

Peripheral Group II Metabotropic Glutamate Receptors (mGluR2/3) Regulate Prostaglandin E₂-Mediated Sensitization of Capsaicin Responses and Thermal Nociception

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Previous studies have shown that group II metabotropic glutamate receptors (mGluRs) are present on the peripheral terminals of primary sensory neurons, suggesting that they might be involved in nociception. In this study, we investigated the modulation of nociception by peripheral group II mGluRs and the molecular basis of this modulation. Subcutaneous injection of a group II mGluR agonist, 2*R*,4*R* 4-aminopyrrolidine-2,4-dicarboxylate (APDC), did not alter thermal sensitivity but blocked prostaglandin E₂ (PGE₂)-induced thermal hyperalgesia. This effect was blocked by (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid, a selective group II mGluR antagonist. In cultured primary sensory neurons, APDC blocked PGE₂-

induced potentiation of capsaicin responses, which was abolished when neurons were pretreated with pertussis toxin. Similar potentiating effects induced by forskolin but not 8-bromo-cAMP were also blocked by the activation of group II mGluRs. These results indicate that peripheral group II mGluRs act via inhibition of adenylyl cyclase to reverse the sensitization of capsaicin receptors and the thermal hyperalgesia induced by PGE₂, and suggest that peripheral group II mGluRs might be targeted for therapeutic intervention in inflammatory pain states.

Key words: capsaicin; DRG; mGluR; VR1; pain; phosphorylation; PKA; prostanoid; PGE₂; inflammation; cAMP

Inflammatory pain, which is a major report by patients with inflammation (Levine and Reichling, 1999), usually manifests as an increased response to painful stimuli (hyperalgesia) and pain sensation to previously innocuous stimuli (allodynia). During inflammation, a number of molecules, including prostaglandins, bradykinin, substance P, and others, are released into the injury site. Many of these inflammatory mediators sensitize primary afferent nociceptors via the cAMP/PKA pathway. Activation of adenylyl cyclase (AC) or PKA produces hyperalgesia, whereas PKA inhibitors reduce hyperalgesia (Taiwo and Levine, 1991). One mechanism by which the cAMP/PKA pathway could induce sensitization is by modulating the principle channels that transduce sensory stimuli.

The vanilloid receptor 1 (VR1), also known as the capsaicin receptor, is a nonselective cation channel expressed predominantly in sensory neurons that functions to integrate a number of pain-inducing stimuli (Caterina et al., 1997; Tominaga et al., 1998). VR1 is essential for inflammatory thermal hyperalgesia, because inflammation-induced thermal sensitization fails to develop in mice lacking VR1 (Caterina et al., 2000; Davis et al., 2000). Prostaglandin E₂ (PGE₂), by activating G_s to activate the cAMP/PKA pathway, enhances capsaicin receptor function (Pitchford and Levine, 1991; Lopshire and Nicol, 1997); this effect may mediate thermal hyperalgesia.

In addition to the well known inflammatory mediators men-

tioned above, glutamate is also released in peripheral tissues during inflammation (Omote et al., 1998; deGroot et al., 2000). Glutamate activates ionotropic glutamate receptors (iGluRs), including NMDA, AMPA, and kainate receptors, as well as G-protein-coupled metabotropic glutamate receptors (mGluRs). Peripheral injection of glutamate induces mechanical and thermal hypersensitivity by activating both iGluRs and mGluRs (Carlton et al., 1995; Jackson et al., 1995; Zhou et al., 1996; Bhawe et al., 2001; Walker et al., 2001).

Based on sequence homology and pharmacological properties, mGluRs are divided into three groups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8). Group I mGluRs are localized on nociceptive primary afferent fibers in the skin; activation of peripheral group I mGluRs enhances nociception (Bhawe et al., 2001; Walker et al., 2001). Group II mGluRs are also expressed on primary afferent terminals (Carlton et al., 2001), but the role of peripheral group II mGluRs in nociception remains unknown. Systemic administration of group II mGluR agonists reduces inflammatory hyperalgesia (Sharpe et al., 2002). We hypothesize that group II mGluR agonists may act through peripheral mechanisms to reduce nociception.

The necessity for the cAMP/PKA pathway in inflammatory hyperalgesia suggests that G_i-coupled receptors might be targeted to reduce pain. Indeed, activation of G_i-coupled μ -opioid and adenosine receptors produces analgesic effects (Joris et al., 1987; Taiwo and Levine, 1990; Karlsten et al., 1992). Because group II mGluRs are coupled to G_i, one mechanism by which group II mGluR agonists could reduce hyperalgesia is by inhibiting the cAMP/PKA pathway. The present study examined this hypothesis both *in vivo* and *in vitro* and demonstrated that peripheral group II mGluRs regulate PGE₂-mediated sensitization, possibly by reversing PGE₂-induced modulation of capsaicin receptors.

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MATERIALS AND METHODS

Behavioral testing. Male ICR mice (6–8 weeks of age; Taconic, Germantown, NY) were allowed to recover for at least 2 d after arrival and habituated for at least 3 hr before experiments. Six mice were tested at approximately the same time on different days and divided into three groups with three different treatments.

Thermal sensitivity was measured as described previously (Bhave et al., 2001). A light beam was applied to the plantar surface of the hindpaw (IITC Life Sciences, Woodland Hills, CA), and the foot withdrawal latency was measured. Before injections, baseline (time 0) was measured three times at an interval of ~10 min and averaged. After injections, withdrawal latency was measured at 30 min, 45 min, 1 hr, 1.5 hr, and 3 hr.

Stock solutions of 100 mM (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) and 0.1 mM (2*s*)-2-amino-2-[(1*s*,2*s*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) (both from Tocris Cookson, Ballwin, MO) were made in 1 and 1.2 eq NaOH, respectively. A stock solution of 10 mg/ml PGE₂ (Sigma, St. Louis, MO) was made in 100% ethanol. All drugs were diluted in 0.1 M PBS, pH 7.4. An equal amount of 100 mM HCl was added when diluting APDC to adjust the pH. All drugs were injected subcutaneously in a volume of 10 μl into the plantar surface of the hindpaw. Appropriate vehicles were prepared as the diluents for each drug.

Cell culture. DRGs were isolated from 4- to 6-week-old ICR mice. Isolated ganglia were placed into 4°C PBS without Mg²⁺ or Ca²⁺ (Invitrogen, Paisley, UK). Ganglia were incubated in 15 U/ml papain/L-cysteine (Worthington, Freehold, NJ) in HBSS (Invitrogen) for 20 min at 37°C, then washed with HBSS three times, followed by a 20 min incubation at 37°C in 1.5 mg/ml collagenase (Sigma) in HBSS. Ganglia were washed with HBSS three times before trituration with fire-polished Pasteur pipets. Dissociated cells were plated at ~3000 cells/well on 12 mm glass coverslips coated with poly-D-lysine and collagen (Sigma). Cells were cultured for 5–6 d at 37°C in humidified air with 5% CO₂. The culture medium contained neurobasal medium, 10% FBS, 100 U/ml penicillin/streptomycin, and 2 mM Glutamax (all from Invitrogen). In experiments in which pertussis toxin (PTX; Sigma) was used, 500 ng/ml PTX was added into the medium the night before.

Calcium imaging. Cells were loaded with 10 ng/ml fura-2 AM (Molecular Probes, Eugene, OR) in HBSS for 1 hr and then washed three times and incubated in fresh HBSS for ~45 min before the experiments were performed. A coverslip was placed in the perfusion chamber (~200 μl volume) and perfused with HBSS at 2 ml/min. Cells were viewed under an inverted 1×70 microscope (Olympus Optical, Tokyo, Japan). Images were captured with a Hamamatsu (Shizouka, Japan) Orca cooled charge-coupled device camera and recorded and analyzed using the SimplePCI software package with the dynamic intensity analysis module (Compix, Tualatin, OR). Traces are all expressed as the ratio of fluorescence emission at an excitation wavelength of 357 and 380 nm, respectively. All experiments were performed at room temperature.

Drug application. (2*R*,4*R*)-APDC, LY341495, and PGE₂ stock solutions were made as described above. Capsaicin (Sigma) stock solution was made in ethanol. Forskolin (Sigma) stock solution was made in DMSO. IBMX (Sigma) and 8-bromo-cAMP (Sigma) stock solutions were made in deionized water. All drugs were diluted to final concentrations in HBSS and applied via bath perfusion.

Data analysis. Off-line analysis was performed using Microcal Origin (Microcal Software, Inc., Northampton, MA). Data are expressed as means ± SEM. Treatment effects were analyzed by one-way ANOVA followed by Tukey's *post hoc* multiple comparisons using GraphPad Prism (GraphPad Software Inc., San Diego, CA). The time course of the effects of PGE₂ was analyzed by two-way ANOVA followed by Bonferroni post-tests. Error probabilities of *p* < 0.05 were considered statistically significant.

RESULTS

Activation of peripheral group II metabotropic glutamate receptors blocks prostaglandin E-induced thermal hyperalgesia

To test whether peripheral group II mGluRs can regulate thermal nociception, APDC, a selective group II mGluR agonist, was injected subcutaneously into the plantar surface of a mouse hindpaw. As shown in Figure 1*b*, APDC did not alter baseline thermal withdrawal latency, which is consistent with previous reports (Walker et al., 2001; Sharpe et al., 2002). To test the

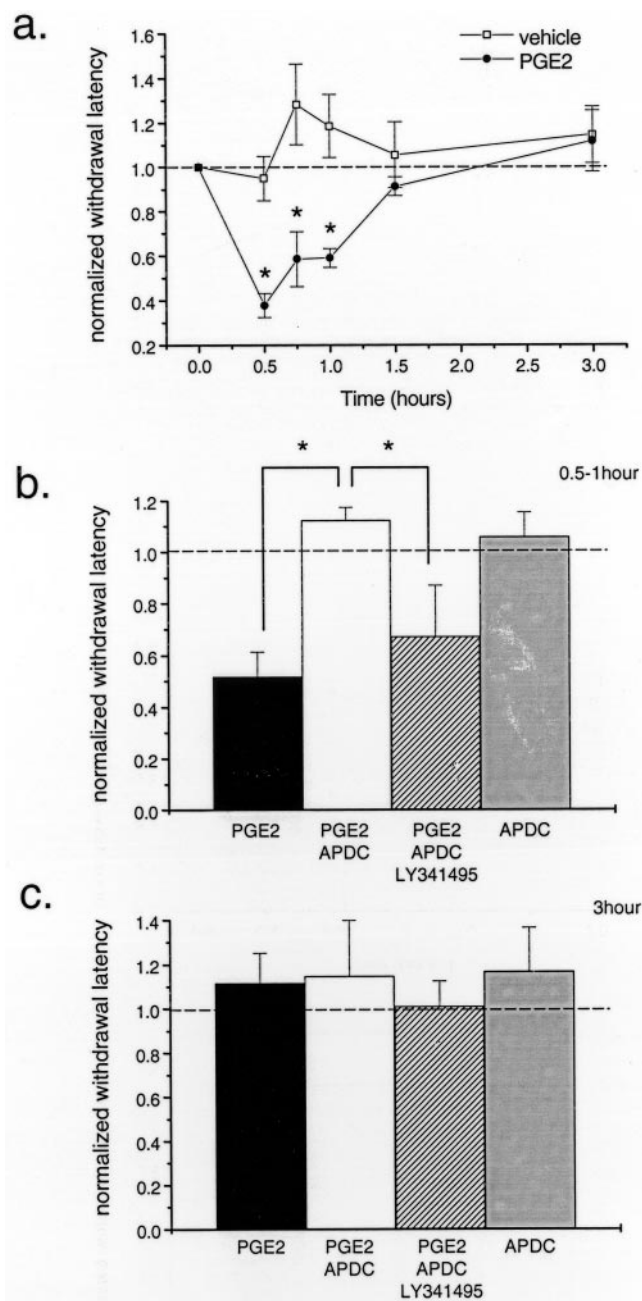


Figure 1. Activation of peripheral group II mGluRs blocks PGE₂-induced thermal hyperalgesia. *a*, PGE₂ (100 ng in 10 μl) injected subcutaneously into the plantar surface of a mouse hindpaw decreased withdrawal latency to radiant heat applied to the hindpaw. Asterisks indicate time points at which PGE₂ (*n* = 6) was significantly different from vehicle (*n* = 7). *b*, Normalized thermal withdrawal latencies at 0.5, 0.75, and 1 hr were averaged and compared with baseline values. Injection of APDC (20 ng in 10 μl; *n* = 6) had no effect alone, but coinjection of APDC (*n* = 6) blocked PGE₂-induced hyperalgesia. LY341495 (0.2 ng in 10 μl; *n* = 7) blocked the APDC effect. *c*, At 3 hr, all groups were statistically indistinguishable from baseline. In all panels, dashed lines indicate the basal response.

hypothesis that group II mGluRs could play a role in nociception when responses to heat are sensitized by the activation of G_s-coupled receptors, 100 ng of PGE₂ was injected subcutaneously into the plantar surface of the hindpaw to induce thermal hyperalgesia. PGE₂-induced thermal hyperalgesia lasted >1 hr and

peaked at or before 30 min (Fig. 1*a*), which is consistent with previous results (Kerr et al., 2001). Coinjection of APDC with PGE₂ blocked PGE₂-induced thermal hyperalgesia (Fig. 1*b*). LY341495, a selective group II mGluR antagonist, completely blocked the APDC effect (Fig. 1*b*). Three hours after injection, when PGE₂-induced hyperalgesia had recovered, all groups showed no significant difference from the baseline (Fig. 1*c*).

Group II metabotropic glutamate receptors block prostaglandin E₂-induced sensitization of capsaicin responses

The above results suggest that activation of peripheral group II mGluRs does not affect basal thermal sensitivity but blocks PGE₂-induced thermal hyperalgesia. The VR1, or capsaicin receptor, is a key component of the thermal transduction machinery; it is critical for the development of thermal hyperalgesia (Caterina et al., 1997; Tominaga et al., 1998; Davis et al., 2000). PGE₂ sensitizes capsaicin receptors by activating the cAMP/PKA pathway (Pitchford and Levine, 1991; Lopshire and Nicol, 1998). To test the hypothesis that group II mGluRs block PGE₂-induced thermal hyperalgesia by blocking PGE₂-induced potentiation of capsaicin receptor function, we investigated the effects of PGE₂ and APDC on capsaicin responses in cultured mouse DRG neurons.

VR1 is a cation channel that allows Na²⁺ and Ca²⁺ influx when activated by heat, protons, or capsaicin. Therefore, we studied VR1 function by measuring capsaicin-induced calcium influx into DRG neurons. In 20% of DRG neurons, application of capsaicin induced increases in intracellular Ca²⁺ of varying amplitude and duration. Neurons with responses <10 min in duration were included in this study (Fig. 2). Ten minutes after the first capsaicin application, a second identical capsaicin application produced a smaller response than the first, indicating desensitization of capsaicin receptors. The ratio of the amplitude of the second response to that of the first was calculated as the “response ratio.”

As shown in Figure 2, when PGE₂ was applied between the two capsaicin responses, the response ratio was significantly increased ($p < 0.001$; ANOVA). When APDC alone was applied, there was no significant change in desensitization (Fig. 2), which is consistent with our behavioral result that APDC did not change baseline thermal sensitivity. However, when APDC was coapplied with PGE₂, the response ratio was significantly decreased compared with cells treated with PGE₂ ($p < 0.05$; ANOVA). LY341495, a selective group II mGluR antagonist, completely blocked the effect of APDC ($p < 0.05$), suggesting that the effects of APDC were attributable to the activation of group II mGluRs.

Group II metabotropic glutamate receptors block prostaglandin E₂ sensitization of capsaicin responses by activating G_i and inhibiting AC

To test the hypothesis that group II mGluRs function by activating G_i, we treated cells with PTX (500 ng/ml overnight) before calcium-imaging experiments. PTX induces ADP ribosylation of G_i and impairs the interaction of G_i with receptor–ligand complexes, uncoupling receptors from downstream second messenger systems (Hsia et al., 1984; Moss and Vaughan, 1984). Treatment with PTX did not affect capsaicin responses or the effects of PGE₂, whereas the effect of APDC was abolished (Fig. 3).

Previous experiments suggest that group II mGluRs function by activating G_i. To test the hypothesis that APDC blocks PGE₂-induced sensitization by inhibiting AC, we investigated whether activation of group II mGluRs could block the effects

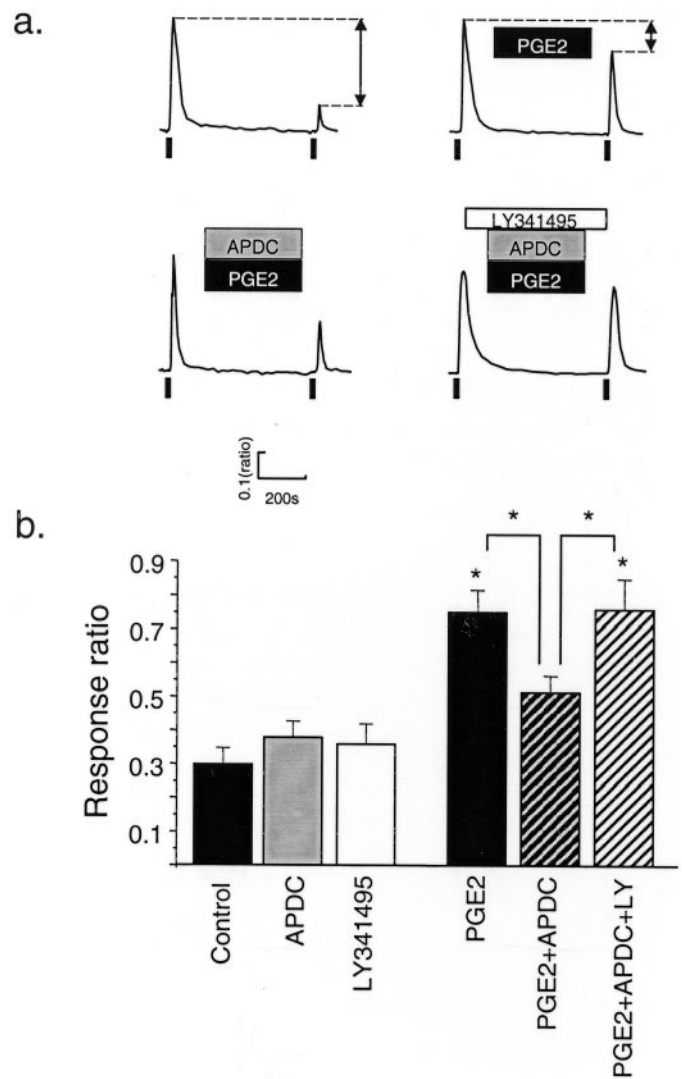


Figure 2. Group II mGluRs block PGE₂ enhancement of capsaicin receptor function. *a*, Representative traces of capsaicin-induced calcium responses recorded from cultured DRG neurons; 20 nM capsaicin was applied twice (bars below the traces; 21 sec each), inducing pronounced desensitization of the calcium response. PGE₂ (200 nM, 7 min) decreased this desensitization of capsaicin responses (note the arrows compared with control). APDC (10 μ M) blocked the PGE₂ effect, and LY341495 (100 nM) blocked the APDC effect. Dashed lines show the peaks of the responses. *b*, Means \pm SEM for the data shown in *a*; $n = 28$ –60; $*p < 0.05$.

of forskolin. Forskolin directly activates AC (Birnbauer et al., 1983), and this effect of forskolin can be inhibited by activation of G_i (Hildebrandt et al., 1982). cAMP synthesized by AC is rapidly metabolized within cells. To increase and prolong the effect of forskolin, we coapplied IBMX, an inhibitor of cAMP phosphodiesterase. Application of forskolin plus IBMX produced effects similar to those of PGE₂ (Fig. 4). Application of APDC blocked the ability of forskolin to enhance capsaicin responses.

The results described above suggest that group II mGluRs block sensitization by PGE₂ or forskolin at or downstream of AC. Because IBMX is present, group II mGluRs were not acting on phosphodiesterases. To rule out the possibility that group II mGluRs are acting on PKA or other downstream effectors, we used 8-bromo-cAMP, a nonhydrolyzable analog of cAMP, to

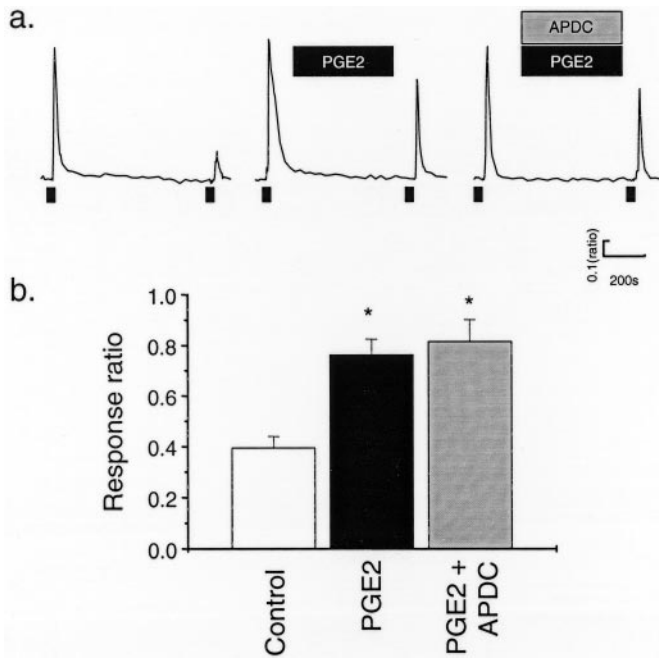


Figure 3. Group II mGluRs block PGE₂ enhancement of capsaicin receptor function via a PTX-sensitive G-protein. *a*, Representative traces of the effect of PGE₂ (200 nM, 7 min) in the presence and absence of APDC (10 μM) in cells treated with PTX (500 ng/ml, overnight). In PTX-treated cells, PGE₂ enhanced capsaicin responses, but APDC failed to block the PGE₂ effects. Bars below traces represent time of capsaicin application. *b*, Means ± SEM; *n* = 51–73; **p* < 0.05.

modulate capsaicin receptors. 8-Bromo-cAMP produced effects similar to those of PGE₂ (Fig. 4). However, the effects of 8-bromo-cAMP were not blocked by APDC, suggesting that group II mGluRs are not acting on PKA or its downstream targets.

DISCUSSION

Agonists of group II mGluRs have been shown to act systemically as analgesics (Sharpe et al., 2002). Furthermore, these drugs depress excitatory synaptic transmission in the substantia gelatinosa (Gerber et al., 2000) and block capsaicin-induced central sensitization in spinothalamic tract neurons (Neugebauer et al., 2000). The observation that group II mGluRs are expressed on peripheral terminals of primary sensory neurons suggests that they might also act peripherally to produce antinociceptive effects. Here we have shown that group II mGluRs do not affect basal thermal nociception, but that activation of these receptors blocks the development of hyperalgesia induced by PGE₂.

The fact that group II mGluRs couple to G_i and the fact that the cAMP/PKA pathway is critical in the sensitization of nociception led us to hypothesize that the mechanism by which group II mGluRs block PGE₂-induced thermal hyperalgesia is via inhibition of AC. PGE₂ significantly decreased desensitization of capsaicin-induced Ca²⁺ influx in sensory neurons. Activation of group II mGluRs did not change capsaicin responses but significantly attenuated PGE₂-induced sensitization, which is consistent with our findings that APDC had no effect on basal thermal sensation but did block PGE₂-induced hyperalgesia. PTX treatment abolished this APDC effect. In agreement with previous reports that PGE₂ exerts its effects via the cAMP/PKA pathway, application of forskolin or 8-bromo-cAMP also sensitized capsaicin responses. Group II mGluR activation blocked the effects of

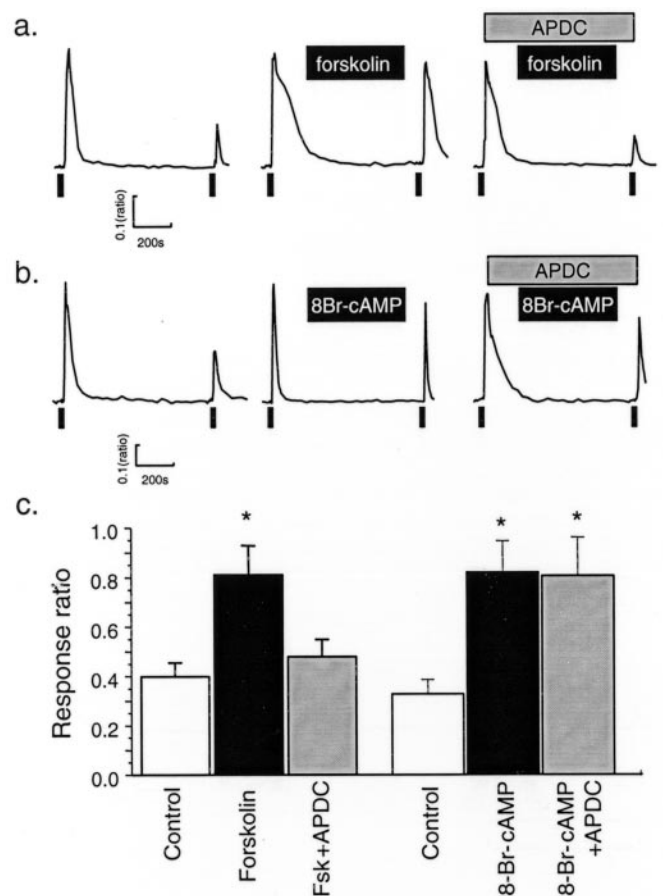


Figure 4. Group II mGluRs block sensitization induced by the activation of AC but not sensitization induced by the direct activation of PKA. *a*, Representative traces of the effect of forskolin (50 μM) plus IBMX (100 μM) (7 min) in the absence and presence of APDC (10 μM). Forskolin produced an enhancement of capsaicin responses similar to that of PGE₂, which was blocked by APDC. *b*, Representative traces of the effect of 8-bromo-cAMP (100 μM) (7 min) in the absence and presence of APDC (10 μM). 8-bromo-cAMP produced an enhancement of capsaicin responses similar to that of PGE₂, and this enhancement was not reduced by APDC. Bars below traces represent time of capsaicin application. *c*, Means ± SEM; *n* = 30–47 for each group; **p* < 0.05. Fsk, Forskolin.

forskolin plus IBMX but not the effect of 8-bromo-cAMP. These results indicate that group II mGluRs block PGE₂-induced sensitization by activating G_i and inhibiting AC.

Although there are many G_i-coupled receptors, their inhibitory effects on AC were characterized primarily in heterologous expression systems in which overexpression of a protein might produce nonphysiological effects. Only in a very few cases have the physiological functions of a G_i-coupled receptor been shown to be attributable to the inhibition of AC. Group II mGluRs are coupled to G_i. In the CNS, the mechanism of these effects is poorly understood, despite the fact that they have been shown to produce various effects, including presynaptic modulation of synaptic transmission (Macek et al., 1996) and regulation of long-term depression and long-term potentiation (O’Leary and O’Connor, 1998; Otani et al., 1999). The present study indicates that peripheral group II mGluRs attenuate PGE₂-induced thermal hyperalgesia and suggests that this effect is mediated by blocking PGE₂-mediated enhancement of capsaicin responses. Our results also indicate that this was attributable to the activation of G_i and inhibition of AC after group II mGluR activation.

Many inflammatory mediators, including PGE₂ (Cui and Nicol, 1995; Hingtgen et al., 1995; Mnich et al., 1995), prostaglandin I₂ (Murata et al., 1997), adenosine (Sawynok et al., 1997), serotonin (Taiwo and Levine, 1992), and 8*R*,15*S*-dihydroxyeicosatetraenoic acid (Levine et al., 1986), activate the cAMP/PKA pathway to induce hyperalgesia. Group II mGluR agonists are likely to inhibit the production of cAMP induced by other inflammatory mediators and, accordingly, might reduce the nociceptive sensitization induced by these agents.

It is interesting to note that the expression of group II mGluRs changes in several pain models. L-Acetylcarnitine, an analgesic used to treat neuropathic pain, acts by upregulating mGluR2 in DRG neurons (Chiechio et al., 2002). UV-irradiation-induced peripheral inflammation increases the expression of mGluR3 mRNA in the dorsal horn (Boxall et al., 1998). mGluR3 mRNA is increased in the cerebral cortex of monoarthritic rats (Neto et al., 2001). Although our results suggest that activation of peripheral group II mGluRs can fully block PGE₂-induced thermal hyperalgesia, it would be interesting to investigate whether they have more significant effects in prolonged pain models.

We have shown recently that peripheral group I mGluRs also modulate nociception. Peripherally applied (*RS*)-3,5-dihydroxyphenylglycine, a selective group I mGluR agonist, dose-dependently induces thermal hyperalgesia; antagonists of group I mGluRs can prevent and attenuate formalin-induced pain (Bhave et al., 2001). The present data indicate that peripheral group II mGluRs play an opposing role in modulating nociception. During inflammation, glutamate is released into the peripheral injury site and is thought to play a role in inducing inflammatory pain. Although it is unknown whether group I mGluRs and group II mGluRs are colocalized in the same peripheral sensory afferents, the activation of group II mGluRs might act as a balancing mechanism to counteract some of the effects of glutamate. It would be interesting to investigate whether group II mGluRs provide tonic inhibition of nociception and whether inhibition of group II mGluRs would result in more significant nociceptive sensitization. As mentioned above, the expression of group II mGluRs increases in several pain models; this might act as a protective mechanism to prevent additional sensitization of nociception in situations in which nociceptive thresholds are already decreased. Additional experiments will need to be performed to investigate whether glutamate acts to induce hyperalgesia at the beginning of inflammation while at the same time producing an analgesic effect during prolonged inflammation when there are more group II mGluRs present.

Our study is the first to demonstrate a role for peripheral group II mGluRs in the modulation of nociception. These results do not rule out the possibility that group II mGluRs also modulate inflammatory pain through central mechanisms. However, our results indicate that activation of peripheral group II mGluRs completely blocks the thermal hyperalgesia induced by PGE₂. In addition to the induction of inflammatory pain, PGE₂ might also contribute to the maintenance or progression of inflammatory pain, because inflammation can be accompanied by a sustained increase in PGE₂ levels (Davies et al., 1984). Peripheral group II mGluRs can potentially be targeted to control inflammatory pain while at the same time avoiding the possible CNS side effects of systemic administration.

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