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Group II metabotropic glutamate receptor activation on peripheral nociceptors modulates TRPV1 function

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Abstract

Transient receptor potential vanilloid 1 (TRPV1) receptors are critical to nociceptive processing. Understanding how these receptors are modulated gives insight to potential therapies for pain. We demonstrate using double labeling immunohistochemistry that Group II metabotropic glutamate receptors (mGluRs) are co-expressed with TRPV1 on rat dorsal root ganglion (DRG) cells. In behavioral studies, intraplantar 0.1 μ M APDC, a group II agonist, significantly attenuates capsaicin-induced nociceptive behaviors through a local effect. The APDC-induced inhibition of capsaicin responses is blocked by 1 μ M LY341495, a group II antagonist. At the single fiber level, nociceptor responses to capsaicin are significantly decreased following exposure to APDC and this effect is blocked by LY341495. Finally, activation of peripheral group II mGluRs inhibits forskolin-induced thermal hyperalgesia and nociceptor heat sensitization, suggesting group II receptors are negatively coupled to the cAMP/PKA pathway. The data indicate that group II mGluRs and TRPV1 receptors are co-expressed on peripheral nociceptors and activation of mGluRs can inhibit painful sensory transmission following TRPV1 activation. The data are consistent with group II and TRPV1 receptors being linked intracellularly by the cAMP/PKA pathway. Peripheral group II mGluRs are important targets for drug discovery in controlling TRPV1-induced nociception.

1. Introduction

The transient receptor potential vanilloid 1 (TRPV1) receptor is considered a molecular integrator of chemical and physical stimuli (Caterina et al., 1997; Tominaga et al., 1998). These receptors are localized almost exclusively on nociceptors and when activated, they can result in intense painful sensations (Caterina and Julius, 2001). TRPV1 knock-out (KO) mice have relatively normal responses to acute noxious thermal and mechanical stimulation. In pain models, the TRPV1 KO's develop formalin-induced pain behaviors, carrageenan-evoked mechanical hyperalgesia and nerve-injury-induced mechanical hyperalgesia, (Davis et al., 2000; Caterina et al., 2000; Bolcskei et al., 2005). However, they show an obvious lack of heat hyperalgesia following carrageenan-induced inflammation (Davis et al., 2000; Caterina et al., 2000; Bolcskei et al., 2005) and a significant reduction in the thermal and mechanical hyperalgesia following a mild burn injury (Bolcskei et al., 2005). Thus, modulation of the TRPV1 receptor could be key to controlling pathophysiological pain.

Activation of group II metabotropic glutamate receptors (mGluRs) has been shown to play a role in reducing spinal cord injury pain (Mills et al., 2002), neuropathic pain (Simmons et al., 2002; Chiechio et al., 2002) and various types of inflammatory pain (Sharpe et al., 2002; Simmons et al., 2002; Yang and Gereau, 2003). Importantly, behavioral studies demonstrate

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group II activation can block prostaglandin E₂ (PGE₂)- and carrageenan-induced mechanical allodynia (Yang and Gereau, 2003) as well as intradermal capsaicin (CAP)-induced central sensitization of dorsal horn cells (Neugebauer et al., 2000).

The demonstration that primary sensory neurons express group II mGluRs (Carlton et al., 2001b) and their activation results in a significant reduction in PGE₂-induced potentiation of CAP responses (Yang and Gereau, 2002) offered the first suggestion that peripheral group II mGluRs might be an important target for the development of novel peripheral analgesics. In the present study, we further investigate peripheral group II mGluRs expressed on cutaneous nociceptors and their role in modulating TRPV1 function. We demonstrate using double labeling immunohistochemistry that group II mGluRs co-localize with TRPV1 receptors on small to medium diameter dorsal root ganglion (DRG) cells, providing a morphological basis for interaction of these receptors. We show that intraplantar injection of group II agonists inhibits CAP- and forskolin (FK)-induced nociceptive behaviors. *In vitro* electrophysiological recordings in the glabrous skin show that group II agonists attenuate CAP-induced excitation of nociceptors and FK-induced heat sensitization. Some of these data have been previously presented in abstract form (Zhou and Carlton, 2005; Du and Carlton, 2005).

2. Results

2.1. Co-localization of Group II mGluR and TRPV1 in DRG

Single- and double-labeled profiles were counted in DRG sections from two L5 ganglia from two rats. Immunohistochemical staining for either receptor resulted in a homogenous reaction product that filled the cytoplasm but did not stain the nucleus (Fig. 1). The counts demonstrated that $39 \pm 7\%$ of neuronal profiles were labeled positively for mGluR2/3 and $42 \pm 5\%$ were labeled positively for TRPV1. Of neuronal profiles expressing mGluR2/3, all (100%) were double labeled for TRPV1. In contrast $93 \pm 5\%$ of the TRPV1 cells also expressed mGluR2/3. The mean diameter of single mGluR2/3- and TRPV1-labeled cells was 21.8 ± 3.7 and 21.7 ± 3.6 μm , respectively; for double-labeled profiles it was 21.8 ± 3.7 μm .

2.2. Group II mGluR modulation of CAP-induced pain behaviors

Intraplantar injection 0.1% CAP ($n = 6$) evoked pain behaviors including flinching and L/L and co-injection of 0.1 μM APDC (a group II agonist) with CAP ($n = 6$) significantly reduced both of these behaviors (Fig. 2 A and B, $p < 0.05$). Injection of the hind paw with the group II antagonist LY + APDC, followed by CAP ($n = 6$) blocked the APDC effect. These rats showed nociceptive behaviors that were no different from rats injected with CAP alone. The APDC reduced CAP-evoked behaviors through local activation of group II receptors since injection of APDC in one hind paw did not affect the behavior evoked by CAP injected in the other hind paw (Fig. 2A and B, APDC contra). The time courses are illustrated in Fig. 2C and D. Intraplantar injection of APDC alone (Du et al., 2008) or 1 μM LY alone produced little or no behavioral response (not significantly different from PBS injection).

2.3. Group II mGluR modulation of CAP-induced activity in nociceptors

Application of 0.05% CAP to the receptive fields of C-mechanoheat (CMH) units ($n = 10$) induced a robust excitation that persisted for the 2 min application period. Activity increased from a background discharge rate of 0.04 ± 0.02 imp/s to 0.33 ± 0.14 imp/s for CAP alone (Fig. 3A, $p < 0.05$). Application of LY alone for 2 min resulted in a discharge rate of 0.05 ± 0.02 imp/s and this was not different from background. Co-application of 1.0 μM APDC + 0.05% CAP ($n = 13$) attenuated the CAP-induced response (Fig. 3B). The discharge rate (0.11 ± 0.02 imp/s) was significantly reduced compared to CAP alone ($p < 0.05$). Closer inspection of the oscilloscope traces in Figures 4A and B shows that the initial CAP-induced excitation still occurs in the presence of APDC but the later phase of activity during the 2 min application

is greatly attenuated. Co-application of 1.0 μM APDC + 1 μM LY (group II antagonist) with CAP ($n = 8$) blocked the inhibitory effect of APDC, resulting in a CAP-induced increase in discharge rate (0.67 ± 0.22 imp/s) that was not different from CAP alone (Fig 3B). An oscilloscope trace from a representative unit shows that the initial CAP-induced excitation as well as the later phase of activity is restored in the presence of LY (Fig. 4C).

2.4. Group II mGluR modulation of the cAMP/PKA pathway

In behavioral studies, intraplantar injection of 10 μM FK produced a thermal hyperalgesia; the paw withdrawal latencies (PWLs) of FK-injected rats decreased to $62 \pm 7\%$ of baseline compared to PBS-injected rats which had PWLs that were $95 \pm 3\%$ of baseline (Fig. 5). If 0.5 μM ADPC was injected with FK, it prevented the FK-induced decrease in PWL ($82 \pm 3\%$ of baseline) and thus prevented the heat sensitization. In the skin-nerve preparation, exposure of CMH units to 5 μM FK ($n = 8$) sensitized heat responses evidenced by a reduced threshold (39.14 ± 1.19 $^{\circ}\text{C}$) compared to the control group (43.35 ± 0.79 $^{\circ}\text{C}$, $p < 0.05$) and an increased discharge rate (2.79 ± 0.73 imp/s) compared to control (1.02 ± 0.26 imp/s, $p < 0.05$, Fig. 6A and B). In another group of CMH units, application of FK in the presence of 10 μM H89 (a PKA inhibitor, $n = 7$) prevented FK-induced sensitization as there was no significant change in heat threshold (42.32 ± 0.85 $^{\circ}\text{C}$) or discharge rate (1.41 ± 0.25 imp/s) compared to control. Application of 0.5 μM APDC mimicked the effect of H89, preventing FK-induced sensitization such that there was no significant change in heat threshold (40.87 ± 0.99 $^{\circ}\text{C}$) or discharge rate (1.57 ± 0.55 imp/s) compared to control. Exposure of nociceptors to H89 alone had no effect and application of a low (5 μM) and high (50 μM) concentration of dideoxyforskolin, (an inactive analog of forskolin) had no effect. The data are consistent with group II mGluR producing inhibition through modulation of the cAMP/PKA intracellular pathway.

3. Discussion

These studies demonstrate several key aspects of peripheral group II mGluR function in relation to TRPV1. Double-labeling immunohistochemistry verifies that group II mGluRs are localized on small diameter DRG cells expressing TRPV1 receptors, providing a morphological basis for direct group II mGluR modulation of TRPV1-induced activity. Activation of peripheral group II mGluRs inhibits CAP-induced nociceptive behaviors and excitation and sensitization of nociceptors in a forskolin-dependent manner.

3.1. Group II mGluR co-localization with nociceptive markers

Using double labeling immunohistochemistry, this study provides the first anatomical evidence for co-localization of group II mGluR and TRPV1 receptors on a subpopulation of DRG cells. This is consistent with a previous study showing group II mGluR blockade of PGE₂-induced potentiation of CAP-induced responses in cultured DRGs (Yang and Gereau, 2002). The percentages of single labeled mGluR2/3 and TRPV1 neuronal profiles are consistent with previously published estimates (Guo et al., 1999; Michael and Priestley, 1999; Carlton et al., 2001b; Carlton et al., 2004; Carlton and Hargrett, 2007). Quantification of double-stained profiles indicates that virtually all mGluR2/3-expressing cells also express TRPV1, however, a small population of TRPV1 positive cells do not express mGluR2/3. In addition to being highly co-localized with TRPV1, mGluR2/3 is also localized in sensory neurons expressing other “nociceptive” markers such as isolectin Griffonia simplicifolia (I-B4) (Guo et al., 1999; Carlton et al., 2001b) and P2X3 (Guo et al., 1999). It is unknown if the mGluR2/3-TRPV1 expressing population overlaps with these because triple labeling has not been done, however, there is significant co-localization of TRPV1 with IB4 (~45-78%) (Guo et al., 1999; Aoki Y et al., 2005; Breese NM et al., 2005), purinoreceptor P2X3 (75%) (Guo et al., 1999) and calcitonin gene related peptide (CGRP, 79%) (Guo et al., 1999). Thus, it is likely the group II-TRPV1 expressing cells express other nociceptive markers.

3.2. Group II mGluR modulation of pain behaviors following sensitization

Based on the anatomical studies cited above, there is a high probability that group II mGluRs are co-localized with other receptors that are key contributors to peripheral pain transmission in a variety of pain models. Systemic or intraplantar pretreatment with group II agonists reduces formalin-induced pain behaviors (Simmons et al., 2002; Zhou and Carlton, 2005), prevents carrageenan-induced thermal hyperalgesia (Sharpe et al., 2002), complete Freund's adjuvant-induced mechanical allodynia (Zhou and Carlton, 2005), and PGE₂-mediated thermal hyperalgesia and mechanical allodynia (Yang and Gereau, 2002; Yang and Gereau, 2003). The group II antagonist LY significantly prolongs the mechanical allodynia resulting from PGE₂ and carrageenan injection (Yang and Gereau, 2003). The antagonist actions demonstrate that endogenous activation of group II receptors can impede the peripheral action of inflammatory mediators and/or sensitizing agents. For the first time, this study demonstrates that intraplantar injection of a group II agonist has a robust effect in reducing CAP-induced pain behaviors. Based on our antagonist data, we believe these agonist effects are receptor specific. APDC alone has no effect on nociceptor activity and does not produce nociceptive behavior (Du et al., 2008). APDC used at 0.1-1.0 μ M is very close to the ED₅₀ (0.4 μ M, based on an assay analyzing inhibition of FK-stimulated cAMP in non-neural cells, [Schoepp et al., 1999]). Although group I and III mGluRs are also expressed by primary afferents (Ohishi et al., 1995; Zhou et al., 2001; Azkue et al., 2001; Carlton and Hargett, 2007), it is unlikely that they contribute to these actions because APDC, used at this low concentration, does not bind to group I or group III mGluRs (Schoepp et al., 1996, 1999). The group II antagonist LY does have some affinity for group I and III mGluRs (Schoepp et al., 1999). However, when used at 1 μ M, a concentration that is further diluted (10-100X) before it reaches its target in the skin, LY is unlikely to have any significant effect on Group I or III mGluRs (Kingston et al., 1998).

3.3. Group II mGluR modulation of nociceptor activity

We have reported that peripheral group II mGluR activation does not depress nociceptive behaviors or nociceptor unit responses in non-sensitized states but can depress responses when nociceptors are sensitized by exposure to inflammatory soup (Du et al., 2008). We extend these findings, demonstrating for the first time that group II mGluR agonist-induced inhibition can reduce cutaneous nociceptor activity in response to TRPV1 activation. Although TRPV1 activation contributes to the heat response of nociceptors, this is a brief (10 sec in our paradigm), non-sensitizing heat stimulus. However, chemical activation of TRPV1 with capsaicin is exquisitely painful in humans. Intraplantar injection causes nociceptive behaviors in rodents and an inflammatory response lasting 20-30 min producing primary afferent sensitization. Thus, the data presented here concerning group II mGluR actions are in agreement with those indicating effectiveness only in the sensitized state (Yang and Gereau, 2002; Sharpe et al., 2002; Yang and Gereau, 2003; Du et al., 2008). The single fiber recordings demonstrate that the initial CAP-induced excitation (10-40s following application) is preserved in the presence of the group II agonist but the later activity during the CAP application (60-120 s) is greatly reduced or eliminated. The initial nociceptor excitation is mediated by the activation of non-sensitized TRPV1 receptors. The later activity probably reflects the sensitization of the TRPV1 receptors and it is this activity that is blocked by the group II mGluR agonist. While it is possible that cutaneous APDC is having an inhibitory effect on nociceptors through an indirect pathway (activation of Schwann cells or dermal/epidermal components), this is highly unlikely given that mGlu2/3 receptors have been localized on unmyelinated (C fibers) and small myelinated fibers (presumed A δ) in digital nerves (Carlton et al., 2001b). The data strongly suggest that administration of selective group II agonists may be potent therapeutic agents for prevention of peripheral sensitization and for treatment of inflammatory pain.

3.4. Group II mGluR modulation of the cAMP/PKA pathway

Several lines of evidence indicate both group II mGluR and TRPV1 receptors are linked to the intracellular cAMP/PKA second messenger system. Group II mGluRs are negatively coupled to this system in comparison to TRPV1 receptors, which are positively coupled. For example, activation of group II mGluRs inhibits adenylate cyclase and the cAMP/PKA pathway (Tanabe et al., 1993; Schoepp and Conn, 1993), reduces basal levels of cAMP (Harris et al., 2004) and inhibits FK-stimulated cAMP formation (Schoepp et al., 1995). In contrast, cAMP-PKA activity will enhance CAP-activated currents (Pitchford and Levine, 1991; Lopshire and Nicol, 1998), potentiate TRPV1 sensitization (De Petrocellis et al., 2001; Bhawe et al., 2002) and reduce desensitization of CAP-activated currents (Mohapatra and Nau, 2003; Mohapatra and Nau, 2005). Importantly, in cultured primary sensory neurons, group II mGluR activation inhibits adenylate cyclase, reversing the PGE₂-induced sensitization of TRPV1 receptors (Yang and Gereau, 2002). Thus, it is highly likely that group II mGluRs modulate TRPV1 receptors through the cAMP/PKA pathway. This pathway is forskolin-dependent since the inactive analogue dideoxyforskolin did not result in sensitization of the nociceptors. Group II mGluRs are coupled to other effector systems (Schoepp and Conn, 1993; Schoepp, 1994), as are TRPV1 receptors (Premkumar and Ahern, 2000; Vellani et al., 2001; Numazaki et al., 2002; Olah et al., 2002; Sun R et al., 2007). However, it has been demonstrated that inflammatory hyperalgesia is maintained by PKA, and this maintenance is not dependent on upstream or downstream activity in the cAMP/PKA pathway (Aley and Levine, 1999). Thus, inhibiting PKA is key in controlling hyperalgesia. Our single fiber data demonstrate that APDC will block FK-induced heat sensitization in a manner similar to the PKA inhibitor H89. These data are consistent with the hypothesis that the cAMP/PKA pathway is the intracellular link between the TRPV1 and group II mGluR receptors. Activation of group II mGluRs could down regulate cAMP/PKA, reducing the phosphorylation/upregulation of TRPV1 receptors.

3.5. Conclusion

Using immunohistochemistry we demonstrate that group II mGluRs are highly co-localized with TRPV1 receptors on primary sensory neurons. Activation of these receptors on peripheral cutaneous fibers reduces CAP-induced pain behaviors which is blocked by the group II antagonist LY. At the single fiber level, APDC will block CAP-induced excitation of nociceptors and mimic the effect of H89 on FK-induced heat sensitization, demonstrating that these receptors are linked intracellularly by the cAMP-PKA pathway. These data identify group II mGluRs as important peripheral targets for new analgesics for inflammatory pain.

4. Experimental procedures

All experiments were approved by the University Animal Care and Use Committee and followed the International Association for the Study of Pain (IASP) guidelines for the ethical care and use of laboratory animals (Zimmermann, 1983). Steps were taken to minimize both the number of animals used and their discomfort. All rats were male, Sprague Dawley (SD) obtained from Harlan, Indianapolis, IN.

4.1. Immunohistochemical Staining: Double labeling of DRG sections

Rats (n=2) were transcardically perfused with 4% paraformaldehyde with 0.1% picric acid in 0.1 M phosphate buffer (PB) and the 5th lumbar dorsal root ganglia (L5 DRG) from both sides were removed and placed overnight in PB-sucrose for cyroprotection. Frozen sections were cut at 8 µm on a cryostat, placed on gel-coated microslides and allowed to air dry before immunostaining. Percentages of single- and double-labeled DRG cells were generated from the same sections exposed to two different antibodies made in different species. Slides were incubated in rabbit anti-mGluR2/3 (1-2 µg/ml, Chemicon, Temecula, CA, USA, catalog # AB1553) for 48 h at room temperature. After rinsing in 0.1 M phosphate buffered saline (PBS,

pH 7.4), the slides were placed in biotinylated anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h, rinsed in PBS and incubated in Vectastain Elite ABC peroxidase reagent (avidin-biotin complex, Vector Laboratories) for 1 h. After rinsing in PBS, sections were incubated in fluorescein-labeled tyramide (1:75, Perkin-Elmer Life Science, Boston, MA, USA) for 7 min, followed by a wash in PBS. To inactivate any residual peroxidase from the first ABC reaction, slides were placed in 0.3% H₂O₂ in PBS for 20 min and rinsed in PBS before incubation in goat anti-TRPV1 antiserum (1:8000-10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, catalog # sc-8671) for 48 h at room temperature. After PBS rinses, slides were placed for 1 h in cyanine 3 (Cy3) conjugated anti-goat IgG (1:400, Jackson ImmunoResearch, West Grove, PA, USA). To minimize loss of fluorescent label, all slides were coverslipped with Vectastain® mounting media (Vector Laboratories).

Absorption controls were performed to confirm antibody specificity. The mGluR2/3 antisera was pre-absorbed with a peptide fragment (NGREVV DSTTSSL, C-terminus) synthesized by Sigma Genosys (Woodlands, TX, USA). The TRPV1 antisera was pre-absorbed with a peptide fragment (Santa Cruz Biotechnology) corresponding to amino acids 773-837 near the C-terminal. In each case the appropriate peptide was incubated with the working dilution of the antiserum (100 µg peptide/1 ml diluted antisera) overnight at room temperature. Immunostaining with these solutions resulted in a complete lack of staining of cells in DRG sections. In additional control sections, normal goat serum was substituted for each antibody and the staining done as described above. Again no specific or positive staining was seen in these sections.

To visualize labeled profiles, sections were viewed with an Olympus BX51 microscope and a 20X objective using Fluorescein isothiocyanate (FITC) and Cy3 filter cube sets. A 24 bit-color digitized image of each was captured with a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI) with exposures adjusted automatically with Spot software (v3.1) and adjustments made manually to brightness and gamma to achieve the best signal-to-noise ratio. A neuronal profile was determined to be labeled for either mGluR2/3 and/or TRPV1 by subjective inspection, comparing the amount of fluorescence in the profile to that in surrounding unlabeled profiles and to profiles in control sections. Outlines of each cellular profile were made on a clear mylar sheet taped to the computer screen using a different colored felt tipped marker for mGluR2/3, TRPV1 and unlabeled profiles. Percentages of single-labeled profiles were calculated by dividing the numbers of single-labeled by the total number of DRG cellular profiles $\times 100$. Percentages of double-labeled profiles were calculated by dividing numbers of double-labeled neuronal profiles by number of single-labeled $\times 100$. A minimum of two sections from each animal was analyzed with data presented as the mean $\pm$ SD. Diameters of labeled cells were estimated by summing the length and width of those cells with visible nuclei and dividing by 2.

4.2. Behavioral studies

4.2.1. Habituation—Rats were housed in groups of three in plastic cages with soft bedding under a reversed light/dark cycle of 12 h/12 h. Following arrival at the animal care facility, they were acclimated for at least 3 days before any behavioral testing was initiated. Rats were habituated for CAP testing by placing them on a wire screen platform in Plexiglas cages (8 $\times$ 8 $\times$ 18 cm) for 1 h. Each rat was habituated two times before being placed in an experimental group. The paw withdrawal latency (PWL) from a heat source was measured using the method described by (Hargreaves et al., 1988); however, the radiant heat source was replaced with a solid state laser system. Each rat was habituated for this test by being placed in a plexiglass cage on a glass plate that was 1/4" thick and maintained at 23°C. The animals remained on the glass for at least for 20 min before testing was initiated. The laser system (custom made in-house) consisted of a microprocessor-controlled 980 nm (near infrared) continuous wave,

solid-state laser (4 watt). The laser spot size (measured with Kentek laser alignment paper) was 2 mm in diameter. A PWL of approximately 9 s was obtained with parameters of 4.35 amps and 3.5 watts. Measured at the glass surface, the laser reached 40°C within 0.5 s to a maximum of 52°C in 8 s. The cutoff time was 15 s. A red (670nm) sighting beam was coupled to the invisible laser to allow positioning of the laser on the plantar surface on the hind paw. The sighting beam was controlled independently from the laser so it did not interfere with the thermal testing. The animals were habituated for five days before being placed in an experimental group. Each hind paw was tested once for every 10 minutes for 4 trials during habituation for thermal testing.

4.2.2. Drug Injections—Intraplantar drug injections were performed using a 28-gauge needle attached to 50 µl Hamilton syringe with PE20 tubing. For measurements of spontaneous behaviors (flinching, lifting/licking), rats were not anesthetized for the drug injections. For measurement of evoked behavior (thermal paw withdrawal latency) rats were briefly (2-3 min) anesthetized with 3% isoflurane for the drug injection. In either case, the skin of the paw proximal to the footpads was penetrated and the needle tip advanced about 1 cm to the base of the third toe. Each rat was used only once (except where noted) and the experimenter was unaware of which drug(s) were being injected into each animal.

The group II agonist (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC, Tocris Cookson Ltd., Ellisville, MO, USA) was dissolved in 1N NaOH (made in 100 mM as a stock solution). The selective group II antagonist LY341495, (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY, Tocris Cookson) (Thomas et al., 1996; Schoepp et al., 1999) was dissolved in 1.2 N NaOH (made as a 100 mM stock solution). Forskolin (7-beta-Deacetyl-7-beta-(gamma-N-methylpiperazino)butyl forskolin dihydrochloride, MP Biomedicals, Solon, OH, USA) is an adenylate cyclase stimulator was made in PBS. All stock solutions were diluted with PBS to different concentrations as needed in the studies and buffered to a final pH 7.4.

A capsaicin (CAP, Sigma-Aldrich, St. Louis, MO, USA) stock solution was made by dissolving 1 g CAP in a mixture containing 2 ml ethanol, 0.7 ml Tween-80 and 9.3 ml saline. This solution was heated and stirred for 1 h until the final volume returned to 10 ml, indicating the ethanol had evaporated. This 10% stock solution was diluted with CAP vehicle (7% Tween-80 in saline) to make working dilutions of CAP.

4.3. Electrophysiological Studies

4.3.1. In vitro skin-nerve preparation—Rats were sacrificed with an overdose of CO₂. The glabrous skin from the ankle to the tips of the toes was dissected from the hind paw. The medial and lateral plantar nerves were dissected free and kept intact with the glabrous skin. All muscle and tendon tissues were removed from the preparation. The preparation was placed corium side up in an organ bath and superfused (15 ml/min, 34°C) with an oxygen-saturated, modified synthetic interstitial fluid solution (SIF, in mM: NaCl, 123; KCl, 3.5; MgSO₄, 0.7; CaCl₂, 2.0; Na gluconate, 9.5; NaH₂PO₄, 1.7; Glucose, 5.5; Sucrose, 7.5; and HEPES, 10; pH 7.45 ± 0.05). The plantar nerves were moved into a separate chamber containing a superficial layer of mineral oil and a bottom layer of SIF. The nerves were desheathed and teased apart on a mirror stage. Small filaments from either the medial or the lateral plantar nerve were repeatedly split with sharpened forceps until single unit activity was obtained by recording with gold wire electrode. This preparation was first used to record primary afferent activity in hairy skin (Reeh, 1986) and we have successfully adapted it for recordings from primary afferents innervating glabrous skin (Carlton et al., 2001a; Du et al., 2003; Carlton et al., 2004; Du et al., 2006; Du et al., 2008).

4.3.2. Neurophysiological Recordings—Neural activity was recorded using a DAM80 Differential Amplifier (World Precision Instruments, New Haven, CT, USA). Action potentials were acquired and later analyzed with the CED 1401 (Cambridge, UK) using Spike 2 (v5.08) software. The conduction velocity of each unit was determined by monopolar electrical stimulation (1 ms duration, 1 Hz) at the most mechanosensitive site in the receptive field of each unit using a Teflon-coated steel electrode (5 M Ω impedance, 250 μ m shaft diameter with an uninsulated tip) that was gently lowered into the receptive field. The conduction velocity of each unit was determined from the latency of the action potential and the distance from the stimulating electrode to the recording site. Based on measurements using our *in vitro* chamber with a 34°C bath temperature and isolated sciatic nerves (n=3), the average conduction velocity for C-mechanoheat (CMH) fibers was $\leq 1.2 \pm 0.1$ m/s. This agrees with previous *in vitro* nerve recordings in rat (Koltzenburg et al., 1992; Kress et al., 1992).

4.3.3. Thermal and chemical testing procedures—Only units responding to mechanical probing of the glabrous skin with a blunt glass rod with a clearly defined receptive field were studied in detail. For thermal stimulation, radiant heat was applied to each receptive field by a feedback-controlled lamp (custom made in house) placed beneath the organ bath. The beam was focused through the bottom of the bath onto the epidermal surface of the skin. A thermocouple was placed into the corium above the light beam to measure intracutaneous temperature. A standard heat ramp starting from an adapting temperature of 34°C and rising to 51°C in 10 s was applied to each unit from the epidermal side and 51°C on the epidermal side was equal to 47°C on the corium side. The threshold of the heat response was defined as the temperature evoking the second spike following the initiation of the ramp (Koltzenburg et al., 1992; Kress et al., 1992). To document unit responses to drugs, a small hollow cylinder (5 mm diameter) was placed over the receptive field of each unit. The SIF in the cylinder was replaced with the drug(s) made as described above but final dilutions were made in SIF and buffered to pH 7.40 ± 0.05 . The APDC, LY, CAP, FK and 1,9-dideoxyforskolin (dFK, a forskolin analogue unable to stimulate adenylate cyclase) were purchased as described above; H89, (dihydrochloride:N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl), a PKA inhibitor, was purchased from Calbiochem, La Jolla, CA, USA.

As previously described (Reeh, 1986), the majority of units from normal skin had no spontaneous activity but the mechanical search stimulus (probing with a glass rod) or thermal stimulus sometimes resulted in ongoing activity of a low frequency in some units. As shown in Figure 4A, background was minimal and had little effect on subsequent responses.

4.4. Study Design

4.4.1. Group II mGluR modulation of CAP-induced pain behaviors—To determine the effect of group II mGluR activation on CAP-induced pain behaviors, rats were injected with either 30 μ l of PBS (to keep volume constant) followed 30 min later by 20 μ l of 0.1% CAP (n = 6), 30 μ l of 0.1 μ M APDC followed 30 min later by 20 μ l 0.1% CAP (n = 5), a 30 μ l cocktail containing 0.1 μ M APDC + 1.0 μ M LY (group II antagonist) followed 30 min later by 20 μ l 0.1% CAP (n = 6) or 30 μ l of PBS followed by 20 μ l of LY alone (n = 6). The number of flinches and the time spent lifting/licking (L/L) the injected hind paw were measured in 5 min intervals for 60 min.

4.4.2. Group II mGluR modulation of CAP-induced CMH excitation—To determine whether activation of group II mGluRs attenuated CAP-induced excitation of nociceptors, different populations of CMH units were exposed to 0.05% CAP alone, 0.05% CAP + 1.0 μ M APDC or 0.05% CAP + 1.0 μ M APDC + 1.0 μ M LY for 2 min and unit activity recorded. CAP-induced heat sensitization could not be analyzed due to the fact that CAP ultimately desensitized most CMH fibers.

4.4.3. Group II mGluR modulation of the cAMP/PKA pathway—Group II mGluRs are negatively coupled to Gi/Go proteins which can down regulate cAMP/PKA pathways. A series of experiments were done to determine if group II mGluRs modulate nociceptor activity through this same pathway. In behavioral studies, rats received 20 µl intraplantar injections of 10 µM forskolin (FK, an adenylate cyclase activator, n = 6), PBS (vehicle, n = 5) or a 20 µl cocktail of 10 µM FK + 0.5 µM APDC (n = 6) and PWLs to heat determined 30 and 60 min post-injection. Preliminary studies indicated that at least 10 µM FK was needed to produce heat hyperalgesia in the awake, behaving rat. In *in vitro* studies using the skin nerve preparation, skin flaps were soaked in 5.0 µM FK or dKF for 1 h. Then CMH units were isolated and their receptive fields exposed again to 5.0 µM FK alone, 5.0 µM FK + 0.5 µM APDC or 5.0 µM FK + 10 µM H89 (a PKA inhibitor), or 5.0 or 50 µM dFK (inactive FK analogue) for 5 min and then unit responses to heat analyzed. Heat responses from these groups were compared to a separate group of CMH units soaked in SIF buffer for 1 h (control).

4.5. Statistical analysis

Immunohistochemical data are presented as the mean $\pm$ standard deviation (SD), all other data are presented as mean $\pm$ standard error of the mean (SEM) and SigmaStat v3.1 software was used for statistic analysis. Parametric or non-parametric analyses were used (depending on whether a normality test was passed). The time course of CAP-induced behaviors were constructed by plotting the mean number of flinches or time spent L/L in 5 min intervals as a function of time. The total sum of flinches or of L/L behavior during 60 min was obtained and plotted as bar graphs versus treatment and differences between groups were evaluated using a Kruskal-Wallis test followed by a Student-Newman-Keuls post hoc test. The PWL's in the FK behavioral studies were expressed as percent change from baseline and a one way ANOVA (normality test was passed) followed by a Student Newman-Keuls post hoc test used. For the electrophysiology studies, a Kruskal-Wallis test followed by a Dunn's post hoc test was used when multiple groups were compared. $P < 0.05$ was considered significant.

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Figure 1. Double labeling with immunohistochemistry. The same DRG sections were immunostained with antibodies directed against TRPV1 (A) and mGluR2/3 (B) and the merged images are shown in panel C. Notice that all mGluR2/3-labeled cells also label for TRPV1. However, there is a small population of TRPV1 cells (arrows) which do not label for mGluR2/3. Bar = 50 μ m.

Figure 2.

APDC blocks CAP-induced behaviors. Intraplantar injection of 0.1% CAP results in significant flinching (A) and L/L behavior (B). Co-application of 0.1 μ M APDC + CAP significantly reduces these behaviors. The time course for this effect is shown in panels C and D. Addition of the group II antagonist 1.0 μ M LY prevents the APDC-induced analgesia. Injection of 0.1 μ M APDC in the contralateral hind paw (APDC contra) does not change the ipsilateral CAP-induced behavior, signifying a local effect of the APDC on CAP-induced pain. 1.0 μ M LY has no effect on CAP-induced behaviors. (* p <0.05, significantly different from all other groups, Kruskal-Wallis followed by a Student-Newman-Keuls test).

Figure 3.

APDC blocks CAP-induced nociceptor activity. A) Application of 0.05% CAP alone induces a robust increase in nociceptor discharge rate (*significantly different from background and 1.0 μ M LY alone, $p < 0.05$, Kruskal-Wallis followed by Dunn's post hoc test) B) 1.0 μ M APDC significantly decreases the CAP response and addition of 1.0 μ M LY to the CAP + APDC cocktail reverses the APDC-induced inhibition (*significantly different from all other groups, $p < 0.05$, Kruskal-Wallis test followed by Dunn's post hoc test).

Figure 4.

Responses of 3 representative units following drug applications. A) Application of 0.05% CAP to the receptive field of a CMH fiber induced a robust excitation. B) In another unit, addition of 1.0 μ M APDC (CAP + APDC) decreased the CAP-induced excitation, particularly at 60-120s following application. C) In a third unit, 1.0 μ M LY prevents the APDC effect. D) Standard 10 s heat ramp. All 3 CMH units had an initial heat response (H1) however, following exposure to CAP, some units were desensitized therefore the second heat response (H2) was highly variable.

Figure 5.

Signal transduction pathway involved in the APDC effect: behavioral studies. Compared to intraplantar PBS, 10 μ M FK decreased the PWL to heat stimulation to 62% of baseline at 30-60 min post-injection; 0.5 μ M APDC blocked the FK-induced heat sensitivity (*significantly different from all other groups, $p < 0.05$, one way ANOVA followed by a Student-Newman-Keuls test).

Figure 6.

Signal transduction pathway involved in the APDC effect: electrophysiological studies. CMH units were sensitized by exposure to 5.0 μ M FK, evidenced by a decreased heat threshold (A) and increased spike rate to heat stimulation (B) compared to control. Application of 10 μ M H89 prevented FK-induced heat sensitization. 0.5 μ M APDC mimicked the H89 affect, preventing FK-induced heat sensitization. Exposure to 5 or 50 μ M dFK had no effect. (*significantly different from all other groups, $p < 0.05$, Kruskal-Wallis followed by a Dunn's post hoc test).