cm length of PE-10 tubing (inner diameter, 0.28 mm; outer diameter, 0.61 mm), serving as an inflow cannula, was inserted into the subarachnoid space through an incision in the atlanto-occipital membrane. The cannula terminated in the caudal region of the lumbar enlargement (approximately L4). The spinal cord was superfused with 10 μl of either saline or 5.0×10^{-6} M SP, SAP, or SP-SAP followed by a 5 μl saline flush with a 25-μl Hamilton syringe. Five minutes after spinal superfusion, the cannula was carefully withdrawn and the wound closed with 3-0 silk sutures.

14. Measurements of nocifensive behavior and hyperalgesia produced by intraplantar injection of capsaicin were obtained as described [H. D. Gilchrist, B. L. Allard, D. A. Simone, *Pain* **67**, 179 (1996)]. We and most other pain researchers define hyperalgesia as an increase in withdrawal responses. The capsaicin model of hyperalgesia used in the present experiments was chosen as it has been well characterized, it produces hyperalgesia in humans, and it is the only model of hyperalgesia that can be used in parallel animal and human studies. Capsaicin (Sigma) was dissolved in a vehicle of 7.5% polyxyethylene sorbitan monooleate (Tween-80) and saline, and given into the plantar surface of one hindpaw. Each animal received one injection of 10 μg in a volume of 10 μl. The duration of nocifensive behavior, defined as lifting and guarding the injected paw, was measured for the first 5 min after injection. Withdrawal responses to heat were determined by means of a previously described procedure [K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. A. Joris, *Pain* **32**, 77 (1988)]. Rats were placed under a nonbinding cage on a 3-mm-thick glass plate that was elevated to allow maneuvering of a radiant heat source from below. Controlled radiant heat stimuli were applied to the plantar surface of the hindpaw by means of a 50-W light bulb placed in a custom-built case. The start of each trial activated a timer, and withdrawal latencies to the nearest 0.1 s were measured automatically by a photocell that terminated each trial and stopped the timer upon withdrawal of the paw. Four stimuli, spaced at least 1 min apart, were applied to each hindpaw. Withdrawal latency for each paw was defined as the mean latency of the last three trials. The intensity of the heat was adjusted and maintained to produce withdrawal latencies of about 12 s under normal conditions. Heat hyperalgesia was defined functionally as a decrease in the withdrawal latency. To measure withdrawal responses to mechanical stimuli, we placed rats under a clear plastic cage on an elevated plastic mesh floor (1-cm^2 perforations). A von Frey monofilament with a bending force of 95.0 mN was applied to the plantar surface from below the floor. The stimulus was applied 10 times, each for a duration of 1 to 2 s, at random locations on the plantar surface. The frequency was determined for each hindpaw. Mechanical hyperalgesia was defined as an increase in withdrawal response frequency.
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Preserved Acute Pain and Reduced Neuropathic Pain in Mice Lacking PKCγ

Annika B. Malmberg,* Chong Chen, Susumu Tonegawa, Allan I. Basbaum

In normal animals, peripheral nerve injury produces a persistent, neuropathic pain state in which pain is exaggerated and can be produced by nonpainful stimuli. Here, mice that lack protein kinase C gamma (PKCγ) displayed normal responses to acute pain stimuli, but they almost completely failed to develop a neuropathic pain syndrome after partial sciatic nerve section, and the neurochemical changes that occurred in the spinal cord after nerve injury were blunted. Also, PKCγ was shown to be restricted to a small subset of dorsal horn neurons, thus identifying a potential biochemical target for the prevention and therapy of persistent pain.

Neuropathic pain is a devastating consequence of nerve injury that is characterized by spontaneous, often burning, pain, an exaggerated response to painful stimuli (hyperalgesia), and pain in response to normally innocuous, for example touch, stimuli (allodynia). Neuropathic pain syndromes are among the most difficult to manage. Although the pain produced by tissue injury can usually be controlled by anti-inflammatory drugs and opioids, neuropathic pains such as postherpetic neuralgia, reflex sympathetic dystrophy, and phantom limb pain are often refractory to these treatments.

Some studies suggest that nerve injury leads to neuropathic pain because it triggers an *N*-methyl-D-aspartate (NMDA) receptor–mediated hyperexcitability of dorsal horn neurons in the spinal cord. Events downstream of the NMDA receptor, including activation of various protein kinases, have also been implicated; these are presumed to underlie the persistence of the pain (*1*). Conclusions from these studies, however, are very limited. For example, although there is evidence for a contribution of protein kinase C (PKC), those studies not only used inhibitors that are not specific for PKC but they also provided no information about the contribution of specific isoforms of PKC, at least 10 of which have been identified (*2*). In the present study we examined nerve injury–induced neuropathic pain in mice with a deletion of the gene that encodes for the neuronal-specific (gamma) isoform of PKC.

The deletion (knock-out) of PKCγ produces viable mice with normal appearance. The mice have a slight ataxia, modest impairments in tests of learning and memory (*3*), and some motor incoordination (*4*) that may be related to a defect in elimination of multiple climbing fiber innervation of Purkinje cells (*5*). Although synaptic transmission appears normal, long-term potentiation is impaired (*3*).

In the absence of nerve injury, we found no difference in paw withdrawal responses to thermal or mechanical stimulation in mutant and wild-type mice (Fig. 1). Thus, transmission of acute "pain" messages was intact in the mutant mice. To study pain behavior produced by nerve injury, we tightly ligated one-third to one-half of the diameter of the sciatic nerve; this partial nerve injury produces a neuropathic pain syndrome characterized by a marked and long-lasting reduction in the paw withdrawal threshold to both thermal and mechanical stimulation on the injured side (*6*). In the wild-type mice thermal response laten-

A. B. Malmberg and A. I. Basbaum, Departments of Anatomy and Physiology and W. M. Keck Foundation Center for Integrative Neuroscience, University of California, San Francisco, CA 92143, USA.
C. Chen and S. Tonegawa, Howard Hughes Medical Institute, Center for Cancer Research, and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.
*To whom correspondence should be addressed. E-mail: annikam@phy.ucsf.edu

cies were significantly decreased by the third day after nerve injury; this alteration persisted for the 14-day observation period (Fig. 1A). Compared with the wild-type mice, the mutant mice had a significantly decreased thermal allodynia at all time points after surgery. In the wild-type mice, the latency to withdraw from the heat stimulus decreased to 5 to 6 s from a baseline response of 10 to 11 s; the latency to withdraw in the mutant mice was 8 to 9 s. Nerve injury was not completely without effect in the mutant mice. Compared with values before nerve injury, we recorded a modest, albeit significant, decrease in thermal withdrawal latencies, but the magnitude of the change was much less than what we recorded in the wild-type mice.

Because mechanical hypersensitivity is a predominant symptom of neuropathic pain in patients (even contact of clothes is often intolerable), we also studied the response to mechanical stimulation. Partial sciatic nerve injury in the wild-type mice led to a profound decrease of the threshold for evoking hindpaw withdrawal to a mechanical stimulus (von Frey hair) (Fig. 1B). This mechanical allodynia appeared on the third day after surgery and persisted for the duration of the experiment. In contrast, we found no significant change of the mechanical threshold in the PKCγ mutant mice (Fig. 1B). Thus, PKCγ is essential for the production of mechanical allodynia after nerve injury.

Peripheral nerve injury not only produces a neuropathic pain syndrome but also significantly alters the neurochemistry of the ipsilateral dorsal root ganglion (DRG) and the spinal cord dorsal horn (7). Therefore, we also compared the neurochemical consequences of nerve injury in the wild-type and mutant mice. We focused on substance P (SP), a major neurotransmitter of small diameter nociceptive primary afferents, and on the neurokinin-1 (NK-1) receptor, which is targeted by SP. We also studied neuropeptide Y (NPY), which is found in the dorsal horn in normal animals but not in DRG cells; after nerve injury both NPY mRNA and peptide are expressed in DRG neurons (7). Consistent with many previous studies in the rat, in the wild-type mice we found that partial nerve injury produced a marked decrease in SP and an increase in NPY and NK-1 receptor immunoreactivity in laminae I and II of the dorsal horn ipsilateral to the sciatic nerve injury (Fig. 2). The alteration in the amounts of these neurotransmitter and receptor markers was greatest in the spinal segments that receive primary afferent input from the sciatic nerve (namely, lumbar segments L4 and L5), but the increase in the number of NK-1 receptors also extended several segments rostral and caudal to these sites. However, we found a significantly smaller nerve injury–induced alteration of SP, NK-1, and NPY immunoreactivity in the mutant compared with the wild-type mice (8) (Figs. 2 and 3). In other words, the almost complete failure of the mutant mice to develop the neuropathic pain syndrome after nerve injury was paralleled by a very limited neurochemical reorganization in the dorsal horn of the spinal cord.

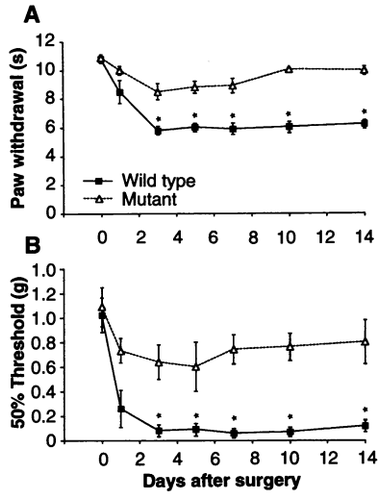

Fig. 1. Before surgery there were no differences in thermal or mechanical thresholds between wild-type and mutant mice. (**A**) In wild-type mice, nerve injury produced a significant reduction in paw withdrawal latencies to a heat stimulus on the injured side [$P < 0.001$, repeated measure analysis of variance (ANOVA)]. This decrease was significantly different from the paw withdrawal latencies in mutant mice. Asterisks indicate a significant difference on the injured side between wild-type and mutant mice (*$P < 0.05$, PLSD Fisher's test). The mutant mice also displayed a modest but significant thermal allodynia ($P < 0.05$, repeated measures ANOVA). No difference in paw withdrawal latencies were observed on the noninjured side in either group. (**B**) Injury to the sciatic nerve produced a significant decrease in paw withdrawal thresholds to von Frey hair stimulation on the injured side in wild-type ($P < 0.05$, Friedman test) but not in mutant mice ($P > 0.05$) compared with baseline thresholds. Asterisks indicate significantly lower thresholds on the injured side in wild-type mice compared with the injured side of mutant mice (*$P < 0.05$, Mann-Whitney test). No change in withdrawal latency was observed on the contralateral side.

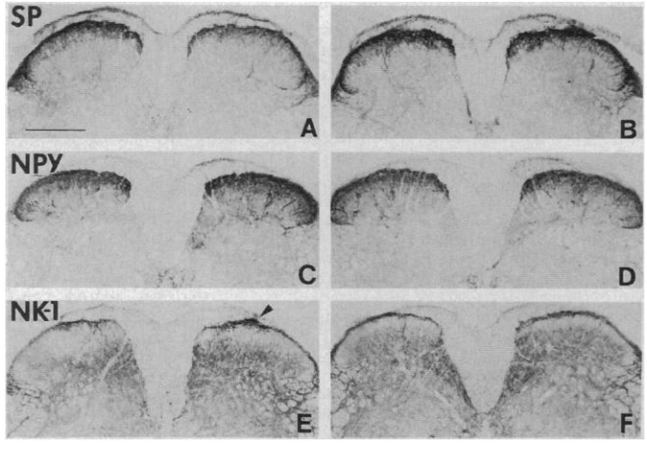

Fig. 2. SP (**A** and **B**), NPY (**C** and **D**), and NK-1 receptor (**E** and **F**) immunoreactivity at the L4 spinal segment of wild-type (A, C, E) and mutant mice (B, D, F). The nerve injury was on the right side. The largest change in SP, NPY, and NK-1 receptor immunoreactivity occurred in the wild-type mice. The increase in NK-1 receptor immunoreactivity was concentrated in the medial part of lamina I (arrowhead). Scale bar, 300 μm.

Because the NK-1 receptor is exclusively located in neurons that are postsynaptic to the nerve injury (9), it provides a marker of transneuronal changes produced by peripheral nerve injury. In contrast, changes in SP and NPY could occur both pre- and postsynaptically (7). It was thus important to specifically evaluate the DRG response to nerve injury. Furthermore, because partial nerve injury produces a variable effect in the DRG, it can be difficult to evaluate quantitatively. Thus, in a second series of experiments, we completely transected the sciatic nerve to produce a maximal response and then examined both the DRG and dorsal horn. As expected, this injury produced maximal neurochemical changes, including a significant decrease in SP and an increase in NPY immunoreactivity in the dorsal horn of the wild-type mice (10). In the mutant mice, however, despite there being a total nerve transection, we again found minimal change in the dorsal horn of the spinal cord (10). In contrast, the neurochemical response of the DRG to injury did not differ in the wild-type and mutant mice. Specifically, in the two groups of mice we recorded comparable decreases in the number of DRG neurons that expressed SP and a comparable up-regulation of NPY-immunoreactive neurons (10). The fact that we found differences in the dorsal horn but not in the DRG suggests that the response of the primary afferent to injury was not altered in the mice that lack PKCγ. Rather, the PKCγ deletion was manifest as a significant reduction of the neurochemical response of postsynaptic neurons to

nerve injury.

Persistent pain states can arise from tissue as well as nerve injury (*11*). A selective deficit is found in the development of inflammation and tissue injury–induced "nociceptive" pain in mice that carry a null mutation in the gene that encodes the neuronal-specific isoform of the type I regulatory subunit (RIβ) of protein kinase A (PKA) (*12*). The latter animals, however, showed no change in the neuropathic pain behavior produced by partial nerve injury. To determine whether PKCγ also contributes to nociceptive pain, we studied the PKCγ mutant mice in an inflammation model produced by hindpaw injection of dilute formalin (*13*). Consistent with acute pain responses being unaffected by the PKCγ deletion, we found that pain behavior in the first phase of the formalin test, which is presumed to result from direct activation of small diameter primary afferent "pain" fibers, did not differ in wild-type and mutant mice (*13*). However, the second, prolonged phase of nociceptive pain behavior, which is driven largely by tissue inflammation, was attenuated (*13*). The PKCγ mutant mice also showed reduced swelling of the formalin-injected paw. In agreement with the reduced inflammation resulting in decreased nociceptive inputs to the central nervous system, in the dorsal horn of the spinal cord ipsilateral to the injury we recorded significantly less Fos immunoreactivity, a marker of neuronal activity, in mutant compared with wild-type mice (*14*). Finally, compared with wild-type mice, we found that the PKCγ mutant mice displayed a significant reduction (44%) of plasma extravasation induced by intradermal injection of capsaicin into the paw (*15*). These results underscore the difference in the pathophysiology of tissue injury– and nerve injury–evoked persistent pain states. Nociceptive pain that results from tissue injury involves both PKA and PKC (and possibly other second messenger systems). In contrast, a full-blown neuropathic pain state can be produced by nerve injury even when PKA RIβ is absent, but deletion of PKCγ prevents the development of this neuropathic pain condition.

Although PKCγ expression can only be detected 7 days after birth and reaches maximal levels by 28 days of age (*16*), the decreased neuropathic pain behavior and neurochemical reactivity in the mutant mice could have resulted from a developmental abnormality that reduced the number of small diameter primary afferent "pain" fibers. To address this possibility, we used electron microscopy to count the numbers of myelinated and unmyelinated axons in the L5 dorsal root of mutant and wild-type mice. We found that neither the morphology nor the numbers of myelinated or unmyelinated axons differed between mutant and wild-type mice (*17*). Thus, the behavioral and neurochemical phenotype is likely to be due to the absence of PKCγ rather than to a developmental change secondary to its deletion.

An important insight into the possible mechanism through which PKCγ influences spinal nociceptive processing was revealed in our subsequent immunocytochemical studies of the distribution of the classical set of PKC isozymes (α, βI, βII, and γ). Specifically, although the first three were distributed rather homogeneously in DRG, in sympathetic ganglia, and in all of the superficial layers of the dorsal horn of the spinal cord, the spinal cord distribution of PKCγ was highly restricted (*18*). It was only found in a subset of interneurons in the inner part of the substantia gelatinosa (lamina II) of the dorsal horn (Fig. 4). Because PKCγ was not detectable in DRG neurons, it is very unlikely that the phenotype observed resulted from long-term changes in the injured primary afferent. In fact, because the neurochemical response of the DRG to injury, namely, decreased amounts of SP and increased amounts of NPY, was not altered in the mutant mice, we conclude that nerve injury–evoked signals were transmitted faithfully to the DRG (presumably by means of retrograde axonal transport) in both wild-type and mutant mice. Furthermore, because primary afferents do not express the NK-1 receptor (*9*), it follows that nerve injury–induced changes in the neurochemistry of the dorsal horn (for example, NK-1 receptor up-regulation) involve PKCγ-containing interneurons that are downstream of the primary afferent. The functional target of these interneurons must include neighboring dorsal horn neurons that express the NK-1 receptor. Although we cannot rule out a contribution of other PKC isoforms, we suggest that PKCγ-mediated phosphorylation of substrate proteins in interneurons of the inner part of the substantia gelatinosa is critical and probably necessary for the full development of the neuropathic pain state produced by peripheral nerve injury.

The concomitant reduction of the behavioral and anatomical response to nerve injury points to the superficial dorsal horn as the critical locus of the PKCγ contribution to neuropathic pain, but it is not clear how a deletion of PKCγ in these interneurons resulted in inflammation deficits. Decreased central sensitization (*19*) could account for the reduction of tissue injury–induced pain, but it could not explain the reduction of neurogenic inflammation. It is conceivable that a deletion of PKCγ in the dorsal horn reduces interneuron-generated dorsal root reflexes (*20*); this would decrease the release of peptides from the peripheral terminals of primary afferents and thus reduce neurogenic inflammation. On the other hand, because inflammation can be influenced by sympathetic and hormonal factors (*21*, *22*), including circulating corticotrophin releasing factor, it is possible that the reduction of tissue injury–evoked pain and neurogenic inflammation resulted from deletion of PKCγ at multiple sites in the central nervous system.

Interneurons of the inner part of lamina II differ considerably from those located dorsally, in lamina I and the outer part of lamina II. Those in the inner part of lamina

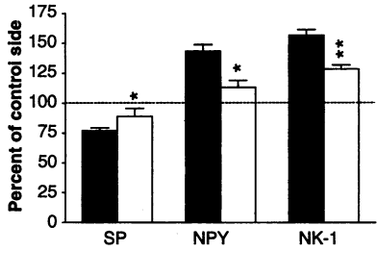

Fig. 3. Nerve injury–evoked changes in SP, NPY, and NK-1 receptor immunoreactivity in the dorsal horn of the spinal cord in PKCγ mutant (white bars) and wild-type (black bars) mice. Data are presented as the mean ratio in percent of immunoreactivity ± SEM between the nerve-injured and the noninjured side at 14 days after the nerve injury (*8*). A value lower than 100% indicates that the injured side contained less immunoreactivity than the noninjured side; a value greater than 100% indicates that there is an increase of immunoreactivity on the injured side. Asterisks indicate significant differences between wild-type and PKCγ mutant mice with PLSD Fisher's test (*$P < 0.05$; **$P < 0.01$).

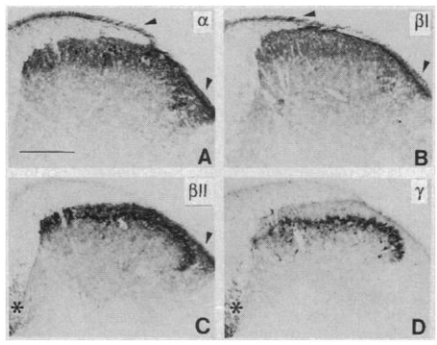

Fig. 4. Distribution of PKCα (**A**), PKCβI (**B**), PKCβII (**C**), and PKCγ (**D**) immunoreactivity in the spinal cord of wild-type mice (*6*). The arrowheads in (A), (B), and (C) point to axonal staining that probably originates in the DRG. Asterisks identify immunoreactivity of axons located in the corticospinal tract, which in rodents is found in the base of the posterior columns. Only the PKCγ staining is confined to interneurons of the inner part of lamina II. Scale bar, 200 μm.

II (where PKCγ is concentrated) receive a selective input from a neurochemically distinct population of unmyelinated primary afferents that express an adenosine triphosphate–sensitive $P2X_3$ receptor, bind the lectin Bandeiraea simplicifolia, and uniquely contain a fluoride-resistant acid phosphatase in their central terminals (*23*). Most importantly, in contrast to the "pain"-responsive neurons of the overlying lamina I and outer lamina II, neurons of inner lamina II respond preferentially to non-noxious inputs (*24*). Thus, PKCγ-regulated changes in the processing of non-noxious inputs by dorsal horn neurons may be critical to the development of neuropathic pain after nerve injury.

From a clinical perspective, the very restricted spinal cord location of the PKCγ-containing interneurons is advantageous. If selective inhibitors of PKCγ can be developed, it may be possible to alleviate nerve injury–induced neuropathic pain states without the profound side effects that are inevitable with nonselective inhibitors of PKC. Moreover, because acute pain responses were not affected in the mutant mice, selective inhibitors of PKCγ would not interfere with the important, protective function that acute pain serves.

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6. Nerve injury was produced by tying a tight ligature around approximately one-third to one-half of the diameter of the sciatic nerve, similar to the approach described for rats by Seltzer and colleagues [Z. Seltzer, R. Dubner, Y. Shir, *Pain* **43**, 205 (1990)]. The general appearance and motor function are normal in the injured mice; occasionally, the mice hold the ligated paw in a protected position when they are not moving. We have not observed autotomy. We assessed mechanical allodynia with von Frey hairs using the up-down paradigm [S. R. Chaplan, F. W. Bach, J. W. Pogrel, J. M. Chung, T. L. Yaksh, *J. Neurosci. Methods* **53**, 55 (1994)], and thermal sensitivity by measuring paw withdrawal latencies to a radiant heat stimulus [K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, *Pain* **32**, 141 (1988)]. In previous studies we found that partial nerve injury produces a significant decrease in thermal latencies for 5 to 6 weeks and mechanical allodynia for more than 2 months. In all studies, the observer was blind to the genotype of the mice. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.
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10. Total nerve transection was performed in halothane-anesthetized mice by ligating and then transecting the sciatic nerve at the mid-thigh level. Fourteen days later, the mice were transcardially perfused with 10% formalin fixative, and the spinal cord and DRG were removed and prepared for immunocytochemistry (*8*). Spinal cord sections and DRG were stained for SP or NPY immunoreactivity. To determine the percentage of immunoreactive cells in the DRG, we counted all cell bodies (with visible nuclei) in six sections of the L4 and L5 ganglia from five wild-type and five mutant mice. We never recorded NPY immunoreactivity in mice without nerve injury or on the contralateral side in mice with nerve injury. In contrast, 14 days after nerve injury in wild-type mice, we found NPY immunoreactivity in 18 ± 2% of the L4 and L5 DRG cell bodies. In the mutant mice 16 ± 2% of DRG cell bodies contained NPY immunoreactivity; the differences were not statistically significant [$P > 0.05$, protected least significant difference (PLSD) Fisher's test]. The density of NPY labeling in the dorsal horn of wild-type mice also increased, to 124 ± 3%, comparing the injured to the uninjured sides (*8*). The mutant mice showed less of an increase (110 ± 3%) at the L4-L5 spinal segment; this difference was statistically significant ($P < 0.05$, PLSD Fisher's test). SP immunoreactivity of the DRG was decreased on the injured sides in both wild-type and mutant mice (9 ± 1% and 8 ± 1% of all DRG cells were SP-immunoreactive); these differences were not significantly different, but both were different from the immunoreactivity of the respective noninjured sides (18 ± 1% and 19 ± 1% of all DRG cells were SP-immunoreactive). In the spinal cord dorsal horn, nerve transection in wild-type mice produced a 35 ± 3% decrease in SP immunoreactivity compared with the noninjured side. In contrast, the mutant mice only showed a 14 ± 4% reduction in dorsal horn SP immunoreactivity on the injured side. The difference was significant ($P < 0.01$, PLSD Fisher's test).
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13. Four wild-type and four mutant mice received a 10-μl intraplantar injection of 2% formalin solution, and the amount of time that the mice licked the injected paw was monitored for 60 min. The incidence of licking was measured in 2-min periods at 5-min intervals. To quantify the magnitude of the inflammatory response, we measured the paw diameter with a spring-loaded caliper (Mitutoyo) 90 min after the formalin injection. There was no difference in licking behavior during the first phase of the formalin test between wild-type (52 ± 6 s) and mutant (41 ± 5 s) mice ($P > 0.05$, *t* test). In contrast, the second phase of the formalin test was significantly reduced ($P < 0.05$, *t* test) in mutant (39 ± 10 s) compared with wild-type mice (90 ± 12 s). The formalin-evoked paw edema was also significantly less ($P < 0.01$, *t* test) in mutant (2.61 ± 0.08 mm) compared with wild-type mice (3.30 ± 0.09 mm).
14. The mice were perfused 90 min after the formalin test, and the spinal cord tissue was prepared for immunocytochemistry as described in (*8*). To quantitate the number of Fos-like immunoreactive neurons, we took photographs of 5 to 10 sections from the L4-L5 spinal segment at low (×4) power on a Nikon Microphot-FXA microscope. The photographs were divided into four segments: laminae I-II, II-VI, V-VI, and the ventral horn. A person blinded to the groups counted the number of Fos-immunoreactive neurons. Five to 10 spinal cord sections were counted per mouse and averaged so that each mouse had a mean value for regional Fos immunoreactivity. There was a significant difference in Fos expression between the two groups ($P < 0.05$, two-way ANOVA comparing group and laminae). Specifically, the mutant mice showed a significant reduction ($P < 0.05$, PLSD Fisher's test) of Fos-positive cells in laminae I and II compared with wild-type mice (41 ± 3 versus 60 ± 4 cells).
15. To study capsaicin-induced plasma extravasation, we anesthetized five mice from each group with 50 mg of pentobarbital per kilogram of body weight and made an intravenous injection of Evan's Blue (10 mg/kg) into a tail vein. Five minutes later, 0.1 μg of capsaicin (8-methyl-*N*-vanillyl-6-noneaneamide; Sigma, St. Louis, MO) in 5 μl of vehicle (10% ethanol, 10% Tween-80, and 80% saline) or vehicle alone was injected intradermally into the dorsal part of the hindpaw, and punches of skin were sampled 30 min later. The Evan's Blue was extracted from the tissue samples by incubation in formamide; extravasated protein was measured spectrophotometrically as described (*21*). In wild-type mice Evan's Blue was extracted from the paw skin at a concentration of 1.1 ± 0.1 μg/ml. Mutant mice showed significantly less ($P < 0.05$, *t* test) extravasated Evan's Blue (0.6 ± 0.1 μg/ml).
16. Two wild-type and two mutant mice were deeply anesthetized with pentobarbital and perfused with a mixture of 3% glutaraldehyde, 3% formaldehyde, and 0.1% picric acid [L. A. Langford and R. E. Coggeshall, *J. Comp. Neurol.* **184**, 193 (1979)]. After the perfusion, the spinal cord with attached dorsal roots was removed and fixed in the same fixative overnight. The following day, L5 dorsal roots were dissected and rinsed in 0.1 M phosphate buffer followed by fixation for 1 hour in 1% OsO_4 and stained in 2% aqueous uranyl acetate for 30 min. The dorsal roots were then dehydrated in ascending concentrations of ethanol, passed into propylene oxide, and embedded in Durcupan resin (Fluka). Finally, the ultrathin sections were placed on single-hole grids coated with butvar and stained with uranyl acetate and lead citrate. The thin sections of the dorsal roots were examined with the electron microscope, and photographs were taken at an original magnification of ×1600. Myelinated and unmyelinated axons from the dorsal roots were counted from montages of electron micrographs. Neither the morphology nor the numbers of myelinated or unmyelinated axons differed between mutant and wild-type mice. Specifically, the number of myelinated fibers was 2542 and 2854 in the two wild-type mice and 2791 and 2712 in the two mutant mice. The number of unmyelinated fibers was 5065 and 5648 in the wild-type mice and 5360 and 5585 in the mutant mice.
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18. Animals were prepared as described in (*8*). Dense PKCγ immunoreactivity was found in lamina IIi and the dorsal corticospinal tract in the spinal cord of wild-type mice. This expression pattern is in agreement with a previous study [M. Mori, A. Kose, T. Tsujino, C. Tanaka, *J. Comp. Neurol.* **299**, 167 (1990)]. No PKCγ expression was found in either the DRG or the superior cervical ganglion. There was no

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Structural Requirements for Glycolipid Antigen Recognition by CD1b-Restricted T Cells

D. Branch Moody, Bruce B. Reinhold, Mark R. Guy, Evan M. Beckman,* Daphney E. Frederique, Stephen T. Furlong,† Song Ye, Vernon N. Reinhold, Peter A. Sieling, Robert L. Modlin, Gurdyal S. Besra, Steven A. Porcelli‡

The human CD1b protein presents lipid antigens to T cells, but the molecular mechanism is unknown. Identification of mycobacterial glucose monomycolate (GMM) as a CD1b-presented glycolipid allowed determination of the structural requirements for its recognition by T cells. Presentation of GMM to CD1b-restricted T cells was not affected by substantial variations in its lipid tails, but was extremely sensitive to chemical alterations in its carbohydrate or other polar substituents. These findings support the view that the recently demonstrated hydrophobic CD1 groove binds the acyl chains of lipid antigens relatively nonspecifically, thereby positioning the hydrophilic components for highly specific interactions with T cell antigen receptors.

Human CD1 proteins are a family of nonpolymorphic transmembrane glycoproteins expressed in association with β_2-microglobulin on the surface of antigen-presenting cells (APCs) (*1*, *2*). Unlike antigen-presenting molecules encoded in the major histocompatibility complex that present peptide antigens to T cells, at least two human CD1 proteins (CD1b and CD1c) mediate specific T cell recognition of bacterial lipid and glycolipid antigens (*3*–*6*). Two classes of CD1-restricted lipid antigens—mycolic acids and phosphoglycolipids such as phosphatidylinositol mannosides (PIMs) or lipoarabinomannan (LAM) (*4*, *5*)—have been identified. To find other antigens presented by the CD1 system, we established additional T cell lines specific for mycobacterial lipid antigens. Analysis of the $CD4^-CD8^-$ $TCR\alpha\beta^+$ T cell line LDN5, isolated from a skin biopsy of a cutaneous reaction to *Mycobacterium leprae* antigen, revealed evidence for a third class of CD1-restricted lipid antigens (*7*).

LDN5 proliferated to only one lipid fraction separated by preparative thin-layer chromatography (TLC) from organic extracts of *M. leprae* and cross-reacted strongly with a lipid of identical retardation factor (R_f) extracted from *M. phlei* (*8*). TLC staining indicated that the lipid contained carbohydrate (anthrone positive) but not phosphate (molybdenum negative), distinguishing this antigen from the two previously described classes of CD1-restricted antigens. Proliferative responses to the purified glycolipid were observed only for LDN5, but not for a panel of 14 other T cell lines, ruling out a nonspecific T cell–stimulating activity (Fig. 1A). LDN5 lysed antigen-pulsed C1R B lymphoblastoid cells transfected with CD1b but not mock-transfected cells, indicating that the antigen-specific response was mediated by CD1b (Fig. 1B).

The structures of the lipid and carbohydrate moieties of the antigenic glycolipid were determined separately. The products resulting from alkaline hydrolysis of the antigen were partitioned and recovered separately from organic and aqueous phases. The organic phase lipids coeluted on high-pressure liquid chromatography (HPLC) with mycobacterial mycolic acids (*4*), and the aqueous phase contained a single product that was identified as glucose by gas chromatography (GC). This composition analysis suggested that the glycolipid antigen was glucose monomycolate (GMM), a previously described mycobacterial cell wall component consisting of a single glucopyranoside residue esterified at its sixth carbon to mycolic acid (*9*).

Electrospray ionization mass spectroscopy (ESI-MS) analysis of the intact glycolipid revealed a predominant ion at a mass-to-charge ratio (m/z) of 1382, corresponding to GMM containing a monounsaturated, C_{80} wax-ester mycolic acid (Fig. 1C) (*10*). GMM was separately isolated from trehalose dimycolate (cord factor) treated with aqueous acid, which released intact GMM by cleavage at the α-glycosidic linkage (*11*). Cord factor–derived GMM stimulated LDN5 with a dose response that was nearly identical to that of the GMM purified directly from *M. phlei* (Fig. 1D). Thus, the antigenic glycolipid recognized by LDN5 was isolated from three independent sources and shown to be GMM, the prototype for a third class of CD1-restricted antigens, mycolyl glycolipids.

We determined the role of the lipid portion of GMM in T cell recognition by isolating GMM from mycobacterial species that differ in mycolic acid composition. *Mycobacterium bovis* BCG, *M. fortuitum*, *M. smegmatis*, and *M. phlei* produce GMMs consisting of glucose esterified to mycolic acids that vary in acyl chain length and the presence or absence of R group substitutions, double bonds, and cyclopropane rings (*12*). LDN5 responded to each of these different GMMs at equivalent doses, indicating that the naturally occurring structural variations of the hydrophobic tails of the antigen were unlikely to determine specific T cell responses (Fig. 2A). This result was definitively confirmed by the CD1b-re-

D. B. Moody, E. M. Beckman, D. E. Frederique, S. T. Furlong, S.A. Porcelli, Lymphocyte Biology Section, Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA.
B. B. Reinhold, S. Ye, V. N. Reinhold, Mass Spectrometry Unit, Boston University School of Medicine, Boston, MA 02118–2394, USA.
M. R. Guy and G. S. Besra, Department of Microbiology, Colorado State University, Fort Collins, CO 80523–1677, USA.
P.A. Sieling and R. L. Modlin, Division of Dermatology and Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90033, USA.

*Present address: Biogen, 14 Cambridge Center, Cambridge, MA 02142, USA.
†Present address:. Zeneca Pharmaceuticals, Wilmington, DE 19803, USA.
‡To whom correspondence should be addressed. E-mail: sporcelli@rics.bwh.harvard.edu