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Syntaxin Modulates Kv1.1 through Dual Action on Channel Surface Expression and Conductance†

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ABSTRACT: The Kv1.1 channel that is expressed throughout the central and peripheral nervous system is known to interact with syntaxin 1A, a member of the exocytosis machinery protein complex. This interaction was previously shown to increase the macroscopic currents of the presynaptic Kv1.1 channel when coexpressed in *Xenopus* oocytes, while it decreased the unitary channel conductance and open probability. This apparent discrepancy has been resolved in this work, using electrophysiological, biochemical, and immunohistochemical analyses in oocytes by overexpression and antisense knockdown of syntaxin. Here, we demonstrate that syntaxin plays a dual role in the modulation of Kv1.1 function: enhancement of the channel's surface expression along with attenuation of single channel ion flux. These findings broaden the scope of channels and transporters that are dually modulated by syntaxin. Although the dual functioning of syntaxin in modulation of Kv1.1 channel activity may seem antagonistic, the combination of the two mechanisms may provide a useful means for fine-tuning axonal excitability and synaptic efficacy.

In the nervous system voltage-gated K^+ channels (Kv)[1] serve a variety of important functions, particularly the control of cell excitability (*1*). Interactions between Kv channels, which are involved in exocytosis, and proteins of the exocytotic machinery complex, the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, have been shown to modulate the properties of the channels (*2*−*7*). Recently, the direct interaction of a Kv channel with syntaxin was shown to enhance transmitter release (*8*−*10*). These interactions are reminiscent of those occurring between some exocytotic proteins and the voltage-gated Ca^{2+} channels which affect the gating of the channels, their efficient plasma membrane targeting, and the efficacy of transmitter release (*11*−*14*).

The Kv1.1 channel, a fast delayed rectifying Kv channel belonging to the *Shaker* family of Kv channels, is expressed throughout the central and peripheral nervous system (*15*, *16*) and has a role in repolarizing the membrane potential in axons and at synaptic terminals (*17*, *18*), thereby modulating axonal excitability and synaptic efficacy.

We showed that Kv1.1 interacts with syntaxin 1A (syntaxin) and SNAP-25, in *Xenopus* oocytes, synaptosomes, and islet β-cells (*2*, *19*). The N-terminus of the channel was shown to be the site of interaction with syntaxin and to mediate its modulation of the channel function (*2*). Specifically, analysis of macroscopic currents in oocytes coexpressing Kv1.1 and syntaxin demonstrated a biphasic dependence on syntaxin concentration. Low syntaxin concentration caused an increase in K^+ current amplitudes, and no apparent increase in cell-surface expression could be detected. In contrast, high syntaxin concentration caused a reduction in current amplitudes, which was coupled to a concomitant decrease in cell-surface channel expression. Therefore, it was suggested that low syntaxin concentration regulates intrinsic channel properties leading to enhanced macroscopic currents, whereas the high syntaxin concentration was assumed to impair the machinery responsible for trafficking of proteins to the plasma membrane (*2*).

Surprisingly, our follow-up study of the effect of low syntaxin concentration on single channels in plasma membrane patches of oocytes demonstrated that low syntaxin decreased the unitary conductance of all of the conductance states of Kv1.1 and decreased their open probabilities (*20*), in an apparent discrepancy with the enhanced macroscopic currents (*2*). This raised the notion that, in contrast to our previous observation (*2*), low syntaxin concentration, possibly within the range of physiologically relevant concentrations, does have a role in

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[1]Abbreviations: Kv channels, voltage-gated K^+ channels; PM, plasma membrane; TEVC, two-electrode voltage clamp; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; AS, antisense; NS, nonsense; ODNs, oligodeoxynucleotides.

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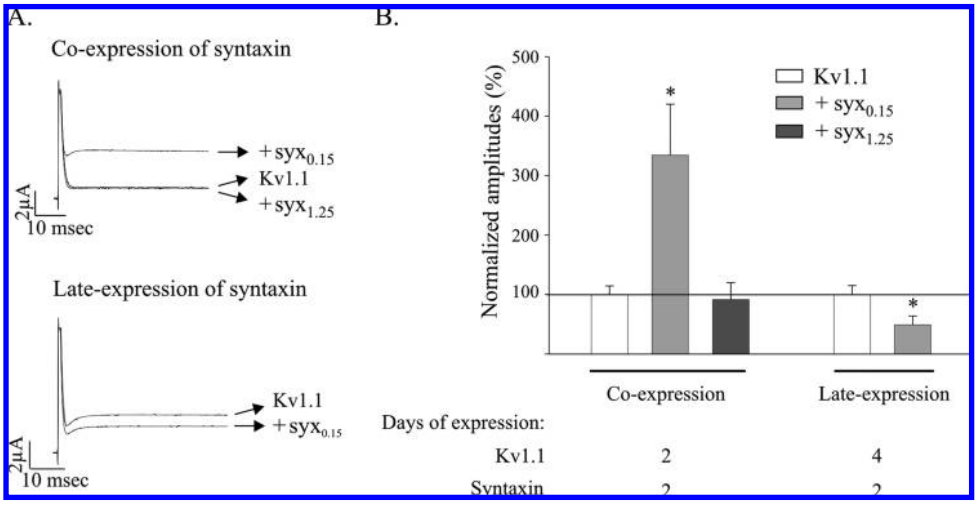

FIGURE 1: Effect of coexpression or late expression of syntaxin on Kv1.1 current amplitudes. Low syntaxin concentration (+$syx_{0.15}$) enhances the current amplitudes upon coexpression, in contrast to high syntaxin concentration (+$syx_{1.25}$), and decreases the current amplitudes upon late expression. (A) Representative Kv1.1 current traces elicited by +50 mV pulses from a holding potential of −80 mV in the absence (Kv1.1) and presence of syntaxin (+syx), either coinjected with the channel (coexpression; upper panel) or injected 2 days after channel injection (late expression; lower panel); currents were measured 2 days after syntaxin injection. One or two syntaxin mRNA concentrations were injected: 0.15 ng/oocyte (+$syx_{0.15}$) and 1.25 ng/oocyte (+$syx_{1.25}$). (B) Normalized current amplitudes measured from oocytes coexpressing (left) or late-expressing (right) syntaxin, as denoted. Bars represent mean values of normalized amplitudes ± SEM; *, $p < 0.05$. Four to18 oocytes were assayed per group.

the enhancement of channel surface expression, which occurs in parallel to its damping effect on single channel activity.

Here, this hypothesis was examined by rigorous measurements of protein expression levels in plasma membranes and macroscopic currents.

The physiological significance of the dual actions of syntaxin, to enhance plasma membrane expression and concomitantly to reduce single channel activity, which might seem antagonistic, is discussed in view of the role of Kv1.1 in presynaptic release.

MATERIALS AND METHODS

Oocytes and Electrophysiological Recording. Oocytes of *Xenopus laevis* were prepared as described (*21*). Oocytes were injected (50 nL/oocyte) with 3−7.5 ng/oocyte Kv1.1 mRNA for biochemical studies and with 0.5 ng/oocyte Kv1.1 mRNA for electrophysiological experiments alone or together with 0.05−2.5 ng/oocyte syntaxin mRNA. For antisense (AS) experiments, two AS oligodeoxynucleotides (ODNs) were used (30 pg/oocyte); AS-linker corresponds to amino acids 163−170 in the linker separating helixes H2B and H3, and AS-H3 corresponds to amino acids 210−220 within the H3 helix of syntaxin 1A. As a control a nonsense (NS) ODN was used (50 pg/oocyte). Detailed sequences are described in ref *2*. Two-electrode voltage-clamp (TEVC) recordings were performed as described (*22*). Currents amplitudes were elicited by stepping up the membrane potential from a holding potential of −80 mV to +50 mV. Net current was obtained by subtracting the scaled leak current elicited by a voltage step from −80 to −90 mV. Oocytes with a leak current of more than 3 nA/1 mV were discarded.

Immunoprecipitation in Oocytes. [^{35}S]Methionine/cysteine metabolically labeled oocytes were subjected to immunoprecipitation (IP) as described (*22*). Briefly, immunoprecipitates by an antibody directed against the Kv1.1 C-terminus (Alomone Laboratories, Jerusalem, Israel) from 1% Triton X-100 homogenates of either plasma membranes (PMs) or internal fractions (Ifs) (separated mechanically, as described in ref *23*) were analyzed by SDS−PAGE on 8% gel. Digitized scans were derived by PhosphorImager (Molecular Dynamics, Eugene, OR), and relative intensities were quantitated by ImageQuant.

Oocyte Plasma-Membrane Patch Preparation and Confocal Microscopy. Plasma-membrane patch preparations and fluorescence labeling were performed as described (*24*). Kv1.1 was labeled with rabbit antibody (1:250; Alomone) and Cy3 donkey anti-rabbit IgG. Results were analyzed by confocal laser scanning microscopy, using a Zeiss instrument.

Statistical Analysis. Data are presented as means ± SEM. Student's *t* test was used to calculate the statistical significance of differences between two populations.

RESULTS

As discussed in the introduction we hypothesized that low syntaxin, in contrast to high syntaxin, enhances channel cell-surface expression, which occurs in parallel to its damping effect on single channel activity; the balance between the two functions of syntaxin dictates the apparent effect on the macroscopic current amplitudes. To address this hypothesis, we first advanced the TEVC analysis of the effect of syntaxin on macroscopic currents of Kv1.1 overexpressed in oocytes (Figure 1). If indeed low syntaxin enhances channel cell-surface expression, its effect to enhance currents will be less prominent if syntaxin is not coexpressed with the channel but is expressed later on, when much of the cell-surface expression of the channels has already occurred. We compared the effects of 2 day expression of low syntaxin (0.15 ng/oocyte; $syx_{0.15}$), which was either coinjected with the channel ("coexpression"; Figure 1) or injected 2 days following

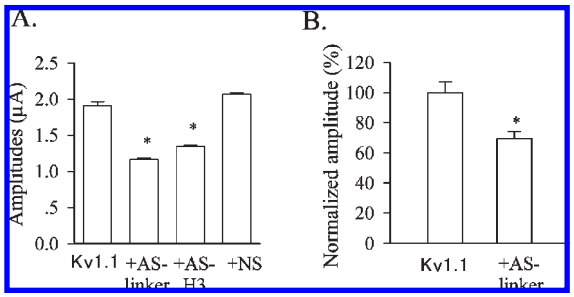

FIGURE 2: Kv1.1 current amplitudes are decreased by antisense ODN knock down of endogenous syntaxin. (A) Effect of two different AS-ODNs, AS-linker and AS-H3, targeting different regions of syntaxin (linker and H3, respectively) on Kv1.1 currents. Currents were elicited as in Figure 1 in oocytes of a single batch expressing Kv1.1 either alone or together with the AS-ODNs (+AS-linker, +AS-H3). A nonsense ODN (+NS) has no effect on current amplitudes. Five to seven oocytes per group were assayed. (B) Normalized and averaged effects of AS-linker on current amplitudes measured in eight batches of oocytes expressing Kv1.1 (41−48 oocytes in each group). *, $p < 0.002$.

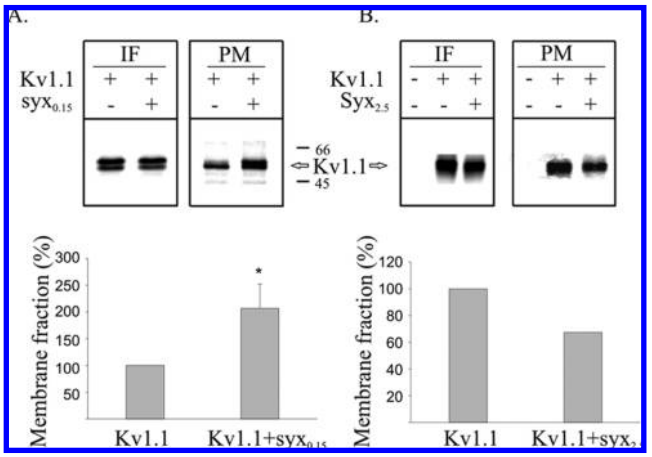

FIGURE 3: Kv1.1 cell surface expression in the presence of different syntaxin concentrations. Low syntaxin increases and high syntaxin decreases Kv1.1 protein PM content. Digitalized PhosphorImager scans of [^{35}S]Met-labeled Kv1.1 immunoprecipitated from homogenates of internal (IF) or plasma membrane (PM) fractions of oocytes, using an antibody targeted against the Kv1.1 C-terminus. Oocytes were injected with Kv1.1 in the presence (+) or absence (−) of either low (syx $_{0.15}$; A) or high (syx$_{2.5}$; B) syntaxin concentration and assayed 2 days later. Bars below scans depict ratios (quantified by ImageQuant and corresponding to the lanes above the bars) of PM to IF Kv1.1 protein content in the absence (Kv1.1) or presence (Kv1.1 + syx$_{0.15}$, A; Kv1.1 + syx$_{0.2.5}$, B) of syntaxin, averaged from three (A) or two (B) independent experiments. *, $p < 0.05$. The apparent different ratios of band intensities within the Kv1.1 doublets (A, right panel, indicating extent of Kv1.1 phosphorylation (*22*)) in the presence and absence of syntaxin were not reproducible.

the injection of the channel ("late expression"; Figure 1). Clearly, the late expression of syx$_{0.15}$, in contrast to its coexpression, did not enhance, but rather decreased, the current amplitudes. Thus, these results substantiated the hypothesis that low syntaxin, alongside its attenuation of single channel current (*20*), enhances the channel's cell-surface expression. In contrast, and according to our hypothesis, a high syntaxin concentration (1.25 ng/oocyte; syx$_{1.25}$) did not enhance the current amplitudes upon its coexpression (Figure 1; this same concentration already decreased the current amplitudes in our previous study (*2*).

Next, we examined the involvement of endogenous syntaxin in Kv1.1 cell-surface expression by measuring current amplitudes in Kv1.1-expressing oocytes in which the endogenous syntaxin was knocked down by AS-ODNs. The efficiency of the AS-ODNs, referred to as AS-linker and AS-H3, in knocking down syntaxin was verified before (*2*). An ODN of the same length and scrambled nucleotide sequence (NS-ODN) was used as control. TEVC analysis of the AS-ODN effects in a single experiment showed that injection of AS-linker or AS-H3 decreased the current amplitudes by 39.3% and 30%, respectively. Injection of NS had no effect (Figure 2A). The normalized averaged reduction in Kv1.1 current amplitude by AS-linker over several experiments was ~30% (Figure 2B). The finding that knocking down endogenous syntaxin caused a decrease in macroscopic currents strengthens the notion that the physiologically relevant effect of overexpressed syntaxin was the one measured in oocytes expressing low syntaxin concentration, where current amplitudes were enhanced, suggesting a physiological role for syntaxin in enhancement of Kv1.1 channel cell-surface expression.

Next, we set out to perform a careful analysis of Kv1.1 cell-surface expression under different experimental conditions, using two approaches. The first approach utilized SDS−PAGE analysis of metabolically labeled Kv1.1 protein, immunoprecipitated from the plasma membrane (PM) or internal fractions (IF, consisting of cytoplasm and intracellular organelles) of oocytes expressing Kv1.1 alone or together with syx$_{0.15}$ or syx$_{2.5}$ for 2 days (Figure 3). In accordance with the TEVC analysis, the amount of channel protein in the PM fraction was significantly increased in the presence of syx$_{0.15}$. To quantify this effect, the PM content of Kv1.1 was normalized to the corresponding IF content and in the presence of syx$_{0.15}$ was found to be 2.10 ± 0.40 of the PM content in the absence of syntaxin (Figure 3A; the channel protein appears as a doublet (*22*, *23*)). In contrast, as shown before (*2*), the Kv1.1 PM content in the presence of syx$_{2.5}$ was only 0.67 of the PM content in the absence of syntaxin (Figure 3B).

The second approach utilized confocal image analysis of immunolabeled Kv1.1 protein in isolated PM patches of oocytes expressing Kv1.1 alone or coexpressing increasing concentrations of syntaxin for 2 days (Figure 4). In the presence of syx$_{0.15}$ and syx$_{0.5}$, the amount of labeling was significantly increased, while at a higher syntaxin concentration, syx$_{1.25}$, the increase was abolished (Figure 4A,B). In a control experiment aimed to ascertain that the labeling was related to the Kv1.1 protein, we preincubated the Kv1.1 antibody with the peptide against which the antibody was targeted and found that, at saturating amounts of the peptide, about 60% of the labeling was diminished (Figure 4C).

Taken together, the biochemical and confocal analyses demonstrate upregulation of Kv1.1 surface expression, which is specific to the presence of low syntaxin concentrations.

DISCUSSION

Kv1 channels play critical roles in regulating resting membrane potential, action potential duration, and neurotransmitter release in mammalian neurons (*25*); therefore, changes in the number and location of surface

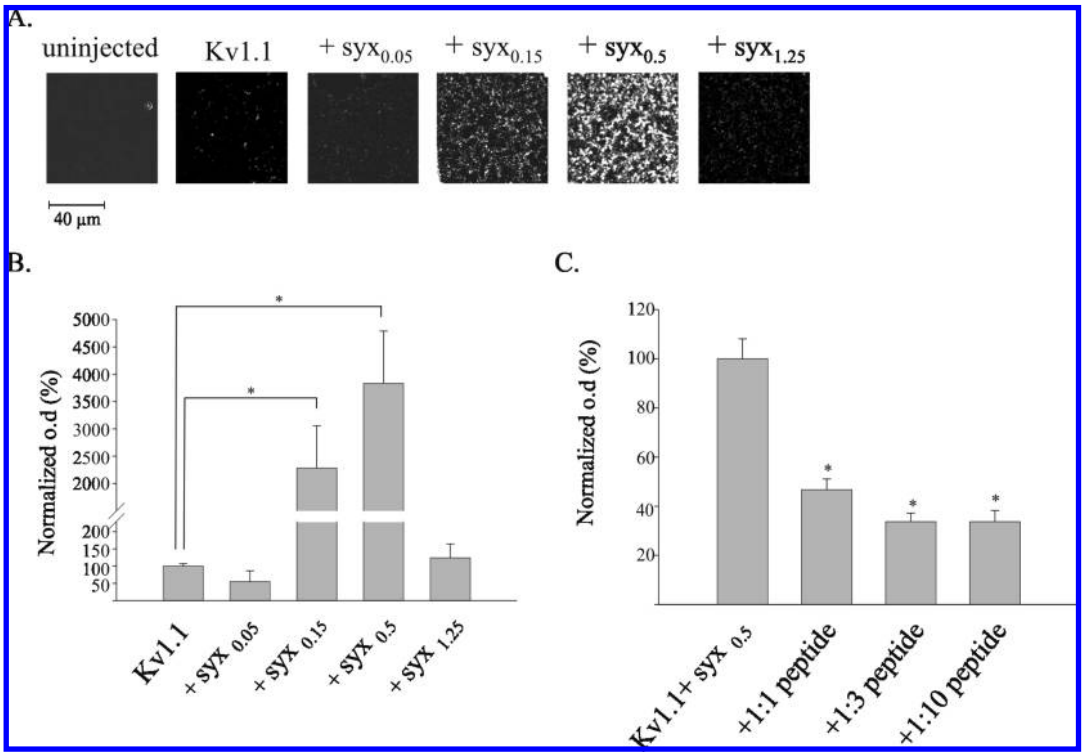

FIGURE 4: Cell surface expression of Kv1.1 in plasma membrane patches of oocytes coexpressing different concentrations of syntaxin. Low syntaxin concentrations increase Kv1.1 cell surface expression. (A, B) Confocal microscopic images (A) and normalized quantification of Kv1.1 protein (B) in PM patches from oocytes either uninjected, Kv1.1-expressing, or coexpressing Kv1.1 with increasing concentrations of syntaxin, measured 2 days after injection. Data for $syx_{0.05}$ and $syx_{1.25}$ are pooled from five oocytes per group from a single batch, and for Kv1.1, $syx_{0.15}$ and $syx_{0.5}$ are from 14 to 23 oocytes per group from three to four batches of oocytes. (C) Preincubateion of the Kv1.1 antibody with different concentrations (as denoted) of the peptide against which the antibody was targeted reduces Kv1.1 labeling. Data are from 5 to 11 oocytes per group from a single batch. Bars represent mean values of normalized od ± SEM; *, $p < 0.05$.

channels can profoundly influence electrical excitability and presynaptic efficacy (*26*). Efficient cell surface expression of some Kv subunits may be promoted by a cytoplasmic C-terminal VXXSL motif (*27*), different glycosylated states (*28*−*30*), binding to the auxiliary β subunit (*31*), and amino acids in deep pore regions (in Kv1 subfamily members) that may be major determinants for channel processing and cell-surface expression (*32*, *33*).

Here, we suggest a novel dual action for syntaxin, in enhancement Kv1.1 cell-surface expression, alongside its documented effect to attenuate single channel activity (*20*). Both actions are induced by low syntaxin concentration. Upon coexpression of syntaxin with Kv1.1, the enhancement of surface expression overrides the attenuation of single channel current, resulting in increased macroscopic currents. However, upon late expression of syntaxin, when many channels have already reached the cell surface, independently of their interaction with syntaxin, channel cell-surface expression becomes a less dominant process, resulting in unmasking the effect of attenuation of single channel current and the ensuing apparent reduction of the macroscopic currents. The enhancement of Kv1.1 cell-surface expression by syntaxin is suggested to be physiologically relevant since antisense knockdown of endogenous syntaxin, in contrast to overexpression of low syntaxin, reduced the macroscopic currents. Indeed, the pronounced channel clustering behavior observed upon coexpression of low syntaxin, demonstrated by patch clamp analysis of single channel behavior (*20*), argues in favor of syntaxin-induced enhancement of fusion of Kv1.1-containing vesicles with the PM. Therefore, we can also assume that the reduction of channel cell-surface expression in the presence of a high concentration of syntaxin can be attributed to an artifact caused by overexpression of a protein involved in the machinery responsible for trafficking of proteins to the plasma membrane.

The concept of dual functioning of syntaxin to modulate both cell surface expression and intrinsic properties has already been established in the case of several channels/transporters. Syntaxin both increases ENaC channels surface expression and decreases their chloride-sensitive currents, the latter presumably by regulating single channel open probability (*34*, *35*). Surface expression of GABA transporter GAT1 is positively regulated via direct interaction with syntaxin, concomitantly to decreased transport rates (*36*). In CFTR, however, plasma membrane insertion is interfered by syntaxin (*37*), in addition to decreased currents and channel open probability (*38*).

This work includes the Kv1.1 channel among the channels/transporters dually regulated by syntaxin. Being expressed in axons and localized to presynaptic terminals where it regulates neurotransmitter release (*25*), the intricate modulation of Kv1.1 function, via effects on channel cell surface expression and gating, provides powerful means for fine-tuning neuronal excitability and neurotransmitter release.

Intriguingly, we have recently established that another Kv channel, Kv2.1, which plays a major role in the regulation of dense-core vesicle- (DCV) mediated release

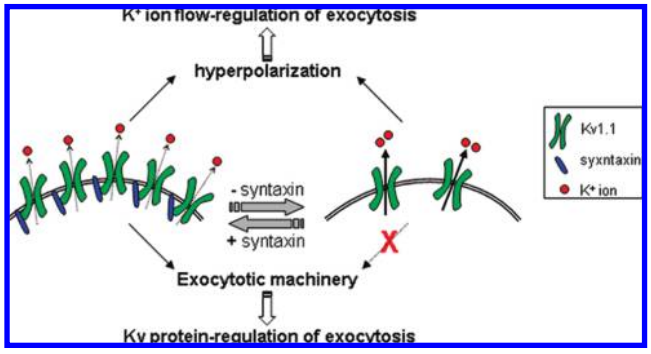

FIGURE 5: Schematic model illustrating consequences of the dual modulation of Kv1.1 by syntaxin, relevant to presyntaptic release. Kv1.1 mediates K^+ ion flow, ensuing in membrane potential hyperpolarization in axons and synaptic terminals where it is located, and consequent attenuation of neurotransmitter release (K^+ ion flow regulation of exocytosis). The interaction with syntaxin (left) confers direct interaction of Kv1.1 with the exocytotic machinery and may consequently influence neurotransmitter release, independent of the K^+ ion flow (yet to be determined; Kv protein regulation of exocytosis). Since the interaction of Kv1.1 with syntaxin increases the number of PM Kv1.1 channel proteins without proportional increase in total K^+ ion flow (because of concomitant damping of ion fluxes through single channels), the Kv protein regulation is achieved without major alteration of the K^+ ion flow regulation of neurotransmitter release.

from neuroendocrine cells (*19*, *39*), acts via its association with syntaxin to directly facilitate release (*9*, *10*). Notably, this novel role is independent of the potassium ion flow through the channel pore, which tends to hyperpolarize the membrane potential and to indirectly limit the opening of voltage-gated Ca^{2+} channels, resulting in release inhibition. It was argued that the two Kv2.1 actions, via its pore and via its association with syntaxin, which might seem antagonistic, in combination reinforce the known requirement for repetitive firing for DCV release. Single action potentials will not produce maximal exocytosis because membrane potential hyperpolarization from Kv channel pore activity will indirectly limit Ca^{2+} influx. However, release in response to repetitive firing that produces sustained Ca^{2+} elevation will be facilitated by the biochemical interaction of the channel with syntaxin.

In view of our previous findings (*2*) that the interaction of Kv1.1 with syntaxin occurs in brain synaptosomes and is dynamic, and associated with neurotransmitter release, we suggest that, in analogy to Kv2.1, the dual action of syntaxin on Kv1.1, although demonstrated only in oocytes, may serve to fine-tune presynaptic efficacy (Figure 5). Thus, an increased number of channel proteins, concomitantly to a reduction in the single channel current, provides means for quite a selective strengthening of a possible (yet to be determined) pore-independent influence of Kv1.1 on presynaptic release, mediated by its association with the exocytotic protein, syntaxin, without concomitant proportional enhancement of total potassium ion flow, which hyperpolarizes membrane potential and inhibits release.

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