1940 Research Article

# Non-conducting function of the Kv2.1 channel enables it to recruit vesicles for release in neuroendocrine and nerve cells

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#### **Summary**

Regulation of exocytosis by voltage-gated  $K^+$  channels has classically been viewed as inhibition mediated by  $K^+$  fluxes. We recently identified a new role for Kv2.1 in facilitating vesicle release from neuroendocrine cells, which is independent of  $K^+$  flux. Here, we show that Kv2.1-induced facilitation of release is not restricted to neuroendocrine cells, but also occurs in the somatic-vesicle release from dorsal-root-ganglion neurons and is mediated by direct association of Kv2.1 with syntaxin. We further show in adrenal chromaffin cells that facilitation induced by both wild-type and non-conducting mutant Kv2.1 channels in response to long stimulation persists during successive stimulation, and can be attributed to an increased number of exocytotic events and not to changes in single-spike kinetics. Moreover, rigorous analysis of the pools of released vesicles reveals that Kv2.1 enhances the rate of vesicle recruitment during stimulation with high  $Ca^{2+}$ , without affecting the size of the readily releasable vesicle pool. These findings place a voltage-gated  $K^+$  channel among the syntaxin-binding proteins that directly regulate pre-fusion steps in exocytosis.

Key words: Kv2.1 channel, Chromaffin cells, DRG neurons, Exocytosis, Vesicle release, Vesicle recruitment

#### Introduction

Before a vesicle can be released, it must go through several maturation steps (Sorensen, 2004): docking (onto the plasma membrane), priming (becoming fusion competent) and fusion (Wojcik and Brose, 2007). During the exocytotic process, only a small subset of vesicles are available for immediate release (on a scale of tens of milliseconds) from the releasable vesicle pool. However, a slower, sustained phase of release - enhanced by elevated levels of cytosolic Ca<sup>2+</sup> – relies on a reservoir of vesicles at different stages of maturation that is recruited during the stimulation in a use-dependent manner (Kits and Mansvelder, 2000; Sorensen, 2004). The precise nature of the processes underlying the maturation stages is unclear (Rettig and Neher, 2002; Sudhof, 2004), but they appear to require, in addition to soluble Nethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, many additional release-regulating proteins (Becherer and Rettig, 2006; Wojcik and Brose, 2007).

The voltage-gated K<sup>+</sup> channel Kv2.1 is commonly expressed in neuroendocrine cells (MacDonald et al., 2001; Wolf-Goldberg et al., 2006) – where it is well-positioned to regulate large dense-core vesicle (LDCV)-mediated hormone release – as well as in soma and dendrites of neurons (Du et al., 1998; Hwang et al., 1993; Kim et al., 2002; Rhodes et al., 1995) – where it might influence the LDCV-mediated release of neuropeptides and neurotrophins. Kv2.1 has classically been viewed as indirectly exerting an inhibitory function on exocytosis by influencing the membrane potential of cells (Dodson and Forsythe, 2004). However, recently, Kv2.1 has been shown to regulate release from neuroendocrine cells directly, independenty of its ion flux, through the specific cytoplasmic

domain C1a that interacts with syntaxin 1A (syntaxin) (Feinshreiber et al., 2009; Singer-Lahat et al., 2008; Singer-Lahat et al., 2007), a protein component of the exocytotic SNARE complex (Hay and Scheller, 1997).

The physical interaction of native Kv2.1 with syntaxin has been shown to be dynamic and also enhanced during the Ca<sup>2+</sup>-triggered phase of exocytosis (Singer-Lahat et al., 2007), whereas its impairment attenuated the release of norepinephrine from pheochromocytoma 12 (PC12) cells (Singer-Lahat et al., 2008). Vice-versa, overexpression of Kv2.1 wild type, but not of a mutant channel that lacks the C1a domain and therefore does not interact with syntaxin, facilitated the release of atrial natriuretic factor (ANF) after elevation of cytoplasmic Ca<sup>2+</sup> levels. Hence, it was suggested that Kv2.1 belongs to the group of proteins that regulate vesicle release by virtue of their direct interaction with syntaxin (Feinshreiber et al., 2009; Mohapatra et al., 2007).

Our study here addresses two important issues concerning the Kv2.1-induced facilitation of vesicle release. First, because the Kv2.1-induced facilitation has been demonstrated only with regard to secretion from neuroendocrine cells, it is intriguing to determine whether it also applies to secretion from the soma in neurons. The importance of somatic release in neurons has been described for several types of neuron (Chen et al., 1995; Dan et al., 1994; Sun and Poo, 1987), including dorsal-root-ganglions (DRGs) (Bao et al., 2003; Huang and Neher, 1996; Zhang and Zhou, 2002). Specifically, regulation of release of pain-related peptides stored in LDCVs in cell bodies of small DRGs (Zhang et al., 1995) may contribute to primary sensation and pain (Zheng et al., 2009). Here, we assess the existence of the Kv2.1-induced

facilitation phenomenon in the soma of small DRGs, of which Kv2.1 currents represent two-thirds of the delayed-rectifier K<sup>+</sup> currents (Ik) (Bocksteins et al., 2009) and which undergo robust Ca<sup>2+</sup>-regulated exocytosis (Huang and Neher, 1996). We demonstrate that Kv2.1 channels in small DRGs physically interact with syntaxin and induce facilitation of release. This indicates that this phenomenon is more universal and is not only limited to neuroendocrine cells.

Second, although several scenarios underlying this phenomenon have been suggested (Feinshreiber et al., 2009), it is still unknown which of the vesicle maturation steps are regulated by the interaction of Kv2.1 with syntaxin. Here, we assess the mechanism underlying Kv2.1-induced facilitation of release in Kv2.1-overexpressing adrenal chromaffin cells and demonstrate that it does not affect the fusion step per se, but rather relies on an enhanced number of fusion-competent vesicles that are recruited during prolonged cytosolic Ca<sup>2+</sup> elevation after emptying of the releasable vesicle pools. This is the first report describing an ion channel to directly regulate mobilization of vesicles, independently of its ion flux.

#### Results

## Kv2.1 facilitates vesicle release in DRGs independently of $K^{\dagger}$ flow but requires the Kv2.1-syntaxin association domain

We have recently demonstrated that Kv2.1 enhances exocytosis in neuroendocrine cells independently of K<sup>+</sup> flow (Singer-Lahat et al., 2008; Singer-Lahat et al., 2007). In this study, we first asked whether this new Kv2.1-facilitated release is also common to neurons or whether it is restricted to neuroendocrine cells. This question was addressed by using freshly prepared small rat DRGs, which release pain-related peptides from their cell bodies. These cells have a round shape and 1 day after their preparation they have only a few processes, making them ideal for voltage-clamping and membrane-capacitance (Cm) measurements (Zhang and Zhou, 2002). Kv2.1 was overexpressed in the neurons (Kv2.1 cells), resulting in increased voltage-dependent outward current densities (recorded in the presence of K+; supplementary material Fig. S1A,B). Changes in Cm evoked by six successive membrane depolarizations were monitored in the presence of Cs<sup>+</sup> (replacing K<sup>+</sup>) in the electrode solution, to exclude involvement of K<sup>+</sup> currents (Fig. 1A). Similar to the effect of Kv2.1 on exocytosis from neuroendocrine cells, a twofold enhancement of exocytosis in the Kv2.1 vs control cells was measured (Fig. 1B). Importantly, the enhancement was not caused by differences in Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels between the Kv2.1 and control cells

In mouse PC12 cells, the Kv2.1-facilitated release was shown to be mediated through the specific cytoplasmic domain C1a (Singer-Lahat et al., 2007), which interacts with syntaxin (Michaelevski et al., 2003). To examine whether a similar mechanism can account for the effect in DRGs, a Kv2.1 mutant that lacks the C1a domain, and which therefore does not interact with syntaxin (Kv2.1ΔC1a) (Singer-Lahat et al., 2007), was overexpressed (Kv2.1ΔC1a cells), resulting in increased voltage-dependent outward current densities (recorded in the presence of K<sup>+</sup>; supplementary material Fig. S1C). Changes in Cm evoked by six successive membrane depolarizations were monitored. In contrast to Kv2.1 cells, vesicle release was not enhanced in Kv2.1ΔC1a cells (Fig. 1C). This indicates that the C1a domain, which can intract with syntaxin, mediates the Kv2.1 effect in DRGs.

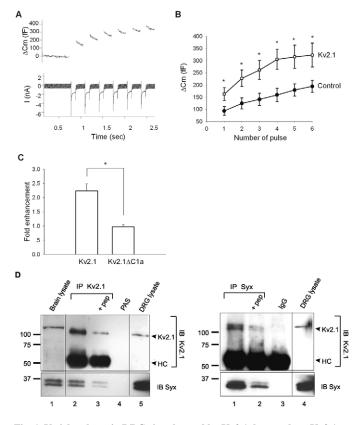


Fig. 1. Vesicle release in DRGs is enhanced by Kv2.1, but not by a Kv2.1 mutant with impaired syntaxin-binding capacity. (A) Representative traces of depolarization (six pulses of 100 mseconds from -70 mV to 0 mV at 2 Hz) induced differences in membrane capacitance ( $\Delta$ Cm) and inward currents (I) in a Kv2.1 cell (upper and lower traces, respectively; CsCl replaced KCl in the electrode solution). (B) Averaged depolarization-induced cumulative  $\Delta Cm$  in control (filled circles; n=14) and Kv2.1 (empty squares; n=17) cells. (C) Averaged enhancement of total  $\Delta$ Cm (measured at pulse number 6) induced by Kv2.1 (data shown in B) and Kv2.1ΔC1a, calculated as the averaged ratio between control and Kv2.1 (six experiments) or control and Kv2.1 $\Delta$ C1a (four experiments; n=9 and n=13, respectively) in each experiment. Data are shown as mean  $\pm$  s.e.m. \*P<0.05. (**D**) Kv2.1 protein interacts with syntaxin in DRGs. DRG lysates were immunoprecipitated (IP) by Kv2.1 (left panel) or syntaxin 1A (Syx; right panel) antibodies in the presence (+pep) or absence of the antigen peptide, as indicated above the lanes. For additional controls, DRG lysates were immunoprecipitated by IgG (irrelevant antibody) or by protein A- Sepharose (PAS) (without IgG), as indicated above the lanes. The immunoprecipitated proteins were separated by SDS-PAGE, western blotted by using syntaxin 1A (Syx) or Kv2.1 antibodies, as indicated at the sides of the blots. HC, heavy chain of the antibodies used. Molecular-weight markers are shown on the left of the blots. For each IP reaction we used lysate of DRG ganglia from two rats. We loaded 0.2% (for Kv2.1 detection) or 0.05% (for syntaxin detection) on DRG lysate (no immunoprecipitation was performed) and 7 µg of rat brain cytosol on brain lysate (no immunoprecipitation was performed). The results shown are representative of three similar experiments.

To verify the physiological relevance of the effect we had established in DRG cells that overexpress Kv2.1 channels, we further set to demonstrate that native Kv2.1 channels interact physically with syntaxin in DRGs. Using an antibody against Kv2.1, we found that syntaxin co-precipitated with the Kv2.1 protein (Fig. 1D, left panel, lane 2). Importantly, the co-precipitation could be blocked by pre-incubation of the antibody with the peptide against which the

antibody had been raised (Fig. 1D, left panel, lane 3). Moreover, the co-precipitation was not apparent when using a control antibody (IgG) (Fig. 1D, right panel, lane 3). To verify the specificity of the co-precipitation, we performed the reciprocal experiments in which Kv2.1 was co-precipitated with syntaxin, using an anti-syntaxin antibody (Fig. 1D, right panel, lane 1). Again, the co-precipitation was blocked by pre-incubation of the antibody with the peptide against which the antibody was raised (Fig. 1D, right panel, lane 2) and was not apparent using IgG control antibody (Fig. 1D, right panel, lane 3). Taking these results together, we concluded that the channel-syntaxin interaction underlies the Kv2.1-induced facilitation of exocytosis. In all, similar to neuroendocrine cells, Kv2.1 facilitation of exocytosis occurs also in DRGs and does not require a functional pore; however, it does require a cytosolic domain that mediates a direct interaction with syntaxin.

#### Kv2.1 facilitates vesicle release in chromaffin cells

Next, we studied the mechanism underlying the Kv2.1-induced enhancement of release, in an attempt to ascribe the effect to specific vesicle maturation step(s). To this end, we chose bovine chromaffin cells, which are used as an unique model system for studying Ca<sup>2+</sup>-triggered exocytosis, and for which the mechanism of exocytosis and vesicle pools are well-characterized (Becherer and Rettig, 2006; Rettig and Neher, 2002; Sorensen, 2004). In addition, chromaffin cells confer the advantage of high-time resolution Cm measurements and amperometric detection of catecholamines (CA) which, together with the existing model of exocytosis (Bruns, 2004), are ideal to clarify the mechanisms of action of Kv2.1. Moreover, based on a preliminary study, we found that Kv2.1-induced facilitation of release also occurs in these cells (Feinshreiber et al., 2009).

First, we characterized the effect of overexpressed Kv2.1 on CA release from chromaffin cells using amperometry, which enables better detection of the possible effects on single-vesicle-fusion kinetics, in contrast to the previous characterization by optical measurements of the bulk release of GFP-tagged ANF in PC12 cells (Singer-Lahat et al., 2007). Expression of endogenous Kv2.1 was undetectable in chromaffin cells (see supplementary material Fig. S2). However, expression of exogenous Kv2.1 resulted in increased voltage-dependent outward currents, similar to those determined in Kv2.1-expressing oocytes (Michaelevski et al., 2002) (supplementary material Fig. S1D,E).

Secretion was triggered by five consecutive stimulations with high-K+solution at 2-minute intervals. Data were recorded continuously and grouped into 5-second bins for analysis (Fig. 2A,C insets). Analysis of cumulative release showed that, similar to PC12 cells, exocytosis in chromaffin cells was facilitated by Kv2.1 (Fig. 2A,C). In response to the first stimulation, release was enhanced ~1.9-fold (38.9±6.4 pC and 21±4.2 pC in Kv2.1 and in control cells; Fig. 2A, left panel). Notably, in response to the second stimulation, both the Kv2.1 and control cells exhibited initial release levels and extents of Kv2.1-induced enhancement that were similar to those observed in response to the first stimulation (approximately twofold: 38.9±8 vs 18.8±3.5 pC; Fig. 2A, right panel), suggesting that the Kv2.1 effect occurs during the prolonged stimulations and reverses in their absence. Similar enhancement (~1.8-fold) persisted in response to subsequent stimulations (Fig. 2B). Furthermore, the Kv2.1-induced enhancement developed with an apparent delay during the prolonged stimulations: the initial secretion during the first 5 seconds of stimulation was only slightly larger in Kv2.1 cells than in controls; the difference only became statistically significant

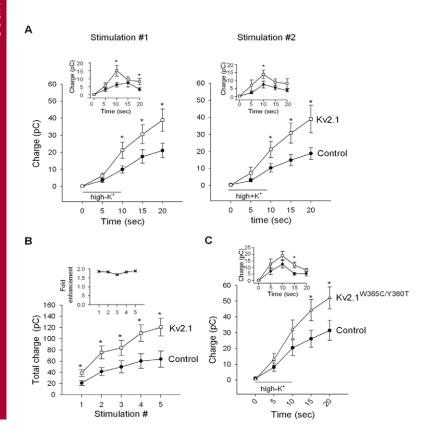


Fig. 2. Kv2.1 enhances release evoked by K<sup>+</sup>-induced depolarizations in a pore-independent manner, measured from chromaffin cells. (A,C) Analysis of cumulative amperometric measurements of charge released in response to two consecutive stimulations of Kv2.1 (n>25) vs control (n>18) cells (insets in A show amperometric recording) and to a single stimulation from Kv2.1 $^{W365C/Y380T}$  (n=21) vs control (n=11) cells (inset in C shows amperometric recording). Release was stimulated by five consecutive 10-second focal applications of high-K<sup>+</sup> solution (bar) spaced at 2-minute intervals. (B) Averaged total charges released in response to all five stimulations. Inset shows Kv2.1 enhancement of release calculated as the ratio between the total cumulative charge released in Kv2.1-expressing vs control cells. Data are shown as mean  $\pm$  s.e.m. \*P<0.05.

after 10 seconds of stimulation (Fig. 2A) (Feinshreiber et al., 2009).

The above stimulation protocol, which uses KCl applications for membrane depolarization, presumably clamps the membrane potential near the reversal potential for K<sup>+</sup> and should, therefore, minimize the impact of the Kv-channel pore (i.e. minimize K<sup>+</sup> currents). This by itself should bring the membrane potential to its resting potential more rapidly and decrease secretion. However, to determine directly whether the K<sup>+</sup> current flowing through the Kv2.1 pore is involved in the facilitation of depolarization-evoked release, a pore mutant (Kv2.1<sup>W365C/Y380T</sup>) with abolished ion conductance (Malin and Nerbonne, 2002) (see supplementary material Fig. S1F) was used. Release for Kv2.1<sup>W365C/Y380T</sup> was enhanced to a degree similar (~1.7-fold) to the wild-type channel (52.0±7.0 vs 31.4±6.3 pC; Fig. 2C), suggesting, as expected, that a functional pore is not required for the Kv2.1-mediated facilitation of release from chromaffin cells. Moreover, enhancement of vesicle release did not arise from changes in membrane potential or intracellular free Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>), as verified in several control experiments (supplementary material Fig. S3). Importantly, the Kv2.1<sup>W365C/Y380T</sup>induced enhancement developed with a delay during the prolonged stimulation, which is similar to that in Kv2.1 (Fig. 2C).

#### Kv2.1-induced facilitation of vesicle release does not arise from an increased charge release during a single fusion event

The Kv2.1-induced facilitation of release could be caused by an increase in the CA secreted per vesicle and/or an increase in the number of fusing vesicles. To discern between the two mechanisms and to evaluate their contributions, we used amperometry to detect the exocytosis of single-fusion events (spikes).

To test for the first mechanism, we analyzed the kinetics of release of single vesicles (Fig. 3). Amperometric events are often preceded by a pre-spike foot (PSF) that reflects the trickle of transmitter through the narrow, slowly expanding fusion pore before its subsequent rapid expansion that allows bulk release of the transmitter in a spike of current (Fig. 3A) (Bruns and Jahn, 1995; Chow et al., 1992). Analysis of PSF characteristics showed that the Kv2.1 channel had no significant effect on foot frequency, average charge released or foot duration (Fig. 3B). In other words, neither the size nor the stability of the PSF changed with expression of Kv2.1.

A subtle, albeit statistically significant  $\sim 1.3$ -fold increase in the rise time ( $t_{rise}$ ) of the spike was observed in the presence of Kv2.1 (Fig. 3C), suggesting a role for Kv2.1 in the initial rate of opening of the fusion pore, which affected the rate of CA release from the fusing vesicle. Nevertheless, spike quantal charge was not changed by Kv2.1 (Fig. 3C). Such alterations in the kinetics and size of amperometric spikes are expected of SNARE-binding proteins (Han et al., 2004). However, these small modifications in the rise time of the amperometric spikes do not seem to underlie the almost twofold enhancement of release by Kv2.1.

## Kv2.1-induced facilitation of vesicle release is accompanied by an increased number of release events

To test for the second mechanism that might underlie the enhanced release, we calculated the average number of spikes per cell that were evoked in response to five stimulations. An ~1.7-fold increase in the number of spikes in Kv2.1 vs control cells was measured (44±5.4 vs 26±3.4; Fig. 4B; representative cells are shown in Fig. 4A). A similar increase (~1.8-fold) was measured in Kv2.1W365C/Y380T vs control cells (32±3.4 vs 18±1.7; Fig. 4C; one stimulation per cell). Remarkably, the fold increases in the number of spikes matched the corresponding fold increases in CA release (Fig. 2). Namely, the increase in the number of releasing vesicles fully accounted for the facilitated release by Kv2.1.

It is reasonable to assume that the initial secretion measured by amperometry in response to depolarization induced by KCl relates to fusion of vesicles from a readily releasable pool (RRP). However, as soon this RRP is depleted, vesicle refilling starts and it is impossible to separate these stages by using this experimental protocol, although we can assume it starts after a few seconds. Nevertheless, the apparent delay in the development of the Kv2.1-induced enhancement (Fig. 2A,C) and the similar extent of this effect observed in response to subsequent stimulations (Fig. 2A,B; see discussion above) implies an involvement of Kv2.1 in the recruitment of vesicles for release during the prolonged stimulations.

## Kv2.1 increases vesicle recruitment during prolonged stimulation

To establish the above hypothesis that Kv2.1 enhances vesicle recruitment, we stimulated exocytosis by flash photolysis of caged  $Ca^{2+}$ , which causes step-like increases in  $[Ca^{2+}]_i$ , enabling

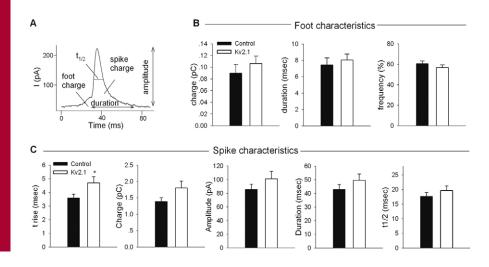


Fig. 3. Effect of Kv2.1 on single pre-spike foot (PSF) and spike parameters in chromaffin cells.

(A) Example of an amperometric single spike with a PSF. (B) Quantitative analysis of single PSF characteristics in control (filled bars) or Kv2.1 (empty bars) cells. Kv2.1 has no effect on foot charge, duration or frequency. (C) Quantitative analysis of single-spike characteristics. Kv2.1 has an effect on rise time (t rise) of the spikes; filled bars, control cells; open bars, Kv2.1 cells. Data are shown as median ± s.e.m. for EGFP (*n*=1081 spikes) and Kv2.1 (*n*=1687 spikes). \**P*<0.05.

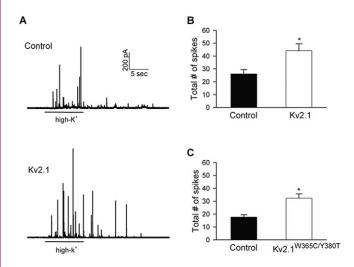


Fig. 4. The number of spikes is increased in chromaffin cells expressing Kv2.1. (A) Representative amperometric recordings in response to 10-second focal applications of high- $K^+$  solution (bar) from control (upper panel) and Kv2.1 (lower panel) cells. Shown are responses to the first out of five consecutive stimulations separated by 2-minute intervals. (B) Averaged total number of spikes recorded in response to five stimulations for control (n=7) and Kv2.1 (n=18) cells. (C) Averaged total number of spikes recorded in response to one stimulation for each control (n=11) and Kv2.1 (n=365C/Y380T (n=21) cells. Data are shown as mean  $\pm$  s.e.m. \*P<0.05.

designation of the released vesicles to distinct pools (Becherer and Rettig, 2006; Zikich et al., 2008).

Control cells displayed a typical biphasic increase in membrane capacitance, in which an exocytotic burst is followed by a sustained phase (component) of secretion (Fig. 5A). Kinetics analysis distinguishes between the burst phase (<1 second after the flash) and the sustained component (>1 second after the flash). The exocytotic burst results from the fusion of vesicles from the RRP (immediately releasing and slowly releasing pools), whereas the sustained phase represents the fusion of vesicles that are recruited and undergo priming from a docked but unprimed pool during the Ca2+ pulse (Ashery et al., 2000; Neher, 2006; Parsons et al., 1995). Overexpression of Kv2.1 did not affect exocytosis during the first second after onset of the Ca<sup>2+</sup> flash (burst component; 266±38 fF in control vs 294±47 fF in Kv2.1 cells; Fig. 5B), in agreement with the amperometric findings, which demonstrate that, during the initial 5 seconds, the amount of secretion is similar in both control and Kv2.1 cells (Fig. 2A,C). This suggests that Kv2.1 does not change the number of fusion-competent vesicles. Rather, it leads to a twofold increase in the sustained component (61±9 fF in control vs 126±33 fF in Kv2.1 cells; Fig. 5C), in accordance with our suggested hypothesis of involvement of Kv2.1 in the recruitment of vesicles for release during ongoing stimulation.

#### **Discussion**

Our study demonstrates that the Kv2.1-induced facilitation of vesicle release, mediated by its cytoplasmic syntaxin-association domain and not its functional pore, is common to both neuroendocrine and nerve cells (Fig. 1). Together with our previous studies (Feinshreiber et al., 2009; Singer-Lahat et al., 2008; Singer-Lahat et al., 2007) this new role for Kv2.1 has been established with regard to both exogenous and endogenous channels, using

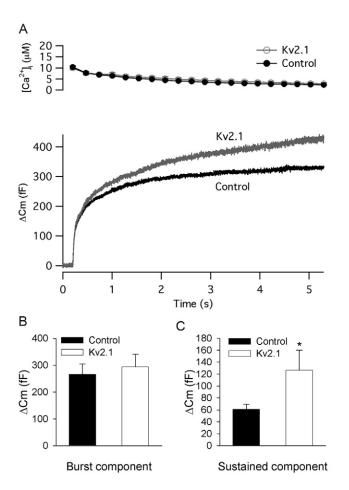


Fig. 5. Kv2.1 expression in chromaffin cells leads to an increased sustained component of secretion. (A) Average  $[{\rm Ca}^{2+}]_{\rm in}$  (upper panel) and membrane capacitance changes ( $\Delta {\rm Cm}$ ; lower panel) in control cells (black) and Kv2.1 cells (grey) in response to flash photolysis of caged  ${\rm Ca}^{2+}$ . (B,C) Average increase in capacitance during the exocytotic burst (0-1 seconds) (B) and the sustained (1-5 seconds) (C) components. Bars represent mean  $\pm$  s.e.m. \*P<0.05.

release paradigms that include stimulations with high-K<sup>+</sup> solution, membrane depolarization and direct [Ca<sup>2+</sup>]<sub>i</sub> elevation, and monitoring techniques that include amperometry, fluorescence, radioactivity and capacitance measurements.

Importantly, new insights into the mechanistic aspect of the Kv2.1-induced facilitation of release are provided by several findings of this study: (1) The similar extent of facilitation throughout several consecutive stimulations, despite the increased depletion of vesicles during preceding stimulations (Fig. 2). (2) The apparent augmentation of release in later phases of the stimulation (Fig. 2). (3) An approximately twofold increase in the total number of release events (Fig. 4), which is comparable to the approximately twofold increase in the released charge (Fig. 2). (4) The lack of a significant effect on single-spike charge (Fig. 3). All these characterisites of the effect of Kv2.1 are compatible with the enhanced vesicle recruitment induced by Kv2.1. Indeed, this notion has been confirmed in experiments using flash photolysis of caged Ca<sup>2+</sup> (Fig. 5). The demonstration of an increased sustained component of secretion in the presence of Kv2.1 – without a significant change in the burst component – indicates that fusion of vesicles that are recruited during the Ca<sup>2+</sup> pulse is enhanced by Kv2.1. This is the first evidence that an ion channel can regulate mobilization of vesicles directly, independently of its ion flux.

This action of Kv2.1, which involves interaction with a key exocytotic protein, might seem antagonistic to its common action through the pore domain. However, the combination of the two mechanisms has been suggested to reinforce the known activity dependence of LDCV exocytosis in Kv2.1-expressing neuroendocrine cells and neurons (Singer-Lahat et al., 2007). Whereas a single action potential will not produce maximal exocytosis – because of the pore function that tends to hyperpolarize the membrane potential and to indirectly limit Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels, release in response to repetitive firing – which causes sustained elevation of intracellular Ca<sup>2+</sup>, will be facilitated.

#### Kv2.1 protein as a regulator of vesicle recruitment

Previous studies describing the exocytosis-facilitating action of Kv2.1 could not discriminate between actions on pre-fusion steps and actions on the fusion process itself (Singer-Lahat et al., 2008; Singer-Lahat et al., 2007) (reviewed in Feinshreiber et al., 2009; Mohapatra et al., 2007). This study rules out a major impact of Kv2.1 on fusion itself; rather, it establishes Kv2.1 as a regulatory protein in pre-fusion processes leading to enhanced refilling of the releasable vesicle pools. During the priming process sequential formation of the trimeric SNARE complex occurs (Becherer and Rettig, 2006; Brunger, 2001; Bruns and Jahn, 2002; Chen and Scheller, 2001; Fasshauer, 2003; Jahn and Sudhof, 1999; Rizo and Sudhof, 2002). It begins with the assembly of the binary t-SNARE complex of syntaxin and SNAP-25, which forms a scaffold for VAMP2 binding (Fasshauer and Margittai, 2004). To enter the t-SNARE complex, the free syntaxin has to undergo a structural change and adopt the open conformation that enables its SNARE motif to associate with those of SNAP-25 (Jahn and Scheller, 2006). Thus, the open conformation of syntaxin can facilitate vesicle priming via formation of t-SNARE complexes (An and Almers, 2004; Fasshauer et al., 1997). Importantly, Kv2.1 has been shown to interact with both the open conformation of syntaxin (Leung et al., 2005) and the t-SNARE complex (Michaelevski et al., 2003; Tsuk et al., 2005), suggesting that Kv2.1 enhances vesicle priming by promoting assembly and/or stabilization of the t-SNARE complex.

Although most studies assume a sequential docking-primingfusion model with distinct and sequential molecular reactions that underly each step, there is new evidence that priming and docking are interlinked at the molecular level. However, there is not much evidence to directly support the assumption that priming is downstream of docking (for a review, see Verhage and Sorensen, 2008) because both steps are regulatory processes necessary for the assembly of the SNARE complex (Wojcik and Brose, 2007). Intriguingly, open syntaxin was also shown to be directly involved in the docking of vesicles to the plasma membrane (Hammarlund et al., 2007). Hence, Kv2.1 may be considered to be involved through its interaction with open syntaxin – not only in regulation of priming but also in that of vesicle docking. The fact that Kv2.1 activity is exerted during ongoing stimulation may arise from the massive disassembly of SNARE complexes during prolong stimulation into monomeric SNAREs and the formation of new trans-SNARE complexes for subsequent fusion (Gladycheva et al., 2007; Hanson et al., 1997).

Notably, it has recently been shown that, in the presence of VAMP2, the t-SNARE complexes are released from Kv2.1 and form ternary SNARE complexes that do not interact with the channel (Tsuk et al., 2008). This suggests a role for Kv2.1 protein in the regulation of vesicle docking and/or priming during LDCV release without any interference in the formation of the ternary SNARE complex. Indeed, stabilization of the acceptor t-SNARE complex is a known mechanism that is shared by several regulatory proteins (e.g. Munc13, Munc18, complexin and synaptotagmin) (Weninger et al., 2008). Kv2.1 may therefore be considered another member of this class of exocytosis-regulating proteins.

#### **Materials and Methods**

#### Plasmid construction

Rat Kv2.1 cDNA was modified by PCR to contain a 5' BamHI site followed by a Kozak consensus sequence and a 3' BssHII site, and inserted upstream of the poliovirus internal ribosome entry site (IRES) by using BamHI and BssHII restriction sites to yield the enhanced green fluorescent protein (EGFP)-tagged Kv2.1-expressing plasmid pSFV1-Kv2.1-IRES-EGFP. A non-conducting Kv2.1 mutant (Malin and Nerbonne, 2002) was generated in pBluescript vector as described previously (Singer-Lahat et al., 2007) and subcloned into the virus as described above to generate pSFV1-Kv2.1W365CY380T-IRES-EGFP. The sequence of each construct was verified by DNA sequencing. The control construct pSFV1-EGFP has been used previously (Yizhar et al., 2004).

#### DRG cell preparation and electrophysiology

Freshly isolated DRGs (15-25 µm in diameter) from postnatal 2- to 4-day old Wistar rats were enzymatically digested with trypsin type 3 (0.5 mg/ml, Sigma) and collagenase 1A (1 mg/ml, Sigma) in the presence of DNase 1 (0.1 mg/ml, Sigma) in DMEM. One hour after preparation cells were transfected with Lipofectamine 2000 (Invitrogen) and used the following day. For each reaction 0.5 µg pcDNA3-EGFP alone (control cell) or either 0.75 µg pcDNA3-Kv2.1 (Kv2.1 cell) or 1 µg pCDNA3-Kv2.1ΔC1a (Kv2.1ΔC1a cell) (Singer-Lahat et al., 2007) were used. Cells were voltage-clamped at -70 mV. External medium contained (in mM): 150 NaCl, 5 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose (pH 7.4); the intracellular pipette solution contained (in mM): 153 CsCl, 1 MgCl<sub>2</sub>, 10 HEPES, 4 ATP (pH 7.2). For Kv2.1-expression measurements CsCl was exchanged with KCl. Capacitance measurements were performed using the phase-tracking technique (Fidler and Fernandez, 1989; Neher and Marty, 1982) implemented in Pulse Control 5.0a4 software (Instrutech Corp., Port Washington, NY) that runs on top of the graphical software Igor Pro 3.15 (Wavemetrics, Lake Oswego, OR) on a Macintosh G3 computer equipped with the ITC-16 analog-to-digital converter (Instrutech Corp., Port Washington, NY). The cells were voltage clamped in whole-cell configuration using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA). After manual compensation of membrane capacitance (Cm), lock-in phase angles yielding signals proportional to changes in Cm and conductance were calculated by dithering the series resistance with a 500 k $\Omega$  resistor. The capacitance output was calibrated by a 100 fF change in the Cm compensation setting. After the calibration of both membrane capacitance and conductance, the command sinusoid (1 kHz, 40 mV peak-to-peak amplitude) was created digitally, converted to the analog signal, filtered at 2 kHz with an eight-pole Bessel filter (Brownlee Precision, San Jose, CA), and applied to the cell. The resulting current response contained contributions from all elements of the circuit plus noise. This current response was analyzed by the phase-sensitive detection of pulse-control lock-in amplifier to give a magnitude of the current at two orthogonal phase angles. With the correct phase angle, the part of this current response reflected changes in membrane capacitance (Cm), whereas the other reflected the combined changes of membrane resistance (Gm) and series resistance (Gs). Data analysis was performed using Igor Pro 5.0 software (Wavemetrics) and custom-written macros.

#### Immunoblot analysis

Rat brain, adrenal gland medulla (AGM), bovine brain and bovine chromaffin cells were lysed in buffer containing: 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 10% glycerol, 0.2 mM sodium orthovanadate, 1  $\mu$ M phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/mL leupeptin and 1 mM DTT. All reagents were purchased from Sigma. Lysates were incubated for 30 minutes at 4°C, and centrifuged (12,000 g) for 10 minutes at 4°C. Samples were separated by SDS-PAGE and subjected to western blot analysis by using an antibody against the C-terminus of Kv2.1 (corresponding to amino acid residues 841-857 of rat Kv2.1) (Alomone Labs, Jerusalem, Israel).

#### Co-immunoprecipitation and immunoblotting of DRG cells

Dorsal root ganglia isolated from postnatal 1- to 3-day old rats were solubilized in ice-cold buffer containing (in mM): 150 NaCl, 50 Tris-HCl, 5 EDTA, 1 EGTA, 1% freshly prepared y3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid

(CHAPS) (Boehringer Mannheim) supplemented with protease inhibitors cocktail (Boehringer Mannheim), for 1 hour at 4°C, and were centrifuged for 25 minutes at 4°C at 10,000 g. The collected supernatant was incubated for 1 hour with protein-A-Sepharose (PAS) beads and centrifuged again to clear the lysate, followed by incubation at 4°C for 4 hours with an antibody against the C-terminus of Kv2.1 or against an N-terminal peptide of syntaxin 1A (polyclonal; Alomone Labs, Jerusalem, Israel). The antibodies were prebound to PAS beads, in the absence or presence of the corresponding antigen peptides against which the antibodies were generated (antibody-to-peptide ratio was 1:2 for Kv2.1 and 1:0.1 for syntaxin 1A). Following the incubation, the bound proteins were thoroughly washed three times in PBS with 0.2% CHAPS. Special precaution was taken to avoid nonspecific interactions with syntaxin or Kv2.1 adhering to PAS beads. Such adhesions were minimized by including 1% BSA in the reaction and 5% glycerol in the final washing step. Immunoprecipitated proteins from DRG cells taken from two rats (for each reaction), and DRG and rat brain cytosol lysates were separated by 10% SDS-PAGE and subjected to western blotting by using antibodies against Kv2.1 (Alomone Labs) and syntaxin 1A (monoclonal anti HPC-1; Sigma Israel, Rehovot, Israel), using the ECL detection system (Pierce Protein Research Products, Thermo Scientific).

#### Chromaffin cell preparation and infection

Isolated bovine adrenal chromaffin cells were prepared and cultured as described previously (Ashery et al., 1999). Cells were used 2-3 days after preparation. Infections with pSFV1-Kv2.1-IRES-EGFP, pSFV1-Kv2.1<sup>W365C/Y380T</sup>-IRES-EGFP or control construct pSFV1-EGFP were performed on cultured cells at least 24 hours after plating. Detection of EGFP signals was done 6-12 hours after infection with the control EGFP construct or 12-18 hours after infection with the Kv2.1 constructs (a plasma-membrane protein) and performed by using an Axiovert25 (Zeiss, Germany) microscope with a filter set for EGFP (EXFO, Ontario, Canada).

### $[\text{Ca}^{2+}]_i$ measurements during high-K\*-solution stimulation and current-clamp recordings

[Ca<sup>2+</sup>]<sub>i</sub> was measured by dual-wavelength ratiometric fluorometry with Fura-2 acetoxymethyl ester (AM) (Molecular Probes). Cells were incubated with 2 µM Fura-2AM for 45 min in the incubator before being used for experiments. To facilitate loading of the AM indicators, the mild detergent pluronic F-127 was added to Fura-2AM in a 1:1 ratio immediately before incubation (Groffen et al., 2006). fluorescence excitation light was generated by a monochromator (TILL Photonics, Munich, Germany) into the epifluorescence port of an IX-50 Olympus microscope equipped with a 40× objective (UAPO/340; Olympus, Tokyo, Japan). Fura-2AM was excited at 350 nm and 380 nm and detected through a 500-nm long-pass filter (TILL Photonics). The resulting fluorescence signal was measured by using a photomultiplier. The illumination area was reduced to cover only the diameter of the cell. Background fluorescence was measured at 350 nm and 380 nm, and subtracted from the corresponding 350 nm and 380 nm fluorescence values of the cells. To convert the ratio (R) of the fluorescence signals at both wavelengths into [Ca<sup>2+</sup>]<sub>i</sub>, a calibration curve was constructed, where each data point corresponded to the average ratio obtained during in-vitro measurements using solutions with free Ca<sup>2+</sup> buffered to the respective concentrations (Groffen et al., 2006). The same stimulation protocol as in the amperometry experiments was used, with the same stimulation solutions used for external K<sup>+</sup> and high-K<sup>+</sup> concentration.

For membrane-potential measurements, cells were subjected to whole-cell current clamp recordings. Recordings were sampled at 5 kHz and filtered at 2 kHz. Cells were stimulated with a high- $K^+$  solution, as described in the amperometry measurements. Analyses were performed by using clampfit9 software (Axon CNS Molecular Devices). Given values represent mean  $\pm$  s.e.m.

#### Amperometry measurements and single-spike analysis in chromaffin cells

A constant voltage of 800 mV vs an Ag<sup>+</sup>/AgCl reference was applied to the electrode and the amperometric current was recorded with a VA-10 amplifier (npi Electronics GmbH, Tamm, Germany). The amperometric currents were filtered at 1 kHz, digitized using a Digidata 1322A analog-to-digital converter and monitored online with Clampex 9 software package (Axon CNS Molecular Devices, Sunnyvale, CA). Sampling rate was 10 kHz. The tip of the carbon fiber was pressed gently against the cell surface. External solutions contained (in mM): 146 NaCl; 2.4 KCl; 2.5 CaCl<sub>2</sub>; 1.2 MgCl<sub>2</sub>; 10 NaHCO<sub>3</sub>; 10 HEPES-NaOH plus 2 mg/ml D-glucose, pH 7.3 (osmolarity was adjusted to 300 mOsm). Amperometric spikes were analyzed using a newly developed algorithm (MATLAB, The MathWorks Inc., Natick, MA) that separates spikes from background noise, thereby eliminating the need for filtering (R.F. and U.A., unpublished). On the basis of the noise levels of the experiment (dynamic threshold algorithm), spikes smaller than 5 pA were considered as noise. A median value for each parameter was taken from every cell.

#### Amperometry statistical analysis

Vectors of the medians were assessed for normal distribution by using normplot and boxplot. If the vectors did not distribute normally, we performed a log operation and Mann-Whitney test. For vectors distributed normally, the equality of variances was assessed by using Bartlett's test. For unequal variances, the unequal *t*-test was applied. For equal variances, we used Student's *t*-test. Accordingly, the following statistical analyses were performed for the foot parameter charge (Mann-Whitney),

duration (Mann-Whitney) and number (Student's *t*-test), and for the spike parameters  $t_{\rm rise}$  (log, Student's *t*-test, P=0.04), charge (log, Student's *t*-test, P=0.088), amplitude (log, Student's *t*-test), duration (Student's *t*-test) and  $t_{1/2}$  (log, Student's *t*-test).

#### Photolysis of caged Ca2+ and [Ca2+]i measurements in chromaffin cells

Flashes of UV light were generated by a flash lamp (T.I.L.L. Photonics), and fluorescence excitation light was generated by a monochromator (T.I.L.L. Photonics). The monochromator and flash lamp were coupled by using a Dual Port condenser (T.I.L.L. Photonics) into the epifluorescence port of an IX-50 Olympus Optical microscope equipped with a 40× objective (UAPO/ 340; Olympus Optical). Fura2FF was excited at 350 and 380 nm and detected through a 500 nm long-pass filter (T.I.L.L. Photonics) (Nili et al., 2006). The flash duration was 1-2 mseconds and we continued to photolyse with the monochromator for 5 seconds.

#### Membrane-capacitance measurements from chromaffin cells

Conventional whole-cell recordings and capacitance measurements were performed as described previously (Yizhar et al., 2004; Nili et al., 2006) and analyzed by using Igor Pro (Wavemetrics Inc.). The external bath solution contained (in mM): 140 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES and 2 mg/ml glucose pH 7.2 (320 mOsm). For flash photolysis experiments, the internal pipette solution consisted of (in mM): 105 Cs glutamate, 2 MgATP, 0.3 GTP, 33 HEPES, 0.33 Fura2FF (TefLabs, Austin, TX) (300 mOsm). The basal Ca<sup>2+</sup> was buffered by a combination of 4 mM CaCl<sub>2</sub> and 5 mM NP-EGTA (Graham Ellis-Davis; Drexel University College of Medicine, PA) to give a free  $[Ca^{2+}]_i$  of 400 nM (Yizhar et al., 2004; Nili et al., 2006). The analysis and comparison were always performed from pairs of control and KV2.1-overexpressing cells from the same batch of cells. We excluded from the analysis cells that had a leak above 50 pA, basal Ca<sup>2+</sup> concentrations above 500 nM, post flash Ca<sup>2+</sup> above 30  $\mu$ M, access resistance above 20 M $\Omega$ , or cells that showed spontaneous changes in membrane capacitance that exceeded 10% of the cell surface area.

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