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REVIEW —*Current Perspective*—

In Vivo Molecular Signal Transduction of Peripheral Mechanisms of Pain

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ABSTRACT—Although we have obtained a number of pharmacological tools and mutant mice lacking specific genes related to the pain, the distinct molecular basis of the pain-producing mechanism has remained to be fully clarified since we have been using conventional paradigms of the nociception test that may drive multiple endogenous molecules affecting nociception at the same time. Here, I will introduce a new paradigm of the nociception test. In this test, we focused on polymodal C-fibers by measuring nociceptive flexor responses induced by the peripheral application of a single species of nociceptive molecule. In addition, we identified the site of drug actions on nociceptor endings by the fact that the nociception was abolished by the intrathecal pretreatment with antisense oligodeoxynucleotide for receptors. Throughout experiments using this paradigm of the nociception test, it was firstly revealed that substance P, a major neurotransmitter of polymodal C-fibers, directly stimulates nociceptor endings through activation of $G_{q/11}$ and phospholipase C, followed by Ca^{2+} influx through plasma membrane-bound inositol trisphosphate receptors, and that bradykinin and histamine, both endogenous representative pain-producing substances, share this mechanism. Another unique mechanism is through G_i-coupled receptors such as receptors for nociceptin (orphanin FQ) or kyotorphin (tyrosine-arginine). The latter mechanism was found to be mediated through a substance P release from nociceptor endings. Future studies including some modifications of this paradigm should be also clinically useful for neuropathic pain research as well as understanding of pain physiology.

Keywords: Nociceptin, Substance P, Nociceptor, Pain, Inositol trisphosphate receptor

The nerve endings of primary afferent neurons that transduce pain or nociceptive sensation are called nociceptors. Nociceptors located on the peripheral end of axonal processes, which arise from cell bodies in dorsal root ganglia (or in the trigeminal ganglion), transduce a variety of stimuli into action potentials. The axons associated with nociceptors are only lightly myelinated or unmyelinated and have relatively fast and slow conduction velocities, respectively. Therefore, there are relatively fast and slow pain pathways. In general, the lightly myelinated Aδ nociceptors respond either to intense mechanical or mechanothermal stimuli. Other unmyelinated C fiber polymodal nociceptors tend to respond to thermal, mechanical and chemical stimuli. Under the inflammatory condition, C fiber polymodal nociceptors are stimulated by various chemical mediators such as bradykinin (BK) from plasma, histamine (His) from mast cells, serotonin (5-HT) from platelets, substance P (SP) from C fiber nociceptors and prostaglandins from various cells. In addition, polymodal C fiber has unique properties that they possess SP as a neurotransmitter and are selectively degenerated by capsaicin treatment. Thus, C fiber polymodal nociceptors have been focused on as good targets for studying the molecular and cellular basis of inflammatory pain. Most recently, the vanilloid receptor has been cloned and was found to be a target receptor for capsaicin and heat (1, 2). These reports also accelerate our concerns about the molecular and cellular basis of pain mechanisms through polymodal nociceptors.

The study of molecular mechanisms of pain has been extensively carried out using in vitro models, where the cellular signaling of BK, a representative pain-producing substance, has been characterized in isolated dorsal root ganglion (DRG) cells (3) or neuronal cell lines expressing

the B_2-type BK receptor such as neuroblastoma × glioma hybrid (NG108-15) cells (4). It is now becoming accepted that the cellular events including the depolarization through non-selective cation currents and the hyperpolarization-depolarization through Ca^{2+}-dependent and voltage-dependent K^+ currents are events downstream of the B_2 receptor, including activations of $G_{q/11}$ and phospholipase C (PLC), followed by inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} mobilization and diacylglycerol (DAG)-stimulated protein kinase C (PKC) activation (5). However, the major problems in these strategies using isolated cells may be derived from the fact that the molecular events in the cells may not necessarily reflect the ones in the 'nociceptor endings' since it is well known that signaling molecules working in nerve endings are often different from those in the cell body.

Most recently we provided a new strategy to analyze the in vivo signaling in polymodal nociceptor endings by a simple but unique peripheral nociceptive test (6–9). In this test, the paw of the hind limb in mice are attached with two polyethylene cannulae, which are filled with pain-producing substances, analgesics or drugs affecting signaling, as shown in Fig. 1. The nociceptive flexor responses were monitored through a transducer and recorder. In the nociceptive test using this paradigm, BK given intraplantarly (i.pl.) showed potent dose-dependent flexor responses in the range from 20 fmol to 20 pmol, and they were completely abolished by a B_2 receptor antagonist, HOE140, through another cannula (6). Similar potent responses were also obtained with SP (8) and His (H. Ueda et al., in preparation), each in an antagonist-reversible manner. The specificity of the receptor subtype involved was confirmed when the antisense oligodeoxynucleotides (AS-ODN), but not missense ODN (MS-ODN), was intrathecally given. In such experiments, we carried out intrathecal (i.t.) injections of AS-ODN for receptors to reduce the protein expression in the cell bodies and nerve endings of DRG neurons, but not in peripheral cells such as mast cells, macrophages and blood vessels. In an example of SP-induced peripheral nociception, the nociception was completely abolished by repeated i.t. injections of 10 μg of AS-ODN for SP (NK1) receptor at 5, 3 and 1 day before the nociception test, but not by the MS-ODN treatment (M. Inoue et al., in preparation). Although it is unlikely that the AS-ODN treatment affects the NK1 expression in peripheral cells, another possibility could not be excluded that the change in NK1 receptor expression in the dorsal horn of spinal cord is involved in the blockade of SP-induced peripheral nociception since SP is a representative pain transmitter from polymodal C-fiber neurons. In order to evaluate the contribution of central NK1 expression, we tested

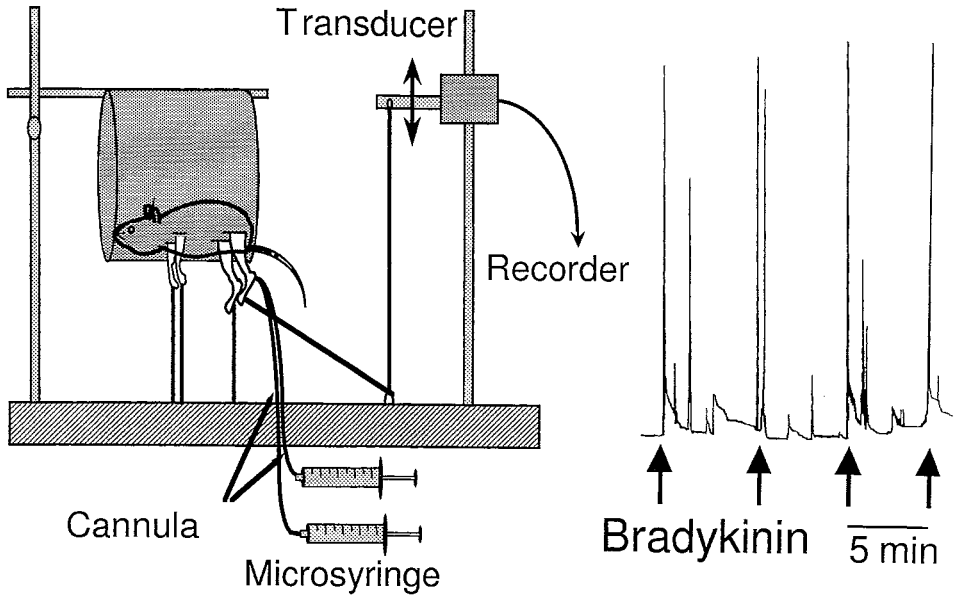

Fig. 1. Apparatus for the peripheral nociception test in mice (modified from ref. 9). Two cannulae were inserted into the planta of the hind limb of mice, and drug injection through a cannula was carried out in a volume of 2 μl, as shown in the left panel. In most experiments, one cannula was filled with a pain-producing substance, while the other was filled with drugs affecting this action such as analgesics or antagonists. Representative flexor responses by the hind limb, which was given 2 pmol of bradykinin every 5 min, are shown in the right panel. The maximal flexor responses after the drug injection were used as nociceptive responses. Such bradykinin-responses are usually constant in this paradigm of experiments for more than 30 min. Other details of the protocol were described previously (7).

the central nociceptive responses by i.t. injection of SP, characterized by scratching, biting and licking (SBL responses). Fortunately, there was no significant change in the central SP-nociception by the i.t. pretreatment with AS-ODN (M. Inoue et al., in preparation). This finding could be explained by the fluorescence microscopical observation following i.t. injection of FITC-labeled AS-ODN for NK1 receptor, in which the fluorescence was predominantly observed in DRG neurons after i.t. injection, much rather than in gray matter neurons of the spinal cord (Fig. 2). Thus, the strategy using i.t. pretreatment with AS-ODN was found to be very useful for the identification of the site of drug actions on sensory primary endings.

Another advantage is related to the fact that the nociceptive responses upon repeated challenges of pain-producing substances are reproducible in a single animal as shown in Fig. 1; this makes it possible to quantitatively evaluate some affecting drugs. Thereby extensive pharmacological studies to observe in vivo signaling could be carried out. As a result, SP-induced peripheral nociception was found to be mediated through NK1, $G_{q/11}$ and PLC (8). In such pharmacological mechanisms, neither the calphostin C-sensitive PKC-mechanism nor the thapsigargin-sensitive Ca^{2+}-mobilization is involved. Instead, araguspongine E (or xestospongin C, see ref. 10), an allosteric antagonist for $InsP_3$ receptor (11), and EGTA, a Ca^{2+}-chelating agent completely blocked these actions. As it is unlikely that EGTA enters into nerve endings, the stimulated $InsP_3$ receptors in plasma membranes of nerve endings may mediate Ca^{2+} influx (Fig. 3). We have previously proposed this hypothesis from biochemical approaches (12). Synaptosomes enriched with nerve ending particles were lyzed and the resulting presynaptic plasma membranes were incubated with $^{45}Ca^{2+}$ to form resealed vesicles containing $^{45}Ca^{2+}$. Obtained inside-out and outside-out resealed vesicles were then perfused on the filter. The addition of $InsP_3$ and Ins 1,3,4,5-tetrakisphosphate ($InsP_4$), but not $InsP_2$, InsP or Ins, evoked $^{45}Ca^{2+}$ release, possibly from inside-out type vesicles. The subcellular distribution studies revealed that the preparation derived from synaptic plasma membranes was the best for this $InsP_3$-mediated $^{45}Ca^{2+}$ transport through resealed vesicles. In contrast, in saponin-permeabilized synaptosomes, no significant $InsP_3$-induced $^{45}Ca^{2+}$ release from intrasynaptosomal microsomes was observed. Taken together, these findings strongly suggest that $InsP_3$ receptor may exist in the presynaptic plasma membranes and play a role in Ca^{2+} transport in nerve endings. As we have obtained quite similar results with BK and His, it is very likely that $InsP_3$ produced by BK, SP or His receptors causes generator potentials through Ca^{2+} influx in presynaptic nerve endings (8).

Through this strategy we have recently proposed a striking concept of peripheral pain transmission (7). It is

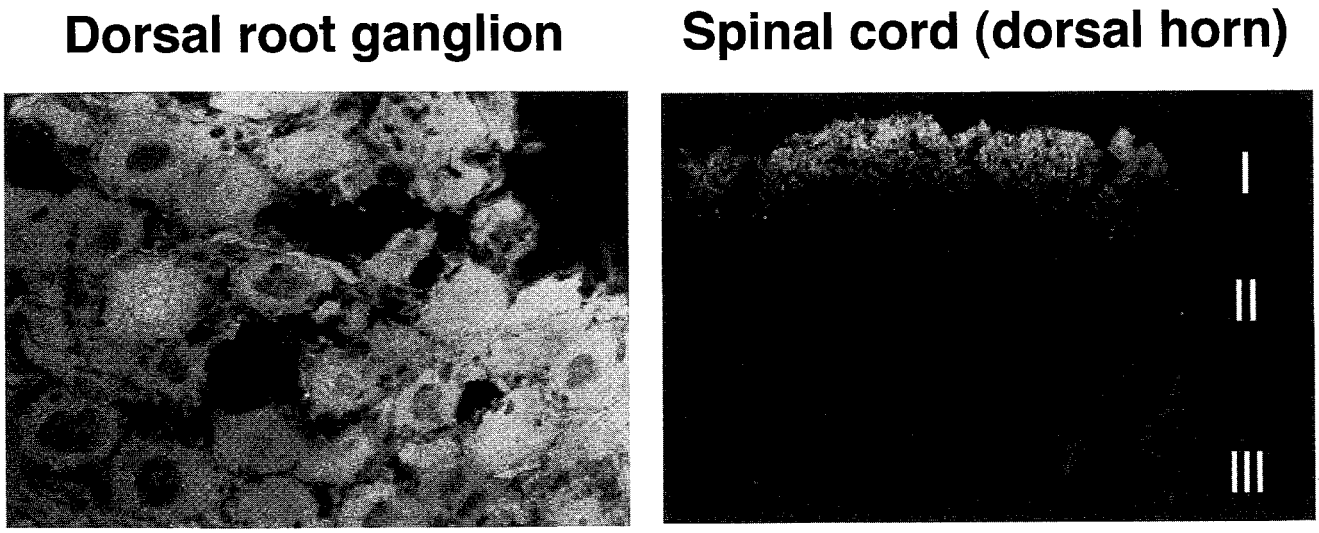

Fig. 2. Selective diffusion of intrathecally injected FITC-labeled NK1 receptor AS-ODN into DRG rather than spinal cord. The result represents a photograph of fluorescence microscopy. Frozen sections of the DRG and spinal cord of mice were fluorescence microscopically observed 30 min after intrathecal injection of 10 μg FITC-labeled AS-ODN for mouse NK1 receptor, (FITC)CGTTATCCATTTTGGCGGGC. The fluorescence in DRG decreased as time goes by, while that in the spinal cord did not change. Although the primary SP polymodal C fibers are known to form synapses at the level of Rexed II, as indicated in the right panel of the figure, there was no significant signal there. The present data was obtained by I. Shimohira, M. Inoue and H. Ueda (unpublished data).

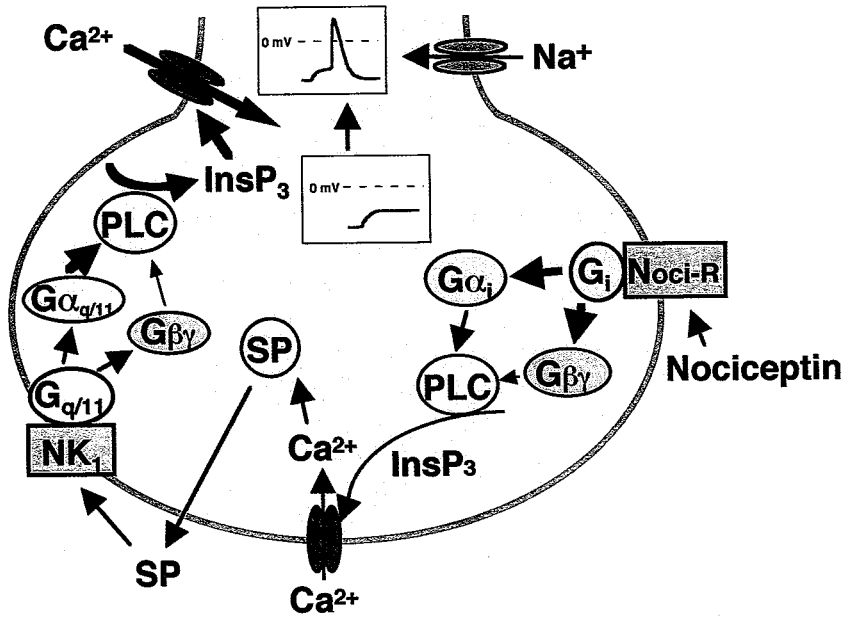

Fig. 3. Proposed model of peripheral pain transmission produced by low doses of nociceptin through a SP-release from nociceptor endings of polymodal C fibers. Details are described in the text.

that extremely low doses of nociceptin (or orphanin FQ) elicits the peripheral nociception through a SP release from nociceptor endings. Nociceptin has been recently discovered as an endogenous peptide ligand for opioid receptor-like orphan receptor (13–15). Although this peptide and the receptor have some similarities in amino acid to dynorphin A and opioid receptors, respectively, it shows anti-opioid or hyperalgesic actions in some nociception tests, while it has analgesic actions in other tests (16, 17). As the dose-dependency of nociceptin in our peripheral nociception test was observed in the range from 0.01 to 3 fmol, it was found to be 1,000–10,000 times more potent than those with BK, SP and His. This action was abolished in mice pretreated with AS-ODN for nociceptin receptor or in nociceptin receptor knock-out mice (18). The mechanisms that underlied these actions were obviously different from those of BK, SP and His since they were abolished by pertussis toxin (PTX, i.pl.)-treatment. This finding was further supported by the fact that the i.t. pretreatments with AS-ODN for α-subunit of G_{i1} blocked the nociceptin-induced flexor responses. From the findings that this nociception was blocked by i.pl. injection of botulinum toxin A or NK1 antagonists or by i.pl. capsaicin pretreatment, which is expected to deplete SP in nociceptor endings, it is evident that the nociceptin-induced response is attributed to the SP-release from nociceptor endings. The use of mutant mice with a targeted disruption of the tachykinin 1 gene (19) confirmed this hypothesis.

The next important issue is to determine what mechanism is involved in the nociceptin-induced SP release. The pharmacological studies using this nociception test only speculate the involvement of G_i-PLC mechanisms (20). Here we also would like to introduce our unique concept for kyotorphin (tyrosine-arginine)-induced met-enkephalin release in the brain (21), in the biochemical approach using resealed vesicles, as mentioned above (12), since low doses of kyotorphin (0.1–100 fmole, i.pl.) also elicit a potent peripheral nociception through a SP release (H. Ueda et al., in preparation). We measured the presynaptic receptor-mediated $^{45}Ca^{2+}$ transport. The addition of kyotorphin caused $^{45}Ca^{2+}$ release, possibly through an $InsP_3$ production in outside-out type resealed vesicles. The pretreatment of lysed membranes with PTX or PLC inhibitor abolished this kyotorphin action, while it was recovered by the reconstitution of PTX-treated membranes with purified G_{i1}, but not G_o. Thus, it is evident that G_{i1}-PLC mechanisms may also contribute to the neurotransmitter release from nerve endings, as shown in our working hypothesis (Fig. 3). In this hypothesis, the stimulation of nociceptin receptor mediates the activation of G_i and PLC, followed by $InsP_3$-gated Ca^{2+} transport and SP release. Released SP causes a similar PLC activation and $InsP_3$-gated Ca^{2+} transport leading to a generator potential. The difference between G_i-coupled receptor-mediated SP-release and $G_{q/11}$-coupled receptor-mediated generator potential may be attributed to the magnitude and duration of Ca^{2+} transport into nociceptor endings. As shown in the cases with the *Xenopus* oocyte expression system measuring Ca^{2+}-gated chloride

currents (22, 23), G_i-mediated currents are weaker in amplitude, but more sustained than $G_{q/11}$-mediated ones. In other words, the latter one is stronger, but thereby rapidly desensitized. Although details remains unclear, the stronger potency of Ca^{2+} transport may be related to the production of generator potentials. Indeed we have observed that relatively lower doses of SP or BK cause peripheral nociception through a SP release in our system. To determine the level of Ca^{2+} transport appropriate for a SP release should be interesting as the next subject for investigation.

Here I mentioned the nociception evoked by extremely low doses (fmole levels) of nociceptin or kyotorphin through an activation of G_i and SP release. However, we also found that these compounds showed wide dynamic range of bell-shaped dose-response curves in the present paradigm of experiments. In other words, higher doses of these compounds (nmole levels) did not show any nociceptive responses, but rather showed analgesic effects through inhibition of BK-responses (ref. 6; M. Inoue et al., in preparation). Since it was known that the stoichiometry of receptor-G_i coupling is much bigger than that of receptor-$G_{q/11}$ coupling (24), such unexpected biphasic responses may be explained by the speculation that abundant G_i, in an active form that possesses a weak intrinsic activity for PLC (23), inhibits $G_{q/11}$-PLC coupling. However, we have many experiments to do to prove this working hypothesis. One of the important studies would be to analyze the receptor-receptor cross-talk in reconstitution experiments using baculovirus/insect cells expressing PLC, G_i, $G_{q/11}$ and their coupled receptors.

The paradigm using nociceptive flexor responses as our test has proven very useful for achieving a better understanding of the in vivo signaling in nociceptors. The study of the in vivo signaling of some other important pain-related substances, prostanoids through G_s (25), ATP and proton through cation channels (26, 27) should be done in the near future. Molecular mechanisms in neuropathic pain is also one of the most important next targets for pain research. I hope that some modifications of this paradigm will be useful for neuropathic pain research.

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