Syntaxin-1A Binds the Nucleotide-binding Folds of Sulphonylurea Receptor 1 to Regulate the $K_{\text{ATP}}$ Channel*

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ATP-sensitive potassium ($K_{\text{ATP}}$) channels in neuron and neuroendocrine cells consist of a pore-forming Kir6.2 and regulatory sulfonylurea receptor ($\text{SUR1}$) subunits, which are regulated by ATP and ADP. SNARE protein syntaxin 1A (Syn-1A) is known to mediate exocytic fusion, and more recently, to also bind and modulate membrane-repolarizing voltage-gated K$^+$ channels. Here we show that Syn-1A acts as an endogenous regulator of $K_{\text{ATP}}$ channels capable of closing these channels when cytotoxic ATP concentrations were lowered. Botulinum neurotoxin C1 cleavage of endogenous Syn-1A in insulinoma H1T-T15 cells resulted in the increase in $K_{\text{ATP}}$ currents, which could be subsequently inhibited by recombinant Syn-1A. Whereas Syn-1A binds both nucleotide-binding folds (NBF-1 and NBF-2) of SUR1, the functional inhibition of $K_{\text{ATP}}$ channels in rat islet $\beta$-cells by Syn-1A seems to be mediated primarily by its interactions with NBF-1. These inhibitory actions of Syn-1A can be reversed by physiologic concentrations of ADP and by diazoxide. Syn-1A therefore acts to fine-tune the regulation of $K_{\text{ATP}}$ channels during dynamic changes in cytosolic ATP and ADP concentrations. These actions of Syn-1A on $K_{\text{ATP}}$ channels contribute to the role of Syn-1A in coordinating the sequence of ionic and exocytic events leading to secretion.

All the excitable cell types, including neurons, muscles, and endocrine cells, are equipped with $K_{\text{ATP}}$ channels (1). The $K_{\text{ATP}}$ channel couples the intracellular metabolic state to electrical activity at the plasma membrane to regulate a number of cellular events such as hormone secretion, muscle contraction, and neuron excitability (1). The dominant $K_{\text{ATP}}$ channels in neurons and neuroendocrine cells make up an octameric protein complex comprised of four Kir6.2 subunits, which are members of the inward rectifying K$^+$ channel family, and four SUR1$^1$ subunits (1–3). Each SUR1 protein contains two NBFs, NBF-1 and C-terminal NBF-2 (3). Much work has been done to elucidate the actions of ATP and ADP on this channel complex (3–9), which, however, remains insufficient to fully explain the regulation of the $K_{\text{ATP}}$ channel (1). Toward this, the neuroendocrine pancreatic islet $\beta$-cell has been the best studied physiologic model (1–4). In the $\beta$-cell, glucose entry into the cell increases the ATP/ADP ratio, which closes the $K_{\text{ATP}}$ channels (4). This leads to membrane depolarization, which opens voltage-dependent Ca$^{2+}$ channels (Ca$_V$), allowing Ca$^{2+}$ to enter the cell and interact with Ca$^{2+}$-sensitive proteins, which eventually leads the SNARE proteins to effect exocytosis (10, 11). Membrane repolarization effected by the opening of voltage-dependent K$^+$ ($K_\text{v}$) channels then closes Ca$_V$, to terminate exocytosis. This sequence of ionic and exocytic events in the $\beta$-cell parallels those of neurons (11).

The role of SNARE proteins in the plasticity of neuro- and neuroendocrine secretion extends beyond exocytotic fusion (10, 11) and is now known to also directly regulate not only Ca$_V$ channels (12, 13), but more recently, the $K_\text{v}$ channels (14, 15) as well. Here, we have used the neuroendocrine $\beta$-cell models to examine whether SNARE proteins could regulate $K_{\text{ATP}}$ channel closure, which would trigger the sequence of ionic events leading to exocytosis. The SURs are members of the ATP-binging cassette protein superfamily, which includes the CFTR CI–channel (16, 17). Since SNARE protein,Syn-1A, binds CFTR (17), we speculated that SUR1 might have similar interactions with SNARE proteins. Whereas Syn-1A binds the N-terminal tail of CFTR (17), we surprisingly found that Syn-1A binds SUR1 at its NBF-1 and NBF-2 domains. Since NBF-1 and NBF-2 are nucleotide-regulated domains, this Syn-1A binding would likely influence ATP and ADP regulation of this channel.

**MATERIALS AND METHODS**

Cell Culture and Transfection—H1T-T15 cells (P. Robertson, Seattle, WA), passages 75–85, grown in Petri dishes (RPMI 1640 medium supplemented with 20 mM glutamine, 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin) were transfected with plasmids (in pcDNAs, Clontech) containing cDNAs of full-length Syn-1A, or with the light chain of BoNT/C1; or Syn-1A-transfected cells (14, 18). H1T-T15 cells have a >90% probability of picking up multiple plasmids (14, 18). Transfection efficiency at 48 h was ~30–40% as determined by visualization of the co-expressed GFP, which serves to identify the transfected cells for electrophysiologic studies.

**Islet $\beta$-Cell Isolation**—Pancreatic islets were isolated from male Sprague-Dawley rats by collagenase digestion and dispersed into single nucleotide-binding folds; CFTR, cystic fibrosis transmembrane conductance regulator protein; GFP, green fluorescent protein; EGFP, enhanced GFP; Glyb, glybenclamide; HIT, hamster insulin-secreting tumor; IP, immunoprecipitation; F, farad; Ca$_V$, voltage-dependent Ca$^{2+}$-channels; $K_\text{v}$, voltage-dependent K$^+$ channels.
cells by treatment with 0.01% trypsin in Ca2+- and Mg2+-free phosphate-buffered saline as described previously (14). Islet cells were plated on glass coverslips in 35-mm dishes and cultured in 2.8 mM glucose (with 7.5% fetal calf serum, 0.25% sodium, 100 μg/ml streptomycin). For intracellular dialysis experiments, islet cells were cultured for 1–2 days before electrophysiological recordings.

Electrophysiology—Single islet β-cells and HIT-T15 cells were studied using the whole-cell patch-clamp technique as reported previously (14, 18). Thin-walled (1.5 mm) borosilicate glass microelectrodes were pulled with a two-stage Narishige (Tokyo, Japan) micropipette puller and heat-polished. The typical tip resistance was 2–4 MΩ. The external solution contained 140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 10 mM HEPES, pH 7.3, and as indicated, 2 mM MgCl2 or 1 mM MgCl2. The internal patch pipette solution contained 140 mM NaCl, 10 mM MgCl2, 10 mM HEPES, 1 mM EGTA, pH 7.4, as indicated, 0.1% bovine serum albumin, 0.2% glutathione, and 0.1% Triton X-100.

RESULTS

Syntaxin-1A Regulates KATP Channels—Using the whole-cell patch clamp technique (14, 18), we first identified the KATP channels in the insulinoma HIT-T15 cells by using glybenclamide (Glyb), the sulfonylurea KATP channel inhibitor. Glyb (0.1 μM) greatly inhibited the HIT-T15 KATP currents (Fig. 1, A and B) recorded at low ATP (0.3 mM) concentration (n = 3). Dialysis of GST-Syn-1A (1 μM) into the HIT-T15 cells also greatly inhibited the KATP currents (Fig. 1, A and B; n = 10). As a control, we used HIT-T15 cells dialyzed with GST (1 μM), which had no effect on KATP currents when compared with the pipette solution (data not shown). Fig. 1C shows the time course of GST-Syn-1A inhibitory effect on the KATP currents under the low ATP (0.3 mM) concentration. Just after the formation of the whole-cell configuration (t = 0 min), the KATP currents remained low for ~2 min (asterisk) for both control and GST-Syn-1A-treated cells. After 2 min, control currents increased steadily as the cell interior equilibrated with the low ATP pipette solution so that the KATP channels would open. At t = 10 min, the control KATP current amplitude exhibited a vigorous increase to 169.0 ± 15.9 pA (n = 5), whereas the current amplitude of the cells dialyzed with GST-Syn-1A showed only a small increase to 65.4 ± 9.7 pA (n = 5). Fig. 1D shows the summary bar graph in which the KATP currents were normalized to cell capacitance (pA/pF) to eliminate the variations in cell size. Glyb (0.1 μM) inhibited KATP current amplitude by ~85% (n = 3), whereas GST-Syn-1A (1 μM) reduced KATP currents by ~65% (n = 5) of the control current (61.9 ± 8.8 pA/pF; n = 5).

To determine whether the effect of Syn-1A is at the plasma membrane or cytosolic level, we examined HIT-T15 cells expressing the full-length Syn-1A (aa 1–266) (20), which included the transmembrane domain, thereby specifically targeting Syn-1A to the plasma membrane (18). This Syn-1A overexpression (>3-fold by Western blot) exhibited very similar inhibition of the KATP whole-cell currents (data not shown) as the dialysis of cytosolic Syn-1A (aa 1–266) shown in Fig. 1. These results indicate that the active Syn-1A domain that inhibits this KATP channel involves only the cytoplasmic domain (aa 1–266) and not the transmembrane domain (aa 267–288) of Syn-1A.

We have postulated that the closed state of this KATP channel is attributed not only to cytosolic ATP levels but possibly also to the levels of endogenous Syn-1A. To examine whether Syn-1A acts as an endogenous inhibitor of the KATP channel, we expressed bovine neurotoxin C1 (BoNT/C1) in HIT-T15 cells, which would specifically cleave the endogenous Syn-1A (21). Since HIT-T15 cells very reliably pick up multiple plasmids (>90%), we co-expressed GFP to identify the BoNT/C1-expressing cells (18). Confocal microscopy showed that the membrane Syn-1A signal in the BoNT/C1-expressing cells (GFP-containing, indicated by arrows) was greatly reduced (Fig. 1E, lower panels) when compared with the endogenous Syn-1A levels of the adjacent cells that did not express BoNT/C1. As the BoNT/C1-expressing cells did not show an increase in the cytosolic Syn-1A signal, this suggests that the cleaved Syn-1A fragments likely undergo cytosolic proteolysis since the Syn-1A monoclonal antibody (Sigma) was generated against the full-length protein and would have recognized either the membrane-bound or the cytosolic fragments of Syn-1A. Since BoNT/C1 could also cleave SNAP-25 in some cell types (22), we also labeled these cells with anti-SNAP-25 antibody (Fig. 1E, upper panels) but saw no difference in the membrane SNAP-25 signals between the BoNT/C1-expressing cells (GFP-containing, indicated by arrows) and the cells that did not express GFP. This result indicates specific proteolysis of endogenous Syn-1A by BoNT/
C1. Next, we examined the whole-cell \( K_{\text{ATP}} \) currents of these BoNT/C1-expressing cells (Fig. 1, A and B), which was \(-155\%\) \((n = 5)\) of the control cells (Fig. 1D), indicating that the \( K_{\text{ATP}} \) currents were indeed being inhibited by the endogenous Syntaxin-1A. Dialysis of GST-Syn-1A \((1 \mu M)\) into these BoNT/C1-expressing cells (Fig. 1A, BoNT/C1+Syn-1A) reduced the \( K_{\text{ATP}} \) current by \(-70\%\) \((n = 8)\) as compared with the BoNT/C1-expressing cells, a reduction similar to the one observed with GST-Syn-1A.
in the control cells. The cytosolic ATP concentration in these cells was lowered to 0.3 mM ATP to reduce ATP blockade of the $K_{ATP}$ channel. The observed Syn-1A inhibition of the $K_{ATP}$ channels may be due to an increase in the sensitivity to ATP-mediated inhibition, or it may be independent of ATP inhibition. More studies would be required to distinguish these possibilities.

**Syntaxin-1A Binds SUR1 at its NBF-1 and NBF-2—Syn-1A actions on these $K_{ATP}$ channels could be mediated by binding to the SUR1 or Kir6.2 subunits. GST-Syn-1A bound to agarose beads pulled down SUR1 from solubilized rat brain synaptic membranes, which was identified with a specific antibody generated against the SUR1 C terminus (19) (Fig. 2A). The major cytoplasmic domains of SUR1 are NBF-1 (aa 696–893) and C-terminal NBF-2 (aa 1358–1544) (3). Fig. 2B shows that GST-NBF-1 and GST-NBF-2 bound to agarose beads pulled down native Syn-1A from rat brain synaptic membranes. To negate the possibility of a coprecipitation of unknown ternary proteins present in the brain, we demonstrated that GST-NBF-1 and GST-NBF-2 pulled down recombinant Syn-1A but not control GST or recombinant SNAP-25 (data not shown). Fig. 2C shows the reciprocal study of immunoprecipitation of Syn-1A (GST cleaved off by thrombin), which pulled down GST-NBF-1 (lane 3) and GST-NBF-2 (lane 5) and not GST (lane 1). The coprecipitated NBFs were identified by Coomassie Blue staining as the only proteins eluting at the same sizes as the control proteins (lanes 4 and 6). Fig. 2D examines the time course of binding (4 °C), showing similar rapid binding kinetics of both NBFs to Syn-1A, in which Syn-1A binding was detectable at 2 min (first time point, ~20% of maximal binding), reaching maximal binding at 1–2 h. Half time ($t_{1/2}$) was ~15.2 and ~9.3 min for NBF-1 and NBF-2, respectively. Fig. 2E examines (i) the concentration dependence of GST-NBF-1 (i) and GST-NBF-2 (ii) binding to Syn-1A (500 pmol, 4 °C, 2 h). Top, representative blots; bottom, summary of three experiments.

**Syntaxin-1A Inhibition of the $K_{ATP}$ Channel Is Mediated by Its Binding to NBF-1—The binding of Syn-1A to NBF-1 and NBF-2 of SUR1 suggests that Syn-1A inhibition of the HIT-T15 $K_{ATP}$ channels may be mediated by interactions with either one of the NBFs. Therefore, we examined the functional interactions of Syn-1A with each NBF using rat islet $\beta$-cells, which would be a better physiologic model. The rat islet $\beta$-cell $K_{ATP}$ channels (Fig. 3, A–C) behaved very similarly as the HIT-T15 cell $K_{ATP}$ channels (Fig. 1, A–D). Specifically, under low ATP (0.3 mM ATP) concentrations, the external application of Glyb (0.1 mM) and dialysis of GST-Syn-1A (1 μM) into islet $\beta$-cell
inhibited the $K_{ATP}$ currents (Fig. 3A). The time course of GST-Syn-1A inhibition of rat islet $\beta$-cell $K_{ATP}$ channels (Fig. 3B) was in fact very similar in pattern to those observed in the HIT cell study (Fig. 1C). When normalized to cell capacitance (pA/pF; Fig. 3C), Glyb (0.1 $\mu$m) and GST-Syn-1A (1 $\mu$m) inhibited rat islet $\beta$-cell $K_{ATP}$ control currents (65.4 ± 9.7 pA/pF; n = 12) by 85% (9.7 ± 1.7 pA/pF; n = 3) and 61% (25.6 ± 5.7 pA/pF, n = 10), respectively. These values are remarkably similar to the HIT cell studies (Fig. 1).

Next we examined the effects of GST-NBF-1 and GST-NBF-2 on the inhibitory actions of Syn-1A (Fig. 3, A and C). GST-NBFs would be expected to bind the endogenous Syn-1A, which would either prevent the formation of or disrupt the complex already formed by endogenous Syn-1A with the $\beta$-cell SUR1, both of which would increase $K_{ATP}$ currents. Since GST-NBF-1 (1 $\mu$m) and GST-NBF-2 (1 $\mu$m) alone only slightly but not significantly increased $\beta$-cell $K_{ATP}$ currents to 77.8 ± 14.6 pA/pF (n = 6) and 72.6 ± 16.3 pA/pF (n = 8), respectively, this supports the latter possibility, that the pre-existing endogenous Syn-1A may have formed stable complexes with the NBFs of many of the endogenous SUR1 proteins, and these complexes may be resistant to disruption by the exogenous GST-NBFs. BoNT/C1 (Fig. 1) would disrupt such complexes upon cleavage of the endogenous Syn-1A. The exogenous NBFs could still pull down Syn-1A from the rat brain (as in Fig. 2A) since Syn-1A is more abundant and generally distributed in the brain than SUR1, and hence, an excess of brain Syn-1A would be available to bind the exogenous NBF proteins. More importantly, when dialed together with GST-Syn-1A (1 $\mu$m), GST-NBF-1 (NBF-1+Syn-1A) completely blocked the inhibitory effect of GST-Syn-1A on the $K_{ATP}$ currents (90.2 ± 16.6 pA/pF; n = 10) (Fig. 3C), which was not significantly different from NBF-1 alone. This is because GST-NBF-1 was premixed with GST-Syn-1A in the pipette solution, which would block the exogenous GST-Syn-1A from binding the free endogenous $\beta$-cell SUR1 to inhibit the $K_{ATP}$ channel. However, GST-NBF-2 did not prevent the inhibitory effects of GST-Syn-1A (NBF-2+Syn-1A), whose $K_{ATP}$ currents remained reduced at 31.9 ± 7.4 pA/pF (n = 4), a value close to the inhibition caused by GST-Syn-1A used alone. These results suggest that Syn-1A seems to interact functionally more with NBF-1 than with NBF-2 to inhibit the $\beta$-cell $K_{ATP}$ channels, at least under these experimental conditions. However, NBF-1 and NBF-2 exist as a heterodimer in the intact cell (1, 5), which, when taken with our result in Fig. 2 showing that Syn-1A binds NBF-1 and NBF-2 with similar affinity, supports an important role of NBF-2, either by its direct interaction with Syn-1A and/or by its influence on NBF-1 interactions with Syn-1A.

**ADP and Diazoxide Can Antagonize Syntaxin-1A Inhibition of $K_{ATP}$ Channels**—Since ADP is the physiologic antagonist to ATP-mediated inhibition of the $K_{ATP}$ channels (25), we examined whether ADP could antagonize the inhibitory effect of Syn-1A on islet $\beta$-cell $K_{ATP}$ channels (Fig. 4, A and B). MgADP (0.3 mM ADP in the pipette solution with 0.3 mM ATP) elicited a $-135\%$ increase (154.3 ± 48.0 pA/pF; n = 8) in $K_{ATP}$ currents from control values (65.4 ± 9.7 pA/pF, n = 12), consistent with a similar study performed on Xenopus oocytes (26). Additional ADP could also be further generated from the hydrolysis of the 0.3 mM ATP. $K_{ATP}$ current amplitude resulting from dialysis of
MgADP (0.3 mM) along with GST-Syn-1A (1 μM) was reduced to 60.6 ± 16.2 pA/pF (n = 8), which is similar to the control currents. This is in contrast to a much more reduced $K_{\text{ATP}}$ current caused by Syn-1A, where cytosolic ADP levels were not raised (Fig. 3). These results indicate that Syn-1A reduced the sensitivity of the $K_{\text{ATP}}$ channel to opening by MgADP, or vice versa, that MgADP reduced the sensitivity of the $K_{\text{ATP}}$ channel to closing by Syn-1A. A possible explanation for these results is that ADP could displace ATP binding to NBF-1 (6, 24). Even at 0.3 mM ATP, there is sufficient ATP to bind the NBFs at micromolar concentrations (6, 24). Taken together, these results suggest that the Syn-1A interactions with the NBFs might include the binding to the Walker A and B motifs or at least be influenced by these domains (25, 26).

The $K_{\text{ATP}}$ channel opener, diazoxide, opens $K_{\text{ATP}}$ channels even under high cytosolic ATP concentrations by its high affinity interaction with SUR1 (5, 25–27). In Fig. 4C (open bars), 3 mM ATP in the pipette solution closed $K_{\text{ATP}}$ channels in rat islet β-cells (10.8 ± 3.8 pA/pF, n = 8), and the addition of GST-Syn-1A (3 mM ATP + Syn-1A) caused a further albeit small and insignificant reduction of $K_{\text{ATP}}$ currents (6.2 ± 0.8 pA/pF, n = 8). At low ATP (0.3 mM ATP) concentrations (open bars), $K_{\text{ATP}}$ currents were 59.1 ± 14.3 pA/pF (n = 3), but when GST-Syn-1A (1 μM) was present (0.3 mM ATP + Syn-1A), the $K_{\text{ATP}}$ currents were reduced to 20.3 ± 6.6 pA/pF (n = 4). The subsequent addition of diazoxide (200 μM) to these cells (Fig. 4C, solid bars) opened the $K_{\text{ATP}}$ channels to the same extent either in the absence or in the presence of GST-Syn-1A (1 μM), whether the cytosolic ATP concentration was high (3 mM ATP) or low (0.3 mM ATP). Specifically, at 3 mM ATP, diazoxide increased $K_{\text{ATP}}$ currents to 30.7 ± 12.8 pA/pF (n = 8) and 33.2 ± 8.1 pA/pF (n = 4) in the absence and in the presence of GST-Syn-1A, respectively. At 0.3 mM ATP, diazoxide caused the $K_{\text{ATP}}$ currents to increase to 91.8 ± 10.8 pA/pF (n = 3) and 80.3 ± 12.9 pA/pF (n = 4) in the absence and in the presence of GST-Syn-1A, respectively. Diazoxide can therefore completely prevent GST-Syn-1A inhibition of the $\beta$-cell SUR1/K$_{\text{ATP}}$ channel, but higher ATP concentrations could reduce this diazoxide effect. This effect of diazoxide to fully reverse the Syn-1A inhibition is contrasted to only by a reduced sensitivity of the Syn-1A-inhibited $K_{\text{ATP}}$ channels to ADP-mediated opening (Fig. 4, A and B).

**DISCUSSION**

Our studies show that Syn-1A binds to the NBF-1 of neuroendocrine β-cell SUR1 (Fig. 1), which leads to the inhibition of the $K_{\text{ATP}}$ channel even when cytosolic concentrations of ATP are low. However, as cytosolic ATP concentrations rise, the inhibitory effect of Syn-1A is small relative to direct ATP inhibition of the Kir6.2 subunit (8, 9). This inhibition of the $K_{\text{ATP}}$ channel by Syn-1A may reduce the efficacy of the rising cytosolic ADP concentrations (Fig. 4) generated by ATP hydrolysis to open the $K_{\text{ATP}}$ channel. These results raise the intriguing
possibility that Syn-1A does not simply inhibit the $K_{ATP}$ channel but may also provide a “braking” mechanism on the $K_{ATP}$ channel opening during dynamic changes of ATP and ADP concentrations. This regulation may be lost when Syn-1A levels are reduced, as is the case in a number of diseases (28–31).

Our results showing the effects of BoNT/C1-induced reduction of HIT-T15 cell levels of Syn-1A on the $K_{ATP}$ currents (Fig. 1) may be of relevance to the pathogenesis of a number of neuro- and neuroendocrine diseases in which the cellular levels of Syn-1A are severely perturbed (28–31). In Williams syndrome, a multisystem disorder, the observed hemizygous deletion of Syn-1A is believed to cause or at least contribute to the severe neurological symptoms (28). The frontal cortex of patients with schizophrenia and depression, whose cause of death was suicide, were noted to exhibit elevated Syn-1A levels as compared with patients who died of other causes (29). These reports suggest that abnormalities in Syn-1A and the complexes formed with its interacting proteins could provide the molecular substrates for abnormalities of neural connectivity (28, 29). Our work suggests that the molecular substrates for abnormalities of neural connectivity were suicide, were noted to exhibit elevated Syn-1A levels as compared with patients who died of other causes (29). These

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