Mechanisms Underlying the Scratching Behavior Induced by the Activation of Proteinase-Activated Receptor-4 in Mice

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A role for proteinase-activated receptor-4 (PAR-4) was recently suggested in itch sensation. Here, we investigated the mechanisms underlying the pruriceptive actions of the selective PAR-4 agonist AYPGKF-NH_2 (AYP) in mice. Dorsal intradermal (i.d.) administration of AYP elicited intense scratching behavior in mice, which was prevented by the selective PAR-4 antagonist (pepducin P4pal-10). PAR-4 was found to be coexpressed in 32% of tryptase-positive skin mast cells, and AYP caused a 2-fold increase in mast cell degranulation. However, neither the treatment with cromolyn nor the deficiency of mast cells (WBB6F1-$Kit^{W/Wv}$ mice) was able to affect AYP-induced itch. PAR-4 was also found on gastrin-releasing peptide (GRP)-positive neurons (pruriceptive fibers), and AYP-induced itch was reduced by the selective GRP receptor antagonist RC-3095. In addition, AYP evoked calcium influx in ~1.5% of cultured DRG neurons also sensitive to TRPV1 (capsaicin) and/or TRPA1 (AITC) agonists. Importantly, AYP-induced itch was reduced by treatment with either the selective TRPV1 (SB366791), TRPA1 (HC-030031), or NK1 (FK888) receptor antagonists. However, genetic loss of TRPV1, but not of TRPA1, diminished AYP-induced calcium influx in DRG neurons and the scratching behavior in mice. These findings provide evidence that PAR-4 activation by AYP causes pruriceptive itch in mice *via* a TRPV1/TRPA1-dependent mechanism.

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INTRODUCTION

Itch is a common symptom of dermatological and systemic diseases such as atopic dermatitis and cholestasis (Ikoma *et al.*, 2006). Chronic itching remains without satisfactory treatment and lowers patients' quality of life (Weisshaar *et al.*, 2006). Studies using pruritogenic spicules obtained from cowhage (*Mucuna pruriens*) have revealed the existence of pruriceptive afferents distinct from the well-known histaminergic pathway (Davidson *et al.*, 2007; Johanek *et al.*, 2007; Namer *et al.*, 2008). The active component of cowhage is mucunain, a cysteine protease that acts as an activator of protease-activated receptors (PARs) (Shelley and Arthur, 1955; Reddy *et al.*, 2008). PARs are a subfamily of G-protein-coupled receptors, named PAR-1 to 4, that are activated by the proteolytic cleavage of their extracellular domain (Vergnolle, 2009).

With the discovery of PAR-2 involvement in itch, great progress has been made in terms of understanding the pathophysiological basis of itching (Steinhoff *et al.*, 2003). PAR-2 (Costa *et al.*, 2008, 2010) and, more recently, PAR-4 (Kempkes *et al.*, 2014) were suggested to mediate itch. PAR-4 is expressed on rodent sensory neurons (Asfaha *et al.*, 2007; Auge *et al.*, 2009) and can be activated by several endogenous proteinases and synthetic hexapeptides (Fu *et al.*, 2014). Interestingly, the itch-causing agent mucunain cleaves PAR-4 more potently than PAR-2 (Reddy *et al.*, 2008). Furthermore, it was shown that cathepsin S, an endogenous cysteine protease that shares sequence homology with the mucunain active site, evokes itch in humans via activation of both PAR-2 and PAR-4 (Reddy *et al.*, 2010). Indeed,

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Abbreviations: AYP, AYPGKF-NH_2; DRG, dorsal root ganglion; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; PAR-4, proteinase-activated receptor-4; SP, Substance P; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin-1; TRPV1, transient receptor potential vanilloid-1; BBB, blood–brain barrier

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intradermal (i.d.) injection of PAR-4 agonists caused scratching behavior in mice (Tsujii *et al.*, 2008; Akiyama *et al.*, 2009, 2010).

Although evidences suggest a role for PAR-4 in itch, the signaling mechanisms involved in this process are poorly understood. Here we investigated the cellular and molecular mechanisms associated with the scratching behavior induced by the PAR-4-activating peptide AYPGKF-NH_2 (AYP) in mice, and provided data supporting the role of PAR-4 in itch. We show that AYP elicits scratching behavior in mice by activating transient receptor potential (TRP) channels and possibly causing the release of itch-mediating neurotransmitters. These findings highlight the potential of PAR-4 as a target for the development of antipruritic drugs.

RESULTS

PAR-4 activation induces scratching behavior in mice

I.d. administration of the selective PAR-4 agonist AYP, but not the inactive peptide YAPGKF-NH_2 (YAP), elicited scratching behavior when injected into the back of the mouse neck (Figure 1a) with an effective dose ranging from 100 to 500 nmol per site and an estimated mean ED_{50} value (accompanied by 95% confidence limit) of 156 (42–572) nmol per site. The dose of 200 nmol per site was chosen for all the subsequent experiments. AYP-induced scratching behavior was time-dependent, peaking within 10 minutes and decreasing slowly over time, without a significant response at 30 minutes (Figure 1b). Interestingly, the number and the time-course profiles of AYP-evoked scratching bouts were similar to those caused by histamine (Figures 1a and b), a widely known pruritogenic agent. As expected, pretreatment with the selective PAR-4 antagonist pepducin P4pal-10 significantly reduced AYP-induced scratching behavior (Figure 1c). In addition, pretreatment with the nonselective opioid receptor antagonist naloxone, used as an antipruritic control drug, also significantly inhibited AYP-induced response (Figure 1d).

AYP-induced scratching behavior is dependent on GRP-expressing fibers

We found PAR-4 to be expressed in ~32% of all mouse skin mast cells and AYP (200 nmol/site) i.d. injection was able to cause mast cell degranulation; however, AYP-induced itch was not dependent on mast cell product release (Supplementary results and Supplementary Figure S1 online). In addition, we detected PAR-4 on skin sensory neurons as PAR-4 immunoreactivity was colocalized with the neuronal marker PGP 9.5 (Figures 2a and b). PAR-4 was also found in the soma of 47% of mouse dorsal root ganglion (DRG) neurons (247/525). Of those, 39% (95/247), 34% (85/247), and 27% (67/247) were small-, medium-, and large-diameter neurons, respectively. To investigate the phenotype of PAR-4-

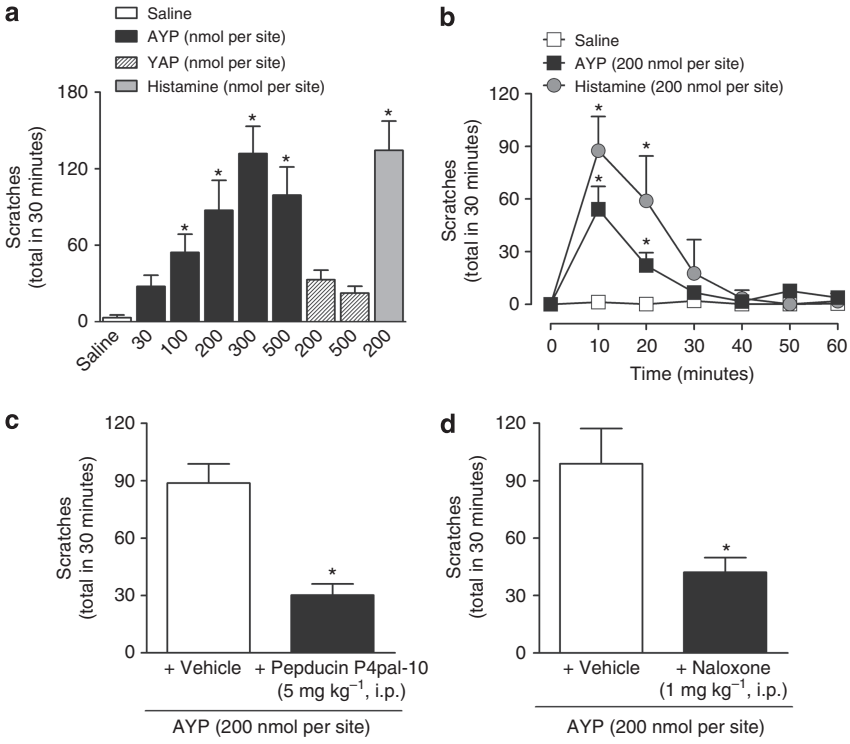

Figure 1. Intradermal injection of AYPGKF-NH_2 (AYP) causes itch-like behavior. (**a**) Scratching behavior elicited by AYP (30–500 nmol per site), histamine (200 nmol per site), or control peptide YAP (200–500 nmol per site). (**b**) Time-course profile of scratching behavior induced by AYP (200 nmol per site) or histamine (200 nmol per site). Effect of (**c**) the proteinase-activated receptor-4 (PAR-4) antagonist pepducin P4pal-10 (5 mg kg^{-1}, i.p., 60 min) and (**d**) the opioid receptor antagonist naloxone (1 mg kg^{-1}, i.p., 30 min) on AYP (200 nmol per site)-induced scratching. Each column represents the mean of 6–8 animals, and the vertical bars represent the SEM. Significant differences (*$P<0.05$) were indicated, as compared with the (**a**, **b**) saline- or (**c**, **d**) vehicle-treated group. (**a**) One-way analysis of variance (ANOVA) followed by Bonferroni's test. (**b**) Two-way ANOVA followed by Bonferroni's test. (**c**, **d**) Student's *t*-test.