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RESEARCH ARTICLE

K.A. Sluka · H. Rees · P.S. Chen · M. Tsuruoka
W.D. Willis

Inhibitors of G-proteins and protein kinases reduce the sensitization to mechanical stimulation and the desensitization to heat of spinothalamic tract neurons induced by intradermal injection of capsaicin in the primate

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Abstract Intradermal injection of capsaicin results in sensitization of spinothalamic tract cells to brushing and pressure applied to the cutaneous receptive field in anesthetized monkeys. A significant increase in background activity also occurs immediately after capsaicin injection that lasts for at least 2 h. A 40–50% decrease in the response to noxious heat stimuli is also observed following capsaicin injection. This study investigated the spinal role of second messengers by extracellularly recording from spinothalamic tract cells and delivering inhibitors of second messenger pathways to the spinal cord by microdialysis. Blockade of protein kinases with the general protein kinase inhibitor, H7 (5.0 mM, $n = 6$), reduced the sensitization of the cells to brush and pressure. Blockade of protein kinase C with NPC15437 (10.0 mM, $n = 10$) reduced the increased background activity and the increased responses to brush. Blockade of protein kinase A with H89 (0.01 mM, $n = 9$) was most effective. H89 reduced the background activity, the increased responses to brush and press, and reversed the decreased response to noxious heat stimuli. Blockade of G-proteins with the general G-protein inhibitor, GDP-β-S (1.0 mM, $n = 9$), reduced the background activity and the responses to brush and pressure without affecting the decreased response to heat. Thus, multiple intracellular messengers appear to be involved in the processing of central sensitization induced by activation of C-fibers following intradermal injection of capsaicin.

K.A. Sluka[1] · H. Rees · P.S. Chen · M. Tsuroka[2] · W.D. Willis (✉)
Marine Biomedical Institute, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1069, USA;
tel: +(409)-772-2103, fax: +(409)-772-4687,
e-mail: wdw@mbian.utmb.edu

Present address:
[1] Physical Therapy Graduate Program, College of Medicine, University of Iowa, 2600 Steindler Bldg., Iowa City, IA 52242-1008, USA
[2] Department of Physiology, Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

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Introduction

Intradermal injection of capsaicin results in sensitization of spinothalamic tract (STT) neurons to peripheral cutaneous stimuli (Simone et al. 1991; Dougherty and Willis 1992). Spinothalamic cells show an increase in baseline activity, as well as an increase in responses to innocuous stimuli such as brushing and pressure (Simone et al. 1991; Dougherty and Willis 1992). The sensitization of dorsal horn neurons induced by capsaicin is blocked by glutamate (GLU) receptor antagonists acting on *N*-methyl-D-aspartate (NMDA) and non-NMDA GLU receptors (Neugebauer et al. 1993, 1994). Additionally, blockade of neurokinin 1 (NK1) receptors results in a reversal of the capsaicin-induced sensitization of STT cells (Palecek et al. 1994). Increased activity of STT cells can be induced by spinal application of *trans*-ACPD, a metabotropic GLU receptor agonist, or by application of phorbol esters to activate the protein kinase C (PKC) transduction pathway (Paleček et al. 1994a, b). Therefore, there is evidence of a possible role of second messenger systems activated through G-protein linked receptors and for PKC in central sensitization of STT cells.

Second messenger systems are thought to play an important role in cellular processing of sensory information. Activation of second messenger pathways can be initiated by a variety of mechanisms. One mechanism involves the binding of a ligand to a cell membrane surface receptor linked to a G-protein. Metabotropic GLU receptors (mGluR1 and mGluR5) and NK1 and neurokinin 2 (NK2) receptors are believed to be linked to stimulatory G-proteins (Guard and Watson 1991; Watling 1992; Holland et al. 1993; Schoepp and Conn 1993) and activate the PKC transduction cascade (Nishizuka 1984; Berridge and Irvine 1989; Guard and Watson 1991). Activation of G-protein-linked receptors could also stimulate the formation of inositol triphosphate (IP3) resulting in mobili-

zation of the intracellular calcium pools (Schoepp and Conn 1993; Bockaert et al. 1993). This would result in an overall increase in calcium and other second messengers such as protein kinases.

Behaviorally, mechanical allodynia and hyperalgesia occur in rats following intradermal injection of capsaicin and these changes last for at least 2.5 h after injection (Kinnman and Levine 1995; Sluka and Willis 1997). Both the mechanical allodynia and the mechanical hyperalgesia induced by capsaicin are reversed by inhibitors of G-proteins and protein kinases (Willis and Sluka 1995). In the formalin test intrathecal administration of inhibitors to PKC can prevent nocifensive behaviors (Coderre and Yashpal 1994). Conversely, PKC activators can increase these nocifensive behaviors (Coderre 1992; Coderre and Yashpal 1994). Few data are available on the central actions of protein kinase A (PKA) on dorsal horn neurons or in nociceptive transmission. However, peripherally local blockade of cAMP can reduce the mechanical hyperalgesia found in diabetic rats (Taiwo and Levine 1989; Ahlgren and Levine 1993).

This study tests the hypothesis that the maintenance of central sensitization of spinothalamic tract neurons involves continual activation of G-proteins and protein kinases. All drugs were administered by microdialysis to decipher the role of the spinal cord in the sensitization of STT cells by intradermal injection of capsaicin.

Materials and methods

All experiments were approved by the Animal Care and Use Committee at our institution.

Anesthesia and experimental set-up

Monkeys (n = 21) were initially anesthetized with ketamine (10 mg/kg, i.m.). This was followed by administration of halothane, α-chloralose (60–90 mg/kg, i.v.) and gallamine triethiodide (20 mg/h, i.v.). Anesthesia was maintained by sodium pentobarbital (5 mg/kg per hour, i.v.). After tracheotomy animals were artificially ventilated to maintain end-tidal CO_2 at approximately 4%. Core body temperature was regulated at approximately 37°C using a thermostatically controlled heating blanket. A laminectomy was performed to expose the lumbar enlargement. A craniotomy was performed for stereotaxic placement of a stimulating electrode into the ventroposterolateral nucleus of the thalamus.

Three microdialysis fibers (Spectrum, 15 kDa cutoff) were positioned in the lumbar enlargement in areas most responsive to stimulation of the lower hindlimb. The fibers were coated with silicone except for a 1-mm gap that was positioned in the ipsilateral gray matter of the dorsal horn of the spinal cord. Artificial cerebrospinal fluid (ACSF; 151.1 mM Na^+, 2.6 mM K^+, 0.9 mM Mg^{2+}, 1.3 mM Ca^{2+},122.7 mM Cl^-, 21.0 mM HCO_3^-, 2.5 mM HPO_4^{2-}, 3.87 mM glucose, bubbled with 95% CO_2, 5% O_2, pH 7.2–7.4) or second messenger inhibitors, dissolved in ACSF, were infused through the microdialysis fiber at a rate of 5 μl/min.

For recording STT cells, a monopolar steel electrode was positioned in the ventral posterior lateral (VPL) nucleus of the thalamus using both stereotaxic coordinates and recordings of responses to stimulation of the dorsal column and hindpaw. The spinal cord was searched for STT cells with a carbon filament electrode by activating STT cells antidromically from the VPL nucleus of the thalamus. The spinal cord is searched in an area relatively close (1–2 mm) to a microdialysis fiber to ensure that the drug would reach the cell in a short period of time. Criteria to establish antidromic activation from the VPL nucleus were: (1) constant latency for the evoked response, (2) ability to follow high-frequency stimulation, and (3) collision of orthodromic spikes with antidromic action potentials. The stored digital records of single-unit activity from STT cells were retrieved and analyzed following each experiment. Background activity was subtracted and then changes in evoked responses calculated.

Administration of drugs

All drugs were administered through the microdialysis fiber (at 5 μl/min), beginning 30 min after injection of capsaicin, for 30 min to 1 h. The inhibitors were dissolved in ACSF and pH was corrected to 7.2–7.4. The following drugs were used: GDP-β-S (guanosine 5'-*O*-(2-thiodiphosphate), a general inhibitor of G-proteins (1.0 mM, n = 9; Sigma, dissolved in 10% dimethylsulfoxide (DMSO) and 90% ACSF); H7 ([1-(5-isoquinolinesulfonyl)-2-methylpiperazine, HCl], a general inhibitor of protein kinases, 5.0 mM, n = 6; Sigma); NPC15437 (2,6-diamino-*N*-([1-oxotridecyl)-2-piperidinyl]methyl)hexanamide, an inhibitor of PKC, 10.0 mM, n = 10; Research Biochemicals); H89 (Carlton et al. 1992j; Molander et al. 1992; Lu and Ho 1992), an inhibitor of PKA (0.01 mM, n = 9; Calbiochem). ACSF (n = 9) was used as a control. Only one drug or ACSF was administered per cell. The concentrations of drugs in the dialysate were presumed to be approximately two to three orders of magnitude higher than the concentrations that reach neurons in the dorsal horn. In the past we have studied the diffusion across the microdialysis fiber in vitro of several similar-sized drugs with quite different chemical properties. The concentration ratio across the microdialysis fiber for all these drugs was between 1% and 4% (Sluka and Westlund 1993a; Sluka et al. 1994).

Since GDP-β-S does not readily cross cell membranes (Kucera and Rittenhouse 1988), it was dissolved in 10% DMSO to permeabilize the cells to allow entry of the drug intracellularly (see Sawada and Sato 1975). DMSO (even at a concentration of 50% in the microdialysis fluid) has no effect on the background activity or the responses of dorsal horn neurons to innocuous or noxious mechanical stimuli (Peng et al. 1996). Concentrations of the inhibitors were based on published dose-response curves, with the dose used being between the ED_{50} and the top of the dose-response curve. This dose was then corrected for diffusion across the fiber by a factor of 100. Thus the concentration of H89, for instance, that crosses the fiber was estimated to be 0.1 μM. Following diffusion through the tissue this would be expected to be much lower. The maximal concentration infused through the microdialysis fiber for: (1) GDP-β-S was 1.0 mM (Taiwo and Levine 1989), (2) H7 was 5.0 mM (Nixon et al. 1991), (3) NPC15437 was 10.0 mM, and (4) H89 was 0.01 mM (Chijiwa et al. 1990). Additionally, concentrations of drugs used for these studies were based on dose-response curves in a previous study in the rat (Willis and Sluka, 1995).

Experimental design

Once an STT cell had been isolated the background activity and responses to mechanical and thermal stimuli were recorded. All cells were characterized by their responses to application of brush, pressure, pinch and squeeze of the skin at the most responsive portion in the receptive field. This was used to classify the cells as low threshold, wide dynamic range or high threshold.

Five sites across the receptive field were defined for cutaneous mechanical stimulation. Brush, pressure and pinch were applied to all sites before and after capsaicin and after administration of inhibitor. An average of all five sites was used for statistical analysis. Heat and cold were applied to one site on the receptive field outside the area of injection by at least 2 cm. Heat was applied for 5 s (fast ramp) in a graded fashion, starting at 41°C and rising in

increments of 2°C to 53°C. Cold was applied continuously (slow ramp) for 60 s with the temperature starting at 36°C and reaching a minimum of 5°C.

Following baseline recordings, capsaicin was injected intradermally into an area within the receptive field. Thirty minutes after injection the background activity and responses to mechanical and thermal stimuli were tested. A second messenger inhibitor was then delivered for 1 h and the cell's responses to cutaneous and thermal stimuli retested. Control cells were tested for responses to all stimuli before, and 30 min, 1 h and 1.5 h after injection of capsaicin while ACSF was infused through the dialysis fiber.

Statistical analysis

A repeated measures ANOVA tested responses in each group. If significance was obtained, post-hoc testing with paired *t*-tests assessed differences across time. A value of $P < 0.05$ was considered significant. All values are given as the mean ±SEM.

Results

Recordings were made from a total of 47 STT cells. Of these, 46 were classified as WDR cells and one as an HT cell. These neurons were located 978–1988 μm (mean 1411 μm) below the surface of the spinal cord. Of these WDR cells 43 became sensitized to peripheral stimuli following capsaicin injection. The HT cell did not sensitize to the capsaicin injection. The background activity of these 43 cells increased significantly from 15.6±1.7 to 24.0±2.1 spikes/s 30 min after injection of capsaicin ($F_{1,42} = 43.75$, $P = 0.0001$). Evoked responses (brush, press, pinch, heat and cold) are presented as the responses with the background activity subtracted. The average responses to brush across the five sites in the receptive field significantly increased from 16.1±1.7 before capsaicin injection to 20.9±2.1 spikes/s 30 min after capsaicin ($F_{1,42} = 9.13$, $P = 0.004$). The responses to pressure also increased significantly following capsaicin injection from 10.9±1.1 to 18.1±2.4 spikes/s ($F_{1,42} = 7.44$, $P = 0.01$). The greatest increase occurred for areas closest to the site of injection. Changes in the responses to pinch varied for different cells, but the average pinch responses were not significantly changed: 25.12±2.9 before capsaicin, 24.7±3.0 spikes/s 30 min after capsaicin ($F_{1,40} = 0.02$, $P = 0.89$). In contrast the responses to heat were significantly decreased in these 43 cells 30 min after capsaicin injection: 25.6±3.0 before capsaicin, 11.3±2.0 spikes/s 30 min after capsaicin ($F_{1,40} = 20.85$, $P = 0.0001$). The responses to a slow application of cold were also significantly decreased following capsaicin ($F_{1.40} = 10.25$, $P = 0.003$). These responses remained decreased throughout the 1.5-h testing period. Responses to cold before capsaicin averaged 37.46±6.6 spikes/s for all cells and decreased to an average of –3.27±6.9 spikes/s. However, if cells were grouped according to treatments the decreased response to cold stimuli did not remain significant for all groups and no drug appeared to have an effect on the changes to cold stimuli. Therefore, if all cells are considered before and after injection of capsaicin there are increases in background activity, increased responses to innocuous mechanical stimuli and decreased responses to thermal stimuli (heat and cold). The following will consider only those changes that remained significantly changed once the cells were separated by group: background activity, brush, pressure and heat responses.

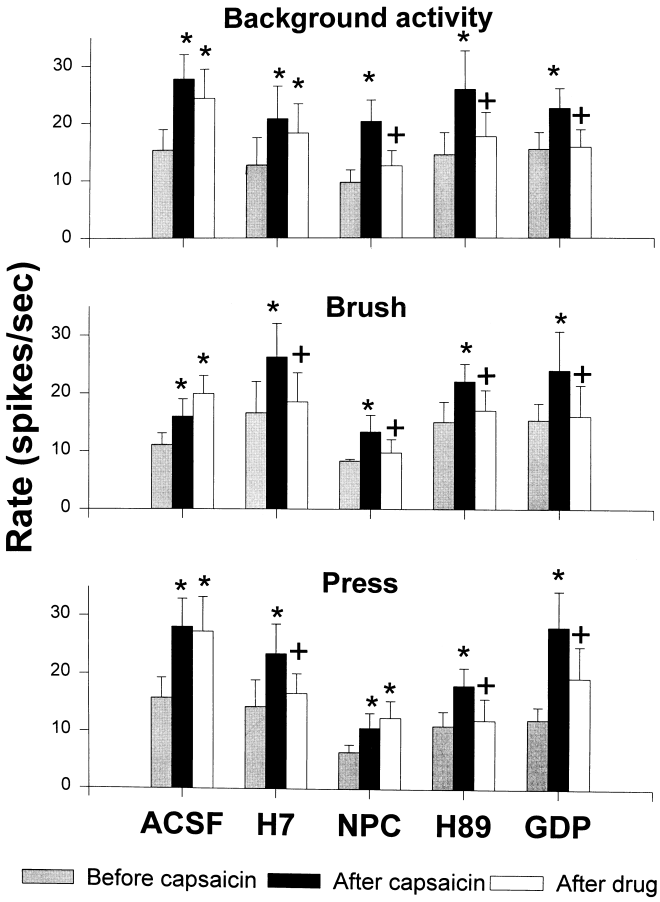


Fig. 1 Bar graphs summarizing the response of the cells before capsaicin (*gray bars*), after capsaicin (*black bars*) and after administration of drug (*open bars*). Artificial cerebrospinal fluid (ACSF) was used as a control for comparison. The background activity and the responses to brush and press applied to the cutaneous receptive field are shown. $^{*}P < 0.05$, significantly different from baseline responses before capsaicin injection. $^{+}P < 0.05$, significantly different from responses after capsaicin injection

Control cells treated with ACSF

Cells from control animals treated with ACSF ($n = 9$) demonstrated significant increases after injection of capsaicin in background activity ($F_{2,18} = 11.44$, $P = 0.001$) and responses to brush ($F_{2,18} = 5.21$, $P = 0.01$) and pressure ($F_{2,15} = 4.34$, $P = 0.05$) applied to the cutaneous receptive field (Fig. 1). These increases were maintained through at least 1.5 h after capsaicin injection. When noxious heat was applied to the cutaneous receptive field (outside the area of injection) after injection of capsaicin there was a significant decrease in the responses of the

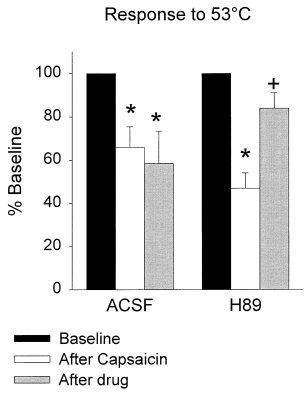


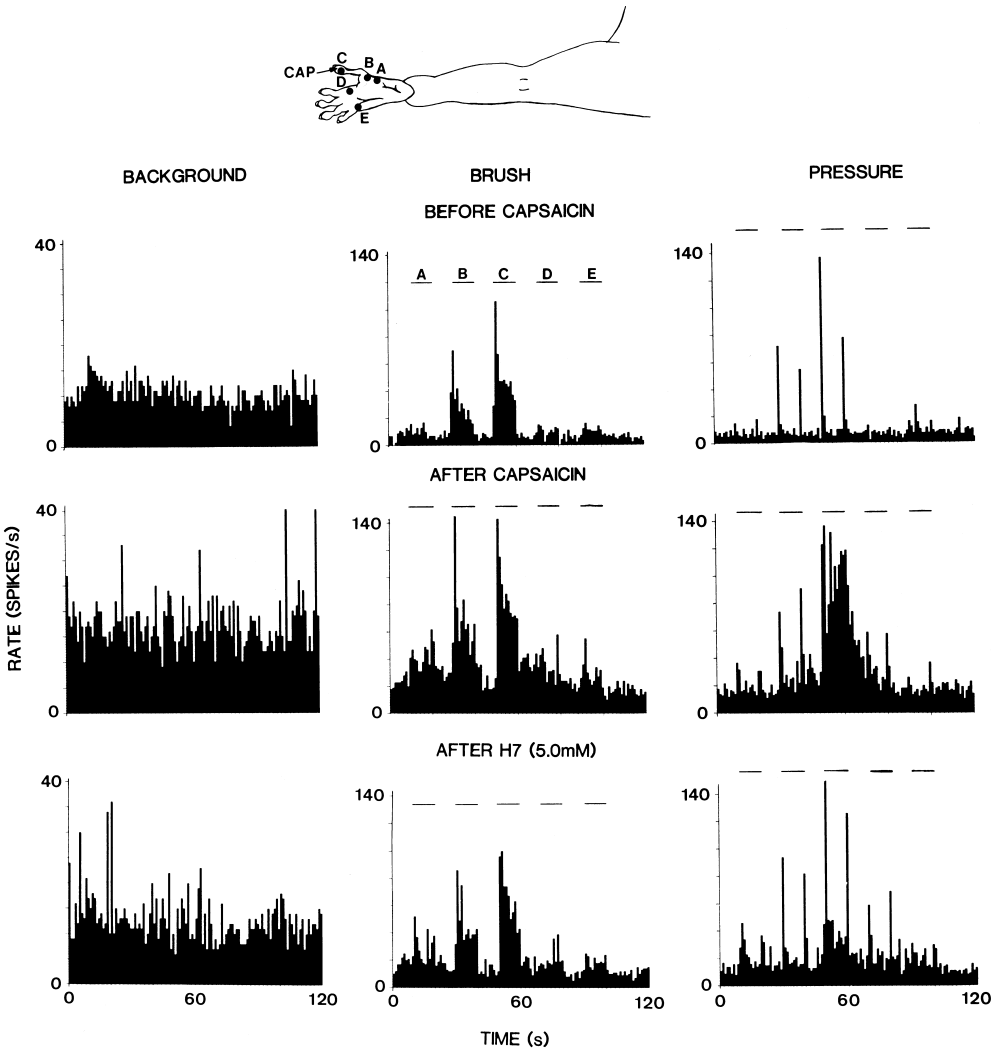
Fig. 2 Bar graphs summarizing the responses to heat (53°C) in all cells treated with ACSF or the protein kinase A inhibitor, H89. Responses are represented as a percentage change from baseline (*black bars*). After capsaicin injection (*open bars*) there is a significant decrease (* $P < 0.05$) in the responses to heat stimuli. Following a 1-h infusion of H89 (0.01 mM) the responses to heat stimuli were increased toward baseline. $^{+}$ Significantly different from responses after injection of capsaicin

neurons when compared with the responses before capsaicin injection ($F_{2,18} = 5.54$, $P = 0.01$). This decrease also was maintained through the 1.5 h testing period (Fig. 2).

Cells treated with the general protein kinase inhibitor, H7

In the groups of cells treated with the general protein kinase inhibitor, H7, there were significant effects for time for changes in background activity ($F_{2,10} = 6.54$, $P = 0.01$), brush ($F_{2,10} = 4.93$, $P = 0.03$), pressure ($F_{2,10} = 4.99$, $P = 0.03$) and heat ($F_{2,10} = 5.01$, $P = 0.03$). There were significant increases from baseline in background activity and the responses to brush and pressure 30 min after injection of capsaicin. In this group of cells there was also a significant decrease in the response of the cells to heat stimuli 30 min following injection of capsaicin. Following microdialysis infusion of H7 (5.0 mM for 30 min to 1 h, $n = 6$) there was a significant reversal in the re-

Fig. 3 Rate histograms from a cell treated with the general protein kinase inhibitor, H7 (5.0 mM), after intradermal injection of capsaicin (*CAP*). Following capsaicin injection (*middle panel*) there were increases in the background activity (*left column*) and in responses to brush (*middle column*) and pressure (*right column*) compared with the responses before injection (*top panel*). Infusion of NPC15437 for 1 h reduced the increased responses to brush and increased background activity without affecting the responses to pressure



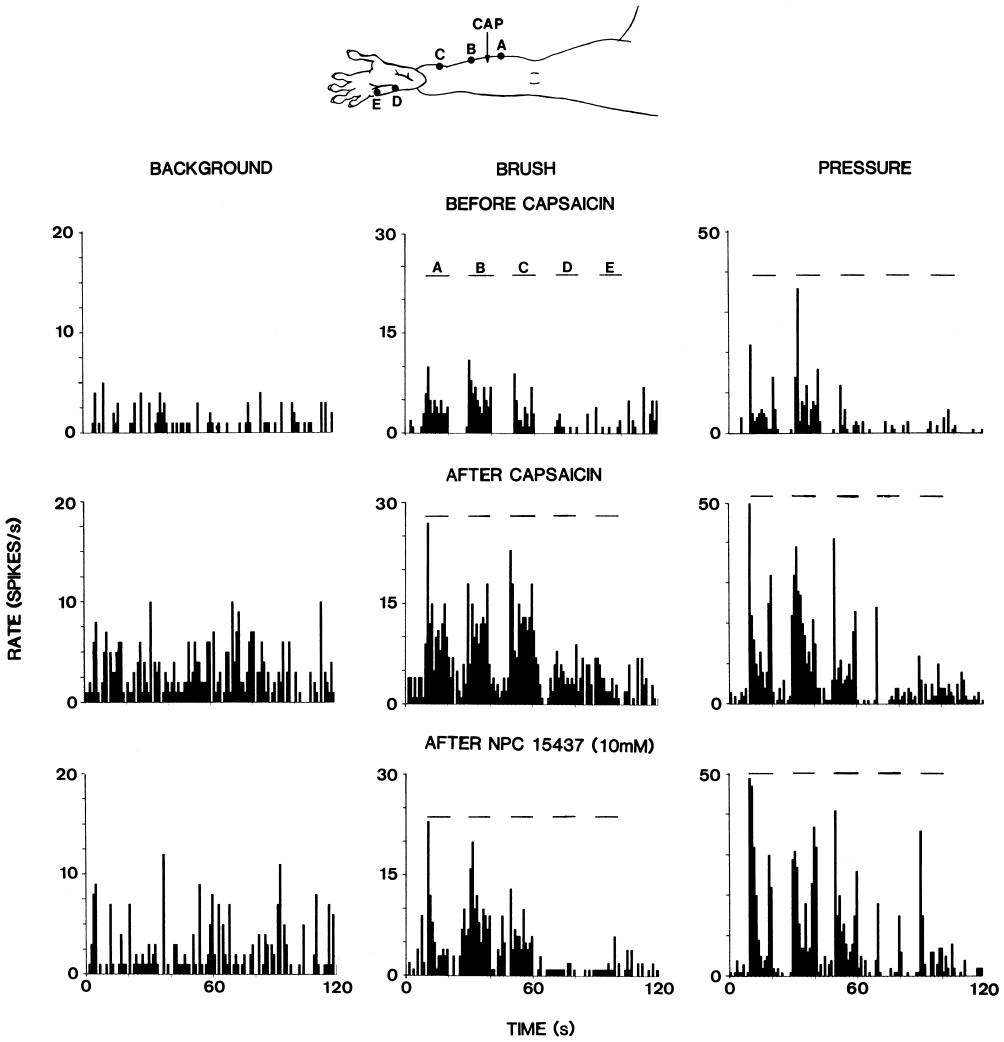


Fig. 4 Rate histograms from a cell treated with the protein kinase C inhibitor, NPC15437 (10.0 mM), after intradermal injection of capsaicin (*CAP*). Following capsaicin injection (*middle panel*) there were significant increases in the background activity (*left column*), and responses to brush (*middle column*) and pressure (*right column*), when compared with the responses before injection (*top panel*). Infusion of NPC15437 for 1 h reduced the increased responses to brush and increased background activity without affecting the responses to pressure

sponse of the STT cells to brush and pressure when compared with responses 30 min after capsaicin injection (Fig. 1). Spinal infusion of H7, however, did not have a significant effect on the increased background activity or on the decreased heat response. The background activity was still significantly increased and the heat response was significantly decreased when compared with baseline responses.

An example of a cell treated with H7 after injection of capsaicin is shown in Fig. 3. The top panels represent the responses of an STT cell before injection of capsaicin. The middle panels show increases in the background activity, brush and press responses of the cell 30 min following injection of capsaicin. The bottom panels represent the changes in the responses of the cell following administration of H7. In this particular cell H7 reduced the brush and pressure responses to close to baseline responses while the background activity was minimally reduced.

Cells treated with a selective PKC inhibitor, NPC15437

In the group of cells treated with the PKC inhibitor, NPC15437, there was a significant effect over time for changes in background activity ($F_{2,18}$ = 11.44, P = 0.001), brush ($F_{2,18}$ = 14.75, P = 0.001), press ($F_{2,18}$ = 4.99, P = 0.03) and heat ($F_{2,10}$ = 5.01, P = 0.03). A summary of the responses of all cells in the group are illustrated in Fig. 1. There was a significant decrease in the responses of the cells to noxious heat stimuli 30 min following injection of capsaicin. Following microdialysis infusion of NPC15437 (10.0 mM for 30 min to 1 h, n = 10), there was a significant reversal in the increased background activity and the increased responses to brush induced by capsaicin injection. Spinal infusion of NPC15437 did not, however, have an effect on the increased response of the cells to pressure or on the decreased heat response. A typical cell is illustrated in Fig. 4 and illustrates changes before and after capsaicin injection and after infusion of NPC15437 for background activity and responses to brush and pressure.

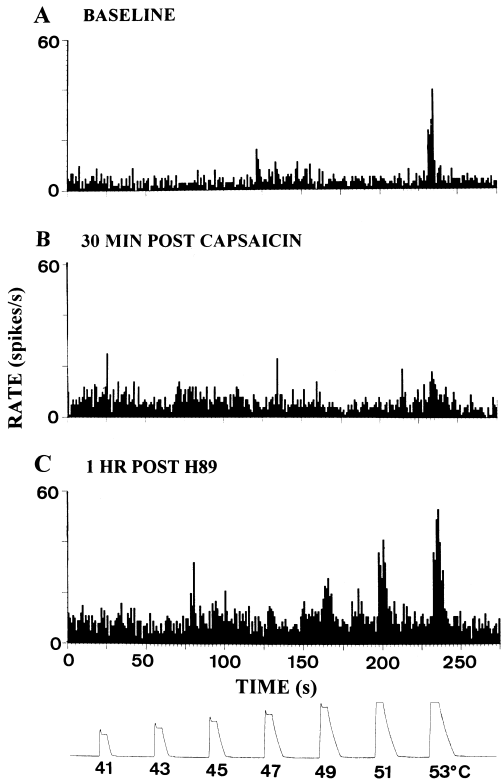


Fig. 5 Rate histograms for the responses to graded heat stimuli from a cell treated with the protein kinase A inhibitor, H89 (0.01 mM), after intradermal injection of capsaicin. After capsaicin injection the responses to heat stimuli were decreased compared with baseline responses. One hour following infusion of H89 the responses to heat returned

Cells treated with a selective PKA inhibitor, H89

In the group of cells treated with the PKA inhibitor, H89 (n = 9), there were significant effects over time for changes in background activity ($F_{2,14}$ = 4.66, P = 0.03), brush ($F_{2,14}$ = 4.66, P = 0.03), pressure ($F_{2,14}$ = 5.43, P = 0.02) and heat ($F_{2,12}$ = 2.91, P = 0.05). Significant increases in background activity and responses to brush and press were observed 30 min following capsaicin injection. There was also a significantly decreased response of the STT cells to noxious heat 30 min following capsaicin injection (Fig. 5). Following microdialysis infusion of H89 (0.01 mM for 30 min to 1 h), there was a significant reversal in the increased background activity and the increased responses to brush and press induced by capsaicin injection (Fig. 1). Spinal infusion of H89 also reversed the capsaicin-induced decrease in the heat response. Figure 5 demonstrates the response of a cell to heat before and after capsaicin injection and after infusion of H89. A response at 53°C occurred in this cell prior to injection of capsaicin. The responses to this heat stimulus averaged 22.6±6.8 spikes/s for all cells in this group. Following injection of capsaicin the responses of the cells to 53°C decreased by more than half to 9.5±6.5 spikes/s. Spinal infusion of H89 resulted in a reversal of the response to heat at 53°C, increasing to 18.3±5.7 spikes/s. Figure 2 summarizes the percentage change from baseline in response to heat at 53°C in animals treated with ACSF as a control and those treated with H89.

Cells treated with the general G-protein inhibitor, GDP-β-S

In the groups of cells treated with the general G-protein inhibitor, GDP-β-S (n = 9), there were significant effects for time for changes in background activity ($F_{2,18}$ = 5.39, P = 0.01), brush ($F_{2,18}$ = 4.25, P = 0.05), pressure ($F_{2,14}$ = 5.66, P = 0.01) and heat ($F_{2,14}$ = 4.31, P = 0.05). Significant increases from baseline in background activity, brush and pressure occurred by 30 min following injection of capsaicin (Fig. 1). There was also a significant decrease in the responses of the cells to heat stimuli 30 min following injection of capsaicin. Following microdialysis infusion of GDP-β-S (1.0 mM for 30 min to 1 h) there was a reversal in the increased background activity of the cells and the responses of the cells to brush and pressure (Fig. 1). Spinal infusion of GDP-β-S did not, however, have an effect on the decreased heat responses.

Discussion

The results of the present study demonstrate a role for PKC, PKA and G-proteins in the maintenance of the sensitization of STT cells after intradermal injection of capsaicin. Different effects were observed between specific second messenger inhibitors. For example, treatment with a PKC inhibitor reduced the increased background activity and the responses to brush (innocuous stimuli) but had no effect on the responses to pressure or heat. The PKA inhibitor, however, reduced the sensitization of the STT cells, i.e., increased background activity, increased responses to brush and press and also reversed the decreased responses to heat stimuli. GDP-β-S reversed the increased background activity and the increased responses to brush and pressure applied to the receptive field without having an effect on the decreased responses to noxious thermal stimuli. The increased responses to brush and pressure induced by intradermal injection of capsaicin are thought to be reflective of a decrease in mechanical threshold and thus representative of secondary mechanical allodynia and hyperalgesia. We have demonstrated in behavioral experiments that intradermal injection of capsaicin results in secondary allodynia and hyperalgesia (Sluka and Willis 1997). Thus, modification of activity in signal transduction pathways can affect the sensitization of cells to mechanical stimuli as well as the responses to heat stimuli.

The same doses of inhibitors were tested in awake behaving rats on mechanical hyperalgesia induced by intradermal injection of capsaicin (Sluka 1996; Sluka and Willis 1997). The drugs were delivered by microdialysis to both the ipsilateral and contralateral dorsal horn. The

threshold to mechanical stimulation was assessed bilaterally and remained the same on the contralateral side after injection of capsaicin or infusion of the compound. This indicates that at the doses applied to the dorsal horn there was no effect on sensation in the normal animals. These same doses, however, were able to reduce the secondary mechanical hyperalgesia induced by intradermal injection of capsaicin observed on the ipsilateral side. In this experiment the drugs were not tested in non-sensitized STT neurons. We cannot exclude a possible effect of these inhibitors in normal animals. However, based on our previous behavioral experiments, these doses of inhibitors (Sluka 1996; Sluka and Willis 1997), delivered by microdialysis, appear to be specific for the sensitized state and do not affect normal responses in animals.

Protein kinase involvement in pain transmission

The protein kinase inhibitors used in the current study have been shown to bind nonselectively to other protein kinases when given in high enough concentrations. H7 is clearly a nonselective protein kinase inhibitor (Nixon et al. 1991). However, H89 is more selective for PKA than for PKG or PKC. The IC_{50} of H89 for PKA is 0.05 μM and that for PKG is 0.5 μM (Chijiwa et al. 1990). It has 1000-fold less affinity for PKC as compared with PKA (Chijiwa et al. 1990). NPC15437 has been shown to be selective for PKC (IC_{50} = 19 μM) with no binding to PKA or calcium-calmodulin dependent kinase at concentrations up to 300 μM (Sullivan et al. 1992). The doses chosen for the inhibitors are based on a 100-fold drop across the microdialysis fiber (Sluka and Westlund 1993a; Sluka et al. 1994). It is expected that degradation and diffusion within the tissue would further reduce the concentration at the target neurons. Thus the maximal concentration that would cross the fiber is below the IC_{50} for the other kinases that might have nonselective effects. Therefore, although nonselective effects with H89 on PKG are possible, we have interpreted these results on the assumption that H89 and NPC15437 are selective for PKA and PKC, respectively, at the doses delivered to the vicinity of STT neurons.

There is good evidence to support the involvement of PKC in the process of central sensitization and the transmission of nociceptive signals. For example, in unsensitized dorsal horn neurons, activation of PKC by phorbol esters or injection of PKC intracellularly enhances both NMDA and non-NMDA GLU currents and increases responses of STT neurons to innocuous stimuli (Gerber et al. 1989; Chen and Huang 1991, 1992). Several models of pain which are associated with sensitized neurons show an involvement of PKC. An increase in membrane-bound PKC in the dorsal horn occurs in peripheral neuropathy (Mao et al. 1992b), following application of mustard oil (Munro et al. 1994) or in the formalin test (Yashpal et al. 1995). This increase in membrane-bound PKC can be prevented by GM1 gangliosides (Mao et al. 1992a,b). Furthermore, nocifensive behaviors associated with peripheral neuropathy or formalin injection can also be prevented by PKC inhibitors (Mao et al. 1992a; Coderre 1992; Coderre and Yashpal 1994; Yashpal et al. 1995). The current study demonstrated that only the increased response to brush in STT cells was reduced to baseline levels with a PKC inhibitor. Although responses to brush are not considered nociceptive, the increased response to brush in STT neurons may be the mechanism for allodynia – a painful response to innocuous stimuli.

The role of PKA in nociception is not well studied. Activation of the cAMP pathway would result in activation of PKA within the cell. We have demonstrated that blockade of the cAMP transduction pathway can reduce secondary mechanical hyperalgesia and allodynia induced by intradermal injection of capsaicin (Sluka 1996; Sluka and Willis 1997). An increase in cAMP has been shown to occur through activation of CGRP receptors in hepatocytes and smooth muscle (Bushfield et al. 1993; Schini-Kerth et al. 1994; Santicioli et al. 1995; Sun and Benhishin 1995). CGRP has been shown to be involved in animal models of pain (Donaldson et al. 1992; Garry and Hargreaves 1992; Kuraishi et al. 1988; Satoh et al. 1992; Smith et al. 1992; Donnerer and Stein 1992; Sluka et al. 1992; Sluka and Westlund 1993b). Peripherally, the mechanical hyperalgesia found in diabetic rats is blocked by local subcutaneous administration of an inhibitor of cAMP (Taiwo and Levine 1989; Ahlgren and Levine 1993). Additionally, intrathecal injections of cAMP analogs reverse the antinociception induced by μ and δ opioid receptor agonists (Wang et al. 1993). Thus, these studies and the results of the current study suggest that increasing cAMP can contribute to hyperalgesia and central sensitization. Therefore, inhibiting the actions of PKA would reduce central sensitization and hyperalgesia.

Interactions between protein kinases have been demonstrated in a variety of cell types. For example, activation of PKC enhances cAMP accumulation in various cell types (Rosengurt et al. 1987; Yoshimasa et al. 1987; Sugden et al. 1987), thereby increasing activation of PKA. Jiang et al. (1992) also suggest that either PKA or PKG cross-activate the other kinase. This is based on the fact that forskolin, which increases cAMP, will activate PKG in addition to PKA. If these interactions exist in dorsal horn neurons, activation of one protein kinase could set off a cascade of events that include activation of other protein kinases. Activation of several different protein kinases could amplify the signal initiated by the peripheral insult and be manifested as central neuronal sensitization and consequent behavioral manifestations of pain. Thus, as in the present study, reduction in central sensitization of central neurons could be accomplished by inhibitors of several different protein kinases.

G-protein involvement in pain transmission

The G-protein inhibitor, GDP-β-S does not readily cross cell membranes (Kucera and Rittenhouse 1988). For this

reason it was dissolved in 10% DMSO to permeabilize the cells enough to allow entry of the drug intracellularly. Using a similar protocol in behavioral studies in rats, animals were injected with capsaicin and then treated by microdialysis with GDP-β-S in 10% DMSO (Sluka and Willis 1997). In these animals there was a significant reversal of the capsaicin-induced allodynia. As a control another group of animals received GDP-β-S without DMSO following injection of capsaicin. In this group there was no change in behavioral responses, i.e., the animals still demonstrated mechanical hyperalgesia and allodynia. Additionally, 10% DMSO in ACSF had no effect on the behavioral responses. Thus, the effects of GDP-β-S on reducing the responses of STT cells are thought to be specific for GDP-β-S and it is presumed that GDP-β-S binds to intracellular sites.

In the inactive state, G-proteins consist of α, β and g subunits with GDP bound to the α subunit (Dunlap et al. 1987)). When a ligand binds the receptor extracellularly, there is an exchange of GTP for the bound GDP resulting in dissociation of the G-protein into the active subunit α-GTP and βγ (Ui and Katadata 1989). G-proteins can either activate or inhibit second messenger systems through α_s or α_i subunits. In addition G_o proteins have been found in neural tissue and α_o has been linked to regulation of calcium channels (Hescheler et al. 1987). The inhibitor used in this study is a general G-protein inhibitor that competes with GTP for its binding site on the α subunit (Dunlap et al. 1987). Thus GDP-β-S could have actions on either inhibitory or stimulatory G-proteins (Dunlap et al. 1987). Acting through a stimulatory G-protein on excitatory neurons, blockade with GDP-β-S would reduce the activation of second messenger systems resulting in decreased sensitization of dorsal horn neurons. The consequence would be a decrease in the behavioral manifestations associated with a peripheral insult. Alternatively, it is possible that GDP-β-S acts on inhibitory G-proteins located on inhibitory interneurons. Blockade of an inhibitory G-protein would also decrease the behavioral manifestations and central sensitization.

Peripherally, activation of G-proteins has been demonstrated in the dorsal root ganglion cells and their peripheral terminals in response to noxious stimuli. Bradykinin-induced excitation of cultured dorsal root ganglion cells is blocked by administration of a G-protein inhibitor (McGuirk and Dolphin 1992). The mechanical hyperalgesia found in diabetic rats is blocked by local subcutaneous administration of an inhibitor of G-proteins, GDP-β-S (Ahlgren and Levine 1993).

Centrally, there is a great deal of evidence to support involvement of spinal G-protein-linked metabotropic glutamate, neurokinin and CGRP receptors in processing nociceptive information. Sensitization of dorsal horn neurons can be reversed by a metabotropic glutamate receptor antagonist, L-AP3 (Young et al. 1994; Neugebauer et al. 1994). In fact, mechanical hyperalgesia in rats can be induced by coadministration of *trans*-ACPD (metabotropic glutamate receptor agonist) and AMPA (non-NMDA glutamate receptor: Meller et al. 1993). Blockade of NK1 or CGRP receptors reverses sensitization of STT cells or dorsal horn cells induced by capsaicin or inflammation (Dougherty et al. 1992, 1994; Neugebauer et al. 1993, 1995, 1996). Behaviorally, NK1 receptor antagonists or antibodies to CGRP have been shown to decrease nocifensive behaviors induced by formalin, capsaicin or inflammation (Yamamoto and Yaksh 1991; Sakurada et al. 1993; Sluka et al. 1997). Therefore, there is indirect evidence for a role of second messenger systems activated through G-protein-linked neurokinin, CGRP and metabotropic GLU receptors in nociception. Recently we demonstrated that there was a decrease in the mechanical allodynia and hyperalgesia produced by intradermal injection of capsaicin by spinal administration of the G-protein inhibitor, GDP-β-S (Sluka and Willis 1997). This is in agreement with the present study that demonstrated a reversal of the increase in the evoked responses to brush and pressure in STT cells following intradermal injection of capsaicin.

Conclusions

The current study demonstrated that sensitization of STT cells to intradermal injection of capsaicin can be reversed by administration of inhibitors to specific protein kinases or to inhibitors to G-proteins. In addition, the desensitization to heat that occurs after capsaicin injection is reversed by inhibition of protein kinase A. Parallel behavioral studies in rats have demonstrated similar results in which secondary mechanical hyperalgesia and allodynia induced by capsaicin were reversed by inhibitors to protein kinases or to G-proteins (Sluka and Willis 1997). Thus, there is a complicated network of intracellular messengers that are activated as a result of prolonged nociceptive input that contribute to central sensitization.

References

Ahlgren SC, Levine JD (1993) Mechanical hyperalgesia in streptozotocin-diabetic rats. Neuroscience 52: 1049–1055

Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. Nature 341: 197–205

Bockaert J, Pin J, Fagni L (1993) Metabotropic glutamate receptors: an original family of G-protein-coupled receptors. Fund Clin Pharmacol 7: 473–485

Bushfield M, Savage A, Morris NJ, Houslay MD (1993) A mnemonical or negative-co-operativity model for the activation of adenylate cyclase by a common G-protein-coupled calcitonin-gene-related neuropeptide (CGRP)/amylin receptor. Biochem J 293: 229–236

Carlton SM, Westlund KN, Zhang DX, Willis WD (1992) GABA-immunoreactive terminals synapse on primate spinothalamic tract cells. J Comp Neurol 322: 528–537

Chen L, Huang LYM (1991) Sustained potentiation of NMDA receptor-mediated glutamate responses through activation of protein kinase C by a μ opioid. Neuron 7: 319–326

Chen L, Huang LYM (1992) Protein-kinase-C reduces Mg^{2+} block of NMDA-receptor channels as a mechanism of modulation. Nature 356: 521–523

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide. J Biol Chem 5267: 5272

Coderre TJ (1992) Contribution of protein kinase C to central sensitization and persistent pain following tissue injury. Neurosci Lett 140: 181–184

Coderre TJ, Yashpal K (1994) Intracellular messengers contributing to persistent nociception and hyperalgesia induced by L-glutamate and substance P in the rat formalin pain model. Eur J Neurosci 6: 1328–1334

Donaldson LF, Harmar AJ, Mcqueen DS, Seckl JR (1992) Increased expression of preprotachykinin, calcitonin gene-related peptide, but not vasoactive intestinal peptide messenger RNA in dorsal root ganglia during the development of adjuvant monoarthritis in the rat. Mol Brain Res 16: 143–149

Donnerer J, Stein C (1992) Evidence for an increase in the release of CGRP from sensory nerves during inflammation. Ann N Y Acad Sci 657: 505–506

Dougherty PM, Willis WD (1992) Enhanced responses of spinothalamic tract neurons to excitatory amino-acids accompany capsaicin-induced sensitization in the monkey. J Neurosci 12: 883–894

Dougherty PM, Palecek J, Paleckova V, Sorkin LS, Willis WD (1992) The role of NMDA and non-NMDA excitatory amino-acid receptors in the excitation of primate spinothalamic tract neurons by mechanical, chemical, thermal, and electrical stimuli. J Neurosci 12: 3025–3041

Dougherty PM, Palecek J, Paleckova V, Willis WD (1994) Neurokinin 1 and 2 antagonists attenuate the responses and NK1 antagonists prevent the sensitization of primate spinothalamic tract neurons after intradermal capsaicin. J Neurophysiol 72: 1464–1475

Dunlap K, Holz GG, Rane SG (1987) G proteins as regulators of ion channel function. Trends Neurosci 10: 241–244

Garry MG, Hargreaves KM (1992) Enhanced release of immunoreactive CGRP and substance-P from spinal dorsal horn slices occurs during carrageenan inflammation. Brain Res 582: 139–142

Gerber G, Kangrga I, Ryu PD, Larew JSA, Randic M (1989) Multiple effects of phorbol esters in the rat spinal dorsal horn. J Neurosci 9: 3606–3617

Guard S, Watson SP (1991) Tachykinin receptor types: classification and membrane signalling mechanisms. Neurochem Int 18: 149–165

Hescheler J, Rosenthal W, Trautwein W, Schultz G (1987) The GTP-binding protein, G_o, regulates neuronal calcium channels. Nature 325: 445–447

Holland LN, Goldstein BD, Aronstam RS (1993) Substance P receptor desensitization in the dorsal horn: possible involvement of receptor – G protein complexes. Brain Res 600: 89–96

Jiang H, Shabb JB, Corbin JD (1992) Cross-activation: overriding cAMP/cGMP selectivities of protein kinases in tissues. Biochem Cell Biol 70: 1783–1789

Kinnman E, Levine JD (1995) Involvement of the sympathetic postganglionic neuron in capsaicin-induced hyperalgesia in the rat. Neuroscience 65: 283–291

Kucera GL, Rittenhouse SE (1988) Inhibition by GDPbS of agonist-activated phospholipase C in human platelets requires cell permeabilization.

Kuraishi Y, Nanayama T, Ohno H, Minami M, Satoh M (1988) Antinociception induced in rats by intrathecal administration of antiserum against calcitonin gene-related peptide. Neurosci Lett 92: 325–329

Lu J, Ho RH (1992) Evidence for dorsal-root projection to somatostatin-immunoreactive structures in laminae I-II of the spinal dorsal horn. Brain Res Bull 28: 17–26

Mao J, Hayes RL, Price DD, Coghill RC, Lu J, Mayer DJ (1992a) Postinjury treatment with GM1 ganglioside reduces nociceptive behaviors and spinal-cord metabolic-activity in rats with experimental peripheral mononeuropathy. Brain Res 584: 18–27

Mao JR, Price DD, Mayer DJ, Hayes RL (1992b) Pain-related increases in spinal-cord membrane-bound protein-kinase-C following peripheral-nerve injury. Brain Res 588: 144–149

McGuirk SM, Dolphin AC (1992) G-protein mediation in nociceptive signal transduction: an investigation into the excitatory action of bradykinin in a subpopulation of cultured rat sensory neurons. Neuroscience 49: 117–128

Meller ST, Dykstra CL, Gebhart GF (1993) Acute mechanical hyperalgesia is produced by coactivation of AMPA and metabotropic glutamate receptors. Neuroreport 4: 879–882

Molander C, Hongpaisan J, Grant G (1992) Changing pattern of c-Fos expression in spinal-cord neurons after electrical-stimulation of the chronically injured sciatic-nerve in the rat. Neuroscience 50: 223–236

Munro FE, Fleetwood-Walker SM, Mitchell R (1994) Evidence for a role of protein kinase C in the sustained activation of rat dorsal horn neurons evoked by cutaneous mustard oil application. Neurosci Lett 170: 199–202

Neugebauer V, Lucke T, Schaible HG (1993) *N*-Methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists block the hyperexcitability of dorsal horn neurons during development of acute arthritis in rats knee-joint. J Neurophysiol 70: 1365–1377

Neugebauer V, Lucke T, Schaible H-G (1994) Requirement of metabotropic glutamate receptors for the generation of inflammation-evoked hyperexcitability in rat spinal cord neurons. Eur J Neurosci 6: 1179–1186

Neugebauer V, Weiretter F, Schaible H-G (1995) Involvement of substance P and neurokinin-1 receptors in the hyperexcitability of dorsal horn neurons during development of acute arthritis in rat's knee joint. J Neurophysiol 73: 1574–1583

Neugebauer V, Ruemenapp P, Schaible H-G (1996) Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn neurons during development of acute inflammation. Neuroscience 71: 1095–1109

Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308: 693–698

Nixon JS, Wilkinson SL, Davis PD, Sedgwick AD, Wadsworth J, Westmacott D (1991) Modulation of cellular processes by H7, a non-selective inhibitor of protein kinases. Agents Actions 32: 188–193

Paleček J, Paleckova V, Dougherty PM, Willis WD (1994a) The effect of phorbol esters on the responses of primate spinothalamic neurons to mechanical and thermal stimuli. J Neurophysiol 71: 529–537

Paleček J, Paleckova V, Dougherty PM, Willis WD (1994b) The effect of trans-ACPD, a metabotropic excitatory amino acid receptor agonist, on the responses of primate spinothalamic tract neurons. Pain 56: 261–269

Peng YB, Lin Q, Willis WD (1997) Involvement of protein kinase C in responses of rat dorsal horn neurons to mechanical stimuli and periaqueductal gray descending inhibition. Exp Brain Res (in press)

Rosengurt E, Murray M, Sachary I, Collins M (1987) Protein kinase C activation enhances cAMP accumulation in Swiss 3T3 cells: inhibition by pertussis toxin. Proc Natl Acad Sci USA 84: 2282–2286

Sakurada T, Katsumata K, Yogo H, Tan-No K, Sakurada S, Kisara K (1993) Antinociception induced by CP 96,345, a non-peptide NK-1 receptor antagonist, in the mouse formalin and capsaicin tests. Neurosci Lett 151: 142–145

Santicioli P, Morbidelli L, Parenti A, Ziche M, Maggi CA (1995) Calcitonin gene-related peptide selectively increases cAMP levels in the guinea-pig ureter. Eur J Pharmacol 289: 17–21

Satoh M, Kuraishi Y, Kawamura M (1992) Effects of intrathecal antibodies to substance-P, calcitonin gene-related peptide and galanin on repeated cold stress-induced hyperalgesia: comparison with carrageenan-induced hyperalgesia. Pain 49: 273–278

Sawada M, Sato M (1975) The effect of dimethyl sulfoxide on the neuronal excitability and cholinergic transmission in *Aplysia* ganglion cells. Ann N Y Acad Sci 243: 337–357

Schini-Kerth VB, Fisslthaler B, Busse R (1994) CGRP enhances induction of NO synthase in vascular smooth muscle cells via a cAMP-dependent mechanism. Am J Physiol H2483-H2490

Schoepp DD, Conn PJ (1993) Metabotropic glutamate receptors in brain function and pathology. Trends Pharmacol Sci 14: 13–20

Simone DA, Sorkin LS, Oh U, Chung JM, Owens C, Lamotte RH, Willis WD (1991) Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. J Neurophysiol 66: 228–246

Sluka KA (1996) The role of the cyclic AMP transduction cascade in mechanical allodynia and hyperalgesia induced by intradermal injection of capsaicin in rats. Neurosci Abstr 22: 1813

Sluka KA, Westlund KN (1993a) Centrally administered non-NMDA but not NMDA receptor antagonists block peripheral knee joint inflammation. Pain 55: 217–225

Sluka KA, Westlund KN (1993b) Behavioral and immunohistochemical changes in an experimental arthritis model in rats. Pain 55: 367–377

Sluka KA, Willis WD (1997) The effects of G-protein and protein kinase inhibitors on the behavioral responses of rats to intradermal injection of capsaicin. Pain (in press)

Sluka KA, Dougherty PM, Sorkin LS, Willis WD, Westlund KN (1992) Neural changes in acute arthritis in monkeys. III. Changes in substance-P, calcitonin gene-related peptide and glutamate in the dorsal horn of the spinal-cord. Brain Res Rev 17: 29–38

Sluka KA, Willis WD, Westlund KN (1994) Inflammation-induced release of excitatory amino acids is prevented by spinal administration of a GABAA and not by a GABAB receptor antagonist in rats. J Pharmacol Exp Ther 271: 76–82

Sluka KA, Milton MA, Willis WD, Westlund KN (1997) Differential roles of neurokinin 1 and neurokinin 2 receptors in the development and maintenance of heat hyperalgesia induced by acute inflammation. Brit J Pharmacology (in press)

Smith GD, Harmar AJ, Mcqueen DS, Seckl JR (1992) Increase in substance-P and CGRP, but not somatostatin content of innervating dorsal-root ganglia in adjuvant monoarthritis in the rat. Neurosci Lett 137: 257–260

Sugden D, Vanecek J, Klein DC, Thomas TP, Anderson WB (1987) Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature 314: 359–361

Sullivan JP, Connor JR, Shearer BG, Burch RM (1992) 2,6-Diamino-*N*-([1-oxotridecyl)-2-piperidinyl]methyl)hexanamide (NPC 15437): a novel inhibitor of protein kinase C interacting at the regulatory domain. Mol Pharmacol 41: 38–44

Sun Y-D, Benhishin CG (1995) Effects of calcitonin gene-related peptide on cyclic AMP production and relaxation of longitudinal muscle of guinea pig ileum. Peptides 16: 293–297

Taiwo YO, Levine JD (1989) Contribution of guanine nucleotide regulatory proteins to prostaglandin hyperalgesia in the rat. Brain Res 492: 400–403

Ui M, Katadata T (1989) GTP-binding proteins as transducers of receptor-mediated cellular signalling. In: Segewa M, Endo M, Ui K (eds) Physiology and pharmacology of transmembrane signalling. Elsevier, Oxford, pp 3–11

Wang JF, Ren MF, Xue JC, Han JS (1993) Cyclic AMP mediates mu and delta, but not kappa opioid analgesia in the spinal cord of the rat. Life Sci 52: 1955–1960

Watling KJ (1992) Nonpeptide antagonists herald a new era in tachykinin research. Trends Pharmacol Sci 13: 266–269

Yamamoto T, Yaksh TL (1991) Stereospecific effects of a non-peptidic NK1 selective antagonist, CP-96,345: antinociception in the absence of motor dysfunction. Life Sci 49: 1955–1963

Yashpal K, Pitcher GM, Parent A, Quirion R, Coderre TJ (1995) Noxious thermal and chemical stimulation induce increases in ^{3}H-phorbal 12,13-dibutyrate binding in spinal cord dorsal horn as well as persistent pain and hyperalgesia, which is reduced by inhibition of protein kinase C. J Neurosci 15: 3263–3272

Yoshimasa T, Sibley DR, Bovier M, Lefkowitz RJ, Caron MG (1987) Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. Nature 327: 67–69

Young MR, Fleetwood-Walker SM, Mitchell R, Munro FE (1994) Evidence for a role of metabotropic glutamate receptors in sustained nociceptive inputs to rat dorsal horn neurons. Neuropharmacology 33: 141–144