

Role of Protein Kinase A in the Maintenance of Inflammatory Pain

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Although the initiation of inflammatory pain (hyperalgesia) has been demonstrated to require the cAMP second messenger signaling cascade, whether this mechanism and/or other mechanisms underlie the continued maintenance of the induced hyperalgesia is unknown. We report that injection of adenylyl cyclase inhibitors before but not after injection of direct-acting hyperalgesic agents (prostaglandin E_2 and purine and serotonin receptor agonists) resulted in reduction in hyperalgesia, evaluated by the Randall–Selitto paw-withdrawal test. In contrast, injection of protein kinase A (PKA) inhibitors either before or after these hyperalgesic agents resulted in reduced hyperalgesia, suggesting that hyperalgesia after its activation was maintained by persistent PKA activity but not by adenylyl cyclase activity. To evaluate further the role of PKA activity in the maintenance of hyperalgesia, we injected the catalytic subunit

of PKA (PKACS) that resulted in hyperalgesia similar in magnitude to that induced by the direct-acting hyperalgesic agents but much longer in duration (>48 vs 2 hr). Injection of WIPTIDE (a PKA inhibitor) at 24 hr after PKACS reduced hyperalgesia, suggesting that PKACS hyperalgesia is not independently maintained by steps downstream from PKA. In summary, our results indicate that, once established, inflammatory mediator-induced hyperalgesia is no longer maintained by adenylyl cyclase activity but rather is dependent on ongoing PKA activity. An understanding of the mechanism maintaining hyperalgesia may provide important insight into targets for the treatment of persistent pain.

Key words: adenylyl cyclase; cAMP; hyperalgesia; pain; protein kinase A; prostaglandin E_2

Tissue injury results in the production of inflammatory mediators, several of which sensitize primary afferent nociceptors (Martin et al., 1987; Schaible and Schmidt, 1988; Davis et al., 1993; Rueff and Dray, 1993), resulting in hyperalgesic pain (tenderness) (Collier and Schneider, 1972; Moncada et al., 1975; Ferreira et al., 1978; Ferreira, 1981). Inflammatory mediators such as prostaglandin E_2 (PGE_2), adenosine, and serotonin (5-HT) act directly on primary afferent nociceptors to decrease the activation threshold and to increase the response to a constant intensity stimulus (Taiwo and Levine, 1989, 1990, 1992; England et al., 1996; Gold et al., 1996a). A substantial literature suggests that PGE_2 -, 5-HT-, and adenosine-induced hyperalgesia, as well as hyperalgesia induced by tissue damage, is initiated by activation of the adenylyl cyclase (AC)–cAMP–protein kinase A (PKA) second messenger cascade (Taiwo et al., 1989; Taiwo and Levine, 1990, 1991, 1992; Pitchford and Levine, 1991; Khasar et al., 1995; England et al., 1996; Malmberg et al., 1997). Voltage-gated sodium currents may be modulated by PKA. Although the majority of voltage-gated sodium currents modulated by PKA are inhibited, it has been shown that PKA is able to enhance the tetrodotoxin-resistant sodium current (England et al., 1996; Cardenas et al., 1997; Gold et al., 1999), a sodium current found primarily in nociceptors (Akopian et al., 1996; Sangameswaran et al., 1996), that is thought to underlie inflammatory mediator-induced sensitization of nociceptors (England et al., 1996; Gold et al., 1996a; Cardenas et al.,

1997) and hyperalgesia (Khasar et al., 1999). Thus, agents that inhibit AC and cAMP-dependent protein kinase (PKA) prevent induction of hyperalgesia by PGE_2 , CGS21680, and 8-hydroxy-2-(di-*N*-propylamino)tetralin (8-OH-DPAT).

Because of the important ramifications for treatment of chronic inflammatory pain, we investigated whether ongoing activity in the cAMP second messenger pathway contributes to maintenance as well as initiation of hyperalgesia and, if so, which steps in the cAMP second messenger cascade determine duration of the hyperalgesia.

MATERIALS AND METHODS

Animals. Experiments were performed on male Sprague Dawley rats (200–250 gm; Bantin-Kingman, Fremont, CA). Animals were housed in groups of two under a 12:12 hr light/dark cycle. Food and water were available *ad libitum*. All behavioral testing was done between 10:00 A.M. and 4:00 P.M. Experiments were performed under approval of the Institutional Animal Care Committee of the University of California, San Francisco.

Behavioral testing. The nociceptive flexion reflex was quantified with a Basile Analgesymeter (Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the rat's hindpaw. The mean baseline threshold was determined as the mean of three readings before the administration of the test agent. The mean baseline threshold, before treatments, for the rats used in these experiments was 109.0 ± 0.45 gm (mean \pm SEM; $n = 284$ paws). Mechanical threshold was redetermined at three time points (15, 20, and 25 min) after treatments. The mean of these three readings was considered to be the paw-withdrawal threshold because of drug administration, and this value was used to calculate the percentage change from the baseline threshold for each paw.

The onset of hyperalgesia was determined by assessing 1, 2, 3, 4, and 5 min after injection of a hyperalgesic agent.

Drug administration. The agents used in this study were PGE_2 (Sigma, St. Louis, MO); WIPTIDE, 8-OH-DPAT, CGS21680, and SQ22356

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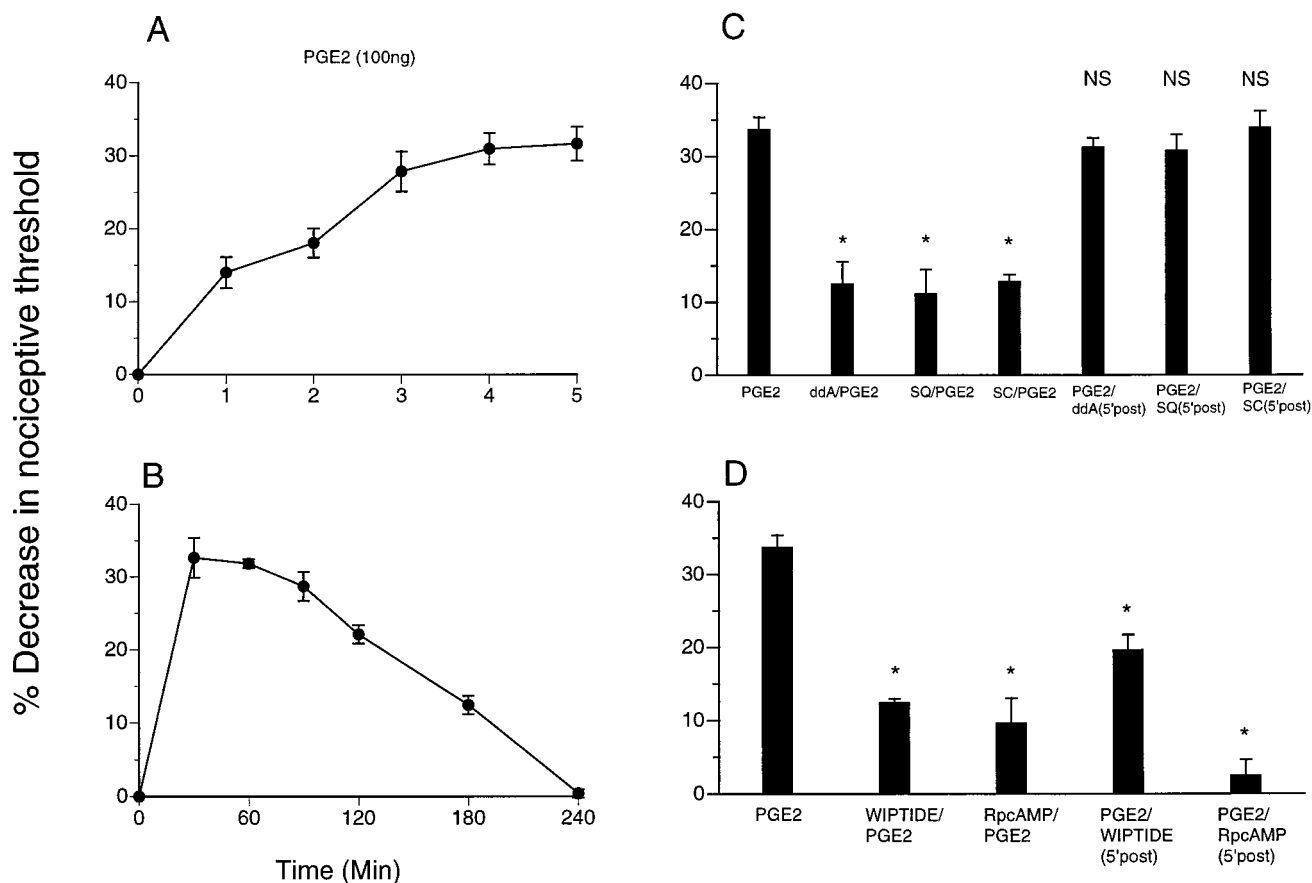


Figure 1. *A*, Latency of onset of PGE₂-induced mechanical hyperalgesia in the hindpaw of the rat ($n = 12$). *B*, Time course of PGE₂-induced mechanical hyperalgesia ($n = 12$). *C*, Effect on PGE₂-induced mechanical hyperalgesia ($n = 22$; $p < 0.05$) of the adenylyl cyclase inhibitors ddA and SQ22356 (SQ) when administered before [ddA/PGE₂, $n = 8$; $*p < 0.05$; SQ/PGE₂, $n = 6$; $*p < 0.05$; SC19220 (SC)/PGE₂, $n = 6$; $*p < 0.05$] or 5 min after (PGE₂/ddA, $n = 10$; $p > 0.05$; PGE₂/SQ, $n = 8$; $p > 0.05$) intradermal administration of PGE₂. *D*, Effect of PKA inhibitors [WIPTIDE and RpcAMPS (RpcAMP)] when administered before (WIPTIDE/PGE₂, $n = 10$; $*p < 0.05$; RpcAMP/PGE₂, $n = 6$; $*p < 0.05$) or 5 min after (PGE₂/WIPTIDE, $n = 10$; $*p < 0.05$; PGE₂/RpcAMP, $n = 6$; $*p < 0.05$) intradermal administration of PGE₂. NS, Not significant.

(Research Biochemicals, Natick, MA); protein kinase A catalytic subunit (PKACS) and RpcAMPS (Calbiochem, La Jolla, CA); dideoxyadenosine (ddA) (courtesy of Dr. Johnson, State University of New York, Stony Brook, NY); and SC19220 (a generous gift from G. D. Searle). The selection of the doses used was based on the dose–response curves determined in this or previous studies (Aley and Levine, 1997, 1998). The stock solution of PGE₂ (1 μ g/2.5 ml) was prepared in 10% ethanol, and further dilutions were made in saline; the final concentration of ethanol was $\leq 1\%$. PKACS and WIPTIDE were dissolved in saline. All drugs were administered intradermally in a volume of 2.5 μ l/paw. For test agents with low cell membrane permeability (WIPTIDE and PKACS), coinjection of 2 μ l of distilled water, to produce hypo-osmotic shock and facilitate cell permeability to these agents, was used (West and Huang, 1980; Burch and Axelrod, 1987; Leidenheimer and Harris, 1991). When combinations of agents were used, they were administered from the same syringe in such a way that the agent mentioned first reached the intradermal site first; similarly, the distilled water preceded agents with low cell membrane permeability. The agents were separated in the syringe by a small air bubble to prevent their mixing in the syringe.

To enhance our ability to analyze a specific second messenger system (that is, to ensure the greatest specificity in block of function), we have (1) used multiple inflammatory mediators known to be direct-acting hyperalgesic agents producing hyperalgesia by the cAMP–PKA second messenger signaling pathway, (2) used the most specific blockers available, (3) used more than one specific blocker of a single target in the second messenger pathway, and (4) used blockers at multiple sites in the same second messenger pathway.

Statistics. Data are presented as mean \pm SEM; statistical significance was determined by ANOVA followed by Scheffe's *post hoc* test, and $p < 0.05$ was considered statistically significant.

RESULTS

Injection of PGE₂ (100 ng) resulted in a significant decrease in the nociceptive threshold that reached peak magnitude by 5 min (Fig. 1*A*). Injection of an adenylyl cyclase inhibitor, ddA or SQ22356, before PGE₂ reduced PGE₂-induced hyperalgesia; however, if injected 5 min after PGE₂, ddA or SQ22356 had no effect on the established PGE₂ hyperalgesia (Fig. 1*C*). Similarly, injection of the E-type prostaglandin receptor antagonist SC19220 before but not 5 min after PGE₂ attenuated PGE₂-induced hyperalgesia (Fig. 1*C*). Injection of a PKA inhibitor, WIPTIDE or RpcAMPS, before PGE₂ also inhibited PGE₂-induced hyperalgesia (Fig. 1*D*); however, unlike the cyclase inhibitors, WIPTIDE or RpcAMPS injected 5 min after PGE₂ was also able to inhibit PGE₂ hyperalgesia. These results suggest that, 5 min after its induction, PGE₂ hyperalgesia is maintained by PKA activity and that adenylyl cyclase activity is no longer required.

To evaluate the generalizability of this role of PKA in the maintenance of inflammatory hyperalgesia, we determined the effect of adenylyl cyclase and PKA inhibitors on hyperalgesia

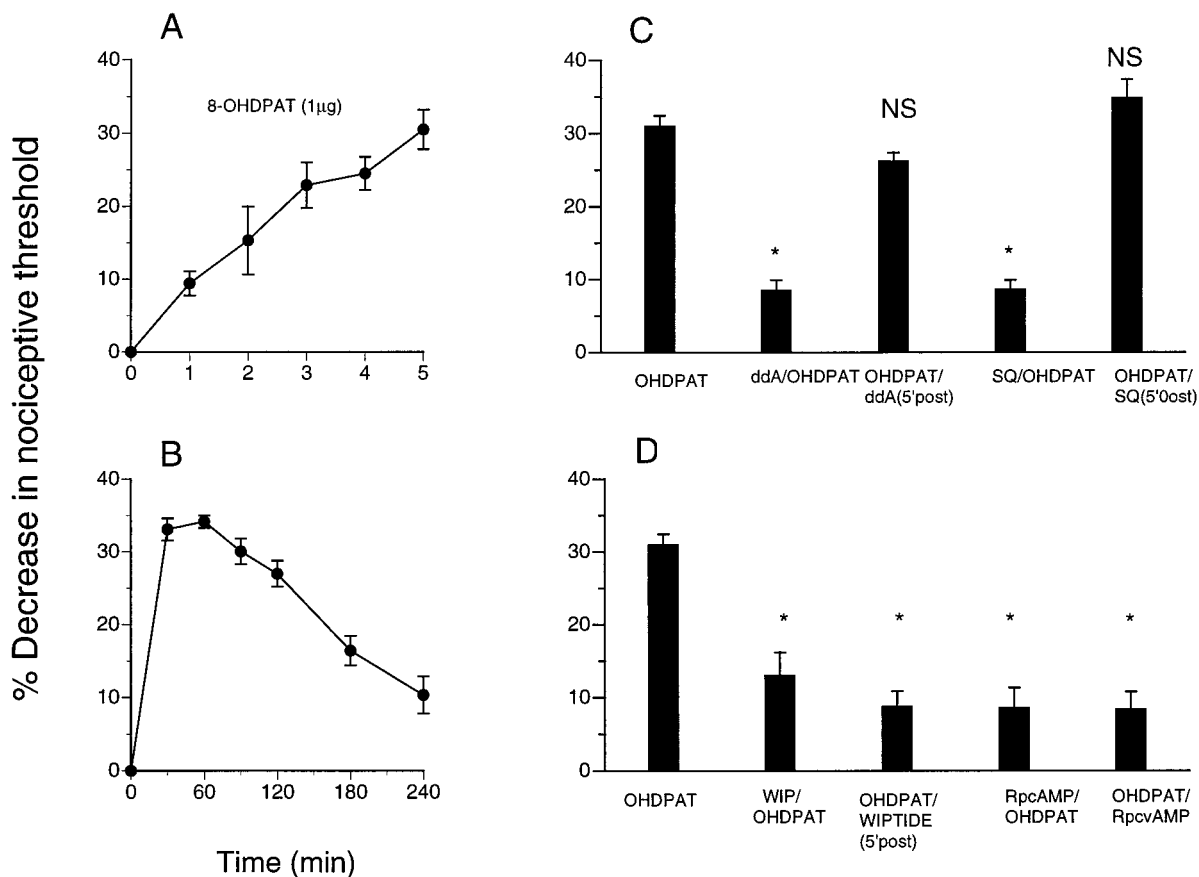


Figure 2. *A*, Latency of onset of direct-acting serotonin agonist [8-OH-DPAT (OHDPAT)]-induced mechanical hyperalgesia in the hindpaw of the rat ($n = 6$). *B*, Time course of 8-OH-DPAT-induced mechanical hyperalgesia ($n = 6$). *C*, Effect on 8-OH-DPAT-induced mechanical hyperalgesia ($n = 6$; $p < 0.05$) of the adenylyl cyclase inhibitors *ddA* and SQ22356 (*SQ*) when administered before (*ddA*/OHDPAT, $n = 6$; $*p < 0.05$; *SQ*/OHDPAT, $n = 6$; $*p < 0.05$) or 5 min after (OHDPAT/*ddA*, $n = 10$; $p > 0.05$; OHDPAT/*SQ*, $n = 6$; $p > 0.05$) intradermal administration of 8-OH-DPAT. *D*, Effect of PKA inhibitors [WIPTIDE (*WIP*) and *RpcAMPS* (*RpcAMP*)] when administered before (*WIP*/OHDPAT, $n = 6$; $*p < 0.05$; *RpcAMP*/OHDPAT, $n = 6$; $*p < 0.05$) or 5 min after (OHDPAT/WIPTIDE, $n = 12$; $*p < 0.05$; OHDPAT/*RpcvAMP*, $n = 6$; $*p < 0.05$) intradermal injection of 8-OH-DPAT.

induced by two other direct-acting hyperalgesic agents, 8-OH-DPAT (5-HT_{1A} serotonin receptor agonist) and CGS21680 (A₂ adenosine receptor agonist) (Taiwo and Levine, 1990, 1992). Injection of 8-OH-DPAT and CGS21680 resulted in a significant decrease in nociceptive threshold (Figs. 2, 3, respectively). Hyperalgesia induced by 8-OH-DPAT or CGS21680 was fully established by 5 min. Injection of the adenylyl cyclase inhibitors *ddA* and SQ22356 immediately before 8-OH-DPAT and CGS21680 resulted in reduced hyperalgesia; however, if injected 5 min after 8-OH-DPAT and CGS21680, *ddA* and SQ22356 had no effect on established hyperalgesia. Injection of a PKA inhibitor, WIPTIDE or *RpcAMPS*, before 8-OH-DPAT or CGS21680 also inhibited induction of hyperalgesia (Figs. 2, 3); injected 5 min after 8-OH-DPAT or CGS21680, WIPTIDE or *RpcAMPS* was still able to inhibit hyperalgesia.

To determine whether “exogenous,” persisting PKA activity mimics inflammatory mediator-induced hyperalgesia, we injected the PKACS that can be introduced to an intracellular location by a preceding injection of distilled water to produce hypo-osmotic shock (Burch and Axelrod, 1987; Taiwo and Levine, 1989; Khasar et al., 1995). Injection of PKACS produced hyperalgesia similar in magnitude to that produced by PGE₂ but lasting at least 48 hr (Fig. 4). Injection of WIPTIDE before PKACS inhibited PKACS hyperalgesia (Fig. 4). WIPTIDE was also able

to reverse PKACS hyperalgesia when injected 5 min, 6 hr, or 24 hr after PKACS.

DISCUSSION

All steps of the cAMP second messenger cascade have been shown to be necessary for the induction of hyperalgesia by direct-acting agents. However, our results indicate that at 5 min after injection, hyperalgesia is maintained by PKA and is independent of upstream activity in the cAMP–PKA second messenger cascade. The fact that WIPTIDE can inhibit PKACS-induced hyperalgesia when administered at any point during its time course suggests that the hyperalgesia is not maintained by persistence of the changes in the cell downstream from PKA, such as long-lasting enhancement of ion channels, such as the tetrodotoxin-resistant sodium current, the probable downstream target for PKA in nociceptors mediating sensitization (England et al., 1996; Gold et al., 1996a; Cardenas et al., 1997) and hyperalgesia (Khasar et al., 1999).

Furthermore, because administration of PKACS (essentially an excess of PKA, devoid of its regulatory subunit) produces prolonged hyperalgesia, with a time course consistent with that of metabolic degradation of PKACS (Lee and Steinberg, 1996), the observed time course of hyperalgesia after PGE₂ seems to reflect the time course of PKA activation. That PKACS hyperalgesia

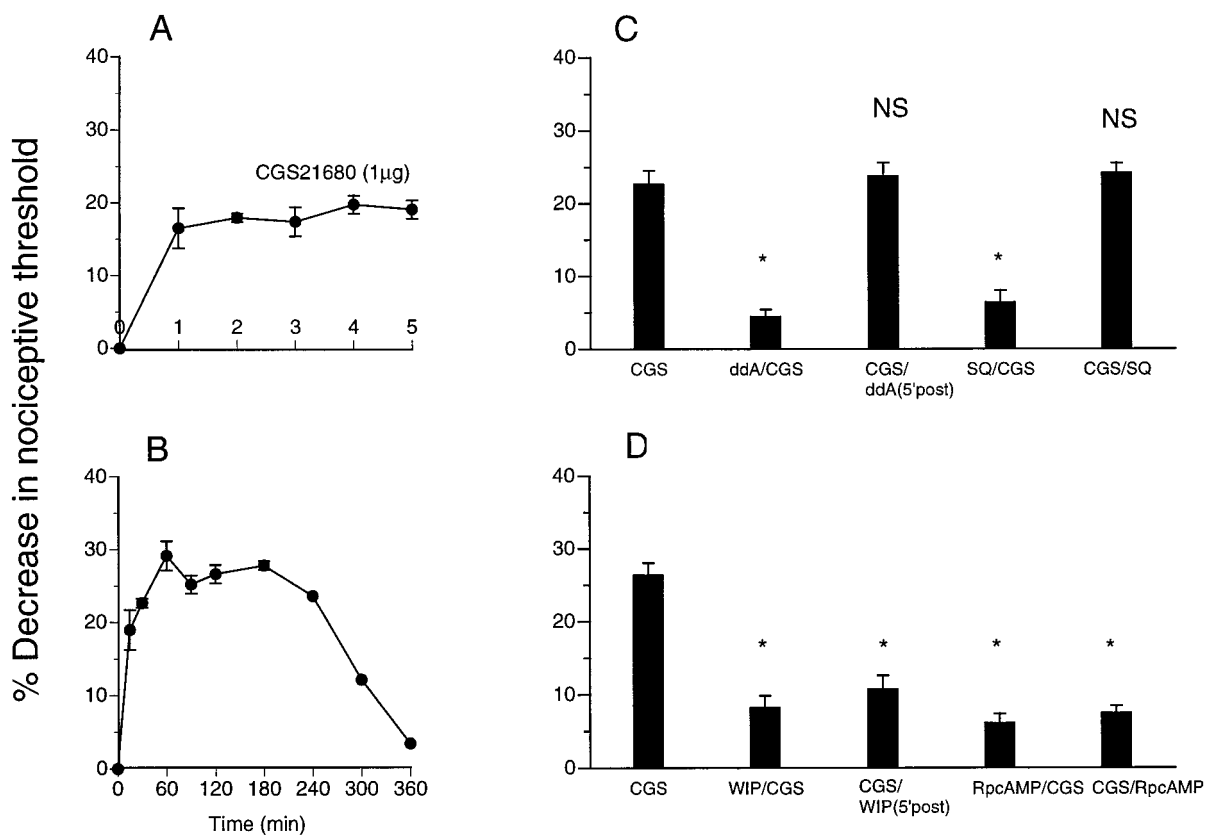


Figure 3. *A*, Latency of onset of direct-acting adenosine agonist [CGS21680 (CGS)]-induced mechanical hyperalgesia in the hindpaw of the rat ($n = 6$). *B*, Time course of CGS21680-induced mechanical hyperalgesia ($n = 6$). *C*, Effect on CGS21680-induced mechanical hyperalgesia ($n = 6$; $p < 0.05$) of the adenylyl cyclase inhibitors *ddA* and SQ22356 (*SQ*) when administered before (*ddA/CGS*, $n = 6$; $*p < 0.05$; *SQ/CGS*, $n = 6$; $*p < 0.05$) or 5 min after (*CGS/ddA*, $n = 6$; $p > 0.05$; *CGS/SQ*, $n = 6$; $p > 0.05$) intradermal injection of CGS21680. *D*, Effect of PKA inhibitors [*WIPTIDE* (*WIP*) and *RpcAMPS* (*RpcAMP*)] when administered before (*WIP/CGS*, $n = 6$; $*p < 0.05$; *RpcAMP/CGS*, $n = 6$; $*p < 0.05$) or 5 min after (*CGS/WIP*, $n = 10$; $*p < 0.05$; *CGS/RpcAMP*, $n = 10$; $*p < 0.05$) intradermal injection of CGS21680.

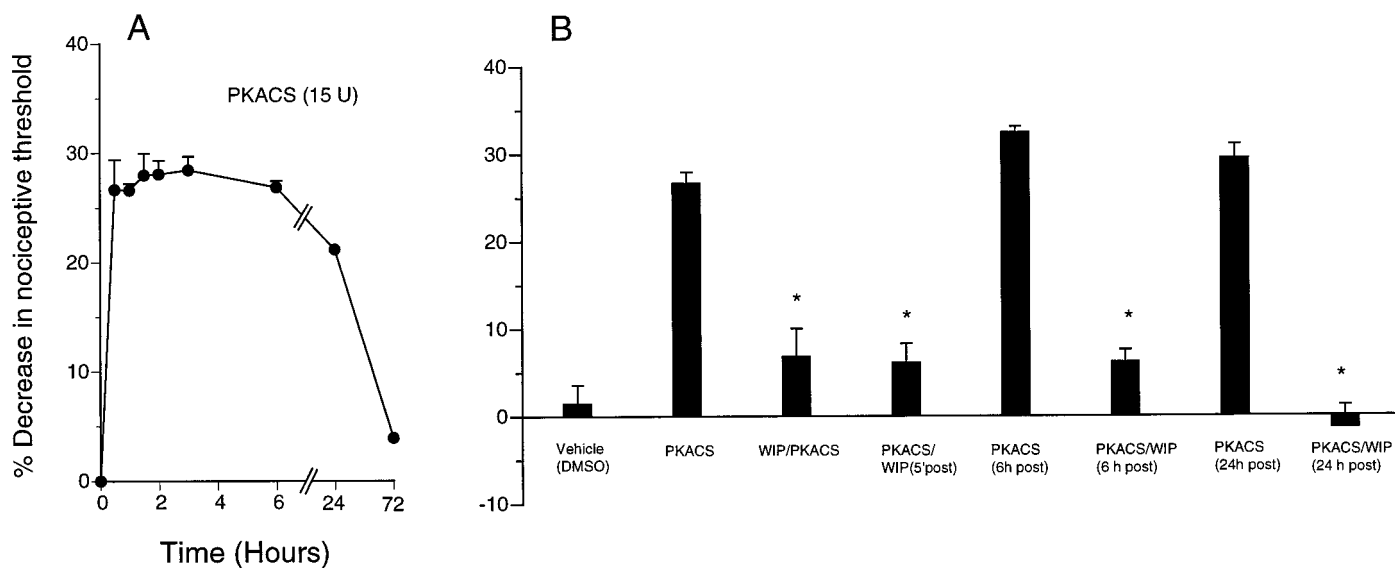


Figure 4. *A*, Time course of PKACS (15 U; $n = 12$)-induced hyperalgesia is shown. *B*, Thirty minutes after intradermal injection of PKACS ($n = 12$) but not its vehicle (DMSO; $n = 6$), significant hyperalgesia ($p < 0.05$) is induced. *WIPTIDE* (*WIP*) injected before (*WIP/PKACS*, $n = 6$; $*p < 0.05$) or 5 min [*PKACS/WIP* (5' post), $n = 6$; $*p < 0.05$], 6 hr [*PKACS/WIP* (6 hr post), $n = 6$; $*p < 0.05$] or 24 hr [*PKACS/WIP* (24 hr post), $n = 6$; $*p < 0.05$] after PKACS inhibited PKACS-induced hyperalgesia.

could be reversed by PKA inhibitors even 24 hr after onset of hyperalgesia further suggests that mechanisms downstream from PKA activity are not effective in the maintenance of the observed hyperalgesia.

If cAMP levels remain persistently elevated, cells undergo changes in their A kinase signaling system; some cells alter the rate of degradation of PKA subunits, and some change the stability of the messages encoding subunits (Spaulding, 1993; Dunbar and Kalinski, 1994; Garrel et al., 1995; Spatz, 1995; Knutsen et al., 1997). Therefore, prolonged hyperalgesia after a sustained exposure to hyperalgesic inflammatory mediators may result from prolonged exposure to cAMP and consequent independently persisting activity of the PKA catalytic subunit because of changes in the ratio of catalytic to regulatory subunits. For example, in nociceptor-like neurons in *Aplysia* (Clatworthy and Walters, 1993a,b; Dulin et al., 1995; O'Leary et al., 1995), PKA plays an important role in the facilitation that mediates both short- and long-term sensitization (Kandel and Schwartz, 1982; Bailey and Kandel, 1993; Byrne et al., 1993). After exposure to the facilitating neurotransmitter serotonin, protein kinase A activity becomes persistently elevated at basal cAMP levels, because of a decrease in the ratio of the concentration of regulatory to catalytic subunits (Sweatt and Kandel, 1989; Chain et al., 1995). Our demonstration that administration of PKACS produces prolonged hyperalgesia, compatible with its rate of metabolism, suggests that a modification of its metabolism could contribute to chronic hyperalgesic states.

In summary, we have provided evidence for the first time that maintenance of hyperalgesia involves a mechanism distinct from the initiating mechanism. Understanding of the mechanisms maintaining hyperalgesia can provide rationales for novel targets for the treatment of chronic inflammatory pain. Specifically, the results of the present study suggest that continued exposure to inflammatory mediators may not be needed to maintain chronic hyperalgesic pain and, therefore, that the most effective site for therapeutic intervention may not be the cell surface receptor in primary afferent nociceptors but the responsible intracellular second messengers. This observation might also underlie the development of resistance to agents that block the production of hyperalgesic inflammatory mediators, such as the nonsteroidal anti-inflammatory drugs, inhibitors of the synthesis of prostaglandins.

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