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·Original Article·

Activation of mammalian target of rapamycin contributes to pain nociception induced in rats by BmK I, a sodium channel-specific modulator

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ABSTRACT

The mammalian target of rapamycin (mTOR) pathway is essential for maintenance of the sensitivity of certain adult sensory neurons. Here, we investigated whether the mTOR cascade is involved in scorpion envenomation-induced pain hypersensitivity in rats. The results showed that intraplantar injection of a neurotoxin from *Buthus martensii* Karsch, BmK I (10 μg), induced the activation of mTOR, as well as its downstream molecules p70 ribosomal S6 protein kinase (p70 S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), in lumbar 5–6 dorsal root ganglia neurons on both sides in rats. The activation peaked at 2 h and recovered 1 day after injection. Compared with the control group, the ratios of p-mTOR/p-p70 S6K/p-4E-BP1 in three types of neurons changed significantly. The cell typology of p-mTOR/p-p70 S6K/p-4E-BP1 immuno-reactive neurons also changed. Intrathecal administration of deforolimus, a specific inhibitor of mTOR, attenuated BmK I-induced pain responses (spontaneous flinching, paroxysmal pain-like behavior, and mechanical hypersensitivity). Together, these results imply that the mTOR signaling pathway is mobilized by and contributes to experimental scorpion sting-induced pain.

Keywords: BmK I; mTOR; p70 ribosomal S6 protein kinase; 4E-binding protein 1; pain; dorsal root ganglion

INTRODUCTION

Mammalian target of rapamycin (mTOR) is a critical downstream target of the phosphatidylinositol-3 kinase (PI3K) pathway. As a major serine/threonine protein kinase, mTOR controls mRNA translation and protein synthesis by phosphorylating its downstream molecules p70 ribosomal S6 protein kinase (p70 S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1)[1-6]. The key role of mTOR in regulating cell growth and metabolism in eukaryotic cells has been well demonstrated[7, 8]. The activity of the mTOR cascade is up-regulated in many pathological states, such as cancer and neurodegenerative disorders[9, 10]. Increasing evidence has suggested that mTOR is one of the most important pathways that controls neuronal excitability and regulates learning and memory-related long-term plasticity[11, 12].

With recent research on the roles of mRNA translation and protein synthesis in the regulation of nociceptive transmission, it has become clear that mTOR signaling-dependent mRNA translation occurs in primary afferents[13-15]. For instance, the mTOR pathway participates in maintaining the sensitivity of myelinated sensory fibers known to be important for capsaicin-induced mechanical

sensitivity[16]. Both central and local administration of mTOR inhibitors alleviate the persistent mechanical but not the thermal hypersensitivity induced by nerve injury[17, 18]. The anti-nociceptive effect of rapamycin, a selective inhibitor of mTOR, might be due to its elevation of the A-fiber activation threshold[18]. However, studies have shown that the thermal sensitivity of inflamed tissue can also be reduced by the intrathecal (i.t.) application of rapamycin. The diverse effects of mTOR inhibitors on pain behaviors may be due to distinct mechanisms in different animal models. Investigating the role of the mTOR pathway in various kinds of pain states has important implications for understanding the mechanisms of nociceptive plasticity and therefore for therapeutic interventions in chronic pain conditions.

Envenomation by the scorpion *Buthus martensii* Karsch (BmK) induces long-term intense pain[3, 6, 8]. Various BmK neurotoxins targeting membrane channels such as voltage-gated Na^+ channels (VGSCs) and K^+ channels are the prime suspects[19-25]. BmK I, a toxin specific to VGSC receptor site 3, increases Na^+ currents and delays the inactivation of VGSCs[26]. Intraplantar (i.pl.) injection of BmK I induces many pain responses in rats, such as spontaneous reflexes, paroxysmal pain-like behaviors, thermal hypersensitivity, and bilateral (mirror-image) mechanical hypersensitivity[26]. It is therefore considered to be the main contributor to scorpion envenomation-induced pain[26, 27]. Compared to other animal models of pain, the BmK I model has a unique mechanism. Our previous studies showed that the dynamic modulation by BmK I of Na^+ channels on peripheral primary afferent fibers, especially capsaicin-sensitive fibers, contributes to pain sensation[26, 28]. The pain of a scorpion sting is, at least partly, due to peripheral nerve sensitization. Moreover, our results suggest that the expression of VGSC proteins increases in the dorsal root ganglion (DRG) after BmK I administration (unpublished data). But whether the mTOR signaling pathway is involved in the process and whether it is essential for peripheral sensitization remain to be revealed.

In the present study, we investigated (1) whether the mTOR cascade is activated in the DRG after scorpion envenomation, and if so, the time-course and cellular distribution of the activation in the DRG; and (2) whether this pathway contributes to pain behaviors induced by a scorpion sting.

MATERIALS AND METHODS

Animals

All procedures were approved by Shanghai University Committee on Animal Care and Use, and complied with the guidelines for the use of experimental animals approved by the European Commission[29], the Animals (Scientific Procedures) Act 1986 passed by the Parliament of the United Kingdom[30], and the guidelines of International Association for the Study of Pain for pain research in conscious animals[31]. Adult male Sprague-Dawley rats (220–250 g; Shanghai Laboratory Animal Center, Chinese Academy of Sciences) were group-housed (5 per cage) under a 12-h light-dark cycle (lights on at 08:00) at 21–23°C with 50% humidity. Food and water were provided *ad libitum*. All efforts were made to minimize suffering and to reduce the number of animals used.

Preparation and Administration of BmK I

BmK I was purified from crude BmK venom as previously described[32], and its purity was assessed by mass spectroscopy prior to experiments. BmK I solution was prepared at 0.2 μg/μL in physiological saline (0.9% NaCl). Fifty microliters of BmK I solution was injected (i.pl.) into the left hindpaw[26]. Rats with i.pl. injection of 50 μL sterile saline were used as controls in the immunohistochemistry and immunoblot experiments.

Preparation and Administration of Deforolimus

Deforolimus (Selleck Chemicals, Houston, TX), a specific inhibitor of mTOR, was dissolved to 250 μmol/L in a DMSO/saline mixture containing 5% (*v*/*v*) DMSO. Briefly, rats were lightly anesthetized with ether. A volume of 10 μL drug solution or vehicle (as control), was injected (i.t.) by direct lumbar puncture between L5 and L6 with a 27-gauge, 1-inch sterile disposable needle connected to a 25 μL Hamilton syringe 30 min before i.pl. administration of BmK I. Puncture of the dura was indicated by a reflexive tail-flick or formation of an "S" by the tail.

Immunohistochemistry

Rats were anesthetized with pentobarbital sodium (60 mg/kg) and perfused intracardially with 200 mL sterile saline, followed by 400 mL fixative containing 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4). The DRGs from L5–L6 were removed from

both the ipsilateral and contralateral sides, post-fixed in the same fixative for 12 h at 4°C, and then cryoprotected in 0.1 mol/L PBS containing 30% sucrose until they sank to the bottom. Frozen serial coronal sections (14 μm) were cut on a cryostat (Microm HM 525; Thermo Scientific, Walldorf, Germany) and mounted on gelatin-coated glass slides.

We probed for activated mTOR, p70 S6K, and 4E-BP1 in the DRGs. Non-specific binding was blocked by incubation with 5% normal horse serum (S2000; Vector Laboratories, Burlingame, CA) in PBS with 0.3% Triton X-100. To label activated calcitonin gene-related peptide (CGRP)/isolectin-B4 (IB4)/200-kDa neurofilament protein (NF200), sections were incubated for 48 h at 4°C with a mixture of rabbit monoclonal anti-p-mTOR (1:200, Cell Signaling Technology, Beverly, MA)/rabbit polyclonal anti-p-p70 S6K (1:50, Santa Cruz, Dallas, TX)/rabbit monoclonal anti-p-4E-BP1 (1:50, Bioworld Technology, St. Louis Park, MN) and mouse monoclonal anti-CGRP (1:400, ABCam, Cambridge, MA)/anti-IB4 conjugated with FITC (1:400, Sigma, St. Louis, MO)/anti-NF200 (1:500, Santa Cruz). After rinsing with 0.01 mol/L PBS for 15 min, the sections were incubated with a mixture of donkey anti-rabbit IgG conjugated with Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA) and anti-mouse IgG conjugated with FITC (1:200, Jackson ImmunoResearch) in the dark for 6 h at room temperature. Control experiments were performed in parallel. For single detection of activated mTOR, p70 S6K, or 4E-BP1, the sections were incubated with the relevant antibody at 4°C for 48 h and then washed in 0.01 mol/L PBS for 15 min. Then the sections were incubated with the secondary fluorescent antibodies conjugated with Cy3 or FITC in the dark for 6 h at room temperature. After the sections were rinsed in 0.01 mol/L PB, they were coverslipped.

Western Blot

Rats were anesthetized with sodium pentobarbital (60 mg/kg) and decapitated at the indicated time-points after BmK I injection. Samples were homogenized in 50 mmol/L Tris buffer (pH 7.4) containing 0.5% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 3% SDS, protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails I and II (Sigma). After centrifugation at 14 500 rpm for 15 min, the supernatant containing total cellular protein was collected. The protein concentration in each sample was determined using a Bradford assay kit (Sigma). Denatured samples containing equal amounts of total protein (30 μg) were separated by SDS-PAGE. The separated proteins were then transferred to a PVDF membrane.

Membranes were first incubated in 5% non-fat milk overnight at 4°C to avoid nonspecific binding. The following primary antibodies were diluted in PBS with Tween-20 (PBS-T) containing 5% non-fat milk for 5 h at room temperature: rabbit polyclonal antibody against p-mTOR (1:1 000, Cell Signaling Technology), rabbit monoclonal antibody against mTOR (1:1 200, Cell Signaling Technology), rabbit polyclonal antibody against p-p70 S6K (sc-7984-R, 1:1 500, Santa Cruz), rabbit monoclonal antibody against p-4E-BP1 (1:1 000, Cell Signaling Technology), and rabbit polyclonal antibody against actin (sc-1616, 1:1 000, Santa Cruz). After washing, membranes were probed with horseradish peroxidase-conjugated secondary antibody (1:10 000, KangCheng Biotechnology, Shanghai, China) for 2 h at room temperature. Immunoblotting signals were developed with ECL reagent (WBKLS0050; Millipore Bioscience Research Reagents, Temecula, CA), exposed to film, and the bands were scanned and quantified using Quantity One software (Bio-Rad Laboratory, Hercules, CA). All bands were normalized to the corresponding β-actin band.

Behavioral Tests

Behavioral tests were used to evaluate the suppressive effect of deforolimus on BmK I-induced pain responses. The measurement of spontaneous nociceptive responses and paw withdrawal mechanical threshold (PWMT) followed the methods described by Bai *et al.*[26]. Briefly, the spontaneous behaviors were determined by counting the number of flinches in 5 min, the total number of flinches, and the number of paroxysmal pain-like behaviors in 2 h after BmK I administration. Then the rats were placed on a mesh floor and the PWMT was evaluated with a series of von Frey fibers (Stoelting Co., Wood Dale, IL). Fibers were applied to the hindpaw for the same duration of 2–3 s at 10-s intervals. A positive response was indicated by brisk withdrawal or flinching of the tested hindpaw. The PWMT was defined as the lowest force that caused at least five withdrawals out of 10 consecutive applications. The cutoff force was set at 26 g to prevent tissue injury.

Statistical Analysis

All results are expressed as mean ± SEM. Spontaneous response behaviors and Western blots between groups were compared by one-way ANOVA followed by Bonferroni's *post hoc* test. Other comparisons were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test.

RESULTS

Unilateral Injection of BmK I Activates mTOR Cascades in Bilateral DRG

Immunohistochemical results revealed that, in the saline-injected group, the intensity of p-mTOR immunostaining in the DRG was extremely low (Fig. 1A, F). However, in the BmK I-treated groups, p-mTOR-immunoreactive (IR) cells increased markedly in the DRGs on both sides at different time-points (Fig. 1B–E, G–J).

c-Fos expression is recognized as a marker of neuronal excitability because it is often rapidly expressed when neurons fire action potentials[33, 34]. According to the study of Bai *et al*.[35], the spinal expression of c-fos reaches a peak 2 h after BmK I administration. Therefore, we assessed whether the phosphorylation levels of p70 S6K and 4E-BP1, downstream molecules of mTOR, were significantly activated in DRGs at this time. As expected, the reactivity of p-4E-BP1 and p-p70 S6K increased (Fig. 2).

The time-course of phosphorylation in the mTOR cascade was determined by Western blotting of the L5–L6 DRGs on both sides after BmK I injection. A representative result and the standardization ratio statistics of these tests are shown in Fig. 3. In the control group, limited activation of mTOR, as well as 4E-BP1 and p70 S6K, was detected. The average intensities of the relevant bands increased significantly in BmK I-treated rats on both sides and reached a peak at 30 min to 2 h after BmK I injection (Fig. 3B–D, G–I). The total amount of mTOR was not affected by BmK I treatment (Fig. 3E, J).

The Distribution of the mTOR Cascade Differs in Subtypes of DRG Neurons

To determine in which subtypes of DRG neurons the mTOR cascade expression increased, p-mTOR, p-p70 S6K, or p-4E-BP1 was double-stained with the neuronal markers CGRP, IB4, and NF200. CGRP, a marker of peptidergic C nociceptors, and IB4, a marker of non-peptidergic C nociceptors, are used to classify small-diameter DRG neurons[36, 37]. NF200 is used as a marker of myelinated A nociceptors, including large- and medium-diameter DRG neurons[38].

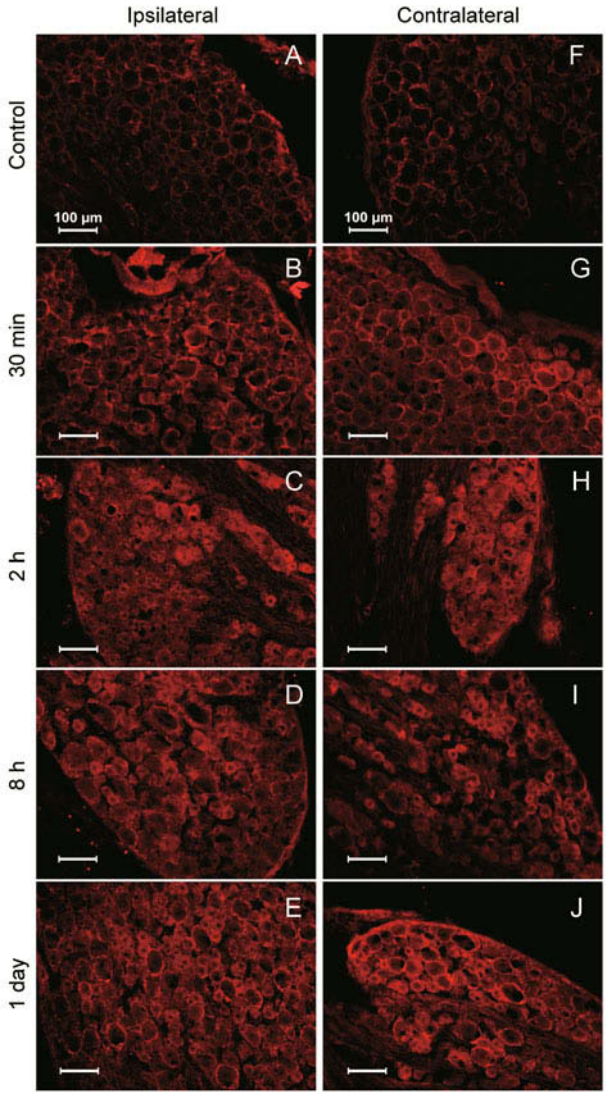

Fig. 1. The spatiotemporal distribution of p-mTOR in rat DRG after i.pl. injection of BmK I. Compared with the saline group (A, F), the BmK I-treated groups showed marked immunoreactivity for p-mTOR in both the ipsilateral (B–E) and contralateral (G–J) DRGs at different time-points.

As shown in Fig. 4, in control animals, 42.8 ± 2.6% of CGRP-positive neurons, 46.9 ± 1.7% of IB4-positive neurons, and 21.7 ± 3.1% of NF200-positive neurons expressed p-mTOR on the ipsilateral side. But 2 h after

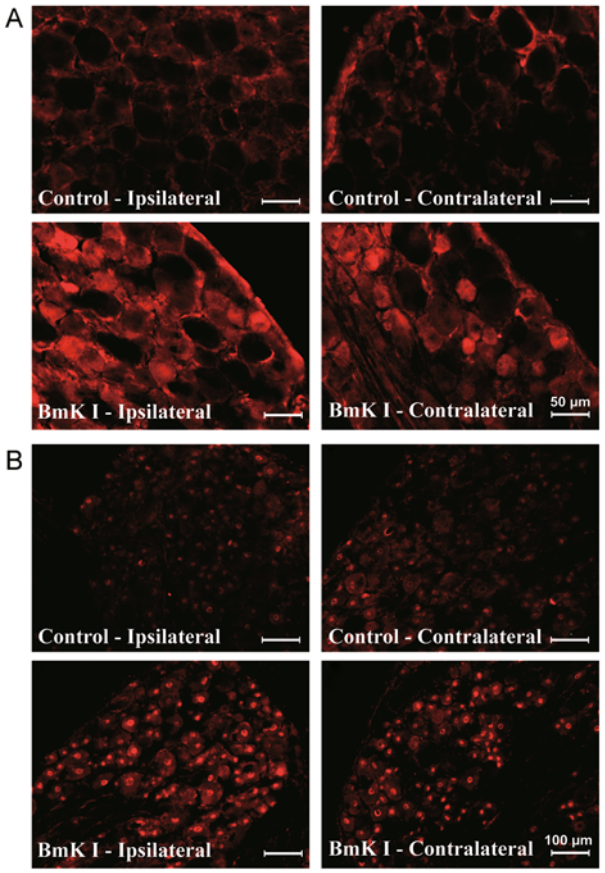

Fig. 2. The spatiotemporal distribution of p-4E-BP1 (A) and p-p70 S6K (B) in rat DRGs 2 h after i.pl. injection of BmK I. Compared with the saline group, the BmK I-treated groups showed marked immunoreactivity on both sides.

BmK I injection, the ratios were increased to 61.3 ± 1.8%, 64.6 ± 1.2%, and 42.4 ± 1.2%, respectively. In addition, on the contralateral side, the ratios were increased from 42.3 ± 3.7%, 47.4 ± 1.4%, and 24.1 ± 2.7% to 68.1 ± 1.9%, 66.9 ± 1.0%, and 47.2 ± 0.7% (Fig. 4B, E, H). On the other hand, the cell typology of p-mTOR IR neurons was also altered. In the control animals, the p-mTOR IR neurons comprised 37.9 ± 2.0% CGRP-positive, 35.5 ± 1.4% IB4-positive, and 20.3 ± 3.2% NF200-positive on the ipsilateral side. But 2 h after BmK I injection, the ratios changed to 43.7 ± 1.7%, 46.6 ± 3.1%, and 30.4 ± 1.8%, respectively. On the contralateral side, the ratios changed from 35.6 ± 2.4%, 31.8 ± 0.9%, and 21.7 ± 2.4% to 45.9 ± 2.7%, 49.5 ± 0.7%, and 34.2 ± 1.2%, (Fig. 4C, F, I).

Similarly, the distributions of p-4E-BP1 and p-p70 S6K also changed after BmK I treatment (Figs. 5 and 6).

Inhibiting the mTOR Cascade Attenuates BmK I-induced Pain Responses

Deforolimus, a specific inhibitor of mTOR, was injected (i.t.) 30 min prior to BmK I administration to investigate the potential involvement of the mTOR pathway in BmK I-induced nociceptive pain behaviors. Compared with the control group, 250 μmol/L deforolimus significantly suppressed the spontaneous pain responses (Fig. 7). The suppression of flinches by deforolimus lasted for >90 min (Fig. 7A). Pre-treatment with deforolimus decreased the total number of flinches from 1066.0 ± 52.7 to 662.0 ± 27.2 (Fig. 7B). The number of paroxysmal pain-like behaviors induced by BmK I in 2 h was suppressed by deforolimus from 5.6 ± 0.7 to 2.0 ± 0.3 (Fig. 7C). Furthermore, the BmK I-induced hypersensitivity was also influenced by deforolimus. Bilateral mechanical hypersensitivity was suppressed at 4 h after BmK I administration. The ipsilateral PWMT increased from 3.7 ± 0.7 g to 7.5 ± 0.6 g (Fig. 7D) and the contralateral value increased from 5.8 ± 0.8 g to 13.0 ± 2.1 g (Fig. 7E).

DISCUSSION

In the present study, we investigated the role of the mTOR cascade in scorpion sting-induced pain behaviors in rats for the first time. We found that i.pl. injection of the main toxin of BmK venom changed the activation level and the cellular distribution of the mTOR cascade in L5–L6 DRG neurons on both sides, showing the involvement of this cascade in scorpion sting-induced nociception. More importantly, an inhibitor of the mTOR cascade attenuated behavioral nociception, showing that the mTOR-dependent mRNA translation pathway is important in the induction of scorpion sting-induced hypersensitivity.

Scorpion Sting Stimulated the mTOR Signaling Pathway in DRG

We found that mTOR, 4E-BP1, and p70 S6K were phosphorylated in DRG neurons on both sides immediately after peripheral injection of BmK I (Figs. 1 and 2). Immunoblot results revealed that the activation peaked at 2 h after BmK I administration (Fig. 3). This raises the questions of why unilateral injection of BmK I induced bilateral activation of the mTOR cascade. There is still no

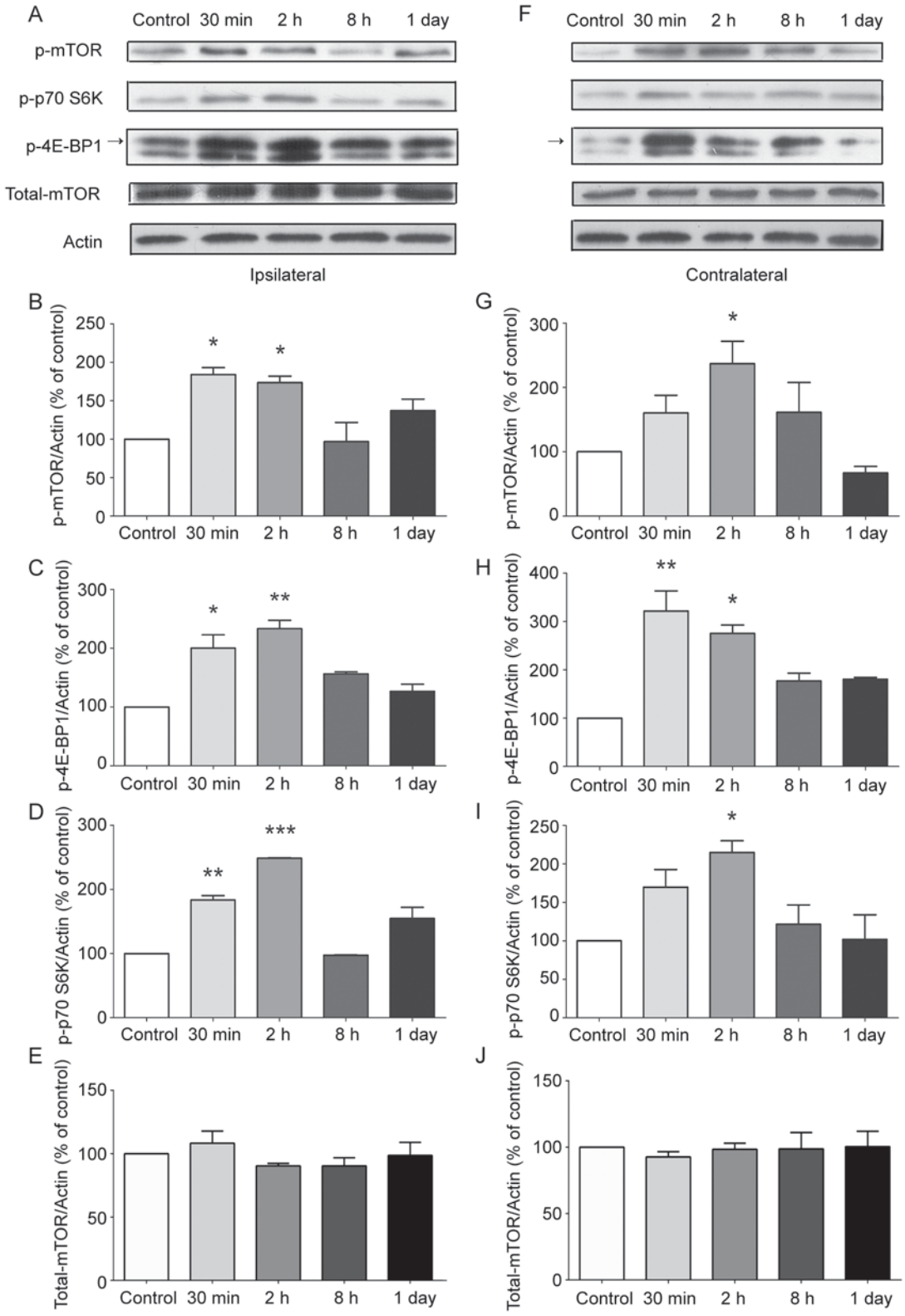

Fig. 3. Representative Western blots and optical band density analysis of p-mTOR, p-p70 S6K, p-4E-BP1, and total mTOR, in L5–L6 DRGs on both sides at different time-points after i.pl. injection of BmK I. **P* <0.05, ***P* <0.01, ****P* <0.001 (*n* = 3).

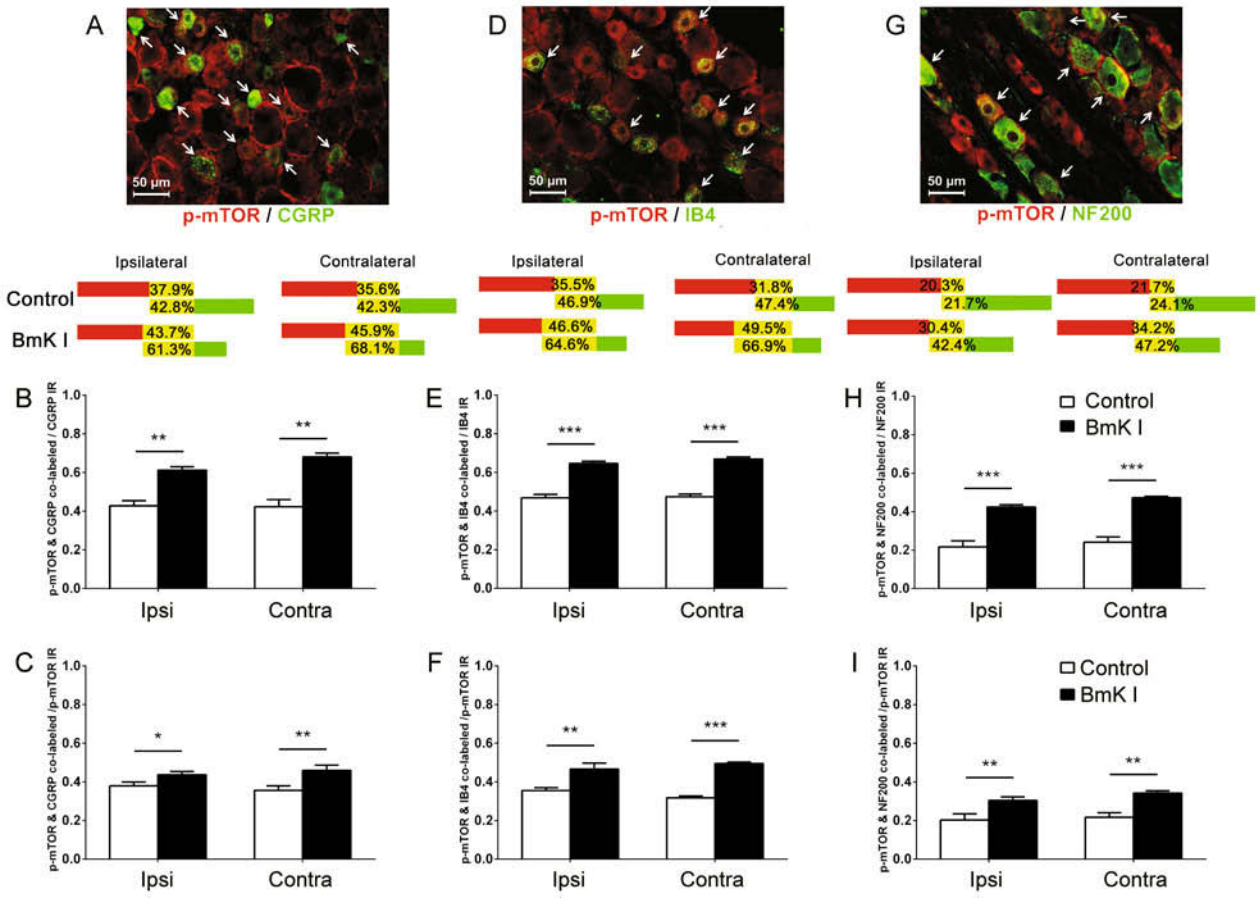

Fig. 4. Cellular localization of p-mTOR immunoreactivity in rat DRG after i.pl. injection of BmK I and quantification of DRG neurons double-stained with p-mTOR and neuronal markers. Arrows indicate co-localization (yellow) of p-mTOR (red) and the respective cell marker (green). **P* <0.05, ***P* <0.01, ****P* <0.001.

direct answer to this question. But what deserves more attention is that bilateral mechanical hypersensitivity was induced by BmK I in rats. The mechanosensitivity peaked at 2–8 h after BmK I administration[26]. As activated mTOR promotes functional protein synthesis, based on the gate-control theory of pain, we suppose that mTOR activated in the contralateral DRG might contribute to the synthesis of proteins and peptides associated with mechanosensitivity.

The DRG is responsible for the bidirectional transmission of information. Nociceptors cannot be excited below the noxious threshold, which suggests that there must be a process to prepare the necessary materials, including proteins and peptides. The mTOR cascade is known to regulate several intracellular activities in response to various stimuli and thereby regulate local mRNA translation[17]. Moreover, the mTOR signaling pathway has been suggested to play roles in hippocampal long-term potentiation[39]. Combining all these findings, we infer that mTOR-dependent intracellular activity might be essential for the peripheral transmission of nociceptive signals induced by a scorpion sting.

Changes in mTOR Cascade Expression in Different Subtypes of DRG Neurons after Scorpion Sting

In this study, we also showed that activated mTOR protein, as well as 4E-BP1 and p70 S6K, increased rapidly after BmK I injection (Figs. 1–3). One more interesting question is in which subtype of DRG neurons are the increased proteins located. There are disputes concerning this question. Some studies revealed that, in response to

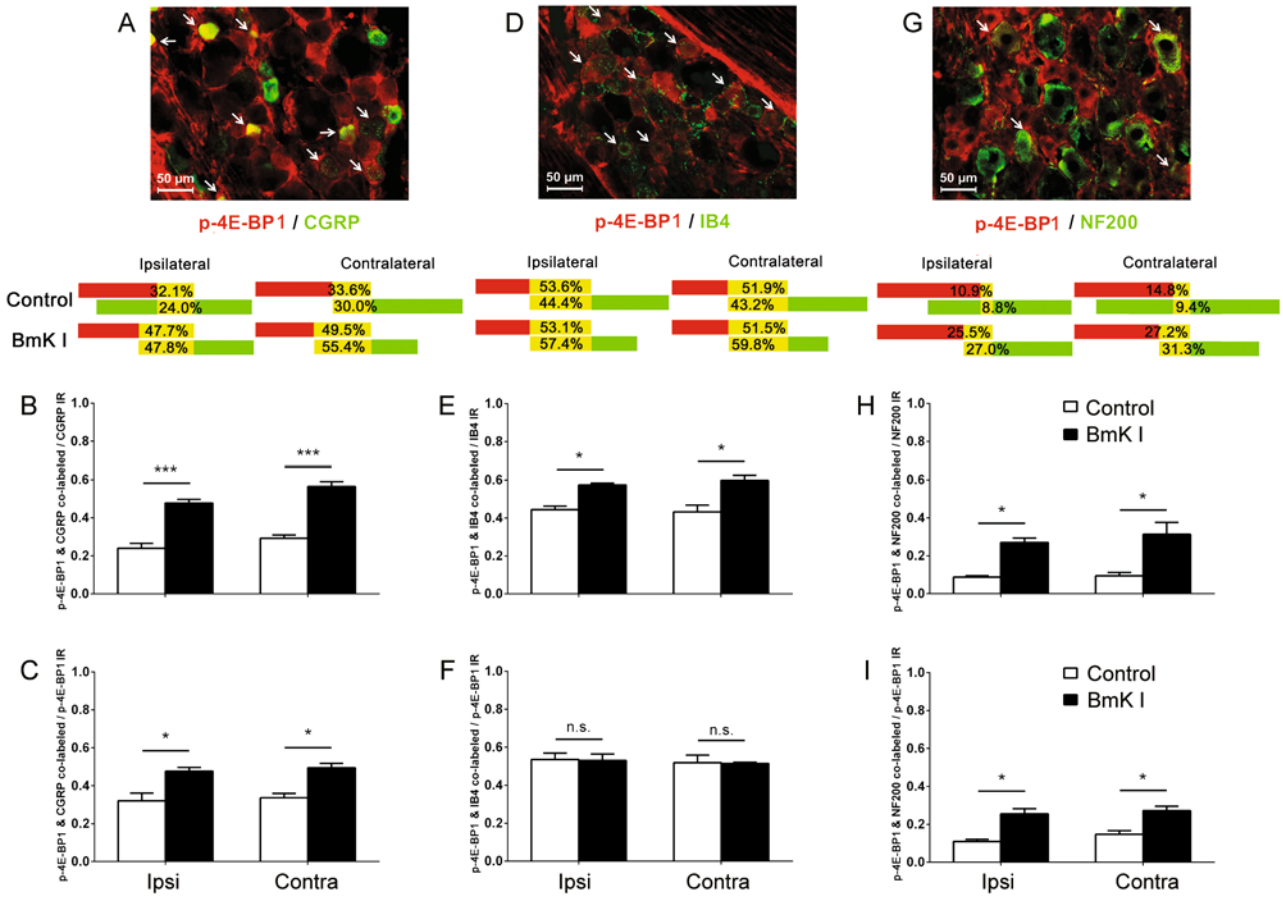

Fig. 5. Cellular localization of p-4E-BP1 immunoreactivity in rat DRG after i.pl. injection of BmK I and quantification of DRG neurons double-stained with p-4E-BP1 and neuronal markers. Arrows indicate co-localization (yellow) of p-4E-BP1 (red) with the respective cell marker (green). ****P* **<0.05, ******P* **<0.001.

capsaicin-induced primary sensitization and spared nerve injury-induced persistent pain, the mTOR cascade is mainly activated in primary afferent A fibers, but scarcely in unmyelinated C fibers[16, 18]. However, in the formalin test, peripheral C fiber-mediated nociceptive transmission onto wide dynamic range neurons is significantly inhibited by rapamycin, whereas the activity of A fibers is not affected[40]. In the present study, with specific neuronal markers, we demonstrated more clearly the up-regulation of mTOR cascade expression in specific subtypes of DRG neurons in a scorpion envenomation-induced pain state. We found that an activated mTOR cascade was present in all three kinds of DRG neurons, especially in CGRP-labeled peptidergic and IB4-labeled non-peptidergic neurons (Figs. 4–6).

The wide difference among these studies is probably because different animal models show diverse symptoms, due to specific mechanisms. For example, capsaicin-induced primary sensitization might be due to the activation of TRPV1 channels located in the skin. BmK I-induced pain-related behaviors are due to both the specific modulation of VGSCs and peripheral inflammation[26].

Moreover, it is well known that different subtypes of DRG neurons have different functions. Our previous study showed that, after BmK I administration, the excitability of rapidly-adapting and type I slowly-adapting low-threshold mechanical A fibers was increased, recorded as an increased frequency and extent of discharge[28]. These findings suggest that the low-threshold mechanical A fibers might contribute to the scorpion sting-induced mechanical hypersensitivity. In the present study, the mTOR cascade

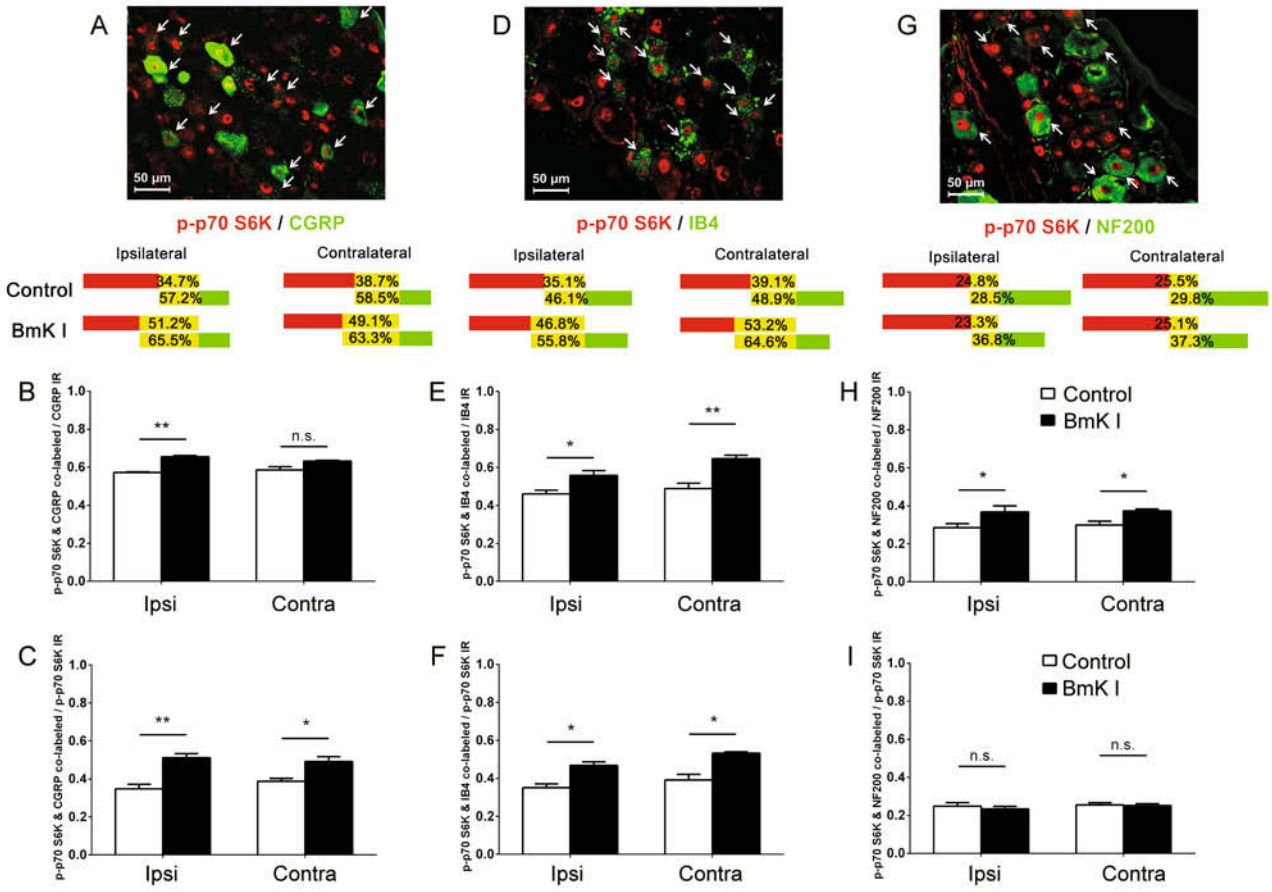

Fig. 6. Cellular localization of p-p70 S6K immunoreactivity in rat DRG after i.pl. injection of BmK I and quantification of DRG neurons double-stained with p-p70 S6K and neuronal markers. Arrows indicate co-localization of p-p70 S6K (red) with the respective cell marker (green). ****P* **<0.05, *****P* **<0.01.

was found to be activated in NF200-labeled large-diameter DRG neurons, which are connected to A fibers, suggesting that mTOR-dependent activity might play an important role in the mediation of pain hypersensitivity.

Suppression of mTOR Cascade Attenuates Behavioral Nociception

Behavioral test results revealed that BmK I-induced pain behaviors were attenuated by pre-administration of deforolimus (i.t., 30 min prior). Several lines of evidence also suggest the involvement of the mTOR cascade in the regulation of neuronal hyperexcitability. Peripheral administration of rapamycin suppresses the pain hypersensitivity induced by capsaicin and formalin, as well as neuropathic pain evoked by spinal nerve ligation[16, 18, 41]. The C-fiber-mediated transmission onto wide dynamic range neurons is inhibited by i.t. administration of rapamycin in the formalin test[40]. Moreover, the upstream molecules PI3K and Akt are strongly increased in DRG neurons after peripheral injury[42, 43]. Inhibition of this signaling prevented pain behaviors, implying that the PI3K–Akt–mTOR cascade is essential for pain behaviors.

In summary, this study provided strong evidence further supporting the role of the mTOR signaling pathway in scorpion sting-induced inflammatory pain, including spontaneous pain response behaviors and mechanical hypersensitivity, indicating that the mTOR cascade in all three types of DRG neurons plays crucial roles in the transmission of peripheral nociceptive information.

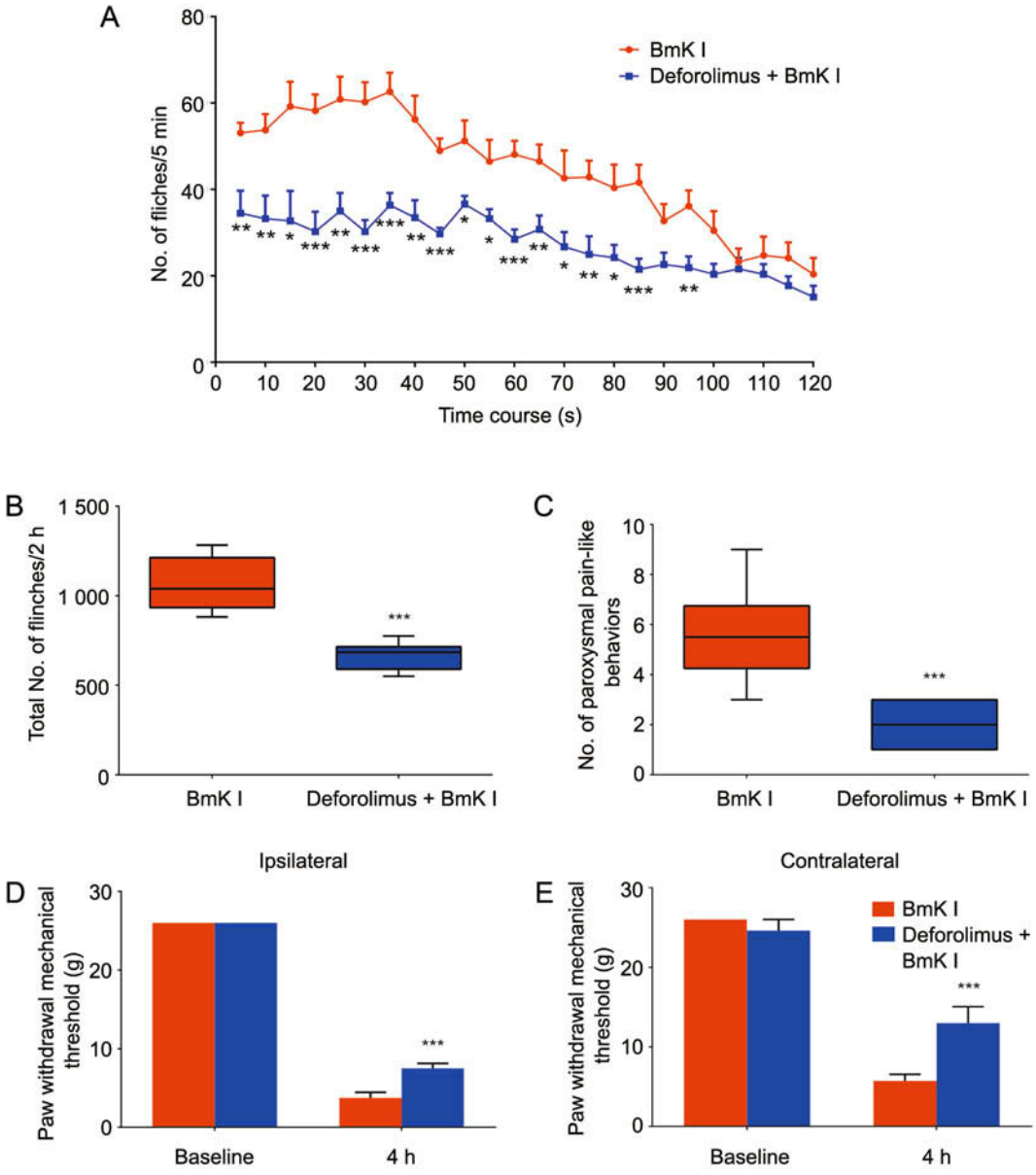

Fig. 7. Suppressive effect of deforolimus on BmK I-induced pain behaviors. A: Flinch behavior was attenuated when rats were pre-treated with deforolimus (250 μmol/L, 10 μL) 30 min prior to BmK I administration. B and C: Suppression of the total number of paw flinches (B) and the total number of paroxysmal pain-like behaviors (C) by deforolimus during 2 h after BmK I injection. D and E: Ipsilateral (D) and contralateral (E) mechanical hypersensitivity was suppressed by deforolimus. *P <0.05, **P <0.01, ***P <0.001 compared with vehicle group. n = 6/group.

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