PKA phosphorylation reshapes the pharmacological kinetics of BmK AS, a unique site-4 sodium channel-specific modulator

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Although modulation of the activity of voltage-gated sodium channels (VGSCs) by protein kinase A (PKA) phosphorylation has been investigated in multiple preparations, the pharmacological sensitivity of VGSCs to scorpion toxins after PKA phosphorylation has rarely been approached. In this study, the effects of BmK AS, a sodium channel-specific modulator from Chinese scorpion Buthus martensi Karsch, on the voltage-dependent activation and inactivation of Nav1.2 were examined before and after PKA activation. After PKA phosphorylation, the pattern of dose-dependent modulation of BmK AS, on both Nav1.2 (α1β1) was reshaped. Meanwhile, the shifts in voltage-dependency of activation and inactivation induced by BmK AS were attenuated. The results suggested that PKA might play a role in different patterns how β-like toxins such as BmK AS modulate gating properties and peak currents of VGSCs.

AMP-dependent protein kinase (PKA) has been long implicated to participate in central or peripheral nociceptive processing as secondary messenger. The activity of VGSCs responsible for initiation and transmission of sensory signals was proved to be subjected to PKA-mediated phosphorylation. However, the regulation patterns of VGSCs by PKA were divergent due to the various VGSCs subtypes and tissue- or cell-type distinct factors fine-tuning the physiological functions such as neuronal information encoding and transmission.

It has been well-documented that PKA phosphorylation could induce conformational change of VGSCs and influence the activity of VGSCs. PKA agonist inhibited the peak currents of VGSCs expressed in CHO cells by 40–50% and reduced the open probability of the channels. Similarly, Na1.2, a brain-type VGSC subtype expressed in Xenopus oocyte was vulnerable to PKA phosphorylation as well. By perfusing cAMP agonist forskolin or isoproterenol (Iso), the intracellular cAMP level was increased leading to activation of PKA phosphorylation pathway in oocytes and inhibition of INa by ~20–30%. In cardiac myocytes, activation of the β adrenergic system using Iso produced variable effects on INa, which were attributed to the activation of PKA.

BmK AS, originated from the venom of scorpion Buthus martensi Karsch (BmK), is a long chain toxin consisting of 66 amino acids cross-linked by four disulphide bridges, which shared highly similarity in sequence only with β anti-insect toxins AaH IT4 (from Androctonus australis Hector, AaH) and BmK AS-1, thereby uniquely being classified as β-like toxins. The pharmacological binding assay revealed that the binding of BmK AS on mammalian or insect nerve preparations could be competently inhibited by BmK IT2, a site-4 VGSC-specific modulator, and therefore, the receptor site of these two toxins may functionally adjacent. However, more evidences regarding to the molecular mechanism underlying the interaction of VGSCs with BmK AS remain to be clarified.

Earlier studies showed that BmK AS could substantially inhibit the Na+ currents (INa) in neuroblastoma B104 cells. Similarly, the reduction on INa was also observed in acutely dissociated small dorsal root ganglion (DRG) neurons from rats in the presence of BmK AS. Later behavioral experiments on animal behavior demonstrated that BmK AS could relieve the nociceptive responses in inflammation pain models. Recently, it is also found that hippocampal injection of BmK AS suppressed pentyleneetetrazole-induced seizures in rat. Thus, BmK AS seems...
to suppress the cell excitability through direct modulation on VGSCs. Unexpectedly, several lines of evidences showed that BmK AS could promote the cell excitability. BmK AS was found to stimulate noradrenaline release from rat hippocampus slices by augmentation of sodium influx. Electrophysiological studies also demonstrated that BmK AS could depolarize the voltage-dependence of fast or slow inactivation, resulting in slower inactivation.

Recent investigations revealed an interesting pharmacological characteristic of BmK AS which modulated the VGSCs in a unique U-shaped dose-dependent manner, i.e. at high concentrations, BmK AS increased the cell excitability, while at low concentrations, BmK AS suppressed the cell excitability. The U-shaped relationship of VGSC modulation may be also dependent on VGSC subtypes or tissue/cell environment.

In light of the potential role of some intracellular micro-environmental factors, such as PKA phosphorylation that was thought to be involving in regulating the channel activity, we examined whether PKA could play a role in BmK AS-driven modulation on Na1.2, the most abundant VGSC subtypes in brain tissues.

**Results**

**PKA phosphorylation reshaped the modulation of BmK AS on INa amplitude.** PKA phosphorylation was achieved by perfusion with β2-adrenergic agonist isoproterenol (Iso) for 20 min following the procedures described by RD. Smith and AL. Goldin. To understand if PKA phosphorylation may bring about any changes in Na1,2 current without co-expression with β2-adrenergic receptor, experimental controls under different concentrations of Iso (10, 100 and 200 μM) were set up before the subsequent tests. The results proved that Iso at experimental range could affect neither the peak Na+ currents (Supplementary Fig. S1, p > 0.05, n = 4–7) nor the voltage-dependent kinetics of Na1.2 (Supplementary Fig. S2). Therefore, 100 μM Iso was chosen to activate the PKA phosphorylation for Na1.2 with the co-expression of β2-adrenergic receptor.

To observe the time course of the peak current change during PKA activation, INa was elicited by a depolarization step of −20 mV at an interval of 1 min lasting for 30 min in the presence of Iso. Baseline was established by perfusion with ND96 solution for 10 min before Iso administration (Fig. 1, open squares).

When applying with Iso, the peak currents of INa decreased gradually, reaching the equilibrium within 5 min (Fig. 1, Black bar, Pre Iso, open circles). After Iso treatment for 20 min, the Na+ current amplitude was reduced by 12.04 ± 1.13% compared to the baseline (Fig. 1, Open circle). The Na+ current did not further decrease, indicating that β2-adrenergic receptors were saturated by Iso in oocytes.

However, application of 500 nM BmK AS resulted in a rapid decay in peak currents of INa in the initial 2.5 min and reached the equilibrium within 10 min (Fig. 1, Grey bar, Pre BmK AS, open triangles). The reduction rate of Ipeak was 35.45 ± 4.57% by BmK AS at the time point of 20 min (Fig. 1, open triangles). The subsequent 100 μM isoproterenol perfusion produced additional 8.53 ± 1.28% reduction on Ipeak (Fig. 1, Black bar, Pro Iso, open triangles). By contrast, if reversing the order of administration by pre-treatment with 100 μM Iso for 20 min, the following 500 nM BmK AS could only inhibit the Ipeak by 18.02 ± 2.36% (Fig. 1, Grey bar, Pro BmK AS, open circles), far less evident when comparing to the pre-treatment of BmK AS. The reduction in INa appeared to be slow and unstable, indicating an attenuated efficacy of BmK AS on Na1.2 after PKA phosphorylation.

Previously, it has been well documented that BmK AS (0.1, 1, 10, 100, 500 nM) modulated the VGSCs in a unique U-shaped dose-dependent manner. To test if after PKA phosphorylation Na1.2 could be modulated by BmK AS in a similar way as seen in the case before, the effects of 1, 10 and 100 nM of BmK AS were examined on Na1.2 before and after PKA activation. Prior to Iso treatment, all the three concentrations of BmK AS could efficiently inhibit the peak currents of INa, but to different extent (Fig. 2), with the most pronounced by 100 nM BmK AS which reduced the Ipeak by 34.04 ± 4.27%, less evident by 1 nM (18.77 ± 2.65%) and the least by 10 nM (12.84 ± 2.33%), in accordance with the trend found in previous study (Fig. 2D). By contrast, this unique nonlinear dose-dependent reduction pattern of BmK AS was altered by pre-treatment with Iso. The reduction rate of BmK AS at three concentrations on INa were changed as follows: 1 nM BmK AS (20.81 ± 3.34%) = 10 nM BmK AS (20.14 ± 3.89%) > 100 nM BmK AS (15.69 ± 2.65%), among which, the inhibitory potency of 100 nM BmK AS was largely attenuated after PKA activation and that of 10 nM BmK AS was slightly enhanced (Fig. 2D).

**PKA phosphorylation reshaped the nonlinear dose-dependent modulation of BmK AS on voltage dependence of activation.** To qualify the pharmacological characters of BmK AS on Na1.2 before and after phosphorylation, samples were selected if the INa peaks at voltages between −20 and −10 mV in the I-V relationship. After perfusion with 100 nM BmK AS for 20 min, the I-V curve of INa was shifted to more negative position. A similar but not significant effect could also be observed in the presence of 1 nM BmK AS, while 10 nM BmK AS induced a slight opposite shift (Supplementary Fig. S3, left). However, when pre-treatment with Iso, the I-V curve of INa was shifted to the depolarized direction by 100 nM BmK AS, while that of 1 nM and 10 nM was shifted to the hyperpolarized positions (Supplementary Fig. S3, right).

Considering the fact that there may be an offset in I-V curve due to the variation in reversal potential (Vrev), the G-V relationship was further assessed to analyze the modulation of BmK AS on voltage-dependent activation of Na1.2 before and after PKA phosphorylation. Consistent with the nonlinear dose-dependent modulation of BmK AS found in I-V relationship, 100 nM BmK AS could competently shift the voltage-dependent activation to more negative potential (ΔV1/2 = 5.54 mV, P < 0.001, Fig. 4A and Table 1) (Fig. 3C, left, Pre AS), while 10 nM BmK AS shifted the activation to the positive direction (ΔV1/2 = 4.89 mV, P < 0.001, Fig. 4A and Table 1). Comparably, PKA activation almost abolished the hyperpolarized shift induced by 100 nM BmK AS on G-V curve after pre-treatment of Iso (Fig. 3C, left, Pro AS). But both 1 nM and 10 nM BmK AS could efficiently shift the voltage-dependent activation to more negative potentials whose V1/2 was decreased by 3.31 mV (P < 0.001,
$eta_1$ subunit has been known to dramatically influence the gating properties of VGSCs, the modulation of BmK AS on voltage-dependent activation was studied in phosphorylated/unphosphorylated Nav1.2 ($\alpha + \beta_1$) in parallel to the Na_v1.2 expressed alone. Distinctively different with the case observed in Nav1.2 alone, BmK AS could move the voltage dependent activation of Nav1.2 ($\alpha + \beta_1$) towards depolarization in a linear dose-dependent way (Fig. 3A–C, right, Pre AS): Prominent for 100 nM ($\Delta V_{1/2} = 6.43$ mV, $P < 0.001$) and $10$ nM ($\Delta V_{1/2} = 6.03$ mV, $P < 0.005$), but less significant for $1$ nM ($\Delta V_{1/2} = 2.05$ mV, $P < 0.05$) (Fig. 4A and Table 1). Similar with Nav1.2 alone, after PKA activation, the modulation of BmK AS on voltage dependent of activation became far less evident (Fig. 3A–C, right, Pro AS), only $10$ nM BmK AS caused depolarized shift on $V_{1/2}$ ($\Delta V_{1/2} = 4.34$ mV, $P < 0.05$) (Fig. 4A and Table 1). Thus, PKA
Figure 3 | Voltage dependence of activation and inactivation for Na$_{\alpha 1.2}$ sodium channels. The voltage-dependence of activation and inactivation are shown for $\alpha$ subunits alone (Left panel) and ($\alpha + \beta 1$) subunits (Right panel).
Table 1 | Parameters for voltage-dependence of activation and inactivation of Na\(_{\alpha}1.2\alpha\) expressed in Xenopus laevis oocytes.

| BmK AS Concentration | Administration* | Activation | Steady-state inactivation |
|-----------------------|-----------------|-----------|--------------------------|
|                       |                 | \(V_{1/2}\) [mV] | \(k_m\) [mV] | \(N\) | \(V_{1/2}\) [mV] | \(k_m\) [mV] | \(N\) |
| 1 nM                  | Control         | -31.51 ± 0.97 | 10.49 ± 0.87 | 23 | -47.16 ± 1.06 | 9.70 ± 0.93 | 22 |
|                       | Pre AS          | -35.04 ± 0.66 | 13.37 ± 0.38 | 10 | -50.20 ± 0.56* | 13.59 ± 0.48* | 12 |
|                       | Pro Iso         | -37.32 ± 2.50* | 17.11 ± 2.70* | 6 | -49.29 ± 0.97 | 12.35 ± 0.91 | 7 |
|                       | Pre Iso         | -30.93 ± 1.18 | 11.26 ± 1.07 | 27 | -47.45 ± 1.32 | 10.49 ± 1.17 | 32 |
|                       | Pro AS          | -35.98 ± 0.88* | 8.24 ± 0.77 | 6 | -47.39 ± 2.68 | 14.67 ± 2.81* | 7 |
|                       | Pro Iso         | -32.30 ± 0.85 | 8.90 ± 0.75 | 5 | -51.37 ± 0.92* | 8.35 ± 0.80 | 8 |
|                       | Pre Iso         | -34.43 ± 0.90 | 9.00 ± 0.79 | 8 | -52.26 ± 0.90* | 9.08 ± 0.80 | 8 |
| 10 nM                 | Pre AS          | -30.93 ± 1.18 | 11.26 ± 1.07 | 27 | -47.45 ± 1.32 | 10.49 ± 1.17 | 32 |
|                       | Pro Iso         | -28.09 ± 3.67 | 18.88 ± 3.67** | 12 | -50.28 ± 0.65* | 9.40 ± 2.67 | 11 |
| 100 nM                | Pre AS          | -36.69 ± 1.19** | 12.91 ± 1.15 | 9 | -46.81 ± 1.24 | 11.42 ± 1.16 | 9 |
|                       | Pro Iso         | -32.17 ± 1.06 | 12.09 ± 0.97 | 10 | -47.52 ± 0.86 | 11.09 ± 0.79 | 10 |
|                       | Pre Iso         | -30.93 ± 1.18 | 11.26 ± 1.07 | 27 | -47.45 ± 1.32 | 10.49 ± 1.17 | 32 |
|                       | Pro AS          | -29.42 ± 0.80 | 10.75 ± 0.72 | 8 | -47.38 ± 0.56 | 10.03 ± 0.50 | 9 |

*Kinetic parameters of Na\(_{\alpha}1.2\)\(_{\alpha}\) after applying with BmK AS of different concentrations (1, 10 and 100 nM) were listed as Mean ± SEM separately.

All the concentrations of BmK AS were applied before (Pre AS) and after (Pro AS) PKA activation through perfusion with 100 μM Iso.

N indicates the number of samples tested.

* *, ** and *** indicates significant difference between control and BmK AS or Iso treated oocytes (*, P < 0.05; **, P < 0.01; ***, P < 0.001; t-test).

phosphorylation largely attenuated the effect of BmK AS on voltage-dependent activation of Na\(_{\alpha}1.2\) either co-expressed with β1 subunit or not.

PKA phosphorylation reshaped the nonlinear dose-dependent modulation of BmK AS on voltage dependence of inactivation. Accordingly, the modulation of BmK AS on steady-state inactivation of Na\(_{\alpha}1.2\)\(_{\alpha}\) was assessed before and after PKA phosphorylation. Generally, the steady-state inactivation of Na\(_{\alpha}1.2\)\(_{\alpha}\) was less sensitive to the modulation of BmK AS compared to that of activation (Fig. 3, left). Among the three dosages of BmK AS applied, only 1 nM BmK AS moderately shifted the value of \(V_{1/2}\) to more negative direction (\(AV_{1/2} = 3.23 \text{ mV}, P < 0.05\)) but following with PKA phosphorylation, the results showed that the modulation of 10 nM BmK AS was substantially enhanced, which hyperpolarized the voltage-dependent steady-state inactivation by 5.30 mV (\(P < 0.01\)) (Table 1, Fig. 3B and 4A). It was of notice that BmK AS at higher concentration (100 nM) did not shift the steady-state inactivation curve before and after PKA phosphorylation.

In contrast to the case of Na\(_{\alpha}1.2\)\(_{\alpha}\), BmK AS had a greater impact on the steady-state inactivation of Na\(_{\alpha}1.2\) (\(\alpha + \beta1\)) in a dose dependent manner. As shown in table 1 and figure 4A, both 10 nM and 100 nM BmK AS could efficiently hyperpolarize the steady-state inactivation (\(AV_{1/2} = 3.03 \text{ mV} \text{ for } 10 \text{ nM}, P < 0.001; AV_{1/2} = 4.04 \text{ mV} \text{ for } 100 \text{ nM}, P < 0.001\)). However, these two concentrations of BmK AS barely had any influence on steady-state inactivation of Na\(_{\alpha}1.2\) (\(\alpha + \beta1\)) after PKA activation, whereas 1 nM BmK AS imposed a slight but significant leftward shift on \(V_{1/2}\) (\(P < 0.05\), Table 1).

For the sodium channels expressed in Xenopus oocytes, the voltage dependent of inactivation splits into two inactivated components, with the faster one whose decaying currents last for several milliseconds and slower one lasts for dozens of milliseconds (see below). It was therefore analyzing the modulation of BmK AS on voltage dependent fast and slow inactivation separately before and after PKA phosphorylation. In oocytes expressed with Na\(_{\alpha}1.2\)\(_{\alpha}\), it was surprised to found that BmK AS hyperpolarized the \(V_{1/2}\) values of fast and slow inactivation in an opposite way. At high (100 nM) and low concentrations (1 nM), BmK AS could induce a remarkable hyperpolarized shift in \(V_{1/2}\) for fast inactivation but not slow inactivation, whereas this shift in slow inactivation was more evident than in fast inactivation by BmK AS at intermediate concentration (10 nM) (Fig. 4B, left, Pre AS; Fig. S4; Table 1). However, the effects of BmK AS on both inactivated state were greatly attenuated after PKA activation (Fig. 4B, left, Pro AS; Fig. S4; Table 1).

Contrary to the difference in BmK AS modulations in voltage-dependent activation between Na\(_{\alpha}1.2\)\(_{\alpha}\) and Na\(_{\alpha}1.2\) (\(\alpha + \beta1\)), both fast and slow inactivation of Na\(_{\alpha}1.2\) (\(\alpha + \beta1\)) was subjected to BmK AS modulation in a linear dose-dependent way. Notably, the extent of hyperpolarized shift in \(V_{1/2}\) induced by 10 nM BmK AS did not increase much compared with the shift induced by 10 nM BmK AS, but both of which were substantially larger than that of 1 nM BmK AS (Fig. 4B, right, Pre AS; Fig. S5; Table 2). Meanwhile, the slow inactivation was more affected by BmK AS at efficient dose range (10 nM and 100 nM) than the fast inactivation. As expected due to the findings mentioned above, the prominent effects of BmK AS for
both fast and slow inactivation were inevitably removed after PKA activation (Fig. 4B, right, Pro AS; Fig. S5; Table 2). PKA phosphorylation reshaped the modulation of BmK AS on open state inactivation kinetics. As mentioned above, the fast and slow inactivated components of Na\(^+\) currents of Nav1.2 responded differentially to BmK AS. To evaluate the fast and slow inactivation kinetics in the presence of BmK AS before and after PKA phosphorylation, we analyzed the time constants and fraction of fast component for inactivation.

For Nav1.2\(\alpha\), at most of the potentials tested, both 1 nM and 100 nM BmK AS could accelerate the open-state fast inactivation by reducing the time constant (\(t_{\text{fast}}\)) to -20 to +20 mV; \(P < 0.1\). In terms of 10 nM BmK AS, it could effectively delay the inactivation by increasing the time constants of slow inactivation (\(t_{\text{slow}}\)) and transform a proportion of Na\(^+\) currents from the fast-inactivation component into the slow-inactivation component at higher depolarized potentials (+10 to +20 mV, \(P < 0.05\)). Hence, the inactivation kinetics of Nav1.2\(\beta\) was modulated by BmK AS in a bidirectional manner. However, when PKA was pre-activated, it was found that the sensitivity of fast inactivation to BmK AS was distinctly attenuated. BmK AS at 1 nM and 100 nM BmK AS could even increase the time constant of slow inactivation at 0 mV and 10 mV respectively (Fig. 5A and 5C, left, Pro AS).

Likewise, the linear dose-dependent modulation of BmK AS on inactivation kinetics of Nav1.2 (\(\alpha + \beta1\)) was reshaped after PKA activation, in agreement with the cases found in voltage-dependent activation and inactivation. Unlike the effect on Nav1.2\(\alpha\), 10 nM BmK AS induced a slight decrease in \(t_{\text{fast}}\) (10 mV, \(P < 0.1\)) but distinctive increase in \(t_{\text{slow}}\) (-30 mV, \(P < 0.05\)) for Nav1.2 (\(\alpha + \beta1\)) at more negative potentials after PKA activation. In the presence of 100 nM BmK AS, the fraction of fast component of inactivation at -30 mV and -20 mV were greatly suppressed, while a substantial increase in \(t_{\text{fast}}\) could also be seen at -30 mV (Fig. 5B and 5C, right).

Figure 4 | Kinetics for voltage-dependent activation and inactivation for Na\(_{\alpha,1.2}\) sodium channels.
Pre AS). However, these changes almost disappeared after PKA activation (Fig. 5B and 5C, right, Pro AS). In addition, it was relevant to note that BmK AS did not change the amount of slowly inactivating steady-state Na⁺ currents featured for Na⁺,2 after depolarization to −20 mV for 10 ms. Unexpectedly, a slight but visible increase in steady-state Na⁺ currents could be induced by 10 nM or 100 nM BmK AS after 10 ms depolarization (P < 0.05) (Fig. 6).

Discussion
The current study demonstrated that PKA activation could reshape the dose-dependent modulation of BmK AS, a β-like scorpion toxin, on Na⁺,2 expressed in oocytes. The finding suggested that the potency of toxins on sodium channel was different before and after PKA phosphorylation.

Based on the previous pharmacological studies of BmK AS on sodium channels, it was concluded that the voltage-dependent activation and inactivation of these VGSC subtypes displayed a U-shaped shift in response to different concentration of BmK AS (0.1, 1, 10, 50, 100, 500 nM)14,15. In the current research, three of the concentrations of BmK AS (1, 10 and 100 nM) were chosen to investigate the pharmacological characteristics of BmK AS on Na⁺,2 with or without β1 subunit. It was found that co-expression of β1 subunit abolished the U-shape dose-dependence of BmK AS. The modulation of BmK AS on Na⁺,2 was attenuated after PKA activation regardless of co-expression with β1 subunit or not. The results suggested that complex interactions might exist among three factors: extracellular scorpion toxin, intracellular PKA, and crossing-membrane β1 subunit.

The results showed that both 1 nM and 100 nM BmK AS could facilitate the activation of Na⁺,2 through negatively shifting the voltage-dependent activation, while 10 nM BmK AS induced a shift in activation to the opposite direction. This nonlinear modulation of BmK AS could also be observed on voltage-dependent fast and slow inactivation of Na⁺,2, in which the inactivation curves were both negatively shifted by 1 nM or 100 nM BmK AS but not by 10 nM BmK AS. Although only three concentrations of BmK AS tested, this nonlinear modulation character of BmK AS was consistent with the U-shaped dose-dependent effects of BmK AS in previous researches.
Figure 5 | Modulation of BmK AS on the inactivation kinetics of phosphorylated/non-phosphorylated Na$_{1.2}$. (A–C) Inactivation time constants and fraction of fast component modulated by BmK AS of each concentration (1, 10 and 100 nM).
However, the direction of shift induced by BmK AS on voltage-dependent activation/inactivation is opposite in two different VGSCs expression systems, a depolarized direction in *Xenopus* oocytes but a hyperpolarized direction in ND7-23 cells (in both expression system, the concentrations of BmK AS applied were the same). Although the opposite direction of shift could be attributed to the different VGSC subtypes, the possibilities of intracellular microenvironment should also be taken into consideration. For example, background PKA activity may be different between systems and therefore cause different effects\(^7\).

In contrast with activation, the hyperpolarized shift of voltage-dependent inactivation of Na\(_{\alpha,1.2}\) by BmK AS could be reproduced on Na\(_{\alpha,1.2}\) \((\alpha + \beta_1)\). Nevertheless, the dose-dependent character of BmK AS modulation on Na\(_{\alpha,1.2}\) \((\alpha + \beta_1)\) was almost different with that of Na\(_{\alpha,1.2}\). The results showed that the efficacy of BmK AS on voltage-dependent activation/inactivation increased in a linear way with the rise of concentrations applied with \((1–100 \text{ nM})\). Meanwhile, in oocytes expressed with Na\(_{\alpha,1.2}\) \((\alpha + \beta_1)\), the shift in activation induced by BmK AS were different from that in oocytes expressed with Na\(_{\alpha,1.2}\) only. This difference could be explained by the co-expression of \(\beta_1\) subunit which has been reported to positively move the voltage-dependence of activation and accelerate the inactivation for Na\(_{\alpha,1.2}\)\(^1\). The disappeared nonlinear modulation of BmK AS and the decreased inactivation time on Na\(_{\alpha,1.2}\) \((\alpha + \beta_1)\) in this study may be caused by the removal of the component of an intermediate inactivation mode brought by *Xenopus* oocyte injection which was substantially reduced through co-injection of \(\beta_1\) subunit\(^19\)–\(^21\).

One interesting finding was that BmK AS at different concentrations may result in different shifts in inactivation kinetics on both Na\(_{\alpha,1.2}\)\(\alpha\) and Na\(_{\alpha,1.2}\) \((\alpha + \beta_1)\) determined with 10 ~ 600 ms-long conditioning pulses (See results). One possibility is that the binding affinity of BmK AS may vary with the duration of depolarized stimuli which was common among scorpion \(\alpha\) toxins but rarely on \(\beta\) toxins\(^22\). However, direct evidences correlating the relationship between binding affinity of BmK AS and different sodium channel states remain to be studied in the future.

The effects of sodium channels on electrical excitability of a neuron depend on both the kinetics and voltage dependence of sodium channel activation and inactivation\(^14\). In accordance with the previous reports, the voltage-dependent activation and inactivation of Na\(_{\alpha,1.2}\)\(\alpha\) were not affected by the PKA regulation\(^19\), which held to be true for the case of Na\(_{\alpha,1.2}\)\(\alpha\) co-expression with the \(\beta_1\) subunit as well. However, PKA phosphorylation was found to largely attenuate the modulation of BmK AS on voltage-dependent kinetics for both Na\(_{\alpha,1.2}\)\(\alpha\) and Na\(_{\alpha,1.2}\) \((\alpha + \beta_1)\). It was of notice that the potency of PKA in reshaping the BmK AS pharmacological efficacy on sodium channel was in relevant to the different concentration of BmK AS, prominent for 1 nM and 100 nM, but less efficient for 10 nM. The inhibition rate on \(I_{\text{Na}}\) and negative shift of steady-state inactivation of Na\(_{\alpha,1.2}\) induced by 10 nM BmK AS were even enhanced after PKA activation (Fig. 1D and Fig. 4B left, Pro AS). This exception was not occurred in the case when \(\beta_1\) subunit was co-expressed with Na\(_{\alpha,1.2}\)\(\alpha\), in which there was a significant attenuation for 10 nM BmK AS-induced negative shifts in inactivation curves before PKA activation (Fig. 4 right, Pro AS). Finally, the remarkable increase in persistent Na\(_{\alpha}\) currents of sodium channel reported previously was hardly observed in our study before and after PKA phosphorylation, which was possibly due to the decrease in BmK AS concentration used (500 nM in those researches)\(^1\)–\(^2\). Based on the observations discussed above, the PKA phosphorylation and BmK AS modulation may be functionally related.

BmK AS belongs to \(\beta\)-like toxins that also includes AaH IT4 from *Androctonus australis* Hector (Loret et al., 1991) and Lqh \(\beta_1\) from *Leiurus quinquestriatus hebraeus* (Gordon et al., 2003). These toxins compete for the excitatory (AaHIT1, BmK IT) and depressant toxin (LqhIT2, BmK IT2) binding sites on sodium channels in insect neuronal preparations, as well as for site-4 of classical \(\beta\)-toxins on rat brain synaptosomes. Therefore, they are classified as “\(\beta\)-like toxins”, but they also compete with moderate affinity with both \(\alpha\) - and \(\beta\)-classical anti-mammalian toxins for their binding site (site 3 and 4) to rat brain synaptosomes\(^29\)–\(^31\). The archetype of the \(\beta\)-like toxins is Ts1, from the Brazilian scorpion *Tityus serralatus*. It was recently demonstrated that this toxin could cooperatively interact with three of four voltage-sensors in Na\(_{\alpha,1.2}\) (domain II, III and IV) with different affinities, leading to opposite pharmacological consequences\(^30\)–\(^32\). The same mechanism might exist and account for the unique pharmacological performance of BmK AS. Furthermore, the pattern of how BmK AS modulates sodium channel and the receptor sites on sodium channels might be conserved among \(\beta\)-like toxins.

Most of the PKA phosphorylation sites were located at DI-II loop and the C-terminal of VGSCs\(^33\),\(^34\) which are right approximate to the ion permeable pore (DI S5–S6 and DIV S5–S6)\(^33\)–\(^34\). The action of PKA phosphorylation depends largely on the five consensus sites located in DI-II loop of VGSCs. In addition, it is reported that the effects of PKA and PKC usually interact molecularly and physiologically, which was possibly owing to the shared phosphorylation

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**Figure 6** | The fractions of residual sodium current after depolarization to \(-20\) mV for 10 ms in *Xenopus* oocytes, before (A) and after PKA phosphorylation (B)\(^*\)\(P < 0.05\); indicating significant difference between the control (white bar) and BmK AS (Dark bar) values.
were clamped at SCIENTIFIC crude venom of BmK as described previously39. The purity of the toxin was confirmed

Methods

Upstream factors involving in regulating sodium channels and excit-

ation currents, voltage-dependent activation and inactivation rate as a

site in DIII (N1466A)35,36. With refer to the pharmacological relevant

recordings were performed using Axon 900A amplifier (MDC, USA) and pclamp

Electrophysiological recording and data analysis

Two-electrode voltage-clamp preparations were made in vitro with SP6 NA–polymerase as the same procedure as that of Nav1.2. The genes encoding the sodium channel

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