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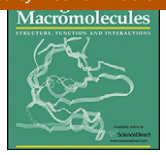

Antinociception of β-D-glucan from *Pleurotus pulmonarius* is possibly related to protein kinase C inhibition

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ABSTRACT

β-D-Glucan, a polysaccharide isolated from an edible mushroom *Pleurotus pulmonarius* (Fr.) Quel., presented antinociceptive activity in mice. This study evaluated the involvement of transient receptor potential (TRP) channels and protein kinase C (PKC) on antinociceptive effect of a (1→3),(1→6)-linked β-D-glucan (GL) in mice. Intraperitoneal administration of GL potently inhibited nociceptive responses induced by intraplantar injections of capsaicin, cinnamaldehyde, menthol, acidified saline and phorbol myristate acetate (PMA). Moreover, Western blot analysis revealed that GL treatment also prevented PMA-induced PKCε activation. Collectively, present results demonstrate that GL could constitute an attractive molecule of interest for the development of new analgesic drugs.

1. Introduction

Phytochemical studies carried out with *Pleurotus pulmonarius* (Fr.) Quel., an edible mushroom used in Chinese cuisine, revealed it contains several polysaccharides, alongside a high amount of proteins, essential amino acids and vitamins, but no lipids or starch [1,2]. Among these polysaccharides are xyloglucan, mannogalactan and β-D-glucan [3–5]. Interestingly, a variety of biological effects have been ascribed to β-D-glucans, namely antitumoral, antioxidant, anti-inflammatory and immunomodulatory properties [6–9]. Recently, our group reported that (1→3),(1→6)-linked β-D-glucan (GL) isolated from *P. pulmonarius* attenuated nociceptive responses, as well as inhibited leukocyte migration and plasma extravasation induced by acetic acid in mice [4]. It was also found to inhibit both phases of formalin-induced licking in mice. Besides, it was demonstrated that GL presented antinociceptive effects in acute and neuropathic pain with participation of ionotropic glutamate receptors and pro-inflammatory cytokine (IL-1β) [10].

Pain is a physiologically relevant sensation necessary to detect and/or prevent injury, but it often outlives its usefulness as a warning system to become chronic and debilitating [11,12]. Pain signaling to the central nervous system is initiated when noxious stimuli excite primary afferent nociceptive C and Aδ fibers, frequently by activation of several types of ionotropic channels and metabotropic receptors [13,14]. In fact, transient receptor potential (TRP) and acid-sensing ion channels (ASIC) participate in generating nociceptive signals in response to various specific noxious stimuli [14–16]. Activity of some of these channels, as well as of other proteins implicated in nociceptive signaling pathways can be upregulated by protein kinase C (PKC) [17–19]. Intraplantar injection of phorbol 12-myristate 13-acetate (PMA), a PKC activator, induces nociceptive responses which are associated with translocation of PKC isoforms from cytoplasmic to membrane of primary afferent nerves, thus indicating that their activation is implicated in peripheral nociception [12,18].

In the present study, we sought to evaluate the effect of GL on the overt nociception triggered by intraplantar protons (activator of ASIC/TRPV1 channels), capsaicin (agonist of TRPV1 channel), cinnamaldehyde (agonist of TRPA1 channel), menthol (agonist of TRPM8 channel) and PMA (a direct activator of PKC) injection into the hind paw. Finally, we also evaluated the participation of the

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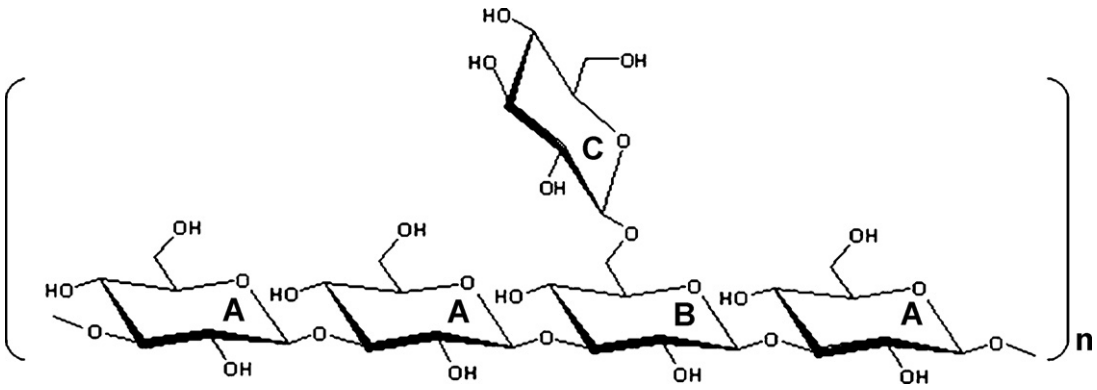

Fig. 1. Chemical structure of the (1→3),(1→6)-linked β-D-glucan isolated from *Pleurotus pulmonarius*.

PKC pathway on the antinociceptive effect of GL on PMA test using Western blot analysis.

2. Materials and methods

2.1. Animals

Experiments were conducted using Swiss mice (25–35 g) of both sexes, housed at 22 ± 2 °C under a 12/12 h light/dark cycle (lights on at 06:00) and with free access to food and water. Male and female mice were distributed equally among groups and acclimatized to the laboratory conditions for at least 1 h before testing. Each animal was used only once. All experiments were performed after approval of the respective protocols by the Institutional Ethics Committee and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [20]. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

2.2. Isolation and characterization of the purified β-D-glucan

The (1→3),(1→6)-linked β-D-glucan (GL) was isolated from the mushroom *P. pulmonarius* (Fr.) Quel. by Smiderle et al. [4]. The polysaccharide fraction was obtained from milled mushroom via hot aqueous extraction, and was submitted to a freeze–thawing process [21]. Partial precipitation took place and centrifugation gave rise to an insoluble fraction, which was treated with aqueous sodium hydroxide at 80 °C and resubmitted to the freeze–thawing process. The soluble fraction then obtained was composed of glucose, and showed a degree of purity greater than 98%. Methylation analysis, controlled Smith degradation, and mono- and bidimensional nuclear magnetic resonance (NMR) spectroscopy confirmed the linkage type and main chain configuration elucidated for this β-D-glucan, which is depicted in Fig. 1. This polysaccharide was estimated to be a high molecular weight (between 1×10^5 and 1×10^6 g/mol) based on previously data published for other *Pleurotus*' glucans [8,22].

2.3. Nociception induced by capsaicin, cinnamaldehyde, menthol and acidified saline

To test if TRPV1, TRPA1, TRPM8 and ASIC receptors constitute potential specific targets for the antinociceptive actions of GL, we tested the effects of the later against nociceptive responses elicited by activators of each channel. To this effect, following systemic treatment with GL or other drugs (see details below), mice received a single 20 μl intraplantar (i.pl.) injection of either capsaicin (5.2 nmol/paw), cinnamaldehyde (10 nmol/paw), menthol (1.2 μmol/paw), acidified saline (2% acetic acid in 0.9% saline, pH 1.98/paw) or the corresponding vehicle, delivered into the ventral surface of the right hind paw. Each animal was then placed, immediately and alone, into a glass cylinder of 20 cm diameter positioned on a platform in front of a mirror to enable full view of hind paws. The amount of time spent licking the injected paw, used as an index of nociceptive behavior intensity, was recorded with a chronometer for 5 min (for capsaicin or cinnamaldehyde) or 20 min (menthol and acid saline). Thirty minutes prior to i.pl. injection of the nociceptive agent, the mice were treated with GL (0.1–100 mg/kg i.p.), ruthenium red (nonselective TRP antagonist, RR, 3 mg/kg i.p.), camphor (TRPA1 antagonist, CAM, 7.6 mg/kg s.c.) or amiloride (the epithelial Na^+ channel blocker, AML, 100 mg/kg i.p.). In another set of experiments, GL (10 and 30 μg/paw) or GF109203X (1 nmol/paw), a PKC inhibitor, was co-administered to the hindpaw together with capsaicin (5.2 nmol/paw), cinnamaldehyde (10 nmol/paw), menthol (1.2 μmol/paw) or acidified saline (2% acetic acid in 0.9% saline, pH 1.98/paw) and the behavioral responses were recorded as described above.

2.4. Nociception induced by PMA

To assess the influence of GL (10, 30 and 100 mg/kg i.p.) on PKC-dependent nociception, nociceptive responses were induced by i.pl. injection of PMA (500 pmol/paw), an activator of PKC. In such experiments, only the licking time accumulated 15–45 min following injection was recorded [23]. Control animals received vehicle (saline, 10 ml/kg i.p.). Procedures, doses and administration routes of the various drugs were chosen on the basis of previous studies [18,23–25] or preliminary experiments.

2.5. Western blot studies

In order to evaluate the effect of GL on PMA-induced PKC activation in hind paw skin, Western blot analysis for PKCε was carried out essentially as described by Ferreira et al. [18]. The mice received GL (100 mg/kg i.p.) or vehicle (saline, 10 ml/kg i.p.) 30 min before i.pl. injection of PMA (500 pmol/paw) or vehicle (phosphate-buffered saline, PBS) into the right hind paw. Fifteen minutes later, the animals were killed with cervical dislocation and the skin and connective tissues of the plantar portion of the injected paw was carefully removed and disrupted using a glass Potter homogenizer in ice-cold buffer containing protease and phosphatase inhibitors (100 mM Tris–HCl, 2 mM EDTA, 2 μg aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 200 mM NaF and 2 mM sodium orthovanadate, pH 7.4). The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The resulting supernatant was further

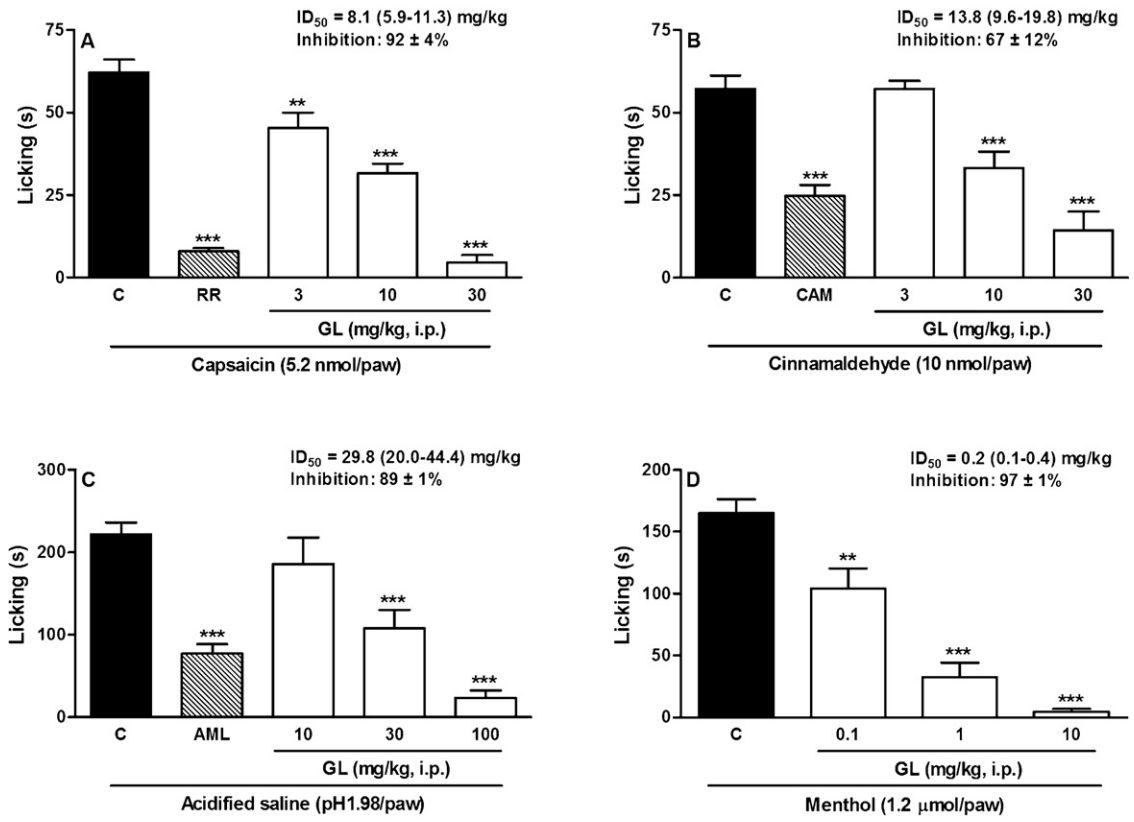

Fig. 2. Effect of GL (3–100 mg/kg i.p.) on hind paw licking induced by i.pl. injection of capsaicin (panel A), cinnamaldehyde (panel B), acidified saline (panel C) or menthol (panel D) in mice. Mice were treated with GL (i.p. at the doses indicated), ruthenium red (RR, 3 mg/kg i.p.), camphor (CAM, 7.6 mg/kg s.c.), amiloride (AML, 100 mg/kg i.p.) or saline (C, 10 ml/kg) 30 min prior to the i.pl. injection. Each column represents the mean ± SEM (n = 6–8). Significance levels when compared to the control group are indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$ (ANOVA followed by Newman–Keuls' test).

centrifuged at 35,000 × g for 30 min at 4 °C. The new supernatant was collected as a cytoplasm-rich fraction, whereas the pellet was resuspended and considered as a membrane-rich fraction. After determining protein content in each sample, using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA), equivalent amounts of protein (50 μg) were mixed in buffer (200 mM Tris, 2.75 mM b-mercaptoethanol, 10% glycerol, 2% SDS, 0.04% bromophenol blue) and boiled for 5 min. Proteins were resolved in 10% SDS polyacrilamide gel by electrophoresis (SDS–PAGE), transferred onto polyvinylidene difluoride membranes according to manufacturer's instructions (Millipore/Amersham Biosciences, Buckinghamshire, UK) and saturated by incubation overnight with 10% nonfat dry milk solution, 60 min incubation with 1.5% gelatin and then incubation with anti-PKCε antibody overnight (diluted 1:1 000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, the membranes were incubated with adjusted peroxidase-coupled secondary antibody (diluted 1:6000, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h. Immunocomplexes were visualized using the ECL chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The optical densities of specific bands were measured with an imaging analysis system (Scion Image for Windows, Frederick, MD, USA) and expressed in arbitrary units.

2.6. Drugs and reagents

Amiloride, camphor, capsaicin, cinnamaldehyde, GF109203X, menthol, phorbol 12-myristate 13-acetate and PBS tablets were from Sigma–Aldrich (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Drugs were dissolved in 0.9% NaCl solution, with the exception of capsaicin, which was dissolved in 10% ethanol in saline, and menthol, which was dissolved in 1.6% ethanol/0.01% Tween 80 in saline. In both of these conditions, the final solutions containing ethanol failed to cause any nociceptive effects *per se* when administered alone.

2.7. Statistical analysis

The results are presented as means ± standard error of the mean (SEM), except the ID_{50} values for GL (i.e., the dose that reduced the nociceptive response by 50% relative to control values), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} values were determined by nonlinear regression from individual experiments using linear regression Graph-Pad software (GraphPad software, San Diego, CA, USA). The percentages of inhibition were calculated for the most effective dose used. Statistical differences between groups were analyzed by one-way ANOVA followed by Newman–Keuls' test. Comparisons between the protein optical densities determined in the Western blot studies were made using two-tailed unpaired Student's t-test. In all cases, differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Effects of GL on nociception induced by capsaicin, cinnamaldehyde, menthol and acidified saline

The i.p. administration of GL (3–30 mg/kg) produced dose-related inhibition of the licking induced by capsaicin (Fig. 2A). GL

Table 1
Effect of GF109203X, a PKC inhibitor, on nociceptive responses of mice to i.pl. injections of capsaicin, cinnamaldehyde, menthol or acidified acid in the hind paw.

Chemical noxious stimulus	% Inhibition
Capsaicin (5.2 nmol/paw)	42 ± 8
Cinnamaldehyde (10 nmol/paw)	n.i.
Menthol (1.2 μmol/paw)	85 ± 6
Acidified saline (pH 1.98/paw)	n.i.

Values represent the mean ± SEM of groups of 6–8 mice. GF109203X (1 nmol/paw) was co-injected with the algogen. n.i.: not inhibited.

displayed a mean ID_{50} of 8.1 mg/kg (95% confidence limits 5.9–11.3) at the 30-min time point and, at 30 mg/kg, inhibited capsaicin-induced responses by 92 ± 4%, an extent similar to that caused by ruthenium red (3 mg/kg i.p.; 87 ± 2%), used as a positive control. Likewise, GL was also effective in reducing nociceptive responses to cinnamaldehyde, acidified saline and menthol, yielding ID_{50}s of 13.8 (9.6–19.8), 29.8 (20.0–44.4) and 0.2 (0.1–0.4) mg/kg, respectively (Fig. 2B, C and D, respectively). Moreover, at 30 mg/kg, GL was as effective as camphor (7.6 mg/kg s.c.) in inhibiting nociception induced by cinnamaldehyde (67 ± 12% vs 57 ± 6% inhibition, respectively; Fig. 2B), and more effective than amiloride (100 mg/kg i.p.) in reducing that caused by acidified saline (89 ± 1% vs 65 ± 5% inhibition, respectively; Fig. 2C). Remarkably, a smaller dose of GL (10 mg/kg i.p.) abrogated menthol-induced hind paw licking entirely (97 ± 1% inhibition; Fig. 2D).

Intraplantar co-administration of GL (10 and 30 μg/paw) did not alter the nociceptive responses induced by capsaicin, cinnamaldehyde, menthol and acidified saline (data not shown). Whereas the GF109203X, an inhibitor of PKC, together with capsaicin or menthol inhibited the nociceptive responses to these algogens by 42 ± 8% and 85 ± 6%, respectively (Table 1). However, co-injection of GF109203X failed to affect nociceptive responses to either cinnamaldehyde or acidified saline (Table 1).

3.2. Effects of GL on PMA-induced nociception and activation of protein kinase C

The i.p. administration of GL (10–100 mg/kg) produced dose-related inhibition of hind paw licking induced by PMA (500 pmol/paw), yielding a mean ID_{50} value of 17.5 (11.0–28.0) mg/kg and full (100%) suppression at the dose of 100 mg/kg (Fig. 3A). Western blot analysis of hind paw skin samples confirmed that i.pl. PMA (500 pmol/site) injection activated PKCε, i.e. induced translocation of PKCε from the cytosolic- to membrane-rich fraction (Fig. 3B and C). Systemic treatment with GL (100 mg/kg i.p.), 30 min prior to PMA injection, abrogated the translocation of PKCε induced by the direct activator of PKC.

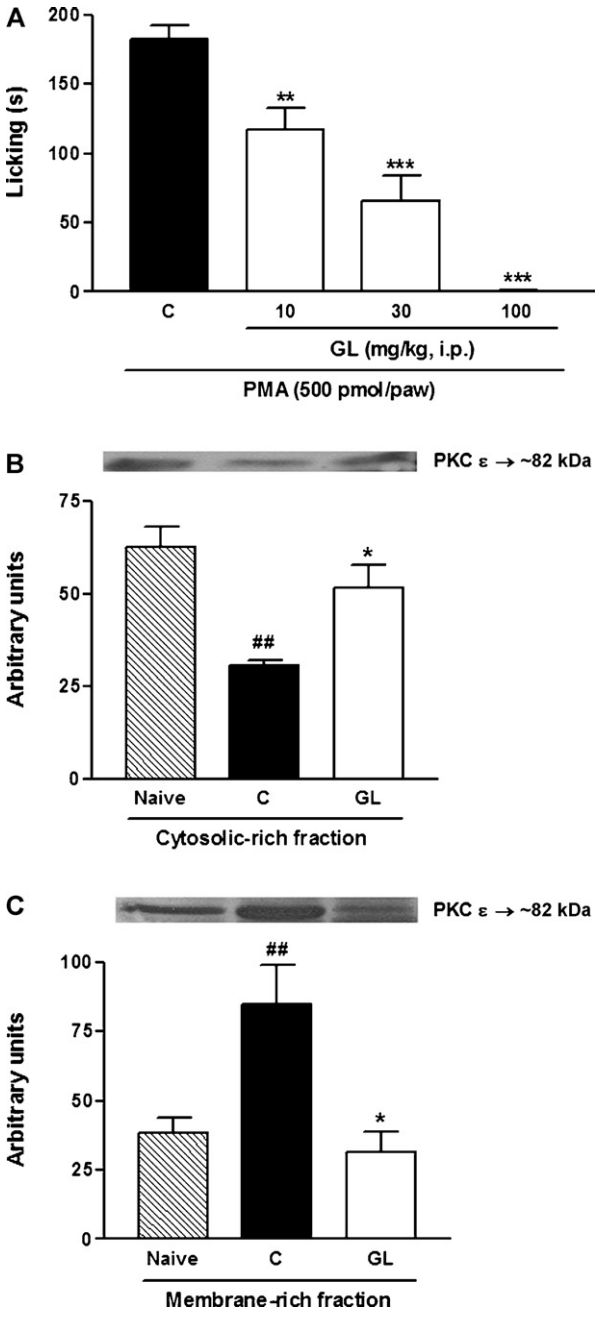

Fig. 3. Effect of GL on PMA-induced hind paw licking in mice (panel A) and *ex vivo* PKCε translocation from cytosolic- to membrane-enriched fractions of hind paw homogenates (panels B and C). GL (10–100 mg/kg) or saline (C, 10 ml/kg) were administered i.p. 30 min prior to i.pl. PMA injection. Nociceptive responses were recorded 15–45 min after i.pl. treatment. Skin samples were collected at 45 min after i.pl. injection, homogenized, processed to yield both fractions and analyzed by Western blot as described in Section 2. Each column represents the mean ± SEM (n = 6–8). Significance levels are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared to the control group and by $^{\#\#}p < 0.01$ when compared to the naive group (ANOVA followed by Newman–Keuls' test in panel A; Student's t-test in panels B and C).

4. Discussion

The results of the current study demonstrate that i.p. administration of GL, a β-D-glucan isolated from the mushroom *P. pulmonarius*, exerts potent antinociceptive effects against nociception induced in the hind paw by capsaicin, cinnamaldehyde, menthol, acidified saline and PMA. Of interest, results obtained *ex vivo* identified PKCε as a potentially relevant molecular target of GL action to suppress nociception in mice.

In a previous study, we observed that GL is endowed with potent antinociceptive activity against abdominal constrictions induced by acetic acid, and promotes potential anti-inflammatory effects including inhibition of plasma extravasation and leukocyte migration into the peritoneal cavity of mice [4]. GL was also shown to inhibit both first and second phases of formalin-induced nociceptive responses in mice. In addition, we have shown that GL inhibits the nociceptive responses triggered by IL-1β and by glutamate, mainly by inhibiting the activation of ionotropic glutamate receptor pathways [10]. Moreover, GL demonstrated an interesting antinociception against neuropathic pain induced by partial ligature of sciatic nerve, however, the mechanisms underlying

these antinociceptive effects remained largely unidentified. A better understanding of the mode of action of GL would enhance the potential therapeutic interest on this naturally occurring β-D-glucan.

Several ion channels play an essential role in the ability of nociceptors (C and Aδ fibers) to detect and discriminate noxious thermal, mechanical and chemical stimuli and convey this sensory information to second order nociceptive neurons of the dorsal horn of spinal cord [16]. In the present study, we observed that GL effectively inhibited nociceptive responses induced by i.pl. injections of capsaicin or cinnamaldehyde, which are highly selective activators/agonists of TRPV1 and TRPA1 channels, respectively, as well as menthol (a preferential TRPM8 channel agonist that can also activate TRPA1 channels at higher concentrations) or acidified saline (which activates TRPV1 and ASIC channels). Importantly, we confirmed that the nociceptive responses elicited by capsaicin, cinnamaldehyde and acidified saline were extensively inhibited by prior treatment with antagonists of their respective specific or preferential targets, i.e. ruthenium red (TRPV1), camphor (TRPA1) and amiloride (ASIC). Due to the lack of a suitable selective antagonist, we were unable to determine if responses to menthol resulted from activation of TRPM8 channels. In addition, the potency of GL in inhibiting cinnamaldehyde-induced nociception is in excellent agreement with that previously observed in the model of formalin-induced hind paw nociception [4], especially considering that formalin is an activator of TRPA1 channels [26]. These results strengthen considerably the evidence that GL is an effective analgesic in rodents.

However, it appears most unlikely that GL induces antinociception by selectively antagonizing the binding of capsaicin, cinnamaldehyde, menthol or protons to their respective target channels, which was supported by the no alteration of nociceptive responses when the GL was co-administered with these activators/agonists. It was demonstrated that capsaicin, stimulating TRPV1 receptor, mediate the release of several neurotransmitters, including glutamate; besides, menthol (TRPM8 agonist) and allyl isothiocyanate (TRPA1 agonist) enhance spontaneous glutamatergic synaptic transmission onto lamina II neurones in spinal cord slices [27–29]. Indeed, we previously demonstrated that GL inhibited the nociceptive responses induced by injection of intrathecal excitatory amino acids [10], suggesting that the GL effect seems to be partially related to inhibition of glutamate release in the dorsal horn after peripheral stimulation of TRPs.

We also observed that GL effectively inhibited nociceptive responses to i.pl. injection of PMA, a direct activator of PKC [18]. Given the extensive evidence implicating PKC in signaling mechanisms leading to nociception and hyperalgesia [30], we hypothesized that this could constitute a potentially relevant target for the antinociceptive action of GL. In this regard, using Western blot analysis, we obtained *ex vivo* evidence that i.pl. PMA injection clearly promoted the translocation of PKCε from the cytosolic- to the membrane-enriched fractions of hind paw skin homogenates, as would be expected of a PKC activator [18]. More importantly, prior i.p. treatment of the animal with GL, at a dose promoting effective analgesia, fully prevented this PMA-induced PKCε translocation. In addition, we found that GF109203X, a selective PKC inhibitor [18], inhibited nociceptive responses induced by i.pl. injection of capsaicin and menthol. However, unlike GL, GF109203X did not influence responses induced by either cinnamaldehyde or acidified saline. As the phosphorylation of TRPV1 by PKC sensitizes this channel to activation by capsaicin [31], blockade of this process by GL might account for the analgesic effect of GL (and GF109203X) against capsaicin-induced nociception. Nonetheless, this does not appear to be a plausible explanation for its inhibitory effects on nociception induced by menthol, cinnamaldehyde or protons (i.e. acidified saline), as PKC actually dephosphorylates and downregulates TRPM8 channels [19], does not contribute to the sensitization of TRPA1 channels [32] and appears to phosphorylate and sensitize only ASIC channels containing ASIC2 subunits [17,33], which are poorly expressed by DRG neurons [34].

It is interesting to note, however, that the antinociceptive profile of action of GF109203X (i.e. more effective against responses induced by menthol than capsaicin and inactive against cinnamaldehyde or acidified saline) was remarkably similar to that of GL, which displayed an ID_{50} against nociception triggered by menthol that was 42-, 72- and 156-fold lower than those obtained against responses induced by capsaicin, cinnamaldehyde and acidified saline, respectively. Therefore, the collected evidence strongly suggests that the antinociceptive effects of GL are associated, at least to a significant extent, to inhibition of PKC activation.

In conclusion, the current study demonstrates that GL, a naturally occurring β-D-glucan, displays pronounced systemic antinociceptive properties in chemical models of nociception models in mice. The mechanisms underlying GL-induced antinociception appear to involve inhibition of PKCε. It remains to be determined if GL also has other potentially relevant targets for its antinociceptive actions and if it is a selective inhibitor of PKCε or can also block activity of other PKC isoforms.

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References

[1] C.G. Wasonga, S.A. Okoth, J.C. Mukuria, C.O. Omwandho, J. Exp. Ther. Oncol. 7 (2008) 147–152.
[2] R. Yatsuzuka, Y. Nakano, S. Jiang, Y. Ueda, Y. Kishi, Y. Suzuki, E. Yokota, A. Rahman, R. Ono, I. Kohno, C. Kamei, Biol. Pharm. Bull. 30 (2007) 1557–1560.
[3] M.F. Moradali, H. Mostafavi, S. Ghods, G.A. Hedjaroude, Int. Immunopharmacol. 7 (2007) 701–724.
[4] F.R. Smiderle, L.M. Olsen, E.R. Carbonero, C.H. Baggio, C.S. Freitas, R. Marcon, A.R.S. Santos, P.A. Gorin, M. Iacomini, Eur. J. Pharmacol. 597 (2008) 86–91.
[5] F.R. Smiderle, L.M. Olsen, E.R. Carbonero, R. Marcon, C.H. Baggio, C.S. Freitas, A.R.S. Santos, G. Torri, P.A. Gorin, M. Iacomini, Phytochemistry 69 (2008) 2731–2736.
[6] T. Mizuno, T. Hagiwara, T. Nakamura, H. Ito, K. Shimura, T. Sumiya, A. Asakura, Agric. Biol. Chem. 54 (1990) 2889–2896.
[7] H.Z. Toklu, G. Sener, N. Jahovic, B. Uslu, S. Arbak, B.C. Yegen, Int. Immunopharmacol. 6 (2006) 156–169.
[8] M. Zhang, S.W. Cui, P.C.K. Cheung, Q. Wang, Trends Food. Sci. Technol. 18 (2007) 4–19.
[9] C.M. Guerra Dore, T.C. Azevedo, M.C. de Souza, L.A. Rego, J.C. de Dantas, F.R. Silva, H.A. Rocha, I.G. Baseia, E.L. Leite, Int. Immunopharmacol. 7 (2007) 1160–1169.
[10] C.H. Baggio, C.S. Freitas, D.F. Martins, L. Mazzardo, F.R. Smiderle, G.L. Sassaki, M. Iacomini, M.C. Marques, A.R.S. Santos, J. Pain 11 (2010) 965–971.
[11] D. Julius, A.I. Basbaum, Nature 413 (2001) 203–210.
[12] K.T. Velazquez, H. Mohammad, S.M. Sweitzer, Pharmacol. Res. 55 (2007) 578–589.
[13] J. Scholz, C.J. Woolf, Nat. Neurosci. 5 (Suppl.) (2002) 1062–1067.
[14] M. Tominaga, Handb. Exp. Pharmacol. (2007) 489–505.
[15] M.J. Caterina, D. Julius, Curr. Opin. Neurobiol. 9 (1999) 525–530.
[16] E.W. McCleskey, M.S. Gold, Annu. Rev. Physiol. 61 (1999) 835–856.
[17] A. Baron, E. Deval, M. Salinas, E. Lingueglia, N. Voilley, M. Lazdunski, J. Biol. Chem. 277 (2002) 50463–50468.
[18] J. Ferreira, K.M. Triches, R. Medeiros, J.B. Calixto, Pain 117 (2005) 171–181.
[19] L.S. Premkumar, M. Raisinghani, S.C. Pingle, C. Long, F. Pimentel, J. Neurosci. 25 (2005) 11322–11329.
[20] M. Zimmermann, Pain 16 (1983) 109–110.
[21] P.A.J. Gorin, M. Iacomini, Carbohydr. Res. 128 (1984) 119–132.
[22] J.C. Santos-Neves, M.I. Pereira, E.R. Carbonero, A.H. Gracher, P.A. Gorin, G.L. Sassaki, M. Iacomini, Carbohydr. Res. 343 (2008) 1456–1462.
[23] J.S. Siebel, A. Beirith, J.B. Calixto, Brain Res. 1003 (2004) 61–67.

[24] E.L. Andrade, A.P. Luiz, J. Ferreira, J.B. Calixto, Neuroscience 152 (2008) 511–520.
[25] R.O. de Campos, A.R.S. Santos, Z.R. Vaz, T.R. Pinheiro, M.G. Pizzolatti, V. Cechinel Filho, F. Delle Monache, R.A. Yunes, J.B. Calixto, Life Sci. 61 (1997) 1619–1630.
[26] C.R. McNamara, J. Mandel-Brehm, D.M. Bautista, J. Siemens, K.L. Deranian, M. Zhao, N.J. Hayward, J.A. Chong, D. Julius, M.M. Moran, C.M. Fanger, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 13525–13530.
[27] M.L. Baccei, R. Bardoni, M. Fitzgerald, J. Physiol. 549 (2003) 231–242.
[28] M. Kosugi, T. Nakatsuka, T. Fujita, Y. Kuroda, E. Kumamoto, J. Neurosci. 27 (2007) 4443–4451.
[29] Y.V. Medvedeva, M.S. Kim, Y.M. Usachev, J. Neurosci. 28 (2008) 5295–5311.
[30] A.L. Souza, F.A. Moreira, K.R. Almeida, C.M. Bertollo, K.A. Costa, M.M. Coelho, Br. J. Pharmacol. 135 (2002) 239–247.
[31] G. Bhave, H.J. Hu, K.S. Glauner, W. Zhu, H. Wang, D.J. Brasier, G.S. Oxford, R.W.t. Gereau, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 12480–12485.
[32] S. Wang, Y. Dai, T. Fukuoka, H. Yamanaka, K. Kobayashi, K. Obata, X. Cui, M. Tominaga, K. Noguchi, Brain 131 (2008) 1241–1251.
[33] E. Deval, M. Salinas, A. Baron, E. Lingueglia, M. Lazdunski, J. Biol. Chem. 279 (2004) 19531–19539.
[34] S. Ugawa, T. Ueda, H. Yamamura, S. Shimada, Brain Res. Mol. Brain Res. 136 (2005) 125–133.