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Involvement of cGMP in Nociceptive Processing by and Sensitization of Spinothalamic Neurons in Primates

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Central sensitization of spinothalamic tract (STT) neurons in anesthetized monkeys after intradermal injection of capsaicin depends in part on disinhibition. Protein kinase C is suggested to participate in this process. The present study shows that the nitric oxide-cGMP (NO-cGMP) signal transduction system also contributes to sensitization of wide dynamic range (WDR) STT neurons located in the deep dorsal horn. The NO-cGMP system was activated by microdialysis administration into the dorsal horn of 8-bromo-cGMP, an analog of cGMP. Sensitization of STT cells by 8-bromo-cGMP increased the responses of deep WDR STT cells to both weak and strong mechanical stimulation of the skin and simultaneously attenuated the inhibition of the same neurons produced by stimulation in the periaqueductal gray (PAG). In contrast, WDR STT cells in the superficial dorsal horn and high-threshold (HT) STT cells in superficial or deep layers showed reduced responses to mechanical stimulation of the skin after infusion of 8-bromo-cGMP, and PAG inhibition of these neurons was unaffected. Sensitization of STT cells and the attenuation of PAG inhibition induced by intradermal injection of capsaicin were prevented by preteatment of the dorsal horn with a guanylate cyclase inhibitor, 1 H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1–one. The results support the hypothesis that activation of the NO-cGMP signal transduction system contributes to the sensitization of WDR STT neurons in the deep dorsal horn and helps explain why intradermal capsaicin injections often fail to sensitize superficial and HT STT cells. The results also support the idea that sensitization of STT cells is produced in part by disinhibition.

Key words: PKG; nitric oxide; guanylate cyclase; capsaicin; sensitization; spinothalamic tract; periaqueductal gray; monkey

It has been proposed that nitric oxide (NO) contributes to the development of hyperalgesia in models of acute and chronic pain (Moore et al., 1991; Haley et al., 1992; Meller et al., 1992a, 1994; Coderre and Yashpal, 1994). The best understood trigger for NO formation in nervous tissue is the opening of NMDA receptor channels, activating NO synthase (NOS) in a Ca^{2+}-dependent manner (MacDermott et al., 1986; Murase et al., 1986; Womack et al., 1988; Bredt and Snyder, 1989). NO then increases the intracellular level of cGMP through activation of soluble guanylate cyclase (Meller et al., 1992a,b; Meller and Gebhart, 1993). In the vascular and nervous systems, cGMP-dependent protein kinases serve as a major effector for NO and cGMP (Meller and Gebhart, 1993; Lincoln et al., 1994). Membrane-permeable cGMP analogs administered intrathecally produce hyperalgesia (Garry et al., 1994a). Furthermore, after intradermal injection of capsaicin, the mechanical allodynia and hyperalgesia are reversed by intraspinal administration of an inhibitor of PKG (Willis and Sluka, 1995). NMDA-induced hyperalgesia is also prevented by pretreatment of the spinal cord with a guanylate cyclase inhibitor (Meller et al., 1992b).

Our laboratory has demonstrated that excitatory amino acids (EAAs) and neuropeptides play an important role in the development and maintenance of central sensitization of primate spinothalamic tract (STT) neurons (Dougherty et al., 1992a,b, 1993, 1994, 1995; Dougherty and Willis, 1992; Sluka et al., 1992; Sorkin et al., 1992). Sensitization of STT cells can be produced by intradermal capsaicin injection or induction of acute arthritis (Simone et al., 1991; Dougherty and Willis, 1992; Dougherty et al., 1992a) and can be blocked by antagonists of NMDA or neurokinin receptors (Dougherty et al., 1992a, 1994). It seems likely that central sensitization of dorsal horn neurons is initiated by the release of EAAs and peptides in the dorsal horn and depends in part on enhanced responses to the synaptic release of EAAs by mechanoreceptive afferent fibers (Dougherty and Willis, 1992; Sluka et al., 1992; Sorkin et al., 1992; Dougherty et al., 1993, 1994, 1995; Neugebauer et al., 1995). On the other hand, we have reported recently that the inhibition of STT neurons induced by the activation of spinal glycine and GABA receptors is reduced when STT cells are sensitized after capsaicin injection or activation of protein kinase C (PKC) (Lin et al., 1996c). Additionally, we found that the inhibition of STT cells produced by stimulating the periaqueductal gray (PAG) is attenuated during central sensitization (Lin et al., 1996b). Because PAG inhibition involves activation of spinal glycine and GABA receptors (Sorkin et al., 1993; Lin et al., 1994), we hypothesize that disinhibition of STT cells might play a role in central sensitization, and that second-messenger systems are involved in this process.

We have now investigated the role of the NO-cGMP signal transduction system in the central sensitization of primate STT neurons. The contribution of changes in spinal inhibition to the central sensitization that is produced by activation of PKG was also examined by testing the inhibitory effects of stimulation in the PAG on STT neurons.

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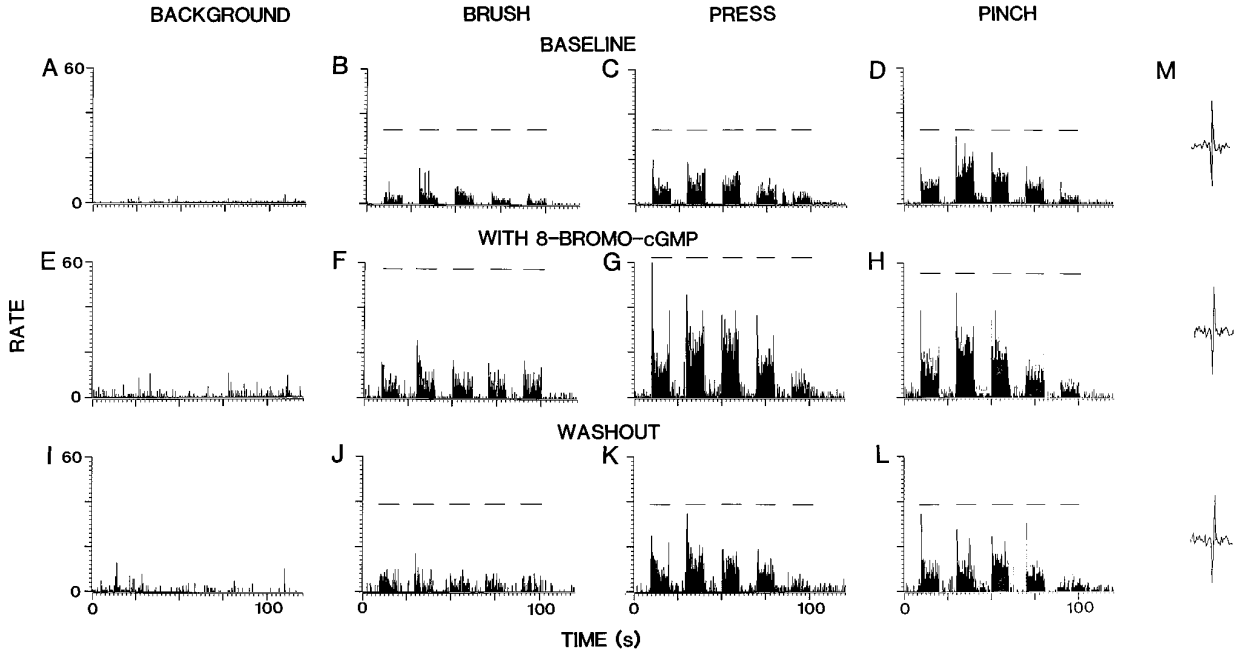

Figure 1. Rate histograms represent the enhanced responses of a deep WDR STT cell during infusion of 8-bromo-cGMP into the spinal dorsal horn by microdialysis. *A–D*, Baseline background activity and responses to mechanical stimuli (*BRUSH*, *PRESS*, and *PINCH*). *Horizontal lines* above histograms show times of application of mechanical stimuli. *E–H*, Increased background activity and responses to cutaneous mechanical stimuli produced by 8-bromo-cGMP infusion. *I–L*, Background activity and responses to mechanical stimuli 1.5 hr after the end of 8-bromo-cGMP administration. *M*, Spikes before, during, and after 8-bromo-cGMP.

A preliminary report has been published previously (Lin et al., 1996d).

MATERIALS AND METHODS

All experiments were approved by the local Animal Care and Use Committee and were consistent with the guidelines of the International Association for the Study of Pain and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Young adult monkeys *(Macaca fascicularis)* weighing 1.5–3.1 kg were sedated with ketamine (10 mg/kg, i.m.), and then anesthetized with a mixture of nitrous oxide, oxygen, and halothane, followed by an intravenous dose of α-chloralose (60 mg/kg). A stable level of anesthesia, as assessed by pupillary constriction, was maintained by intravenous infusion of pentobarbital sodium (5 $mg \cdot kg^{-1} \cdot hr^{-1}$). After a tracheotomy, the animal was artificially ventilated and paralyzed with gallamine triethiodide (20 mg/hr), which was also added to the infusion to maintain paralysis. End-tidal CO_2 and rectal temperature were kept within physiological limits (3.5–4.5% and 37 ± 1°C). A laminectomy was performed to expose the lumbar enlargement and a craniotomy was performed to allow stereotaxic placement of monopolar-stimulating electrodes into the ventral posterior lateral (VPL) nucleus of the thalamus and the PAG, respectively, as described in our previous experiments (Lin et al., 1994, 1996a).

The microdialysis fiber [150 μm inner diameter (i.d.), 9 μm thick wall, 18 kDa molecular cutoff, from Spectrum] was coated with silicone rubber except for a 1 mm gap intended to be the active dialysis zone for drug delivery within the spinal dorsal horn (Dougherty et al., 1992a). In each animal, two or three fibers were passed through the spinal cord with the dialysis zone in the dorsal horn of spinal cord segments L_5–L_7. The positions of fibers were usually located in laminae III–VI, as determined histologically (Sorkin et al., 1988). Artificial cerebrospinal fluid (ACSF), which contained (in mM): 151.1 Na^+, 2.6 K^+, 0.9 Mg^{2+}, 1.3 Ca^{2+}, 122.7 Cl^-, 21.0 HCO_3^-, 2.5 HPO_4^{2-}, and 3.87 glucose, was bubbled with 95% O_2/5% CO_2 before each experiment to reach a pH of 7.4, and was pumped continuously into the fibers with a flow rate of 5 μl/min during the control period and the period when the drug was washed out. Drugs delivered by microdialysis diffuse through at least one spinal segment, without significant leak into the blood and cerebrospinal fluid (Sluka and Westlund, 1993a).

A low-impedance (3–5 MΩ) glass carbon filament electrode was used to record extracellular single-unit discharges in the dorsal horn of the lumbosacral enlargement. STT neurons were searched for in areas close to a microdialysis fiber (within 750μm) to ensure that the drug would reach the cell. Unit activity of STT cells was monitored on storage and digital oscilloscopes and simultaneously fed to a window discriminator interfaced with a data analysis system (CED1401 *plus* linked to a Pentium computer using the Windows 95 operating system) for data storage and later analysis. Individual spike configuration and size were monitored continuously on a digital oscilloscope to confirm that the same cell was registered throughout the experiment. Figure 1*M* shows an example of the spike shape of an STT neuron during an entire experiment. STT cells were isolated using antidromic search stimuli (0.75–1 mA, 200 μsec, at 0.3 Hz) passed through the VPL electrode. The antidromic spikes occurred at fixed latency, showed collision with orthodromic spikes at appropriate intervals, and followed high-frequency (333–500 Hz) stimulus trains.

Second-messenger agents administered through a microdialysis fiber (5μl/min) in the present study included a membrane-permeable analog of cGMP, 8-bromoguanosine-3′,5′-cyclophosphate sodium (8-bromo-cGMP; RBI Inc.), and a selective inhibitor of soluble guanylate cyclase, 1 H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one [(ODQ) from Tocris Cookson, Inc., Bristol, UK]. ODQ was diluted in ACSF to a concentration of 1 mM. The concentration of substances in the dialysis fluid was presumed to be approximately two orders of magnitude higher than the concentration that reached 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 of these drugs was between 1 and 4% (Sluka and Westlund, 1993a; Sluka et al., 1993). Therefore, the final concentration of these two compounds at the site of tested neurons would be in the micromolar range, similar to that used in *in vitro* experiments, in which guanylate cyclase inhibitors have been shown to block selectively the NO-evoked increase in cGMP (Garthwaite et al., 1995). For example, the concentration of 8-bromo-cGMP used in this study was 10 mM. This would result in delivery of a concentration of 10–100 μM at the level of the neurons examined, which is comparable to the concentration used in *in vitro* experiments (Shibuki and Okada, 1991; Ito and Karachot, 1992).

When an STT neuron was isolated, the background activity and responses to mechanical stimuli were recorded. All cells were characterized by their responses to application of brush, pressure, and pinch to the skin of the hindlimb at the most responsive portion of the receptive field. The

responses were used to classify the STT cells as low threshold (LT), wide dynamic range (WDR), or high threshold (HT) (Chung et al., 1986). Five sites across the receptive field were defined for delivery of three sets of mechanical stimuli. Each stimulus was applied for 10 sec followed by a 10 sec pause before the next test site was stimulated. This sequence was followed until each kind of stimulus had been applied to all five sites. Innocuous BRUSH stimuli were delivered by repeated brushing in a stereotyped manner with a camel-hair brush. A firm pressure (PRESS stimulus) with a force of 144 g/mm^2, which is near pain threshold when placed on human skin, was applied to the skin of the receptive field by using a large clip. The small clip (PINCH) exerts a force of 538 g/mm^2 and is distinctly painful when applied to the skin. Care was taken to ensure that the BRUSH responses on each occasion were maximal and that the PRESS and PINCH stimuli were applied to the same marked site. Previous control experiments showed that repeated application of these nondamaging stimuli elicited consistent responses (Owens, 1991; Dougherty et al., 1992b). The inhibitory effects of electrically stimulating the PAG on the responses of STT cells to mechanical stimuli were tested by delivering 1 sec trains of square pulses (200 μsec, 333 Hz) repeated at 2 sec intervals to the PAG at an intensity of 100–400 μA while a 10 sec mechanical stimulus was being applied to one point within the receptive field from which the maximal mechanical response was evoked. Stimulation sites in the PAG were located as described previously (Lin et al., 1994) and were determined histologically to be distributed mostly in the lateral or ventrolateral PAG at the level of the oculomotor or trochlear nuclei.

When control responses were recorded to all test stimuli, the infusion of ACSF was switched to ACSF containing 8-bromo-cGMP for 30–60 min. The tests were repeated during drug infusion. The perfusion fluid was then returned to normal ACSF and another set of tests was made 1–2 hr after drug application was stopped. For testing the effects of the guanylate cyclase inhibitor on central sensitization after intradermal injection of capsaicin, STT cells were separated into two groups. One served as a control group in which no inhibitor was given before capsaicin was injected; for the other group, ODQ was infused for 30–60 min through a microdialysis fiber before capsaicin injection. Capsaicin was then injected intradermally at a site in the receptive field in the same way as that used in our previous studies (Lin et al., 1996b,c). In some cells, after the guanylate cyclase inhibitor had been washed out for a few hours, a second injection of capsaicin was made for the same cell. The responses were tested 15 min before and 15 min after the second capsaicin injection, respectively.

The mean total discharge rate in response to mechanical stimuli applied to the five points across the receptive field was summed, and then the background activity was subtracted to yield a net response value for each type of stimulus. The inhibitory effects of PAG stimulation on cutaneous mechanical stimulation-evoked responses were evaluated by calculating the percentage of inhibition of evoked activity. A repeated-measures ANOVA was used to test differences in the responses and the PAG inhibition 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 ± SE.

RESULTS

Recordings were made from a total of 48 STT neurons in 30 monkeys. The neurons included 43 WDR and 5 HT cells. No LT cells were encountered in the study. The depth of the neurons ranged from 972 to 2143 μm below the dorsal surface of the spinal cord, which corresponds to locations within laminae I-VI (Owens, 1991). The mean depth of recording for the WDR neurons was 1501.3 ± 43.2 μm, and that for the HT neurons was 1422.4 ± 41.1 μm. Twenty-five cells were tested using 8-bromo-cGMP. These cells included 20 WDR neurons and 5 HT neurons. The remaining 23 cells, which were all classified as WDR neurons, were used in experiments to examine the effects of ODQ on the central sensitization produced by capsaicin injection.

Changes in the responses of STT neurons to cutaneous mechanical stimuli produced by intraspinal administration of 8-bromo-cGMP

Two distinctly different types of changes in the responses to cutaneous stimuli were seen in STT cells when the spinal dorsal horn was perfused with the same concentration of 8-bromo-cGMP. The direction of the changes was found to be dependent on how deep an STT cell was located in the dorsal horn and on what category the STT cell belonged to. For example, Figure 1 shows the responses recorded from an STT neuron that was categorized as a WDR cell located 1453 μm from the surface of the spinal cord. When 8-bromo-cGMP was infused into the dorsal horn, the background activity and the responses of this neuron to the BRUSH, PRESS, and PINCH stimuli increased (compare Fig. 1*A–D* with Fig. 1*E–H*). The responses showed partial recovery 1.5 hr after the infusion of 8-bromo-cGMP ended (Fig. 1*I–L*). Comparable observations were obtained for 12 other WDR STT neurons located in the deep dorsal horn.

In contrast, Figure 2 shows the responses of an STT cell that was also categorized as a WDR neuron, but that was located in the superficial dorsal horn. The recording depth was 1030 μm. Infusion of 8-bromo-cGMP resulted in an obvious decrease in the responses to BRUSH, PRESS, and PINCH, with a slight decrease in background activity (compare Fig. 2*A–D* with Fig. 2*E–H*). Partial recovery was observed 2 hr after the termination of the infusion of 8-bromo-cGMP (Fig. 2*I–L*). Similar findings were obtained for six other WDR STT neurons located in the superficial dorsal horn. Five STT cells that were classified as HT neurons and located from 1288 to 1530 μm below the surface of the spinal cord also showed reduced responses to mechanical stimuli when 8-bromo-cGMP was infused into the dorsal horn.

The changes in the responses to mechanical stimuli produced by 8-bromo-cGMP infusion are summarized in Figure 3. Because the same effect was obtained for both HT cells and superficial WDR neurons, the data from these cells were pooled. For the WDR cell population in the deep dorsal horn at depths ranging from 1352 to 2143 μm that are presumably distributed within laminae V–VI (n = 13; Fig. 3*A*), microdialysis infusion of 8-bromo-cGMP resulted in a significant increase in background activity and in the responses to BRUSH, PRESS, and PINCH. In contrast, there was a significant reduction in the responses of HT cells (n = 5; 1288–1530 μm; Fig. 3*C*) and superficial WDR neurons (n = 7; 972–1288 μm; Fig. 3*C*) to BRUSH, PRESS, and PINCH without an obvious change in the background activity. After termination of the infusion, there was partial recovery of the responses.

Changes in the inhibitory effects of stimulation in the PAG produced by intraspinal administration of 8-bromo-cGMP

The effects of intraspinal infusion of 8-bromo-cGMP on the inhibition of responses to mechanical stimuli produced by stimulation in PAG were tested on the same groups of the cells that were used for examining the effects on mechanical stimulation-evoked responses. Figure 4*A* is an example of a recording from a deep WDR neuron (1670 μm). The top row shows the inhibitory effects of PAG stimulation on background activity and on the responses to BRUSH, PRESS, and PINCH. The inhibition was consistent with findings in our previous work (Gerhart et al., 1984; Lin et al., 1994). The baseline percentage of inhibition of the evoked responses was −45.4% for inhibition of BRUSH, −70.6% for PRESS, and −75.6% for PINCH. When the cell was sensitized by 8-bromo-cGMP infusion, the percentage of inhibition decreased to −11.9% for BRUSH, −39.6% for PRESS, and −47.9% for PINCH (*second row*, Fig. 4*A*). Inhibition recovered partially 1.5 hr after the end of drug infusion (*bottom row*, Fig. 4*A*).

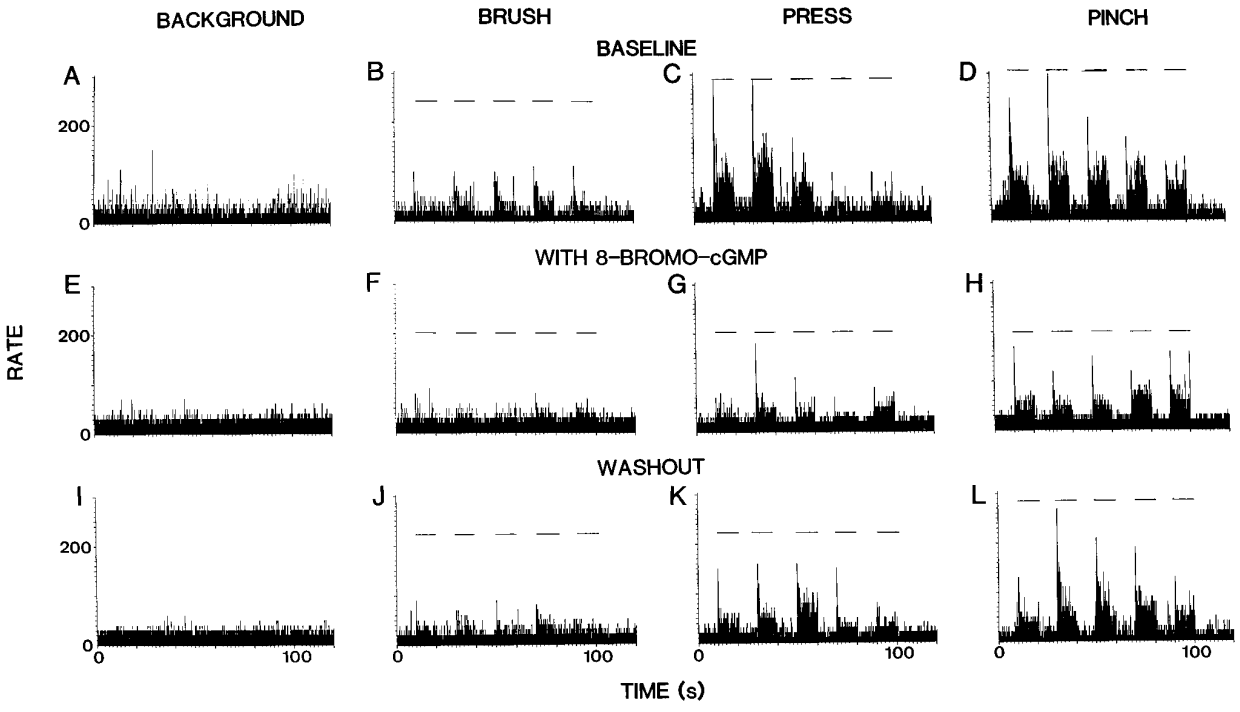

Figure 2. Rate histograms show the reduced responses of a superficial WDR cell produced by infusion of 8-bromo-cGMP within the spinal dorsal horn by microdialysis. *A–D*, Baseline background activity and responses to mechanical stimuli (*BRUSH*, *PRESS*, and *PINCH*). *Horizontal lines* above histograms show times of application of mechanical stimuli. *E–H*, Decreased responses to cutaneous mechanical stimuli produced by 8-bromo-cGMP infusion. *I–L*, Background activity and responses to the mechanical stimuli 2 hr after the end of 8-bromo-cGMP administration.

The effects of PAG stimulation were attenuated significantly during 8-bromo-cGMP application (Fig. 3*B*). Of 13 cells tested, a >25% attenuation of the PAG-induced inhibition of responses to BRUSH, PRESS, and PINCH stimuli was obtained in 11 (84.6%), 8 (61.5%), and 9 (69.2%) cells, respectively. Thus, the attenuation was particularly striking for the inhibition of the BRUSH responses ($p < 0.001$).

For HT and superficial WDR STT cells in which 8-bromo-cGMP infusion reduced responses to mechanical stimuli, 8-bromo-cGMP had no effect on the PAG-induced inhibition. An example of recordings from a superficial WDR neuron (972 μm) is shown in Figure 4*B*. In some of these cells, a potentiation of PAG-induced inhibition was seen, but this did not reach statistical significance. A summary showing the lack of effect of 8-bromo-cGMP is provided by the bar graph in Figure 3*D*.

Effects of a guanylate cyclase inhibitor on sensitization of STT neurons and blockade of PAG-induced inhibition produced by intradermal injection of capsaicin

Capsaicin has been demonstrated to produce central sensitization of STT neurons to innocuous cutaneous stimuli and to attenuate the inhibition of STT neurons induced by stimulation of the PAG (Simone et al., 1991; Dougherty and Willis, 1992; Dougherty et al., 1992a; Lin et al., 1996b). In the present study, one group of WDR STT cells in the deep dorsal horn ($n = 11$) was pretreated with a guanylate cyclase inhibitor, ODQ, by microdialysis, before intradermal injection of capsaicin. The responses to mechanical cutaneous stimuli and PAG-induced inhibition were then compared with those seen in another group of STT cells ($n = 12$) without ODQ pretreatment (Fig. 5).

Figure 5*A* summarizes the effects of ODQ on changes in the responses of STT cells to mechanical stimuli after intradermal injection of capsaicin. In the control group of STT cells, which were not pretreated with ODQ, as shown by the left pair of each set of bars (ACSF), a significant increase in the responses to BRUSH and PRESS (but not PINCH) was observed 15 min after capsaicin injection in all cells tested. In contrast, when ODQ was infused within the spinal dorsal horn while recordings were made from another group of STT cells, as shown by the right pair of each set of bars (ODQ), capsaicin injection failed to evoke an increase in any of the responses to mechanical stimuli.

Figure 5*B* summarizes the effects of ODQ on changes in PAG-induced inhibition evoked by intradermal injection of capsaicin. Consistent with our previous experiments (Lin et al., 1996b), capsaicin injection resulted in a reduction in PAG-induced inhibition of the responses to mechanical stimuli in the control group of STT neurons that were not pretreated with ODQ. A >25% blockade was seen in 10 cells (10 of 12, 83.3%), and the grouped effects reached statistical significance. This is shown by the left pair of each set of bars (ACSF). When the spinal dorsal horn was perfused with ODQ while recording from another group of cells, however, as shown by the right pair of each set of bars (ODQ), capsaicin-induced attenuation of inhibition induced by stimulation of the PAG was prevented completely. In addition, comparison of the baseline values for mechanical responses and PAG inhibition between the two groups of STT cells (open bars in each set of bars) reveals that ODQ itself had no significant effect on the cellular responses to mechanical stimuli or PAG inhibition.

For some STT neurons, it was possible to make two successive injections of capsaicin 2–3 hr apart. Examples are shown in Figures 6 and 7. The spinal dorsal horn was pretreated with ODQ for 30–60 min before the first capsaicin injection. ODQ itself did not have any obvious effect on mechanically evoked responses or PAG inhibition (*second row*, Figs. 6, 7). The first capsaicin injec-

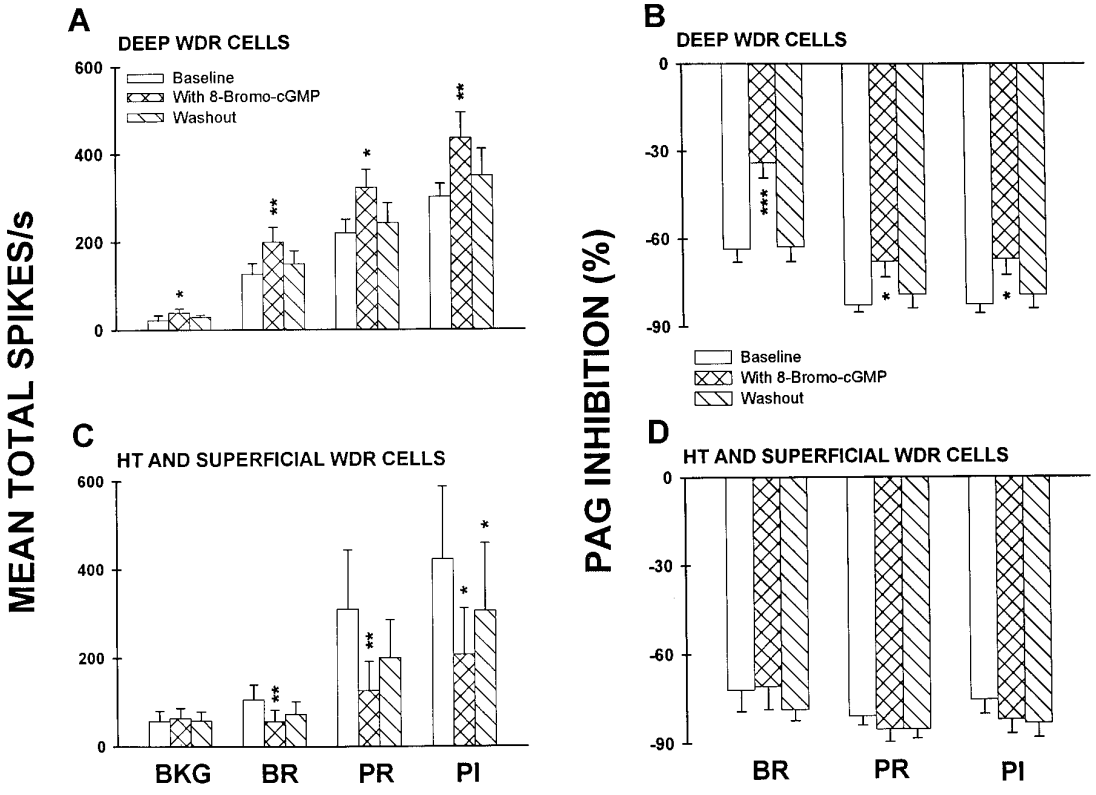

Figure 3. *Bar graphs* summarize the grouped data from STT neurons for background activity, responses to mechanical stimuli, and PAG inhibition when the spinal dorsal horn was perfused with 8-bromo-cGMP. *Panels A* and *B*, Data from deep WDR STT cells ($n = 13$); *panels C* and *D*, data from HT and superficial WDR STT cells ($n = 5$ for HT cells, $n = 7$ for superficial WDR cells). *BKG*, Background activity; *BR*, BRUSH; *PR*, PRESS; *PI*, PINCH. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the baseline level.

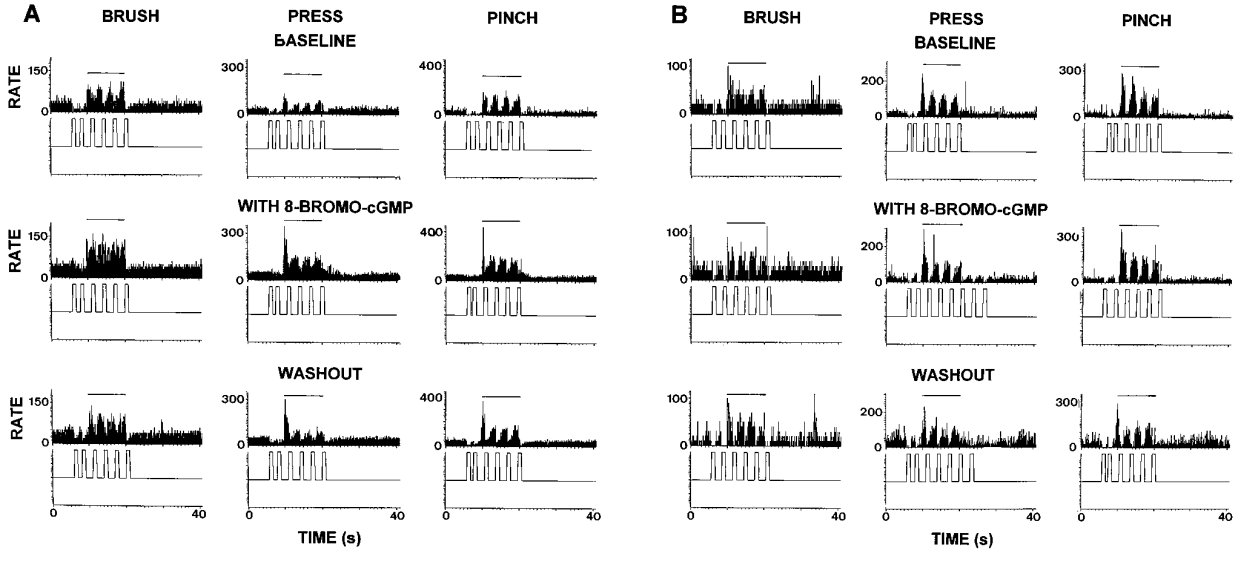

Figure 4. Changes in the inhibition of responses of a deep WDR STT neuron (*A*) and a superficial WDR STT cell (*B*) to mechanical stimuli produced by PAG stimulation when the spinal dorsal horn was perfused with 8-bromo-cGMP. *Top row,* Control effects of PAG stimulation; *second row,* PAG inhibition during 8-bromo-cGMP infusion; *bottom row,* PAG inhibition 1.5 hr after the end of 8-bromo-cGMP infusion. Trains of stimuli applied in the PAG are indicated by *upward-going square waves* below each histogram.

tion, however, did not result in the sensitization of the cell to BRUSH and PRESS or any attenuation of PAG inhibition (*third row*, Figs. 6, 7), although the background activity was increased (*third row*, Fig. 6). ACSF was then infused to wash out any remaining drug for 1.5–2.0 hr, at which time the responses and PAG inhibition were similar to the control values (*fourth row*, Figs. 6, 7). Increased responses to BRUSH and PRESS and a reduced PAG inhibition were then observed after the second dose of capsaicin was injected intradermally, indicating that the effects of ODQ were reversible (*bottom row*, Figs. 6, 7).

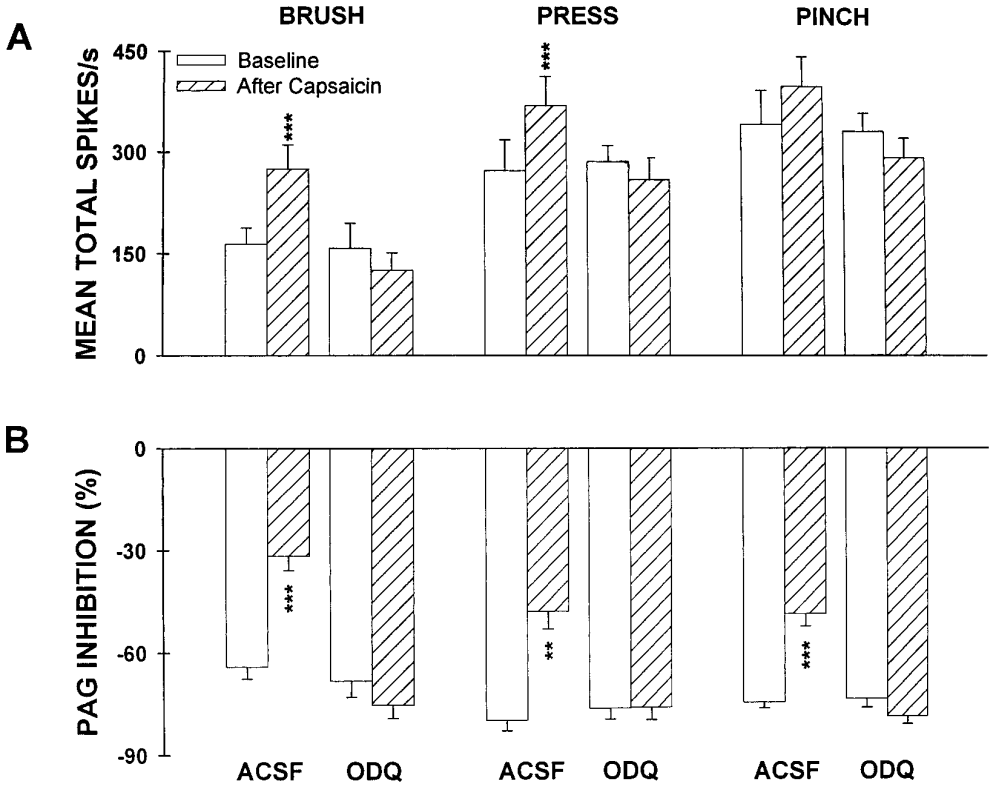

Figure 5. *Bar graphs* summarize the effects of ODQ infusion on the responses of STT cells to mechanical stimuli after capsaicin injection. The responses to mechanical stimulation are shown in *panel A* and PAG inhibition in *panel B*. Cells were divided into two groups. Control responses after capsaicin injection from cells that were not pretreated with ODQ are shown by the *left pair* of each set of *bars* (*ACSF*), and the responses after capsaicin injection when cells were pretreated with ODQ are shown by the *right pair* of each set of *bars* (*ODQ*). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the pre-capsaicin baseline.

DISCUSSION

These experiments demonstrate that administration of 8-bromo-cGMP, a membrane-permeable derivative of cGMP that acts as an analog of cGMP, into the spinal dorsal horn produces long-lasting changes in the responsiveness of STT neurons. The effects vary with the distribution and type of STT cells. STT neurons classified as WDR cells and distributed in the deep layers of the dorsal horn were sensitized during 8-bromo-cGMP infusion. 8-bromo-cGMP decreased the responses to both innocuous and noxious cutaneous stimuli of WDR STT neurons located more superficially and HT STT cells. The inhibition of BRUSH, PRESS, and PINCH responses induced by stimulation of the PAG was attenuated when deep WDR STT cells were sensitized by 8-bromo-cGMP infusion. 8-bromo-cGMP, however, produced no effect on PAG-induced inhibition of HT or superficial WDR STT cells. Furthermore, we found that the enhancement of responses to innocuous stimuli and the attenuation of PAG inhibition of these responses produced by intradermal injection of capsaicin could be prevented by pretreatment of the spinal dorsal horn with a guanylate cyclase inhibitor, ODQ.

Several lines of evidence suggest a role for the NO-cGMP signal transduction system in the development of hyperalgesia. Intrathecal injection of 8-bromo-cGMP resulted in thermal hyperalgesia (Garry et al., 1994a) and increased neuropathic pain-related autotomy (Niedbala et al., 1995). Iontophoretic application of 8-bromo-cGMP onto dorsal horn neurons preferentially enhances responses to noxious stimuli (Radhakrishnan and Henry, 1996). Moreover, an elevated level of immunoreactive cGMP in the dorsal horn was found when hyperalgesia developed after intraplantar injection of carrageenan (Garry et al., 1994b). Behavioral experiments by our group have shown that the allodynia after intradermal injection of capsaicin can be reversed by intraspinal administration of a PKG inhibitor (Willis and Sluka, 1995). These data are consistent with our present results.

We have demonstrated that central sensitization of STT neurons and persistent nociceptive behavior caused by capsaicin injection or arthritis depend on activity at both EAA and neurokinin receptors (Dougherty and Willis, 1992; Dougherty et al., 1992a,b, 1994, 1995; Sluka and Westlund, 1993a,b). It is known that the cGMP level is affected by excitatory pathways that release glutamate (Garthwaite, 1991; Meller and Gebhart, 1993). An increase in cGMP level within the cerebellum was elicited by exogenous glutamate both *in vivo* and *in vitro* (Ferrendelli et al., 1974; Mao et al., 1974; Garthwaite and Balázs, 1978). Glutamate increases cGMP content in cells through activation of NOS, which catalyzes the production of NO from L-arginine (Garthwaite and Balázs, 1978; Garthwaite et al., 1988; Bredt and Snyder, 1989, 1990). This process involves Ca^{2+} influx through receptor-operated ion channels, such as NMDA and some non-NMDA receptors (MacDermott et al., 1986; Garthwaite et al., 1988; Mayer and Miller, 1990; Lerea et al., 1992). The elevated level of NO caused by NOS activation in turn activates soluble guanylate cyclase, thus increasing the intracellular level of cGMP (Knowles et al., 1989; Southam et al., 1991; Bredt and Snyder, 1992).

Immunocytochemical studies have demonstrated NOS in the spinal cords of rats and monkeys (Dun et al., 1992; Zhang et al., 1993). NOS antagonists block nociceptive behavior induced by intraplantar injection of formalin (Moore et al., 1991; Coderre and Yashpal, 1994), decrease the discharges of dorsal horn neurons after formalin injection (Haley et al., 1992), reduce hyperalgesia in a model of neuropathic pain and after intrathecal administration of EAA and SP agonists (Meller et al., 1992a; Radhakrishnan et al., 1995), and prevent hyperalgesia after intrathecal application of NMDA (Kitto et al., 1992). PKG is selectively activated by cGMP or its analogs (Butt et al., 1992; Hartell, 1994). PKG exerts its modulatory effects by phosphorylation of proteins (Lincoln and Cornwell, 1993; Schmidt et al., 1993). Long-term depression of cerebellar Purkinje cells involves desen-

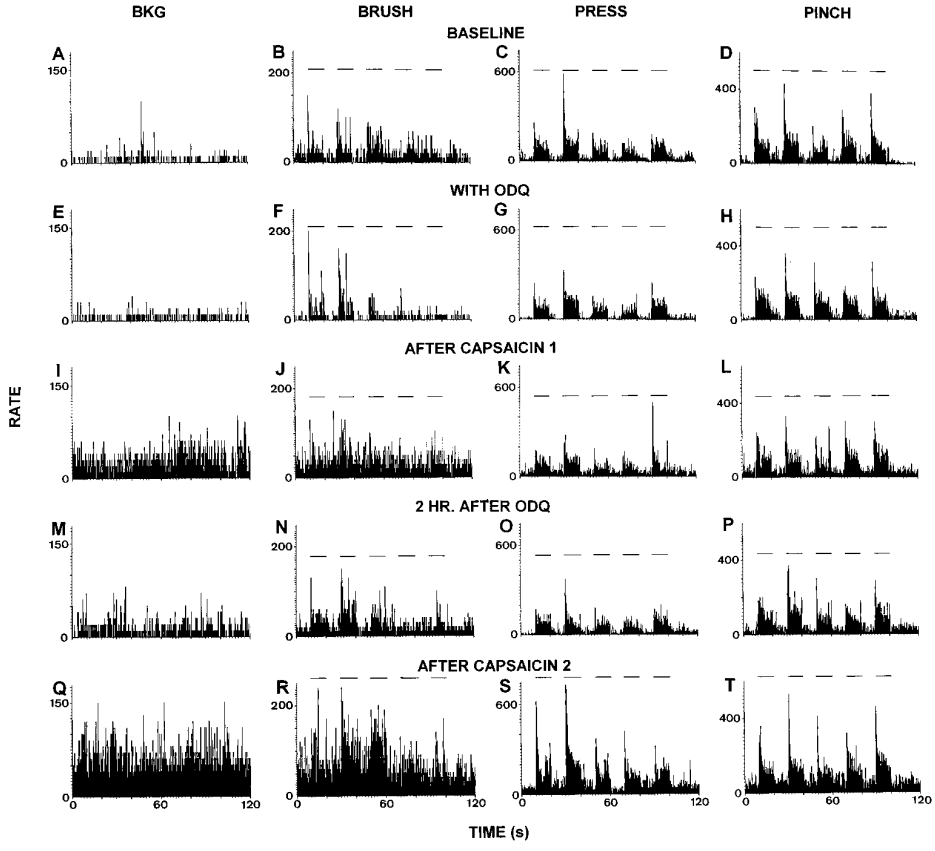

Figure 6. Rate histograms show the responses of an STT cell to mechanical cutaneous stimuli after intradermal injection of capsaicin with and then without pretreatment of spinal dorsal horn with ODQ, respectively. *A–D*, Baseline background activity and responses to mechanical stimuli (*BRUSH*, *PRESS*, and *PINCH*). *Horizontal lines* above histograms represent times of application of mechanical stimuli. *E–H*, Effects of infusion of ODQ within the dorsal horn. *I–L*, Effects of the first capsaicin injection during ODQ infusion by microdialysis. *M–P*, Two hours after the end of ODQ infusion. *Q–T*, Effects produced by the second capsaicin injection when ODQ was washed out.

sitization of AMPA receptors by their phosphorylation after activation of PKG (Ito and Karachot, 1990, 1992). Recently, cGMP-dependent protein kinase I was identified in neurons of spinal dorsal root ganglia and co-expressed with neuronal NOS, providing anatomical evidence that neuron-derived NO could serve to increase cGMP levels and activate cGMP-dependent protein kinase in primary afferent nociceptors (Qian et al., 1996). It can be predicted, therefore, that one of the routes by which the NO-cGMP cascade mediates central sensitization of dorsal neurons is triggered by activation of EAA and neurokinin receptors during noxious stimulation.

We have reported evidence that activation of PKC enhances the responses of STT cells to peripheral stimulation and is involved in the capsaicin-induced sensitization of these neurons (Lin et al., 1996b). Examination of the differences in the effects of activators of PKC and PKG suggests that these protein kinases play somewhat different roles. Microdialysis administration of a phorbol ester, which activates PKC, enhances the responses of STT cells to BRUSH and PRESS, but not to PINCH (Paleček et al., 1994; Lin et al., 1996b). By contrast, spinal infusion of 8-bromo-cGMP, which activates PKG, was found in the present study to increase the responses of STT cells to all three intensities of mechanical cutaneous stimuli. Intradermal injection of capsaicin consistently increases the responses of STT cells to BRUSH and PRESS stimuli, but only in some STT cells does it increase the responses to PINCH. Thus, it appears that PKC is likely to produce the dominant effect in most STT cells after intradermal capsaicin injection, although in some neurons PKG may have a more prominent role. In future experiments, the effects of 8-bromo-cGMP on the responses of STT cells to noxious heat will be examined as a further test of differences between the actions of the PKC and PKG cascades.

It has been suggested recently that disinhibition of STT neurons in response to peripheral stimulation by desensitizing inhibitory amino acid (IAA) receptors, such as glycine and GABA receptors, also contributes to central sensitization (Lin et al., 1996c). Therefore, the change in PAG-induced inhibition can reflect indirectly a functional change in IAA activity because spinal glycine and GABA receptors have been demonstrated to help mediate PAG descending inhibition (Sorkin et al., 1993; Lin et al., 1994). In the present study, microdialysis administration of 8-bromo-cGMP reduced PAG inhibition of WDR STT cells in the deep dorsal horn that underwent central sensitization, but not PAG inhibition of STT cells that were classified as HT or located in the superficial dorsal horn and that were not sensitized. These findings suggest a close association between central sensitization and a reduction in descending inhibition. The inhibitory action of GABA and glycine applied iontophoretically in the vicinity of STT cells is attenuated after intradermal injection of capsaicin or activation of PKC, suggesting a desensitization of IAA receptors on STT cells (Lin et al., 1996c). Presumably this desensitization could be produced by phosphorylation of these receptors by PKC and PKG. In other neural systems, protein kinases phosphorylate certain subunits of IAA receptors, decreasing inhibitory currents (Leidenheimer et al., 1992; Ragozzino and Eusebi, 1993; Rapallino et al., 1993; Vaello et al., 1994). Agents affecting the NO pathway reduce Cl^- currents elicited by GABA in cerebellar granule cells (Zarri et al., 1994). Thus, it is presumed that an attenuation of tonic inhibition of dorsal horn neurons mediated by GABA or glycine receptors could play an important role in central sensitization. Part of these actions appear to be mediated by PKC (Lin et al., 1996b); the present study suggests that PKG may also contribute. In addition, there is an enhancement of the effects of EAAs on glutamate receptors (Chen and Huang, 1991; Dougherty

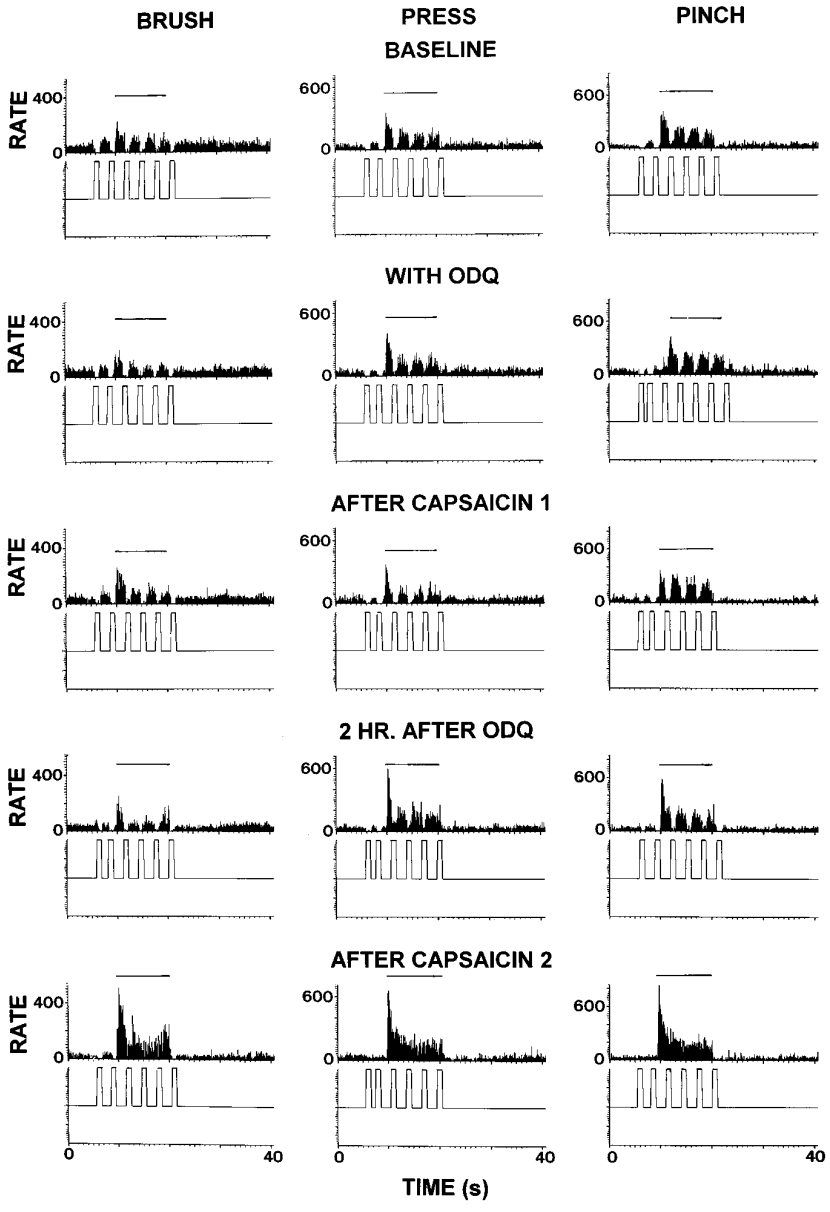

Figure 7. Rate histograms show changes in the attenuation of inhibitory effects of stimulation in the PAG on the responses of an STT cell to mechanical stimuli after intradermal capsaicin injection with and then without pretreatment of spinal cord with ODQ. *Top row*, Control effects of PAG stimulation; *second row*, effects of infusion of ODQ within the dorsal horn; *third row*, PAG inhibition 15 min after the first capsaicin injection during ODQ infusion; *fourth row*, PAG inhibition 2 hr after the end of ODQ infusion; *bottom row*, PAG inhibition 15 min after the second capsaicin injection when ODQ was washed out. Trains of stimuli were applied in the PAG at times indicated by *upward-going square waves* below each histogram. *Horizontal lines* above histograms represent times of application of mechanical stimuli.

and Willis, 1992; Dougherty et al., 1992b). We are currently investigating how activation of the NO-cGMP signal transduction system affects IAA receptors.

The same dose of 8-bromo-cGMP significantly reduced the responses of HT and superficial WDR STT cells to cutaneous mechanical stimuli in this study. One explanation could be that activation of PKG produces a functional change in GABA and glycine receptors on the HT and superficial WDR STT cells that is different from that on the deep WDR STT neurons. GABA and glycine receptors have been demonstrated to be distributed in the most layers of the spinal dorsal horn and an inhibitory effect was always seen in deep and superficial WDR STT cells, as well as HT cells, when these receptors were activated (Willcockson et al., 1984; Carlton et al., 1992; Mitchell et al., 1993; Bohlhalter et al., 1994; Lin et al., 1994, 1996a). Current information, however, fails to provide direct evidence that PKG produces differential effects on IAA receptors in different layers of dorsal horn. On the other hand, capsaicin injections have been shown to fail to sensitize significantly the cutaneous-evoked responses of HT STT cells, in contrast with the usual findings for WDR STT cells (Simone et al., 1991; Dougherty and Willis, 1992). Superficial STT neurons have small receptive fields for high-intensity stimuli (PINCH and heat) and weak responses to low-intensity stimuli (BRUSH), similar to HT cells in the deep dorsal horn (Chung et al., 1986; Al-Chaer et al., 1994; Rees et al., 1995). The responses of superficial dorsal horn neurons to noxious stimulation are facilitated by descending pathways that originate in brainstem nuclei, such as the anterior pretectal nucleus (APTN). Stimulation of the APTN produces antinociception and inhibition of the responses of the nociceptive cells located in deep layers of the dorsal horn (Al-Chaer et al., 1994; Rees et al., 1995). It has been proposed that noxious stimuli excite superficial neurons that activate brainstem nuclei, which in turn inhibit deep neurons of the dorsal horn to reduce the responses to noxious stimuli (McMahon and Wall, 1988; Rees et al., 1995). Therefore, it is reasonable to postulate that the inhibitory effects of cGMP on HT and superficial STT neurons might interfere with this positive feedback pathway and this would attenuate the descending inhibition of deep cells. This presumably

could be another mechanism by which deep neurons are sensitized by the NO-cGMP pathway.

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