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Pharmacological and molecular characterization of the mechanisms involved in prostaglandin E_2 (PGE_2)-induced mouse paw edema

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Running Title: Mechanisms underlying PGE_2-induced edema

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Abbreviations: EP, prostaglandin E_2 receptors; ERK, extracellular-regulated kinase; i.pl., intraplantar; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; MEK, mitogen-activated protein kinase kinase; PBS, phosphate buffer saline; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; protein kinase, PK; PLC, phospholipase C; TRPV1, vanilloid receptor.

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Abstract

The present study evaluated some of the mechanisms underlying PGE_2-induced paw edema formation in mice. Intraplantar (i.pl.) injection of PGE_2 (0.10-10.0 nmol/paw) into the hind paw elicited a dose-related edema formation, with a mean ED_{50} value of 0.42 nmol/paw. The coinjection of selective EP_3 (L826266), but not EP_2 or EP_4 (all 10 nmol/paw), receptor antagonists significantly inhibited PGE_2-induced paw edema. Like L826266, the PGE_2-induced paw edema was markedly reduced by treatment with pertussis toxin and PLC inhibitor U73122. Likewise, the selective NK_1 receptor antagonist FK888 and the antagonist of TRPV1 receptors SB366791 (both 1 nmol/paw) also significantly inhibited PGE_2-mediated paw edema. Conversely, the selective NK_2, NK_3 and CGRP $CGRP_{8-37}$ receptor antagonists all failed to interfere with PGE_2-induced paw edema. The neonatal treatment of mice with capsaicin was also able to reduce PGE_2-induced paw edema. The inhibitors of protein kinase C (PKC) GF109203X and mitogen protein-activated kinases (MAPKs, 30 nmol/paw) JNK (SP600125), ERK (PD98059) and p38 (SB203580), but not PKA markedly decreased the PGE_2-mediated edema formation. The i.pl. injection of PGE_2 (3 nmol/paw) induced a significant activation of MAP-kinases, namely JNK and p38, an effect that was largely prevented by the selective EP_3 receptor antagonist L826266 (10 nmol/paw). Collectively, these findings indicate that edematogenic responses elicited by PGE_2 are mediated by EP_3 receptor activation, also involving the stimulation of PLC, PKC and MAPKs pathways and the participation of TRPV1 and NK_1 receptors. These results make a considerable contribution to our

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comprehension of the mechanisms involved in PGE_2-mediated inflammatory responses in mice.

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Introduction

Inflammation is a complex physiological process which can be defined as a response to cellular and tissue injures caused by infections or physical and chemical stimuli. It is characterized by vasodilatation, increase of blood flow and vascular permeability and cellular recruitment to the inflammatory site. These biochemical and cellular alterations are regulated by inflammatory mediators such as prostanoids (Goulet et al., 2004). Prostanoids consisting of the prostaglandinas (PGs) and thromboxanes (TXs) are rapidly synthesized via the cyclooxygenase pathway in a variety of cells in response to various stimuli such as inflammation (for review see: Bos et al., 2003; Funk, 2001; Hata and Breyer, 2004).

Prostaglandin E_2 (PGE_2), a major cyclooxygenase product, exhibits a broad range of biological actions in diverse tissues through its binding to specific receptors on plasma membrane. These receptors belong to the great family of G protein-coupled receptors and they can be divided into four subtypes (EP_{1-4}), each of which is encoded by distinct genes (Negishi et al., 1995). Whereas EP_1 receptor induces calcium mobilization by phospholipase C activation via G_q protein, EP_2 and EP_4 receptors are known to activate adenylyl cyclase via stimulatory G-protein. On the other hand, the EP_3 receptor reduces cAMP levels as it is coupled to inhibitory G-proteins. In addition, the EP_3 receptors are the only ones to display multiple splice variants identified in several species (for a review, see Kobayashi and Narumiya, 2002; Hata and Breyer, 2004, Bos et al., 2004, Namba et al., 1993).

PGE_2 is generally considered as a key pro-inflammatory mediator, and its role has been extensively studied in several inflammatory events (Ikeda et al.,

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1975; Flower et al.,1976; Raul, 1990). Thus, high levels of PGE_2 have been found in inflammatory exudates, and the injection of PGE_2 directly into tissue has been shown to induce a number of classical sign of inflammation. However, despite the importance of PGE_2 in this process being recognized, the receptor subtypes and the signaling pathways involved in the PGE_2-mediated inflammatory actions remain to be further elucidated. In this regard, using pharmacological and molecular approaches, the present study aimed at investigating the receptor subtypes, as well the possible signal transduction pathways involved in PGE_2-induced paw edema formation in mice.

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Materials and Methods

Animals

The experiments were conducted using male Swiss mice (25 - 35 g) kept in chambers under a 12 h light-dark cycle, with controlled humidity (60 - 80 %) and temperature (22 ± 1 °C). Food and water were freely available. Experiments were performed during the light phase of the cycle. The animals were acclimatized to the experimental laboratory for at least 1 h before testing and were used once throughout the experiments. All experimental protocols used in this study were approved by the Ethics Committee of the Federal University of Santa Catarina (263/CEUA and 23080035336-16/UFSC).

Measurement of mouse paw edema

Experiments were conducted according to the procedures previously described by Campos *et al.* (1995). Briefly, the animals received a 20 µl i.pl. injection, into the right hindpaw, of phosphate-buffered saline (PBS, composition mmol/l: NaCl 137, KCl 2.7, phosphate buffer 10) containing PGE_2 (0.1 - 10 nmol/paw). The left paw received the same volume of PBS and was used as the control. The paw edema formation was measured by means of a plethysmometer (Ugo Basile, Italy) at several time points (15, 30, 45 and 60 min) after the i.pl. injection of PGE_2, and was expressed in µl as the difference between the right and left paws.

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Characterization of the mechanisms involved in PGE_2-induced mouse paw edema

To evaluate the EP receptor subtypes involved in the edematogenic responses induced by PGE_2, animals received an intraplantar (i.pl.) injection of AH6809 (an EP_2 receptor antagonist, 10 nmol/ paw), L826266 (a selective EP_3 receptor antagonist, 10 nmol/paw) or L161982 (an EP_4 receptor antagonist, 10 nmol/paw) coinjected with PGE_2 (3 nmol/paw). In a separate set of experiments, animals received L826266 (0.1 - 10 nmol/paw) 30 min before PGE_2 (3 nmol/paw) injection.

In another experiment to assess the participation of pertussis toxin-sensitive G proteins in PGE_2-paw edema formation, animals were pre-treated with pertussis toxin (10 ng/paw), 20 minutes before PGE_2 (3 nmol/paw) injection. The control group received the same volume (20 μl) of vehicle before PGE_2 administration. In another experimental group, to test whether the PLC was involved in PGE_2-induced paw edema, animals received an intraplantar injection (i.pl.) of PGE_2 (3 nmol/paw) in association with U73122 (1 pmol/paw) a selective PLC antagonist.

In a further experimental group, in order to evaluate the participation of sensorial neuropeptides and vanilloid receptors in PGE_2-mediated mouse paw edema, animals received an intraplantar (i.pl.) injection of the selective NK_1 FK888 (1 nmol/paw), NK_2 SR48968 (0.5 nmol/paw), NK_3 SR142801 (1 nmol/paw) or CGRP $CGRP_{8-37}$ (0.5 nmol/paw) receptor antagonists or the TRPV1 SB366791 (1 nmol/paw) receptor antagonist coinjected with PGE_2 (3 nmol/paw).

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In order to investigate to what extent some groups of kinases are involved in PGE_2-induced edema formation in the mouse paw, we assessed the effects of co-administration of the following selective enzyme inhibitors with PGE_2 (3 nmol/paw): H89 (PKA, 3 nmol/paw), GF109203X (PKC, 3 nmol/paw), SP600125 (JNK, 30 nmol/paw), PD98059 (MEK, 30 nmol/paw) and SB203580 (p38, 30 nmol/paw).

The doses of all inhibitors used in this study were chosen on the basis of literature data or preliminary experiments (Santos and Calixto, 1997; Beirith et al., 2003; Inoue et al., 2003; da Cunha et al., 2004; Ferreira et al., 2004; 2005)

Neonatal capsaicin treatment

To further explore the role of capsaicin-sensitive C fibers in PGE_2-induced paw edema formation, mice were treated during the neonatal period (on the second day of life) with capsaicin (50 mg/kg, subcutaneously) or vehicle alone (10% ethanol, 10% Tween-80 and 80% PBS), as described previously (Gamse R, 1982). Animals were used at 6–7 weeks after the neonatal administration of capsaicin or its vehicle (used as control). In order to discover whether or not the degeneration of capsaicin-sensitive primary afferent C fibers had occurred, we applied the wiping eye test described by Ikeda et al., (2001). Briefly, 50 µl of 0.01% (w/v) capsaicin was instilled into one eye, and the number of wiping motions that occurred in the subsequent 1-min period was counted. The animals that wiped their eyes 5 times or less were used as the neonatal capsaicin-treated group. The edema was induced by i.pl. injection of PGE_2 (3 nmol/paw) and measured as described above.

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Preparation of tissue for western blot studies

The right paws of the mice were isolated at different periods of time (5–60 min) after PGE_2 treatment (3 nmol/paw). In another experimental group, thirty minutes before i.pl. injection of PGE_2 (3 nmol/paw), the animals received an i.pl injection of L826266 (10 nmol/paw). Fifteen min after PGE_2 treatment, the animals were killed and the subcutaneous tissue of the paws was removed.

The subcutaneous tissue of the paws was rapidly removed and homogenized in an ice-cold buffer containing protease and phosphatase inhibitors (100 mM Tris–HCl–pH 7.4; 2 mM EDTA; 2 µg/ml aprotinin, 0.1 mM phenylmethanesulphonyl fluoride, 200 mM NaF and 2 mM of sodium orthovanadate). The homogenate was first centrifuged at 1000 *g* for 10 min at 4 °C. The pellet was discarded and the supernatant was further centrifuged at 35,000 *g* for 30 min at 4 °C. The resulting supernatant was collected as a cytoplasm-rich fraction. Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA). The samples were aliquoted and stored at -80 º C until western blot analysis.

Western blot analysis

In order to assess the possible activation of MAPKs following PGE_2 injection into the mouse paw, western blot analysis was carried out as previously described (Ferreira et al., 2005) with minor modifications. Equivalent amounts of protein (40 µg for JNK and 70 µg for p38 of cytoplasm rich fraction) were mixed with sample buffer (Tris 200 mM, glycerol 10%, sodium dodecyl sulfate 2%, β-mercaptoethanol 2.75 mM and bromophenol blue 0.04%) and boiled for 5 min. Proteins were separated in a 10% sodium dodecyl sulfate-

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polyacrilamide gel by electrophoresis and transferred onto a polyvinilidene difluoride membrane, according to the manufacturer's instructions (Millipore). The membrane was blocked by incubation overnight with a 10% non-fat dry milk solution and then incubated with anti-phosphorylated forms of JNK (phospho-JNK) or p38 (phospho-p38) antibodies. Following washing, the membrane was incubated with adjusted peroxidase-coupled secondary antibodies. The immunocomplexes were visualized using the ECL chemiluminescence detection system (Amersham Biosciences, UK). The membrane was then incubated for 10 min in a stripping buffer at room temperature and re-incubated with an antibody against actin, which served as a loading control.

Drugs and reagents

The following drugs and reagents were used: AH6809 (6-Isopropoxy-9-oxoxanthene-2-carboxylic acid) (Cayman Chemical, MI, USA); capsaicin, $CGRP_{8\text{-}37}$ (calcitonin gene-related peptide fragment 8–37), H89 (N-(2-[p-Bromocinnamylamino]ethyl)--5-isoquinolinesulfonamide), pertussis toxin, PGE_2 and U73122 (1-[6-([17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione) (Sigma, St. Louis, USA); GF109203x (2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide), PD98059 (2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one), SB203580 (4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1*H*-imidazol-4-yl]pyridine), SB366791 (4'-Chloro-3-methoxycinnamanilide) and SP600125 (Anthra[1,9-cd]pyrazol-6(2*H*)-one) (Tocris Cookson, Baldwin, USA); polyclonal antibodies anti-actin and anti-phosphorylated forms of JNK and p38 (Santa Cruz, CA, USA). FK888 (*N*-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-

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methyl-*N*-phenyl-methyl-3-(2-aphthyl)-L-alaninamide) was kindly provided by Fujisawa Pharmaceutical (Osaka, Japan). SR48968 ((*S*)-*N*-methyl-(*N*[4-acetylamino-4-phenylpiperidine)-2-(3,4-dichlorophenyl)butyl]benzamide) and SR142801 ((*S*)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)-piperiden-3-yl)propyl)-4-phenilpipedidin-4-yl)-*N*-methyl-acetamide) were donated by Sanofi Recherche (Montpellier, France). L826266 (2E)-N-[(5-bromo-2-methoxyphenyl)sulfonyl]-3-[5-chloro-2-(2-naphthylmethyl)phenyl]acrylamide and L161982 (N-{[4′-({3-butyl-5-oxo-1-[2-(trifluoromethyl) phenyl]-1,5-dihydro-4H-1,2,4-triazol-4-yl}methyl) biphenyl-2-yl]sulfonyl}-3-methylthiophene-2-carboxamide) were kindly provided by Merck Froost (Kirkland, Canada).

Stock solutions for most drugs (0.1 - 1 M) were prepared in ethanol, except AH6809 and $CGRP_{8-37}$, that were made in a 1% $NaHCO_3$ solution and distilled water, respectively. All drugs were stocked in siliconized plastic tubes and maintained at -18 °C until use. Solutions of these drugs were prepared in PBS (NaCl 137, KCl 2.7 and phosphate buffer 10 mM) to the desired concentration just before use. The final concentration of ethanol did not exceed 0.5%, which alone had no effect on PGE_2-induced edema response. In addition, $NaHCO_3$ alone also had no effect on PGE_2-induced edema response.

Statistical analysis

The results are presented as the mean ± S.E.M. of 4 to 7 animals per group, except the mean ED_{50} or ID_{50} values (i.e. the dose of agonist necessary to produce 50% of the maximal edematogenic response or the dose of antagonists necessary to reduce the agonist response by 50% relative to the control value, respectively), which are reported as geometric means

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accompanied by their respective 95% confidence limits. The ED_{50} or ID_{50} values were calculated by use of the GraphPad Prism software. The percentages of inhibition are reported as mean ± S.E.M. of inhibitions obtained for each individual experiment in relation to the control values. The ED_{50}, ID_{50} and percentages of inhibition were calculated at the peak of PGE_2-induced paw edema (30 min). Statistical comparison of the data was performed by analysis of variance (ANOVA) followed by Dunnett's or Student Newman-Keuls' tests or by Student's unpaired *t*-test, as appropriate. *P* values less than 0.05 ($P < 0.05$) were considered significant.

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Results

PGE_2–induced paw edema formation

The intraplantar injection of PGE_2 (0.1-10 nmol/paw) into the mouse hind paw elicited a dose- and time-dependent edema formation (Figure 1). The edematogenic response induced by i.pl. injection of PGE_2 was evident as early as 15 min after, reaching the maximum at 30 min and remaining significant for up to 60 min. The calculated mean ED_{50} value (and the 95% confidence limits) for this effect was 0.42 (0.29-0.54) nmol/paw and the maximum effect was 80.0 ± 3.0 µl (Figure 1).

Characterization of EP receptor subtypes in PGE_2-induced mouse paw edema

PGE_2-induced paw edema formation was significantly inhibited by coinjection of the selective EP_3 receptor antagonist L826266 (10 nmol/paw), with a percentage of inhibition of 52.9 ± 8.3%. On the other hand, the edematogenic response evoked by PGE_2 was not significantly affected by the co-administration of either EP_2 (AH6809, 10 nmol/paw) or EP_4 (L161982, 10 nmol/paw) receptor antagonists (Figure 2A). However, L826266 was not able to inhibit the edematogenic response induced by PGE_2 at the 15-minute time point. To elucidate whether or not the lack of inhibition was due to antagonist pharmacokinetics we administered it locally 30 minutes before the edema induction. This pretreatment caused an accentuated inhibition (80.8 ± 5.5%) (Figure 2A) of PGE_2-mediated paw edema, an effect which was dependent on the used dose (0.1-10 nmol/paw) (Figure 2B). The mean ID_{50} value calculated using the values obtained with the pretreatment was 0.36 (0.05-1.82) nmol/paw.

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Thus, these results indicate that edematogenic responses caused by PGE_2 are probably mediated mainly through the activation of the EP_3 receptor subtype.

***Participation of* pertussis toxin-sensitive G protein and PLC pathway**

PGE_2-induced paw edema formation was significantly inhibited by pre-treatment with pertussis toxin (10 ng/paw), 20 minutes before PGE_2 injection, by 85.7 ± 5.8% (Figure 3A). Furthermore, PGE_2-induced paw edema was also significantly inhibited by coinjection of the selective PLC inhibitor (U73122, 1 pmol/paw). The inhibition obtained was 76.6 ± 3.3% (Figure 3B).

Assessing the involvement of neurogenic mechanisms in PGE_2-induced paw edema formation

The results in Figure 4A show that pretreatment with capsaicin (50 mg/kg, s.c.) in the neonatal period was able to produce a significant inhibition of the paw edema induced by PGE_2 (66.6 ± 6.6%). The mouse paw edema caused by i.pl. injection of PGE_2 was also significantly reduced by coinjection of the selective NK_1 receptor antagonist FK888 (1 nmol/paw) or the selective TRPV1 receptor antagonist SB366791 (1 nmol/paw). The inhibitions obtained were 61.5 ± 3.8% and 53.8% ± 4.1, respectively. Conversely, coinjection of the selective NK_2 SR48968 (1 nmol/paw), NK_3 (SR14281 (0.5 nmol/paw) or CGRP (CGRP $_{8\text{-}37}$, 0.5 nmol/paw) receptor antagonists had no significant effect on PGE_2-induced paw edema formation (Figure 4B).

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Involvement of PKC, PKA and MAP kinases in PGE_2–induced paw edema formation

Next, we investigated how the activation of some groups of kinases might be implicated in paw edema induced by PGE_2 in mice. Figures 5A and 5B demonstrate that the i.pl. coinjection of the selective inhibitors of p38 SB203580 (30 nmol/paw), JNK SP600125 (30 nmol/paw), ERK PD98059 (30 nmol/paw) and PKC GF109203X (3 nmol/paw), all significantly reduced PGE_2-induced paw edema formation. The percentages of inhibition observed for these drugs were 48.6 ± 2.2%, 51.5 ± 4.9%, 64.0 ± 3.6% and 54.5 ± 5.8%, respectively. In contrast, the coinjection of the selective inhibitor of PKA H89 (3 nmol/paw) had no significant effect on PGE_2 response.

Western blot of MAP Kinases after PGE_2 injection into the mouse paw

Western blot analysis was used in order to investigate further whether the i.pl. injection of PGE_2 activated the MAP kinases JNK and p38, and if so, to what extent the pretreatment with EP_3 receptor antagonist L826266 could prevent this activation.

The i.pl. injection of PGE_2 (3 nmol/paw) resulted in a marked and time-dependent activation of MAPKs. A statistically significant increase in phosphorilation levels of JNK was observed from 5 to 30 min following PGE_2 administration (Figure 6A). Moreover, p38 MAPK phosphorylation was significant from 15 to 60 min after PGE_2 injection (Figure 6B). We did not detect any alteration in actin expression after PGE_2 injection.

The results of Figure 7 indicate that i.pl. injection of PGE_2 resulted in a marked activation of JNK (1.8-fold) and p38 (2.3-fold). Of note, the activation of

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these kinases was markedly diminished by the pretreatment with EP_3 receptor antagonist L826266 (10 nmol/paw). The inhibitions observed were 62.5 ± 12.5 % and 67.8 ± 3.5 % for JNK and p38 MAPK, respectively. However, the activation of JNK and p38 induced by PGE_2 was much more pronounced in these experiments when compared with the initial time-course experiments. These differences are probably due to the fact that we have used different groups of animals.

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Discussion

In the present study we sought to investigate, by the use of pharmacological tools and molecular procedures, the EP receptor subtypes, as well as some of the transducing mechanisms involved in the edematogenic responses mediated by PGE_2 in mice. Our results clearly demonstrate that PGE_2 injection into the mouse paw produces a dose- and time-dependent edema formation at a nanomolar range. These pieces of evidence extend previous studies showing that PGE_2 injection evokes an increase of vascular permeability in the skin of rats, rabbits and humans (Williams and Morley, 1973; Ikeda et al., 1975; Flower et al.,1976; Raul, 1990). Interestingly, the present data allows us to suggest that PGE_2-elicited paw edema in mice is mainly, if not solely, mediated by the activation of EP_3 receptors. This conclusion derives from the results indicating that edema formation induced by PGE_2 injection is markedly prevented by the selective EP_3 L826266, but not the EP_2 AH6809 or EP_4 L161982, receptor antagonists. In fact, it has been recently demonstrated that edema formation and plasmatic extravasation induced by the topical application of AA is found significantly decreased in mice with genic deletion of EP_3 receptors, whereas these responses remain unaffected in knockout mice for EP_1, EP_2 or EP_4 receptors (Goulet et al, 2004) In addition, Yuhki *et al.* (2004) showed that IP, EP_2 and EP_3, but not EP_1 or EP_4, receptor subtypes are largely implicated in exudate formation in the model of pleurisy induced by carregeenan in the mouse. Notably, we have also observed that the EP_3 receptor antagonist (L826266) consistently inhibits carrageenan-induced edema in mice, to the same extent that it is able to reduce PGE_2-mediated edema formation (results not shown). Although our data visibly point to the EP_3

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receptor as being mainly responsible for the edematogenic effect of PGE_2 in the mouse paw, we cannot completely discard the participation of the EP_1 receptor in this response, as no selective EP_1 receptor antagonist is currently available for mice. This possibility remains to be further evaluated in future studies.

The EP_3 receptor is distributed throughout most mouse tissues (Narumiya et al., 1999). Its alternative splicing forms are capable of inducing a broad range of effects (Bos et al., 2004). Murine $EP_{3\alpha}$, $_\beta$ and $_\gamma$ receptors usually activate inhibitory G proteins, but they may also interact with G_s and G_{13} proteins (see for review Hatae et al., 2002; Hata and Breyer, 2004). It was first affirmed that EP_3 receptor would be able to decrease the adenylyl cyclase activity (reducing the cAMP production) through an interaction with the $G\alpha_i$ subunit. Furthermore, the stimulation of PLC by EP_3 receptor was reported to induce the accumulation of IP_3 (inositol triphosphate) and DAG (diacylglycerol) that, in turn, promoted intracellular Ca^{+2} mobilization and PKC activation (Irie et al., 1994). Moreover, it has been hypothesized that $\beta\gamma$ subunits of G_i might stimulate phospolipase C (PLC) and activate $p21^{ras}$ (Burkey and Regan, 1995, Irie et al., 1994). The results of the present study confirm and also extend these previous findings by demonstrating that the pretreatment with pertussis toxin significantly inhibited PGE_2-induced paw edema. Furthermore, PGE_2-induced paw edema formation was significantly inhibited by coinjection of the selective PLC inhibitor U73122. We might assume that one or more of the abovementioned signaling pathways are involved in EP_3 receptor-mediated PGE_2-induced responses in the mouse paw.

Another interesting aspect investigated in the present study was the possible participation of certain groups of kinases in the mouse paw edema

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induced by PGE_2. Our results demonstrate that the selective PKC blocker GF109203X, but not the PKA inhibitor H89, was able to consistently reduce PGE_2-induced mouse paw edema. Additional sets of experiments also indicated the relevance of MAP kinases in our model, as the selective JNK SP600125, ERK PD98059 or p38 SB203580 inhibitors were all markedly effective in reducing PGE_2-induced edema formation. The functional results were confirmed by western blot analysis, which indicated that i.pl. injection of PGE_2 resulted in a marked activation of PKC-α (unpublished results), JNK and p38 MAP kinases in the mouse paw. Of high interest, the increased expression of MAP kinases, but not PKC-α, induced by PGE_2, seems to be driven by the activation of EP_3 receptor subtype, as this response was almost completely prevented by the selective EP3 receptor antagonist. However, we cannot discard the hypothesis that EP_3 receptor may activate other forms of PKC. Therefore, we might suggest that EP_3-mediated PGE_2 edematogenic responses in the mouse paw involve the coordinated activation of PKC, but not PKCα-isoform, and MAP kinases. In this regard, there are reports in the literature demonstrating that stimulation of PKC is able to activate the ERK signaling pathway (Vlahos et al., 2003, Qiu e Leslie, 1994).

The activation of peripheral terminals of sensory fibers, especially C fibers, causes the release of neuropeptides (such as tachykinins) both peripherally and in the dorsal spinal cord (Holzer, 1998). These inflammatory mediators, particularly substance P, act on target cells in the periphery, producing the classic signals of inflammation in a phenomenon known as “neurogenic inflammation” (Holzer, 1998; Richardson and Vasko, 2002). The present study also analyzed the possible role played by sensory fibers in PGE_2-

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induced paw edema. Capsaicin is a pharmacological tool that produces the selective degeneration of C and some Aδ fibres, associated with the irreversible loss of more than 80% of small-diameter sensory neuron cell bodies (see for review Holzer, 1991). Our results demonstrated that neonatal treatment with capsaicin was able to inhibit PGE_2-induced mouse paw edema, suggesting that stimulation of primary sensory fibers is an important event for PGE_2 inflammatory responses. In addition, we showed that co-injection of the selective NK_1 FK888, but not NK_2 SR48969, NK_3 SR 142801 or CGRP $CGRP_{8-37}$ receptor antagonists, significantly reduced PGE_2-evoked edema formation. Consequently, it is possible to conclude that pro-edematogenic effects of PGE_2 in the mouse paw are mediated, at least partially, by the release of tachykinins, probably SP, acting on NK_1 receptors. In fact, White (1996) has shown that PGE_2 is capable of releasing SP from cultured rat sensory neurons. The co-localization of tachykinin and TRPV1 receptors (non-selective cation channels expressed predominantly in small diameter sensory fibers) strongly suggests the involvement of the latter in the PGE_2-induced edematogenic response. The TRPV1 receptor is exogenously activated by the pugent plant-derived compound capsaicin, but it is also endogenously stimulated by protons, heat and some lipid-derived mediators (for review see: Calixto et al., 2005). It is now well known that TRPV1 activation augments the release of neuropeptides in a calcium-dependent manner (Bevan and Geppetti, 1994; Kessler et al., 1999). We found that, at least in part, PGE_2-induced edematogenic response in the mouse paw is associated with the activation of TRPV1. This conclusion derives from the results indicating that the coinjection of the selective TRPV1 receptor antagonist (SB366791) significantly inhibited the PGE_2-induced paw edema.

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Recent evidence has suggested that regulation and activation of TRPV1 are defined by complex mechanisms such as phospholipid-mediated inhibition and phosphorylation (Premkumar and Ahern, 2000; Chuang et al., 2001). Of note, TRPV1 can be modulated by the PKA and PKC phosphorylation (Lopshire and Nicol 1998; Cesare et al., 1999). Interestingly, it has recently been shown that functional interaction of TRPV1 with PGE_2 occurs through a PKC-dependent mechanism by coupling of EP_1 receptor (Moriyama et al., 2005). Thus, our results allow us to suggest that PGE_2 might modulate TRPV1 through the PKC-dependent mechanisms. Whether or not MAP kinases are associated with the activation of sensory fibers following EP_3 receptor activation by PGE_2 remains to be further assessed.

Taken together, the present results provide convincing experimental evidence indicating that PGE_2 induces paw edema in mice via the stimulation of EP_3 receptors, in a process dependent on the activation of TRPV1 and NK1 receptors, as well as PKC and MAP kinases. These findings help to elucidate the possible mechanisms underlying PGE_2-elicited inflammatory responses in mice and point to EP_3 receptor antagonists as possible therapeutic options for treating inflammatory disorders.

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Acknowledgments

We thank the pharmaceutical companies for the kind donations of FK 888, L826266, L161982, SR 48968 and SR 142801 used in this study.

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Footnotes

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Legends for Figures

Figure 1. Dose-response and time course effect of PGE_2-induced paw edema formation in mice. Values represent the differences of volume (in microliters) between vehicle-injected (0.02 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four to seven animals. In some cases, the error bars are hidden within the symbols. Significantly different from control values. ** $P<0.01$ (one-way ANOVA followed by *post hoc* Dunnett's test).

Figure 2. (A) Effect of EP_2, EP_3 and EP_4 receptor antagonists on PGE_2-induced paw edema formation in mice. AH6809 (EP_2 receptor antagonist, 10 nmol/paw), L826266 (EP_3 receptor antagonist, 10 nmol/paw) and L161982 (EP_4 receptor antagonist, 10 nmol/paw) were coadministered with PGE_2 (3 nmol/paw). EP_3 receptor antagonist L826266 (10 nmol/paw) was also administered 30 minutes before PGE_2 injection. (B) Dose response effect for the selective EP_3 receptor antagonist on PGE_2 - induced paw edema in mice. L826266 (0.1-10 nmol/paw) was administered locally 30 minutes before PGE_2 (3 nmol/paw). Values represent the differences of volume (in microliters) between vehicle-injected (0.02 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four to seven animals. Significantly different from control values. ** $P < 0.01$ (one-way ANOVA followed by *post hoc* Dunnett's test).

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Figure 3. Effect of pertussis toxin-sensitive G protein and PLC inhibitor U73211 on PGE_2-induced paw edema formation in mice. (A) Pertussis toxin-sensitive G protein (10 ng/paw) was administered 20 minutes before PGE_2 (3 nmol/paw) injection. (B) U73122 (a selective PLC inhibitor, 1 pmol/paw) was coadministered with PGE_2 (3 nmol/paw). Values represent the differences of volume (in microliters) between vehicle-injected (0.02 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four to seven animals. Significantly different from control values. ** $P < 0.01$ (one-way ANOVA followed by *post hoc* Dunnett's test).

Figure 4. (A) Effect of neonatal treatment with vehicle (1 ml/kg, s.c.) or with capsaicin (50 mg/kg, s.c.) on PGE_2-induced paw edema formation in mice. (B) Effect of selective NK_1, NK_2, NK_3 and CGRP receptor antagonists and selective TRPV1 receptor antagonists on PGE_2-induced paw edema formation in mice. FK888 (a selective NK_1 receptor antagonist, 1 nmol/paw), SR48968 (a selective NK_2 receptor antagonist, 0.5 nmol/paw), SR142801 (a selective NK_3 receptor antagonist, 1 nmol/paw), $CGRP_{8\text{-}37}$ (a selective CGRP receptor antagonist) and SB366791 (a selective TRPV1 receptor antagonist) were coadministered with PGE_2 (3 nmol/paw). Values represent the differences of volume (in microliters) between vehicle-injected (0.02 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four to seven animals. Significantly different from control values. ** $P<0.01$ (Student's unpaired *t*-test or one-way ANOVA followed by *post hoc* Dunnett's test).

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Figure 5. Effect of (A) PKC, PKA and (B) MAP kinase inhibitors on PGE_2-induced paw edema formation in mice. H89 (a selective PKA inhibitor, 3 nmol/paw), GF109203X (a selective PKC inhibitor, 3 nmol/paw), SP600125 (a selective JNK inhibitor, 30 nmol/paw), PD98059 (a selective MEK inhibitor, 30 nmol/paw) and SB203589 (a selective p38 inhibitor, 30 nmol/paw) were coadministered with PGE_2 (3 nmol/paw). Values represent the differences of volume (in microliters) between vehicle-injected (0.02 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four to seven animals. Significantly different from control values. * $P<0.05$, ** $P<0.01$ (one-way ANOVA followed by *post hoc* Dunnett's test).

Figure 6. Western blots showing the time course of p38 (A) and JNK (B) activation in response to i.pl. injection of PGE_2 (3 nmol/paw) into the mouse paw. Mouse paw tissues were obtained from PBS (basal) or PGE_2-injected mice at the indicated time points. Cytolosic levels of phosphorylated p38 (P-p38), phosphorylated JNK (P-JNK) and actin were determined using specific antibodies. Results were normalized by arbitrarily setting the densitometry of the basal group and are expressed as the mean ± SEM (n=3). *$P<0.05$, as compared with basal values (one-way ANOVA followed by Dunnett's test).

Figure 7. Representative images of western immunoblotting and densitometry analyses showing the effect of EP_3 receptor antagonists on PGE_2-induced phosphorylation of p38 (A) or JNK (B) and the internal control actin (C). Thirty minutes before PGE_2 administration, animals received i.pl. injection of L-826266 (10 nmol/paw). Paw tissues were obtained from PBS (basal) mice or from mice

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15 minutes after PGE_2 injection. Cytosolic levels of phosphorylated p38 (P-p38), phosphorylated JNK (P-JNK) and actin were determined using specific antibodies. Results were normalized by arbitrarily setting the densitometry of the basal group, and they are expressed as the mean ± SEM (n=3). $^{\#}P<0.05$, as compared with basal values (B), $^{*}P<0.05$, as compared with vehicle group (one-way ANOVA followed by Student-Newman-Keuls' test).

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FIGURE 1

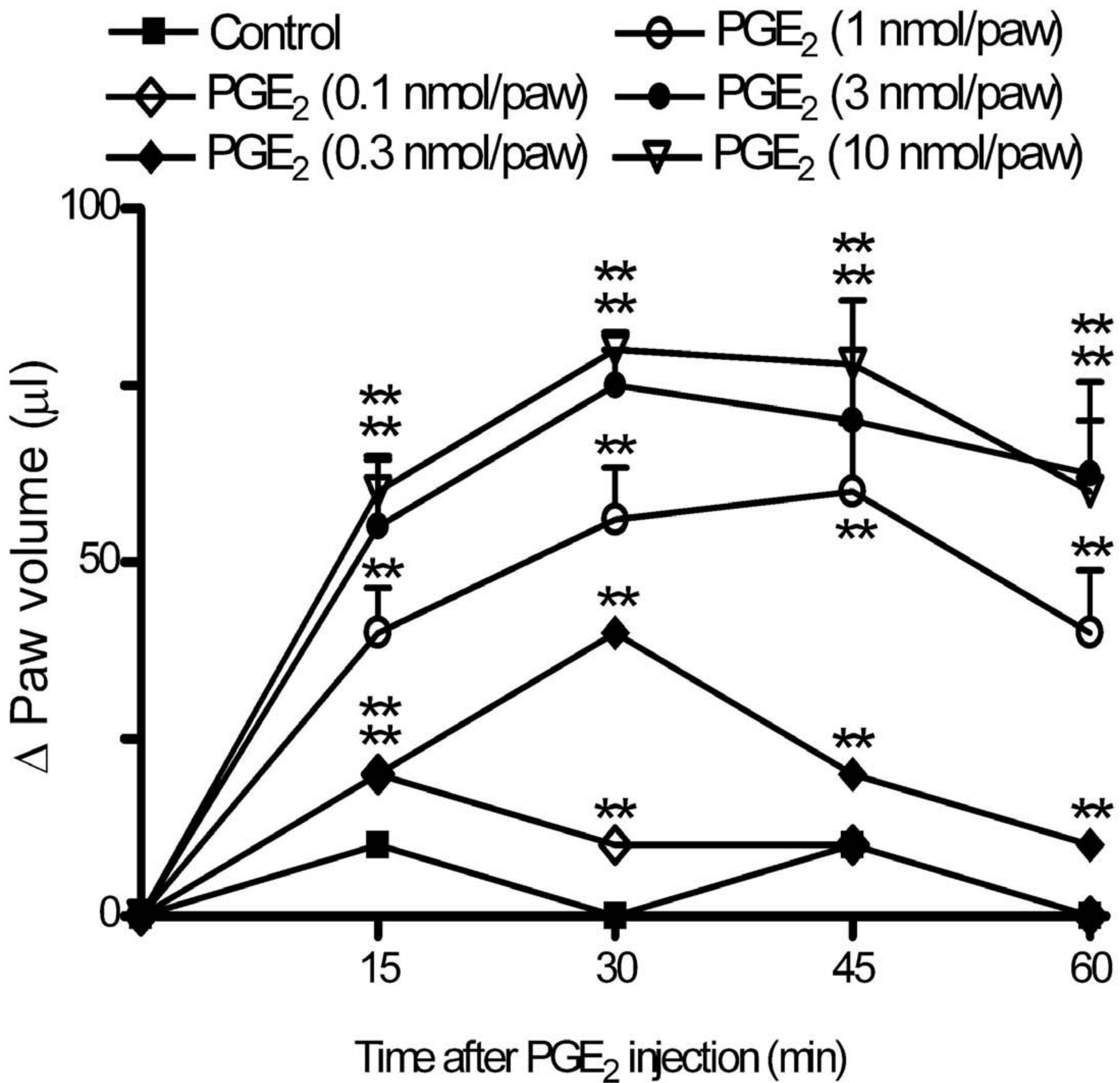

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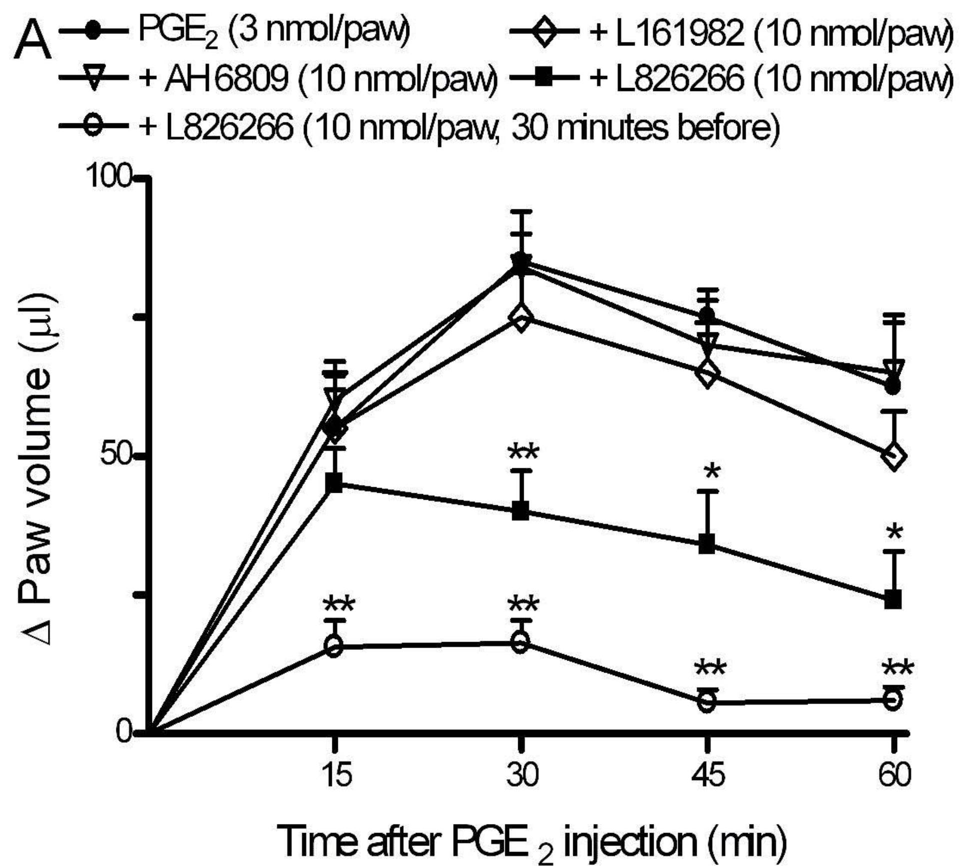
FIGURE 2



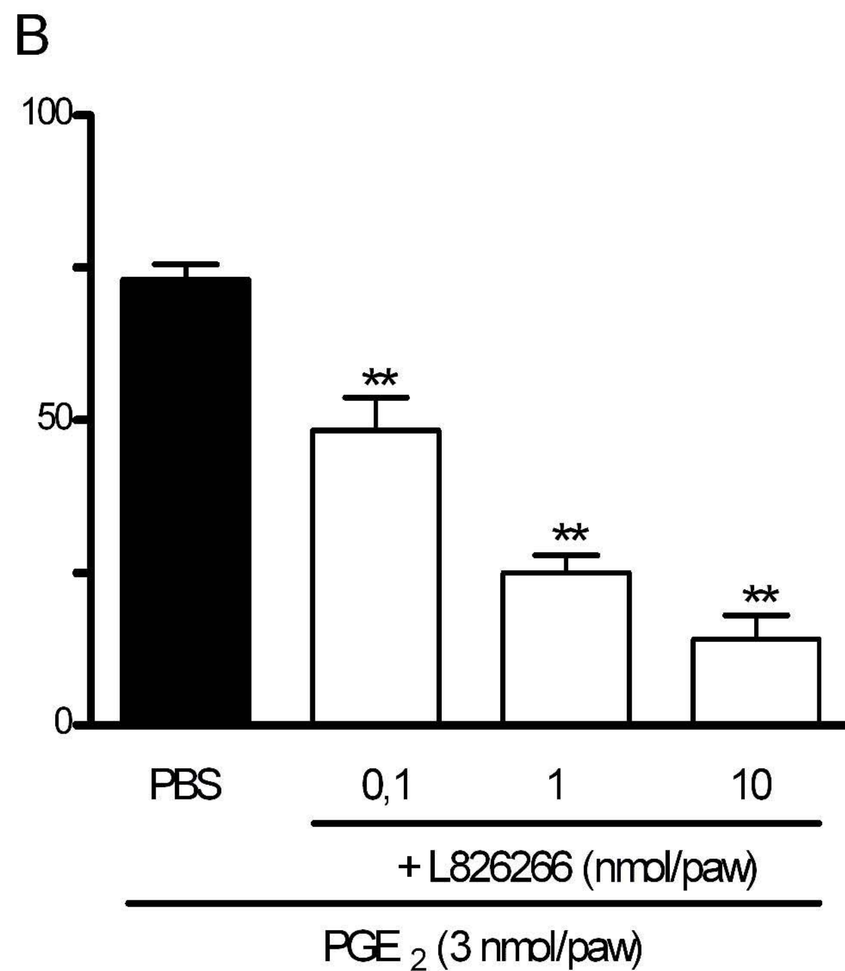


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FIGURE 3

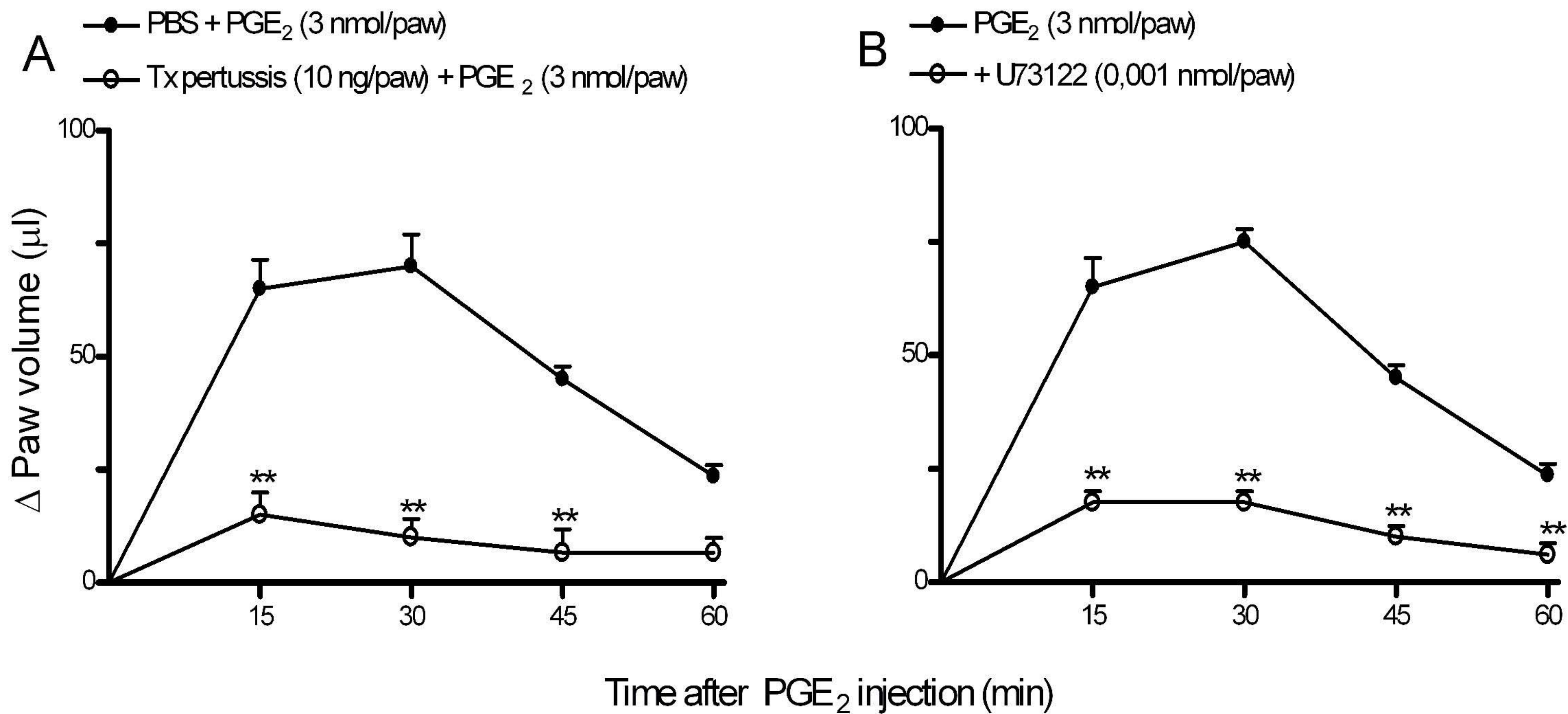

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FIGURE 4

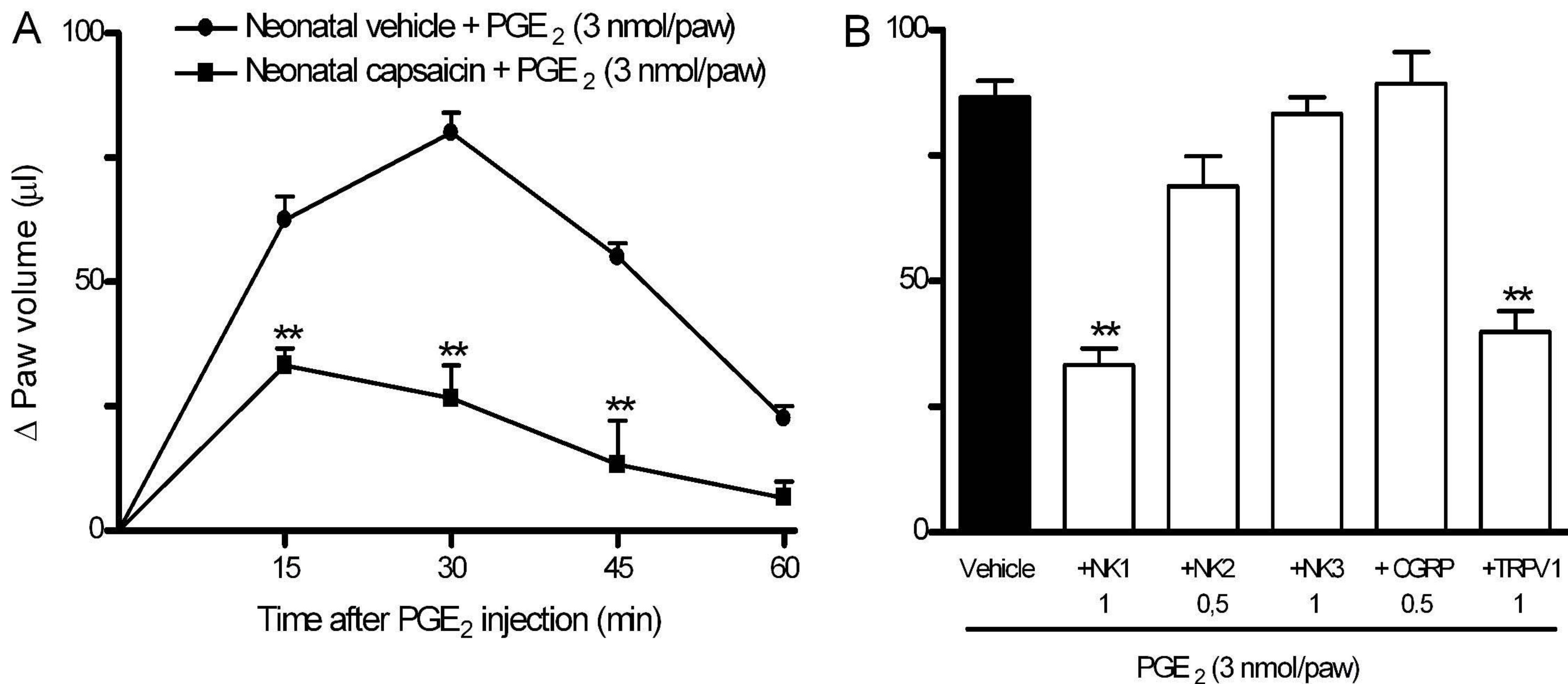

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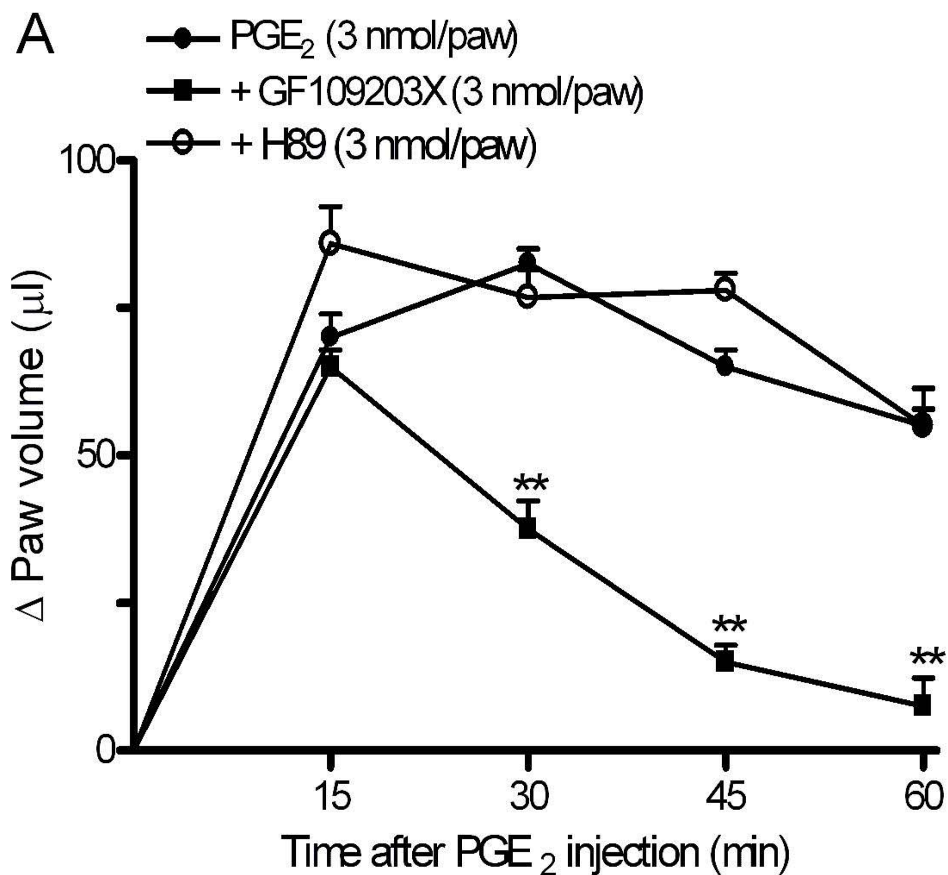
FIGURE 5



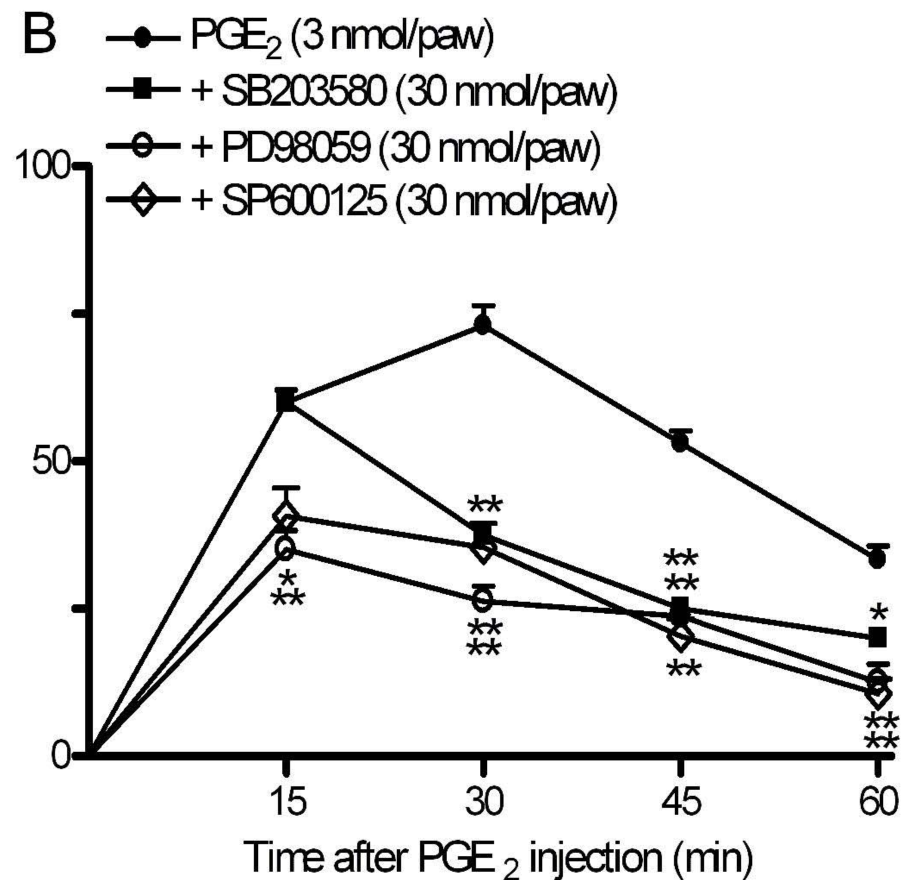


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FIGURE 6

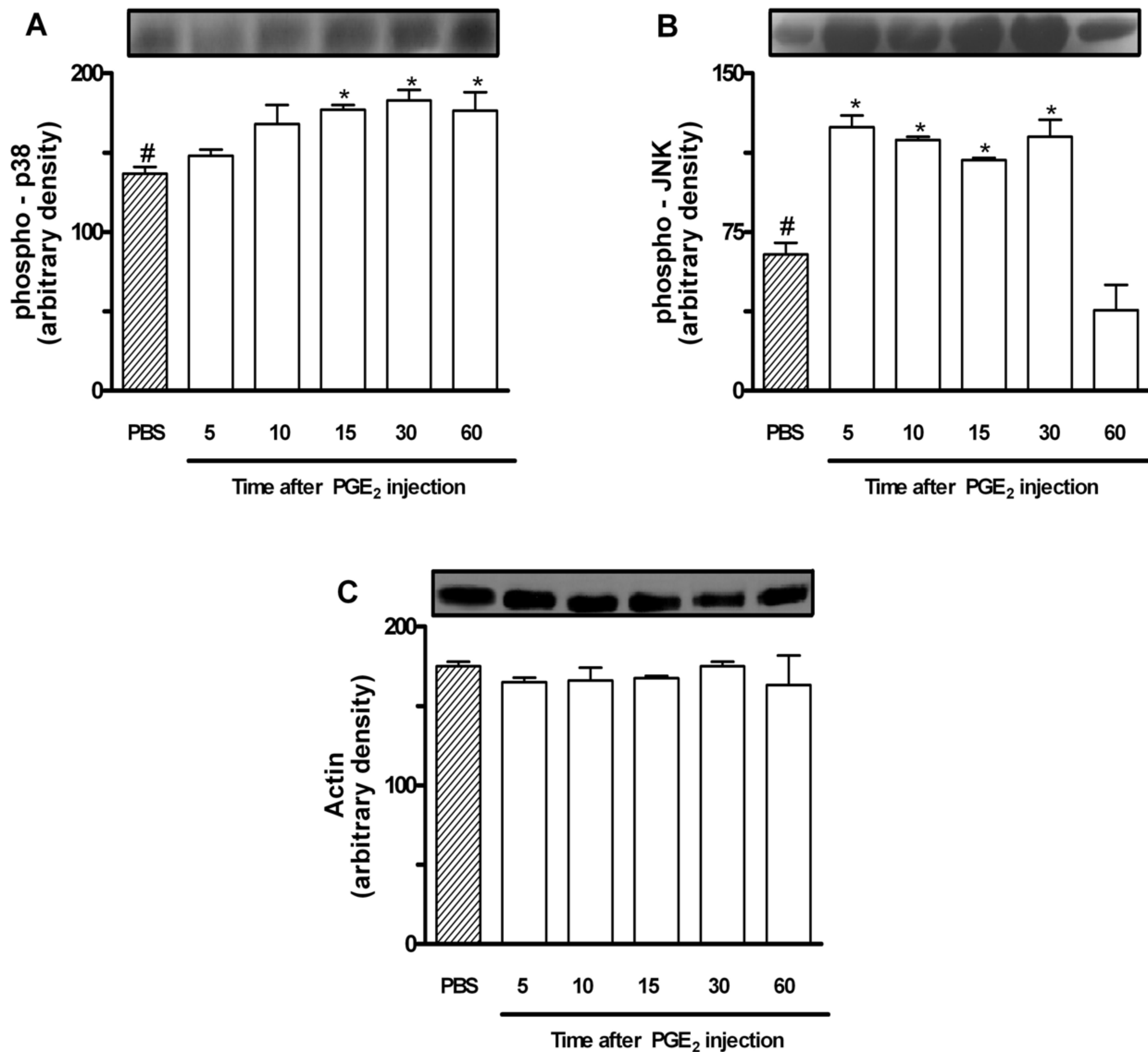

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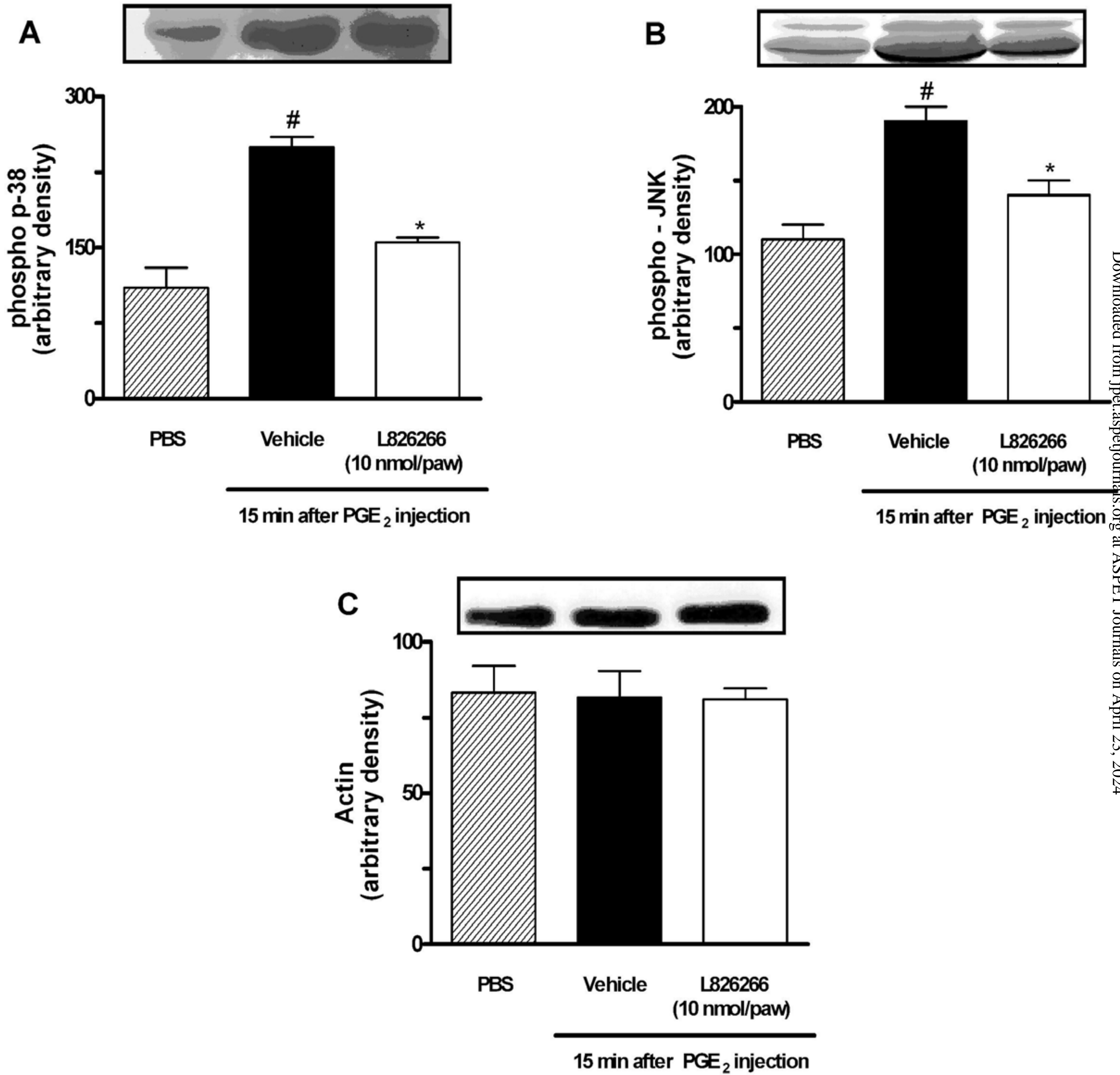
FIGURE 7



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