kir6.2 subunit is the binding site for ATP (11), phosphatidylinositol-4,5-bisphosphate (8, 9), and protons (16, 18-20), whereas the NBFs within the SUR proteins are the binding sites for Mg\(^{2+}\) and ADP (32).

Our recent study had identified a “ternary” protein, Syn-1A, to be present in the plasma membrane of rat cardiac myocytes, which binds cardiac SUR2A protein at its NBF-1 and NBF-2 domains, causing inhibition of K\(_{\text{ATP}}\) channel activity (27). The present study demonstrates that Syn-1A binding to SUR2A and resultant effects on cardiac K\(_{\text{ATP}}\) channel activity are profoundly regulated by the full range of pH encompassing normal physiologic state to mild (exercise) and severe cardiac stress (cardiac ischemia). Although the concentrations of Syn-1A used for the
electrophysiological studies in the cell line (100 nm) and cardiac myocyte (300 nm) seemed relatively high, Syn-1A localized to cardiac plasma membrane (27) may be sandwiched with the channel at such high concentrations. In support of this, botulinum neurotoxin C expression into insulinoma cells to effectively destroy endogenous Syn-1A increased Kir6.2/SUR1 K_ATP currents (26). A similar study of perfusing recombinant toxin into cardiac inside-out patches was not successful in consistently altering K_ATP channel activity (data now shown), likely because of a more stable Syn-1A/SUR2A complex not accessible to toxin cleavage. In addition to pH, our findings also show that Syn-1A binding to SUR2A (in SUR2A-FLAG-expressing and Kir6.2/SUR2A-FLAG-expressing cells) can also be modulated by Mg^{2+} and ATP-induced conformational changes on the SUR2A subunit and not the Kir6.2 subunit. In support of the latter, Syn-1A did not modulate Kir6.2ΔC26 channel activity directly or influence pH regulation of this truncated channel and did not directly bind expressed Kir6.2 or its cytoplasmic domains. Our results therefore suggest that Syn-1A serves as an important integrative link for these dynamically changing metabolic factors (protons, ATP, Mg^{2+}) to finely regulate cardiac K_ATP channel activity in health and disease. Below, we discuss each of the features of this novel study.

Jiang and co-workers (16, 18-20) have shown that pH modulation of Kir6.2 channel activity is independent of SUR and that the full pH sensitivity requires complex interactions between multiple domains within the Kir6.2 subunit. pH and ATP, both acting on distinct sites within the Kir6.2 protein, can reciprocally regulate the sensitivity of K_ATP channels to each other. These effects of pH and ATP are significantly enhanced by the SUR subunit (19), indicating that SUR plays an important role in the fine-tuning of the modulation of the K_ATP channel by these metabolic factors. We show that pH changes could also modulate K_ATP channel activity via a profound influence on Syn-1A binding to the SUR2A subunit. Surprisingly, acidic pH enhanced (rather than reduced) Syn-1A binding to SUR2A, and more remarkably, this “indirect” effect of acidic pH was able to completely overcome and block the direct acidic pH-induced opening of the K_ATP channel. Whether and how protons induce conformational changes to the NBF domains and/or Syn-1A to increase their binding interactions and how the Syn-1A/NBFs interactions transduce the potent inhibitory signal on the Kir6.2 pore subunit would need further studies. An intriguing possibility is that the resulting conformational change on SUR2A may induce further conformational changes on Kir6.2 to reduce its sensitivity to protons as to render the protons ineffective in opening the channel.

Binding of Mg^{2+} to the NBFs of SUR subunits provides coordinate regulation, leading to k_ATP channel activation by antagonizing the inhibitory effect of adenine nucleotides on the Kir6 subunits (11, 32, 33). Our results show that Mg^{2+} enhanced Syn-1A binding to SUR2A at all pH values tested and achieved statistical significance between pH 7 and 7.4. In the presence of both ATP and Mg^{2+}, ATP abolished Mg^{2+}-induced enhancement at the physiologic range (7.4 and 7.0) but not at acidic pHs (6.8-6.0). These results suggest that ATP and Mg^{2+} play important counter-regulatory actions on Syn-1A binding to SUR2A during changes in cytosolic pH. Although pH (6, 7) and ATP concentrations (5-7) fall during cardiac ischemia, the free Mg^{2+} level rises (34-36). Therefore, it appears that all of the changes in metabolic factors occurring during ischemic stress, namely falling pH and ATP levels as well as rising Mg^{2+} concentration, have enhancing effects on Syn-1A binding to SUR2A. Taken together with our findings from the functional studies that enhanced interactions of Syn-1A has inhibitory effects on K_ATP channel opening, we propose a model in which Syn-1A may serve as a “brake” to K_ATP channel activation during periods of metabolic stress.

Under normal physiological conditions, K_ATP channels remain closed in the cardiac myocardium. Conversely, during metabolic stress, falling levels of both ATP and pH would promote channel opening. Although K_ATP channel activation is believed to be cytoprotective via the reduction of Ca^{2+} load and cell excitability (37, 38), the resultant shortening of action potential has also been implicated with the generation of fatal re-entrant arrhythmias (39, 40). Syn-1A inhibition of K_ATP channel activity in acidic tissue may protect against excessive or haphazard K_ATP channel activation that disperses membrane repolarization between ischemic and non-ischemic myocardium, predisposing to heterogeneity of impulse propagation that leads to life-threatening re-entrant arrhythmias (39, 40). In conclusion, our current study demonstrated that acidic pH increases the binding of Syn-1A to SUR2A, which transduces a strong inhibition of the K_ATP channel sufficient to reverse acidic pH-induced K_ATP channel activation. In this manner, Syn-1A may have a paradoxical secondary cardio-protective effect during cardiac ischemia by limiting arrhythmias, serving as a physiological brake to temper the potentially adverse effects caused by haphazard fluctuations of pH, ATP, and Mg^{2+} occurring during conditions of mild (exercise) and severe (cardiac ischemia) cardiac stress.

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