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Neuroscience Letters 349 (2003) 53–57

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**Neuroscience  
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## Impairment of a parabolic bursting rhythm by the ectopic expression of a small conductance $\text{Ca}^{2+}$ -activated $\text{K}^+$ channel in *Aplysia* neuron R15

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Received 13 March 2003; received in revised form 21 April 2003; accepted 28 April 2003

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### Abstract

The electrical properties of neurons are produced by the coordinated activity of ion channels.  $\text{K}^+$  channels play a key role in shaping action potentials and in determining neural firing patterns. Small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{SK}_{\text{Ca}}$ ) channels are involved in modulating the slow component of afterhyperpolarization (AHP). Here we examine whether rat type 2  $\text{SK}_{\text{Ca}}$  (rSK2) channels can affect the shape of the action potential and the neural firing pattern, by overexpressing rat SK2 channels in *Aplysia* neuron R15. Our results show that rSK2 overexpression decreased the intra-burst frequency and changed the regular bursting activity of neurons to an irregular bursting or beating pattern in R15. Furthermore, the overexpression of rSK2 channels increased AHP and reduced the duration of the action potential. Thus, our results suggest that ectopic  $\text{SK}_{\text{Ca}}$  channels play an important role in regulating the firing pattern and the shape of the action potential.

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**Keywords:** Focal epilepsy; Parabolic bursting; Action potential; Afterhyperpolarization; Gene transfer

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R15 is a ‘giant’ neuron found in the right caudal quarter on the dorsal surface of the abdominal ganglion in *Aplysia*, an opisthobranch mollusk. R15 has an endogenous rhythmic bursting activity, which is composed of trains of action potentials separated by inter-burst intervals. R15 has been used as a model cell for studying focal epilepsy, because its bursting activity resembles the paroxysmal depolarization underlying focal epilepsy [12]. Many studies have characterized the  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  currents that contribute to the generation of an autoactive bursting rhythm in R15 [1,9]. Based on the kinetics and voltage dependence of the currents in R15, a number of Hodgkin-Huxley equivalent circuit models of R15 have been developed to simulate its bursting activity and to explain the ionic basis of bursting [1–3].

We have previously studied the ectopic expressions of the specific  $\text{K}^+$  channels, aKv1.1a (a high voltage-activated, transient  $\text{K}^+$  channel of *Aplysia*) and aKv5.1, to determine

their effects on bursting rhythm in R15 [6,9,17]. These studies showed that aKv5.1, a non-inactivating current, completely suppressed R15 bursting activity, whereas aKv1.1a, a shaker-type transient current, failed to influence the spontaneous bursting rhythm of R15. However, the role of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels ( $\text{SK}_{\text{Ca}}$ ) in the bursting rhythm of R15 remains unclear. Adams and Benson [1] argued that the  $\text{SK}_{\text{Ca}}$  is not responsible for the post-burst hyperpolarization or for the intra-burst repolarization of R15. Another simulation model [2] also showed that the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current is not essential for the bursting activity in R15. However, Cingolani et al. [4] showed that the inhibition of endogenous SK2 disrupted regular bursting in rat Purkinje neurons, suggesting that the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current plays a critical role in the regulation of bursting activity. Thus, we asked the question: Would bursting activity be facilitated or inhibited if the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current was introduced in R15? To address this question, we ectopically expressed the rSK2 channel, the most widely expressed member of the small conductance  $\text{SK}_{\text{Ca}}$  family in rat brain [11], by DNA microinjection into the *Aplysia* R15 neurons. We examined the role of  $\text{SK}_{\text{Ca}}$  channels,

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responsible for the slow component of afterhyperpolarization (AHP), on neuronal activity and the shape of the action potential.

*Aplysia kurodai* weighing 100–130 g were purchased from a local supplier in Youngdeok, South Korea and maintained in re-circulating seawater tanks at 14 °C before use. The preparation of the abdominal ganglia and the DNA microinjection into *Aplysia* neurons were performed as described previously [6,8,17]. The entire coding region of the rSK2 was subcloned into BamHI-NcoI sites of the *Aplysia* neuronal expression vector, pNEX $\delta$ F (GenBank accession no. AY229985). pNEX $\delta$ F-rSK2 was co-injected with pNEX $\delta$ -hrGFP [7], a marker of gene transfer. Fig. 1A shows an example of rSK2 expression in the R15 *Aplysia* neuron 8 h after DNA microinjection. First of all, we confirmed the expression of the rSK2 channel by measuring its current with a two-electrode voltage clamp 8 h after injecting pNEX $\delta$ F-rSK2 (Fig. 1B). The amplitude of the expressed steady-state current under normal ASW was  $2.37 \pm 0.22 \mu\text{A}$  ( $n = 3$ ) when measured at the end of a 1 s depolarizing pulse from  $-100$  to  $+60$  mV. This measure represents the sum of the various ionic currents activated during the voltage step. This sum was reduced by 61.3% ( $1.93 \pm 0.43 \mu\text{A}$ ,  $n = 3$ ) in the presence of the venom

apamin, a specific SK2 inhibitor, and by 50.8% ( $1.47 \pm 0.14 \mu\text{A}$ ,  $n = 3$ ) in the presence of  $\text{Na}^+$ - $\text{Ca}^{2+}$  free ASW (460 mM Tris-Cl, 10 mM KCl, 66 mM  $\text{MgCl}_2$ , 10 mM HEPES, pH 7.6). Thus, the apamin- or  $\text{Ca}^{2+}$ -sensitive currents, attributable to the rSK2 current, were  $2.17 \pm 0.30$  or  $1.48 \pm 0.13 \mu\text{A}$  ( $n = 3$ ), respectively. In contrast, apamin- or  $\text{Ca}^{2+}$ -sensitive currents were much smaller ( $0.20 \pm 0.12 \mu\text{A}$ ,  $n = 3$  or  $0.36 \pm 0.07 \mu\text{A}$ ,  $n = 3$ , respectively) in R15 cells expressing GFP alone than in R15 cells expressing rSK2 (Fig. 1C).

We also expressed a GFP-fused rSK2 channel mutant (T387W), in which a threonine residue within the channel conduction pore was replaced by a hydrophobic residue, tryptophan. Although the mutant channels are known to target the cell surfaces when expressed in either *Xenopus* oocytes or mammalian cells, they do not conduct ionic currents (Soh and Park, pers. commun.). However, the overexpression of this mutant channel did not change the current profile ( $0.71 \pm 0.22 \mu\text{A}$ ,  $n = 4$ ) (Fig. 1D), indicating that it did not interfere with endogenous  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents.

Next, we examined the effects of rSK2 overexpression on the bursting pattern of R15. Intracellular recordings and data storage were performed as described previously [8], using sharp microelectrodes filled with 2 M K-acetate, 0.5 M KCl, and 10 mM K-HEPES (10–15 M $\Omega$ ). pNEX $\delta$ F-rSK2 and pNEX $\delta$ -hrGFP were microinjected into R15 immediately after making an initial recording of the neuronal activity. Eight hours after this DNA microinjection, the neuronal activity of the GFP-positive neuron was re-recorded in the absence and in the presence of 1  $\mu\text{M}$  apamin.

In the bursting neuron R15, rSK2 overexpression significantly impaired the bursting rhythm. After rSK2 overexpression in R15, the autoactive parabolic bursting activity dramatically changed to an irregular burst pattern ( $n = 6$ ) or sometimes to a beating pattern ( $n = 2$ ) (Fig. 2A1). The standard deviation of the inter-burst interval increased significantly from  $2.19 \pm 0.27$  to  $3.62 \pm 0.50$  ( $n = 5$ , paired  $t$ -test,  $P < 0.05$ ) after rSK2 expression in each cell showing an irregular bursting pattern. In contrast, the standard deviation of the inter-burst interval did not change significantly after GFP expression in control cells expressing GFP alone (from  $2.19 \pm 0.19$  to  $2.46 \pm 0.22$ ,  $n = 7$ , paired  $t$ -test,  $P > 0.05$ ). The mean value of the burst duration also was increased from  $11.12 \pm 4.90$  s ( $n = 8$ ) to  $101.60 \pm 39.14$  s ( $n = 6$ ) (Fig. 2B1), indicating that rSK2 expression weakened the bursting rhythm. The mean value of the intra-burst spike frequency decreased from  $1.74 \pm 0.15$  to  $0.80 \pm 0.09$  spikes/s (Fig. 2B2). Furthermore, the progressive decrease in the undershoots of spikes, a characteristic of the parabolic bursting pattern, disappeared after rSK2 expression (Fig. 2A1). This decrease in the undershoot may have been caused by a positive shift in the potassium equilibrium potentials, because of extracellular  $\text{K}^+$  accumulation due to the high frequency of the spike trains [5]. Thus, the reduced spike frequency resulting from

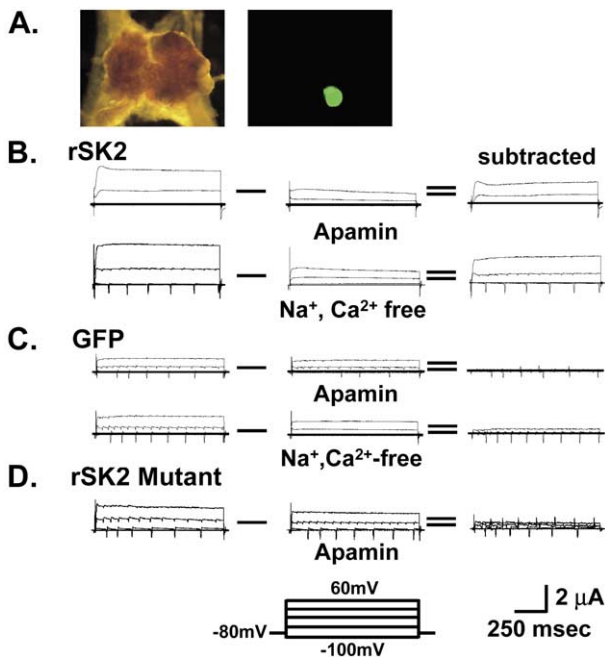


Fig. 1. Membrane currents in *Aplysia* neuron R15 expressing an ectopic rSK2 channel. Cells were voltage clamped at a holding potential of  $-80$  mV and subsequently clamped at potentials ranging from  $-100$  to  $+60$  mV for 500 ms in 40 mV clamping steps. Recordings were performed in ASW,  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ -free ASW, or in the presence of 1  $\mu\text{M}$  apamin for 10 min. (A) Light (left panel) and fluorescence (right panel) microphotographs showing an R15 neuron in the abdominal ganglion expressing GFP. (B) Current traces of GFP-positive neuron expressing rSK2. The subtracted currents represent the rSK2 current. (C) Current traces of neurons expressing GFP alone. (D) Current traces of neurons expressing rSK2 dominant negative mutant.

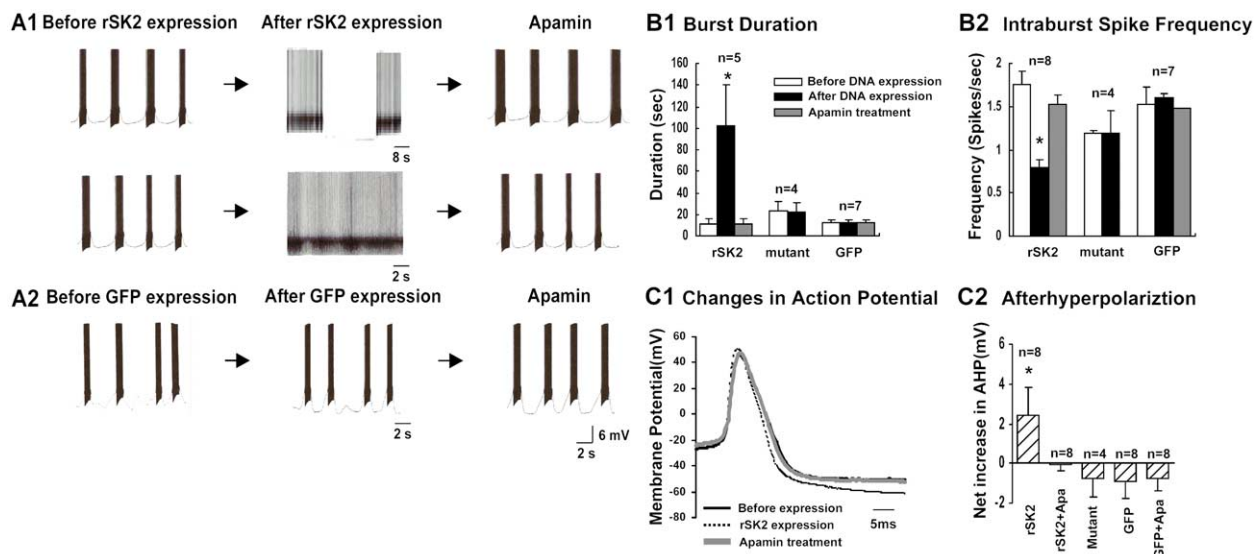


Fig. 2. Effects of rSK2 overexpression on the firing pattern and on the shape of the action potential. (A) Representative traces showing the bursting firing patterns of R15 (A1,A2) before and 8 h after rSK2 (A1) or GFP (A2) expression. (B) Effects of rSK2 overexpression on burst duration (B1) and on the intra-burst frequency of R15 (B2). (B1) The rSK2 overexpression increases the burst duration by inducing an irregular bursting activity in R15 ( $*P < 0.05$ , paired Student's *t*-test). (B2) The intra-burst frequency was changed significantly by rSK2 overexpression ( $*P < 0.05$ , paired Student's *t*-test). (C) Effects of rSK2 overexpression on the shape of the action potential. (C1) The shape of the action potential was changed by rSK2 expression. In addition to an increase in AHP, the duration of the action potential was reduced. The traces show superimposed action potentials before (dotted trace) and after (solid trace) rSK2 expression, and after apamin treatment (gray trace). Apamin treatment reversed both the increase in AHP and the decreased spike duration. (C2) Histogram showing that rSK2 overexpression significantly increased the AHP. AHP was measured from the lowest membrane potential to the threshold of the first spike in a burst at the superimposed figures and the difference was measured as the net increase in AHP ( $*P < 0.05$ , paired Student's *t*-test).

SK2 overexpression during the burst may not allow  $K^+$  accumulation outside the cell. Alternatively, if the decrease in the undershoot resulted from a cumulative inactivation of  $I_K$  [2], the overexpressed SK channels may have compensated for the inactivation of  $I_K$ .

The parabolic bursting rhythm was recovered by treating R15 overexpressing rSK2 with  $1 \mu\text{M}$  apamin, indicating that the impairment of bursting rhythm was due to the activation of expressed rSK2 (Fig. 2A1). In control experiments, the parabolic bursting rhythm of R15 was not impaired by GFP overexpression (Fig. 2A2). Taken together, these results show that rSK2 currents can selectively interrupt the coordinated activation of ionic currents underlying the parabolic bursting activity of R15.

To determine whether endogenous  $SK_{Ca}$  channels contribute to this bursting activity, we treated R15 bursting neurons with  $1 \mu\text{M}$  apamin. This treatment did not affect the regularity of the bursting rhythm (Fig. 2A2,B1,B2), or the shape of the action potential (data not shown) in non-injected R15 or R15 cells expressing only GFP. In addition, we overexpressed rSK2 dominant negative mutant, which can interfere with endogenous  $SK_{Ca}$  channels. However, mutant overexpression did not influence the bursting rhythm of R15 (Fig. 2B1,B2). Taken together, these results suggest that endogenous  $SK_{Ca}$  channels do not significantly influence the rhythmic activities in R15. This finding is consistent with previous reports which proposed that  $Ca^{2+}$ -activated  $K^+$  currents are not involved in the pacemaker potential in the R15 burst cycle [1]. However, we do not rule

out the possibility that endogenous  $SK_{Ca}$  in *Aplysia* may not be effectively blocked by either apamin or dominant negative rSK2.

rSK2 overexpression significantly changed the shape of the action potential in terms of its AHP, duration, rate of rise and peak depolarization. First, the amplitude of AHP was significantly increased compared to control R15 expressing GFP or rSK2 mutant (Fig. 2C1,C2). And, this increase in AHP by rSK2 overexpression was blocked by  $1 \mu\text{M}$  apamin, suggesting that this increase in AHP was due to  $K^+$  current through  $SK_{Ca}$  channels. Second, the duration of the action potential was reduced by rSK2 overexpression (Fig. 2C1), and this was blocked by apamin. In view of the fact that  $SK_{Ca}$  channels are involved in the slow component of AHP, which develops within 10–1000 ms [11,15], it is possible that  $SK_{Ca}$  channels can reduce the duration of the action potential, particularly when a large  $K^+$  current is generated by the overexpressed  $SK_{Ca}$  channels. Third, the rate of rise and the peak depolarization of the action potential were significantly increased by rSK2 overexpression (Fig. 2C1). This change in the rising phase of the action potential is likely to be due to the increased de-inactivation rate of sodium channels by SK2-induced hyperpolarization preceding the initiation of the action potential. By contrast, no significant change in the shape of the action potential was detected after treating control *Aplysia* neurons with apamin.

Then, how does rSK2 overexpression impair the parabolic bursting activity of R15?

A previous study showed that the overexpression of

aKv1.1a (a high voltage-activated, transient  $K^+$  channel) in R15 produces an increase in AHP and a decrease in the spike duration without affecting the bursting activity or intra-burst spike frequency [17]. A simulation study also showed that the transient outward current does not alter the character of the burst [2]. Thus, the specific inhibition of bursting activity by rSK2 overexpression is unlikely to be due to reduced spike duration, and is more likely to be due to rSK2-induced slow AHP that decays with a time constant of  $\sim 150$  ms [14].

It is widely held that slowly activating, voltage-dependent currents play a key role in producing the bursting rhythm of R15. Previous studies on this topic have focused on two transient currents, designated  $I_D$  (depolarizing) and  $I_H$  (hyperpolarizing), both of which increment by a fixed amount after each action potential in a burst [1]. More recently,  $I_{SI}$  (slow inward  $Ca^{2+}$  current) was proposed as a key requirement for bursting activity in an equivalent circuit model, which included the internal  $Ca^{2+}$  concentration and  $Ca^{2+}$  buffer occupancy as state variables.  $I_{SI}$  is a slowly oscillating current that is activated by voltage and inactivated by  $Ca^{2+}$ , and represents the most part of  $I_D$  and  $I_H$  [2]. rSK2 overexpression can block the development of these key currents, i.e.  $I_{SI}$  or  $I_D/I_H$ , during the bursts in many ways. First, the decreased rate of spike generation or spike frequency adaptation produced by rSK2 overexpression may be responsible for the failure of the rhythmic development of these key currents, by interfering with the voltage-dependent activation process and/or the  $Ca^{2+}$ -dependent inactivation process, normally achieved by parabolic high frequency spikes in a burst. Secondly, overexpressed rSK2 may influence the local concentration of  $Ca^{2+}$  near the plasma membrane by the associated calmodulin molecules [10,16]. Finally, overexpressed rSK2 may form heterotetramers [13] with some endogenous  $K^+$  channels to inhibit their function vis-à-vis bursting activity.

However, the two possibilities described above,  $Ca^{2+}$  sequestration by calmodulin and the dominant negative inactivation of endogenous channels by heteromultimerization are unlikely to occur because the inhibition of bursting activity by rSK2 overexpression requires the channel function. Two lines of evidence support this argument. First, inhibition of the channel function by apamin restored the bursting rhythm completely. Second, the bursting rhythm was not affected by the overexpression of the pore-mutant rSK2 channel that interacts with cytosolic calmodulin and targets the cell surface without any detectable ionic currents.

In this study, we overexpressed rSK2 to examine the function of a small-conductance  $SK_{Ca}$  on the shape of the action potential and on the regulation of the rhythmic activities of the bursting neuron *Aplysia* R15. Our results suggest that ectopic  $SK_{Ca}$  channels contribute to an increase in AHP and to the modulation of the firing pattern of bursting neurons, which implies that the high frequency generation of spikes is important for the parabolic bursting

activity of R15. It would be interesting to determine if the rSK2-induced changes in bursting activity and in spike shape can be reproduced by including SK2 current in the equivalent circuit models [1–3] of bursting activity.

## Acknowledgements

The authors wish to thank Dr John P. Adelman (The Vollum Institute, Oregon Health Sciences University) for providing rSK2 cDNA. This work was supported by grants M1-0104-00-0140 from the NRL Program, M1-0108-00-0075 from MOST, No. 1999-1-213-002-5 from KOSEF, and by a BK21 Fellowship from the Korean Ministry of Education.

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