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In Vivo Signal Transduction of Nociceptive Response by Kyotorphin (Tyrosine-Arginine) through $G\alpha_i$- and Inositol Trisphosphate-Mediated Ca^{2+} Influx

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ABSTRACT

Kyotorphin is a dipeptidic neuropeptide (tyrosine-arginine) that has specific receptor coupled to G_i and phospholipase C and elicits Met-enkephalin release. Here, we attempted to demonstrate the in vivo evidence for the presynaptic mechanism by analyzing its nociceptive responses after peripheral application. Kyotorphin elicited potent nociceptive flexor responses at extremely low doses between 0.1 and 100 fmol after the intraplantar injection into the hind-limb of mice. The site of action of kyotorphin-induced responses was identified to be on nociceptor endings, because the responses were markedly attenuated by intrathecal pretreatments with $G\alpha_{i1}$ or $G\alpha_{i2}$ antisense-oligodeoxynucleotides. Similar mechanisms were observed with histamine-induced nociceptive responses, except for the use of different antagonist and $G\alpha_{q/11}$ antisense-oligodeoxynucleotide. Both responses were characterized to be mediated through inositol trisphosphate receptor-gated Ca^{2+} influx, because they were blocked by xestospongin C, an allosteric antagonist for inositol trisphosphate receptor and EGTA, but not thapsigargin. Because the nociceptive responses by compound 48/80 through histamine-release from mast cells were completely abolished by thapsigargin, it is unlikely that the dose of thapsigargin is not sufficient to block both responses. All of these in vivo findings strongly support our previous view that kyotorphin elicits Ca^{2+} influx through inositol trisphosphate receptor located at presynaptic plasma membranes.

Kyotorphin (Kyo), an endogenous neuropeptide (tyrosine-arginine), plays a role in pain regulation in the brain (Takagi et al., 1979; Ueda et al., 1987; Takagi and Ueda, 1988). This peptide administered centrally produced analgesic effects in mice, possibly through a Met-enkephalin release (Takagi et al., 1979; Shiomi et al., 1981). The neurochemical basis of mechanisms suggests that Kyo stimulates its specific receptor, followed by $G\alpha_i$ and phospholipase C (PLC) activations (Ueda et al., 1989). Recently, we observed that this PLC mechanism leads to a Ca^{2+} influx in nerve ending particles or synaptosomes (Ueda et al., 1996). In this report, we propose the hypothesis that inositol 1,4,5-trisphosphate ($InsP_3$) elicits Ca^{2+} transport through plasmalemmal $InsP_3$ receptor but not through intrasynaptosomal Ca^{2+} stores. Because neurochemically dissociated synaptosomes may not be exclusively pure but may contain various other subfractions, however, this hypothesis is required to be further confirmed through more physiological approaches.

Most recently, we developed a simple but sensitive method for evaluating peripheral nociceptive stimulations in mice (Inoue et al., 1998a,b). In this method, pain-producing substances, such as bradykinin (BK) and substance P (SP), showed nociceptive flexor responses when administered intraplantarly (i.pl.) into the hind-limb of mice (Inoue et al., 1997, 1998b). The nociceptive effects of BK were inhibited by nanomolar ranges of Kyo in a Kyo antagonist-reversible and pertussis toxin (PTX)-sensitive manner (Inoue et al., 1997), suggesting that Kyo-induced antinociceptive responses are mediated through its specific receptor. However, our current study shows that extremely low doses (below femtomolar ranges) of nociceptin/orphanin FQ (Meunier et al., 1995; Reinscheid et al., 1995), the endogenous ligand of opioid receptor-like orphan receptor that is coupled to $G\alpha_i$ (Cheng et al., 1997), elicits nociceptive responses in this paradigm of peripheral nociceptive test (Inoue et al., 1998a). This finding suggests the possibility that extremely low doses of Kyo might also elicit nociceptive responses through its receptor and $G\alpha_i$. Here, we report a potent peripheral nociceptive action of Kyo and the in vivo mechanisms through an $InsP_3$-receptor-gated Ca^{2+} influx.

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ABBREVIATIONS: Kyo, kyotorphin; AS-ODN, antisense oligodeoxynucleotide; MS-ODN, missense oligodeoxynucleotide; PLC, phospholipase C; $InsP_3$, inositol 1,4,5-trisphosphate; DRG, dorsal root ganglions; PTX, pertussis toxin; ChTX, cholera toxin; BK, bradykinin; SP, substance P; DPH, diphenhydramine hydrochloride; i.pl., intraplantarly; i.t., intrathecal.

Materials and Methods

Animals. Male ddY mice weighing 20 to 22 g were used in all experiments. Procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983).

Materials. Kyo, Leu-Arg, His, cholera toxin (ChTX) and compound 48/80 were obtained from Sigma Chemical Co. (St. Louis, MO). Bestatin was obtained from Nihon Kayaku (Tokyo, Japan). PTX was obtained from Funakoshi (Tokyo, Japan), Diphenhydramine hydrochloride (DPH) was obtained from Nacalai Tesque (Kyoto, Japan). EGTA was obtained from Dojindo (Kumamoto, Japan), U-73122, U-73343, and thapsigargin were obtained from Funakoshi. Xestospongin C (araguspongine E) was a gift from M. Kobayashi (Kobayashi et al., 1989, 1998). CP-99994 and CP-100263 were generously provided by Pfizer. Kyo, His, PTX, ChTX, EGTA, compound 48/80, CP-99994, and CP-100263 were dissolved in physiological saline, U-73122 and U-73343 were dissolved in 0.1% dimethyl sulfoxide, and xestospongin C was dissolved in 0.01% ethanol. Drugs were administered by i.pl. injection in a volume of 2 μl. To apply different doses, one cannula was filled with increasing concentrations of Kyo or His separated by tiny air spaces. The antisense oligodeoxynucleotide (AS-ODN, 5′-AGA CCA CTG CTT TGT A-3′) for mouse $G\alpha_{i1}$ (Standifer et al., 1996) and its two missense nucleotide (MS1-ODN, 5′-AGC ACA CGT CTT GTT A-3′; MS2-ODN, 5′-AGT CGA TTC GCT CGA A-3′), the AS-ODN (5′-CTT GTC GAT CAT CTT AGA-3′) for mouse $G\alpha_{i2}$ (Standifer et al., 1996) and its MS-ODN (5′-TCT GCT GTA CTA CTA TGA-3′), the AS-ODN (5′-AAG TTG CGG TCG ATC AT-3′) for $G\alpha_{i3}$ (Standifer et al., 1996), the AS-ODN (5′-CGC CTT GCT CCG CTC-3′) for mouse $G\alpha_o$ (Standifer et al., 1996), the AS-ODN (5′-ATG GAC TCC AGA GT-3′) and its MS-ODN (5′-AGT GAC CTC AGG AT-3′) for mouse $G\alpha_{q/11}$, and the AS-ODN (5′-GCC TCA TTG GCA CAA GGG CA-3′) and its MS-ODN (5′-GCT CCA TGT GCA CAG AGG CA-3′) for mouse H_1-type His receptor were synthesized, freshly dissolved in physiological saline, and used for intrathecal (i.t.) injection in a volume of 2 μl on days 1, 3, and 5. On day 6, flexor responses were tested. For Western blot analysis, the following antisera were used: AS/7, which recognizes both $G\alpha_{i1}$ and $G\alpha_{i2}$; QL, which recognizes $G\alpha_{q/11}$; or GC2, which recognizes $G\alpha_o$ (NEN Life Science Products, Boston, MA; each 1:1000 dilution).

Evaluation of Nociceptive Flexor Responses. Experiments were performed, as described earlier (Inoue et al., 1998a,b, Ueda and Inoue, 1999). Briefly, mice were lightly anesthetized with ether and held in a cloth sling with their four limbs hanging free through holes. The sling was suspended on a metal bar. All limbs were tied with strings, and three were fixed to the floor, while the other one was connected to an isotonic transducer and recorder. Mice were lightly anesthetized with ether and a small incision was made in the surface of right hind-limb planta. Two polyethylene cannulas (0.61 mm in outer diameter) filled with drug solution were connected to a microsyringe. Because we used light, soft polyethylene cannulas, they did not fall off the paw during the experiments. The intensity of flexor responses differs from mice to mice, so we used the biggest response among spontaneous and nonspecific flexor responses occurring immediately after cannulation as the maximal reflex. Nociceptive responses were measured after complete recovery (20–30 min) from the light ether anesthesia. Kyo or His injection was administered i.pl. every 5 min unless otherwise stated. In some experiments, Kyo- (or His-) induced nociceptive activity was expressed as the ratio of maximal reflex in each mouse, and in the dose-response experiments, increasing doses of compound were administered every 5-min interval. The average of responses by twice repeated challenges per each dose was evaluated. In other experiments, the effects of test drugs were expressed as the ratio of the response observed over the average of twice repeated control Kyo- (or His)-induced responses obtained in the beginning of experiments. Test drugs affecting Kyo (or His) responses were administered through another cannula immediately after the second control response was measured. Intrathecal pretreatment with neurokinin 1 (NK1) receptor antagonist was performed 30 min before the Kyo challenge.

Western Blot Analysis. SDS-polyacrylamide gel electrophoresis with a 12% polyacrylamide gel and immunoblot analysis were performed as described (Yoshida and Ueda, 1999). Visualization of immunoreactive bands was performed by using an enhanced chemiluminescent substrate for detection of horseradish peroxidase (Super Signaling Substrate; Pierce Chemical Co., Rockford, IL). The intensities of immunoreactive bands were analyzed with NIH Image after the scanning of exposed films.

Statistical Analysis. The data were analyzed using Student's t test after multiple comparisons of the ANOVA. The criterion of significance was set at $P < 0.05$. All results are expressed as the mean ± S.E.

Results

Peripheral Nociceptive Flexor Responses Produced by Kyo and His. In all experiments for Kyo-induced flexor responses, bestatin was injected 5 min before the first challenge of Kyo to protect this peptide from the degradation by aminopeptidases (Ueda et al., 1985). Bestatin (1 nmol i.pl.) itself did not show any significant flexor responses (Inoue et al., 1997). In our previous report (Inoue et al., 1997), 1 nmol of Kyo showed antinociceptive effects against BK responses but did not any significant nociceptive flexor responses by itself. However, when 100 fmol of Kyo was administered i.pl. into the mouse hind-limb, there was a very short-acting, but significant, nociceptive flexor response, as shown in Fig. 1A. Stable flexor responses were obtained on repeated Kyo challenges at 5-min intervals. The mean ± S.E. of Kyo (100 fmol)-induced responses corresponds to a force of 7.01 ± 0.17g (n = 40), and the response to Kyo (0.1 to 100 fmol) was dose dependent with median effective dose (±S.E.) of 11.6 ± 4.0 fmol (n = 5), whereas they started declining from 1 pmol to 1 nmol (i.pl.). Kyo (100 fmol i.pl.)-induced nociceptive responses were completely abolished by 100 fmol of Leu-Arg, a specific Kyo receptor antagonist (Ueda et al., 1989), as shown in Fig. 1B.

Similar nociceptive responses were also observed when a higher dose of His was administered (100 pmol). The median effective dose for His in the present experiments was 28.8 ± 4.6 pmol (n = 5). His (100 pmol)-induced nociceptive responses were also abolished by 200 pmol of DPH, an H_1-type His receptor antagonist (Fig. 1C). The involvement of H_1 receptor in His responses was also confirmed by the experiments using the H_1 receptor AS-ODN. As shown in Table 1, His (10 or 100 pmol)-induced nociceptive responses were significantly blocked by the i.t. pretreatments with AS-ODN but not with MS-ODN. Because AS-ODN administered i.t. is expected to inhibit the expression of the related protein in dorsal root ganglions (DRG) but not in peripheral cells, including mast cells, His responses are very likely through the H_1 receptor on peripheral nerve endings of primary afferent neurons.

The wide dynamic range of Kyo- or His-induced flexor responses suggests that this animal model should be readily amenable to pharmacological interventions and provide a very useful tool for the in vivo analysis of signaling of nociceptive responses by pain-producing substances.

Kyo-Induced Flexor Responses through PTX-Sensitive $G\alpha_i$ on Nociceptor Endings. When the i.pl. injection

of 10 ng PTX was administered after the second Kyo challenge, Kyo responses were rapidly attenuated, and the complete loss of Kyo responses was observed 20 min after the PTX treatment, as previously reported with nociceptin (Inoue et al., 1998a). These results were reproduced in separate experiments, and the PTX blockade was statistically significant, as shown in Fig. 2A. However, the ChTX pretreatment (10 ng i.pl.) had no significant change, whereas it significantly blocked the prostaglandin E_2-induced responses (data not shown). To identify one species of G protein involved in the Kyo signaling, we treated mice with AS- or MS-ODN for various G protein α-subunits to determine the selective blockade of Kyo responses. To be more accurate, His was challenged to determine the positive control response after the final application with Kyo in mice treated with AS-ODNs or MS-ODNs for both $G\alpha_{i1}$ and $G\alpha_{i2}$. We adopted the results only when the response to 100 pmol of His was between 50 and 75% of maximal reflex. The Kyo responses were weakly but significantly attenuated by the i.t. injection of $G\alpha_{i1}$-AS-ODN but not by its $G\alpha_{i1}$-MS1-ODN or $G\alpha_{i1}$-MS2-ODN (Fig. 2B). On the other hand, the responses were markedly attenuated by $G\alpha_{i2}$-AS-ODN but not by its $G\alpha_{i2}$-MS-ODN (Fig. 2C). However, there was no significant change by the AS-ODN for $G\alpha_{i3}$ or $G\alpha_o$. When DRGs treated with various ODNs were analyzed by Western blot analysis, it was revealed that corresponding signals were attenuated by AS-ODNs, but not by MS-ODNs, as shown in Fig. 3. Because the specific antiserum against $G\alpha_{i1}$ is not commercially available, here we used antiserum recognizing both $G\alpha_{i1}$ and $G\alpha_{i2}$. This fact may be related to the finding that the reduction of immunoreactive signal in the case with $G\alpha_{i1}$-AS-ODN was smaller than the case with $G\alpha_{i2}$-AS-ODN (Fig. 3, A, B, and E). We could not detect the $G\alpha_o$ signal in the DRG preparation, whereas the abundant or faint level of $G\alpha_o$ signal was found in the preparation from the brain or spinal cord, respectively (Fig. 3D). These findings suggest that Kyo-induced flexor responses are mediated through $G\alpha_{i1}$ and $G\alpha_{i2}$, which are located on the nociceptor endings.

His-Induced Flexor Responses through PTX-Insensitive $G\alpha_{q/11}$ on Nociceptor Endings. His responses were not affected by either PTX or ChTX (Fig. 2E). Furthermore, the responses were abolished by the i.t. injection of antisense ODN common with $G\alpha_q$ and $G\alpha_{11}$ but not by its MS-ODN (Fig. 2F). The $G\alpha_{q/11}$-AS-ODN, but not its MS-ODN, markedly reduced the immunoreactive signal corresponding to $G\alpha_{q/11}$, as shown in Fig. 3, C and E. Furthermore, these treatments did not affect the signal corresponding to $G\alpha_{i1}$ and $G\alpha_{i2}$, as shown in Fig. 3, C and E. These findings suggest that His-induced flexor responses are mediated through $G\alpha_{q/11}$, which is located on the nociceptor endings.

Possible Involvement of $InsP_3$ and Ca^{2+} Influx into Nociceptor Endings in Kyo- or His-Induced Responses. Kyo-induced responses were markedly inhibited by i.pl. injection of 10 pmol of U-73122, a phospholipase C (PLC) inhibitor, but not by 10 pmol of U-73343, its inactive derivative (Bleasdale et al., 1990), as shown in Fig. 4A. Quite similar results were also observed with His responses (Fig. 4B). These results suggest that PLC activation is involved in the mechanism of both Kyo- and His-induced nociceptive responses.

To investigate the involvement of downstream Ca^{2+}-mobilization mechanisms after a PLC activation, the treatment with xestospongin C, which is an allosteric $InsP_3$ receptor antagonist (Gafni et al., 1997), was performed. As shown in

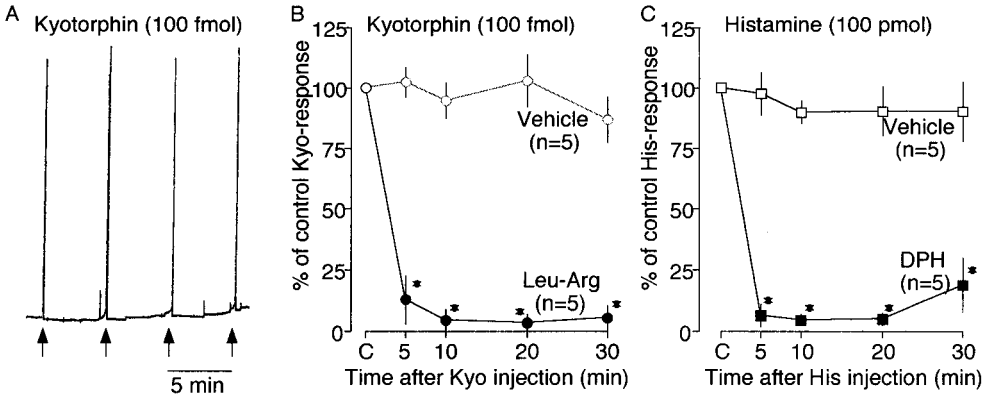


Fig. 1. Kyo- and His-induced nociceptive flexor responses. A, a representative trace of Kyo-induced flexor responses. Kyo (100 fmol) was administered i.pl. consecutively every 5 min, as indicated by the arrow. B, antagonism of Kyo responses by Leu-Arg, a Kyo receptor antagonist. Results represent the percentage of nociceptive responses by Kyo (100 fmol i.pl.) administered at the point indicated in the figure to the average of twice preceding control Kyo responses. Leu-Arg (100 fmol) was administered i.pl. through another cannula immediately after the second control Kyo challenges. *$P < .05$. Each point is the mean ± S.E. from separate five experiments. C, antagonism of His responses by DPH. Details are the same as with Kyo, except for His (100 pmol) and DPH (200 pmol).

TABLE 1

Effects of H_1 receptor AS-ODN or MS-ODN (i.t.) treatment on His-induced nociceptive responses

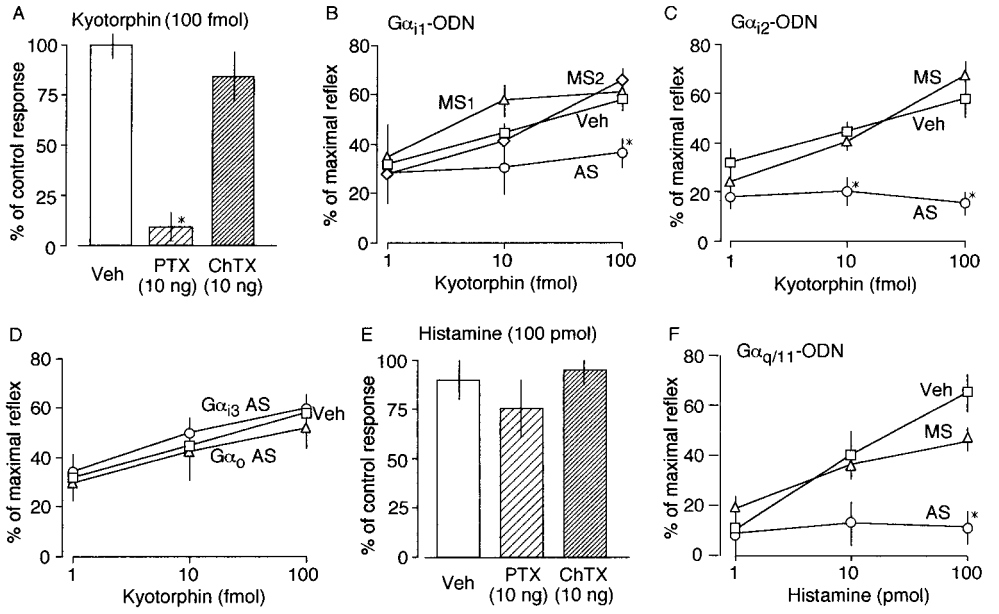
Results are given as the mean ± S.E. from *n* separate experiments. The schedule of ODN treatments is described in *Materials and Methods*.

Dose	Percent of Maximal Reflex					
	Control	*n*	H_1-AS-ODN	*n*	H_1-MS-ODN	*n*
Histamine (1 pmol)	10.2 ± 2.1	5	13.6 ± 5.9	4	3.2 ± 3.2	4
Histamine (10 pmol)	40.3 ± 9.5	5	7.5 ± 7.5*	4	53.1 ± 6.6	4
Histamine (100 pmol)	65.1 ± 7.5	5	10.3 ± 10.2*	4	64.2 ± 6.1	4

* $P < .05$, compared with control.

Fig. 4, C and D, both Kyo and His responses were completely abolished by this treatment at 10 pmol (i.pl.), suggesting the involvement of $InsP_3$ receptor. Because it is well known that $InsP_3$ mobilizes Ca^{2+} from intracellular stores, the treatment with thapsigargin, which depletes Ca^{2+} from the stores (Takemura et al., 1989), was tested. For this purpose, we first attempted to determine the appropriate i.pl. dose of thapsigargin by examining the blockade of compound 48/80-induced nociceptive responses. Because it is well known that compound 48/80 releases His from mast cells through an activation of PLC- and $InsP_3$-induced Ca^{2+} mobilization from intracellular Ca^{2+} stores (Ennis et al., 1980; White et al., 1984), it is expected that this compound elicits nociceptive responses through an action of His. Indeed, compound 48/80 elicited nociceptive responses in a dose-dependent manner (Fig. 5A), and the responses were completely abolished by 200 pmol of DPH (Fig. 5B). The specificity of this antagonism was confirmed by the fact that they were not affected by 40 pmol of HOE140, a specific antagonist for BK B_2 receptor (Wirth et al., 1991), whereas it completely abolished the BK-induced responses (Inoue et al., 1997), as shown in Fig. 5B. When various doses of thapsigargin was administered i.pl., the compound 48/80-induced nociceptive responses were blocked in a dose-dependent manner. Complete blockade was observed when 100 pmol of thapsigargin was used. Therefore, 100 pmol i.pl. of this reagent should be expected to be sufficient to deplete intracellular Ca^{2+} from the stores in various peripheral cells and nerve endings of various neurons in the intraplantar space.

As shown in Fig. 5C, however, Kyo-induced nociceptive



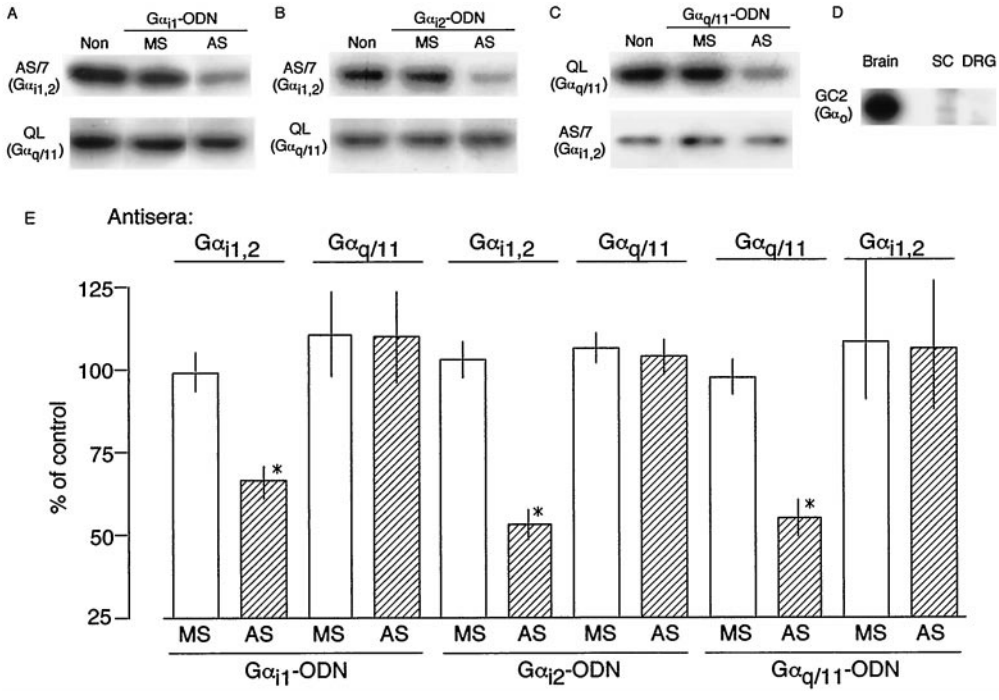


Fig. 2. Different G protein involvements in Kyo and His responses. A and E, effects of PTX (10 ng i.pl.) and ChTX (10 ng i.pl.) treatments on Kyo (100 fmol i.pl.) and His (100 pmol i.pl.) responses. Saline was used instead of toxin as a control (Veh). Results represent the data obtained 20 min after the toxin (or saline) challenge as the mean ± S.E. from five separate experiments (*$P < .05$). B–D and F, effects of antisense oligodeoxynucleotide (AS) or missense oligodeoxynucleotide (MS) for various G protein α-subunits on Kyo or His responses. B, effects of AS and two type of MS (MS1 and MS2) for $G\alpha_{i1}$ on Kyo responses. C, effects of AS and MS for $G\alpha_{i2}$ on Kyo responses. D, effects of AS for $G\alpha_{i3}$ or $G\alpha_o$ on Kyo responses. F, effects of AS and MS for $G\alpha_{q/11}$ on His responses (*$P < .05$). For more details, see the legend to Fig. 1 and Table 1.

Fig. 3. Reduction in G protein α-subunit expression in the DRG by AS-ODN but not by MS-ODN. Mice were administered i.t. injections of AS-ODN (10 μg/2 μl) on days 1, 3, and 5, and their DRGs were isolated on day 6 for Western blot analysis. DRG proteins (30 μg) were separated on an SDS-polyacrylamide gel (12%), transferred to polyvinylidene difluoride, and probed with the indicated antibody. A, DRG samples without treatment (Non), treated with MS2-ODN (MS) or AS-ODN (AS) for $G\alpha_{i1}$, were probed with antisera to AS/7, which recognizes both $G\alpha_{i1}$ and $G\alpha_{i2}$, or QL, which recognizes $G\alpha_{q/11}$. B and C, sample preparation and Western blot analysis were carried out as with A, except for $G\alpha_{i2}$-ODNs (B) and $G\alpha_{q/11}$-ODNs (C). D, samples from forebrain (brain), spinal cord (SC), or DRG were probed with antisera GC2, which recognizes $G\alpha_o$. E, intensity of various bands was analyzed with NIH Image after scanning of exposed film. Results represent the percentage of control signal without ODN treatment (Non) in each Western blot analysis. Data represent the mean ± S.E. from six separate experiments (*$P < .05$).

responses were not affected by this treatment. This result indicates that $InsP_3$ receptor is involved in evoking nociceptive responses through other mechanisms than the Ca^{2+} transport through intracellular stores. Quite similar results were observed in the case with His, as shown in Fig. 5D. His-induced nociceptive responses were not affected by thapsigargin. On the other hand, 2 nmol of EGTA (i.pl.) markedly blocked both Kyo- and His-induced nociceptive responses (Fig. 6, A and B). All of these findings suggest that $InsP_3$ receptor-mediated Ca^{2+} influx, but not Ca^{2+} mobilization from the intracellular stores may be involved in both responses through $InsP_3$.

Blockade of Kyo Responses by i.t. Injection of NK1 Receptor Antagonist. To further characterize the nociceptive neuron involved in Kyo response, the i.t. injection of NK1 receptor antagonist, which recognizes SP, was carried out. The Kyo responses were abolished by 100 pmol of CP-99994, a selective NK1 antagonist, but not by 100 pmol of CP-100263, its inactive isomer (McLean et al., 1993), as shown in Fig. 7. These findings suggest that the primary afferent neurons stimulated by Kyo are SP-containing polymodal C-fibers.

Discussion

The relatively unspecialized nerve endings of primary afferent neurons that initiate pain or nociceptive sensation are called nociceptors (see review, Ueda, 1999). Like other cutaneous and s.c. receptors, they transduce a variety of stimuli into action potentials. Moreover, nociceptors are located on the peripheral end of axonal processes that arise from cell bodies in DRG (or in the trigeminal ganglion). Because peripheral nociceptive axons terminate in unspecialized free endings, it is conventional to categorize nociceptors according to the properties of the axons associated with them. The axons associated with nociceptors are only lightly myelinated or unmyelinated and have relatively fast and slow conduction velocities, respectively. In general, the lightly myelinated nociceptors called Aδ nociceptors respond either to intense mechanical or mechanothermal stimuli, whereas unmyelinated nociceptors called C nociceptors tend to respond to thermal, mechanical, and chemical stimuli and therefore are said to be polymodal. The latter C-fiber polymodal nociceptors could be good targets for studying the molecular and cellular basis of inflammatory pain, because they respond to

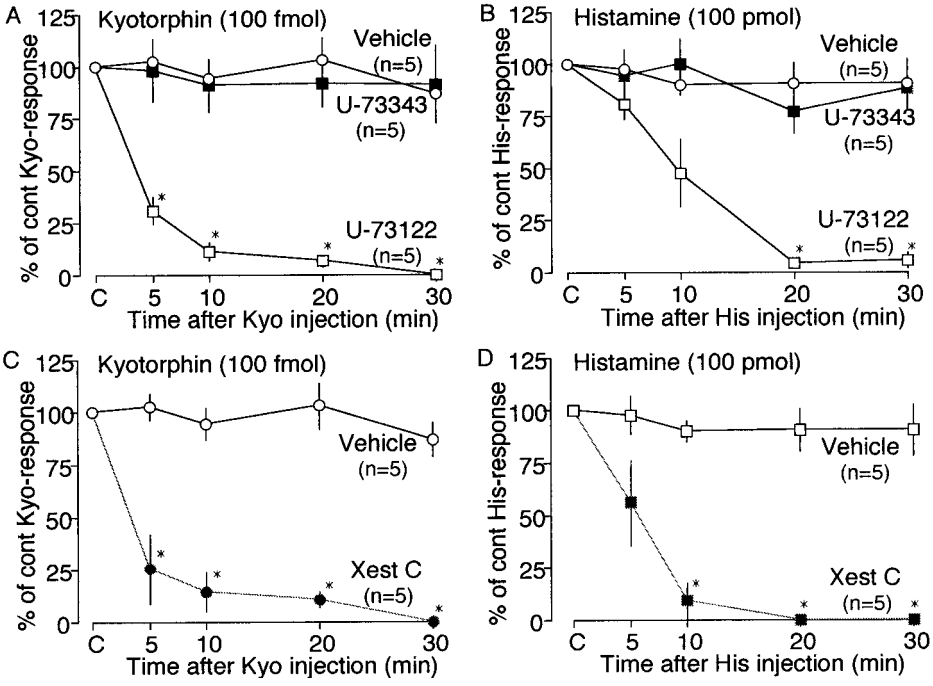


Fig. 4. Involvements of PLC and $InsP_3$ receptor in Kyo and His responses. U-73122 (10 pmol), U-73343 (10 pmol), and xestospongin C (Xest C, 10 pmol) were administered i.pl. immediately after the second control Kyo (or His) challenge. Results represent the mean ± S.E. from five separate experiments (*$P < .05$). For more details, see the legend to Fig. 1.

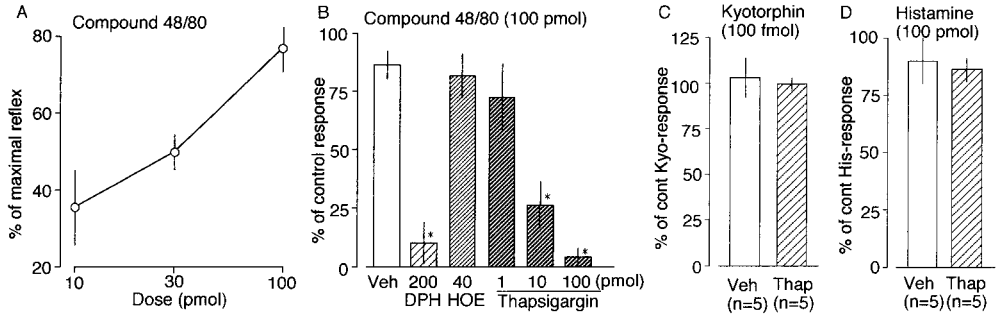


Fig. 5. Pharmacological characterization of compound 48/80-induced nociceptive responses and lack of effects of thapsigargin on Kyo or His responses. A, dose-dependent nociceptive responses by compound 48/80. B, DPH, HOE140 (HOE), or thapsigargin was used at doses indicated. C and D, Kyo or His was used at a dose of 100 fmol or 100 pmol i.pl., respectively. Thapsigargin (Thap) was used at a dose of 100 pmol i.pl. Results (B–D) represent the data obtained 20 min after the antagonist (or saline) challenge as the mean ± S.E. from five separate experiments (*$P < .05$). For more details, see the legend to Fig. 1 and Table 1.

inflammatory mediators, such as BK from plasma, His from mast cells, serotonin from platelets, SP from C-fiber nociceptors, and prostaglandins from various cells. In addition, these neurons possess SP as a neurotransmitter and are selectively degenerated by capsaicin treatment.

The peripheral nociception test used in this study was developed for the purpose of analyzing in vivo signaling mechanisms at the level of C-fiber nociceptor endings. This test has several advantages over many other assays of analgesia, as described previously (Dubuisson and Dennis, 1977; Singh et al., 1983). Briefly, it is sensitive enough to assess very weak and short-acting nociceptive responses induced by a local application of extremely small amounts of pain-producing substance (Inoue et al., 1998a). Second, the nociceptive responses in this test appeared to involve relatively simple molecular and neuronal mechanisms, because they are attributed to the stimulation of identified receptors. Third, because the peripheral nerve endings are far distant from the cell body in DRG, the site of actions of various pharmacological reagents affecting such behavioral responses could be confined to nerve endings. In addition, taking into account that primary afferent neurons (or nociceptors) have bidirectional axon fibers, the in vivo signaling at the peripheral side of such neurons could be also expected on the other, central side.

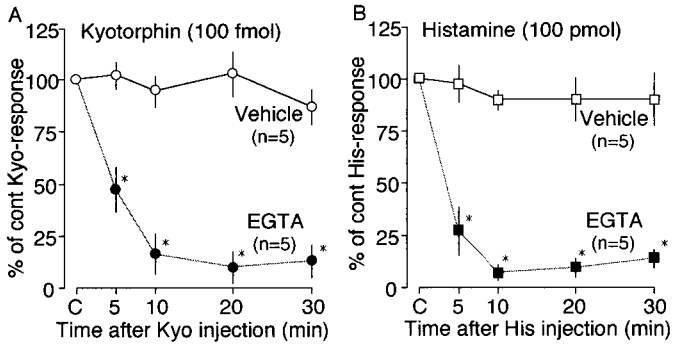


Fig. 6. Blockade of Kyo or His responses by EGTA. EGTA was used at a dose of 2 nmol i.pl. (*$P < .05$). Results represent the mean ± S.E. from five separate experiments. For more details, see the legend to Fig. 1.

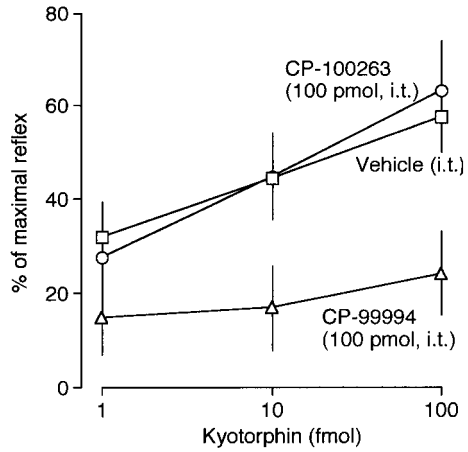


Fig. 7. Blockade of Kyo responses by i.t. injection of NK1 antagonist. CP-99994 (a selective NK1 antagonist) or CP-100263 (an inactive isomer) at 100 pmol was administrated i.t. 30 min before the first challenge of Kyo. Results represent the mean ± S.E. from five separate experiments (*$P < .05$) compared with vehicle-treated mice.

Previously, we have reported that Kyo stimulates its specific receptor, followed by $G\alpha_i$ and PLC activation using GTPase and PLC assays (Ueda et al., 1989). Here we confirmed the signaling of Kyo-induced nociceptive flexor responses using in vivo pharmacological assay. We propose that Kyo-induced flexor responses are mediated by the Kyo receptor on nerve endings of primary afferent neurons through $G\alpha_{i1}$ and $G\alpha_{i2}$, because they were abolished by local application of Leu-Arg or PTX or by i.t. injection of $G\alpha_{i1}$ or $G\alpha_{i2}$-AS-ODN (Figs. 1B and 2, A–C). Because it is unlikely that the injected antisense ODNs reached to the peripheral planta of the hindlimbs and affected peripheral cells, such as mast cells, macrophages, lymphocytes, or vascular cells, it is evident that this treatment inhibited the $G\alpha_{i1}$ and $G\alpha_{i2}$ protein synthesis in primary afferent neurons. Similar results were obtained with His-induced responses, which were blocked by H1 receptor or $G\alpha_{q/11}$-AS-ODN administered i.t. (Table 1 and Fig. 2F). Here we have a question of whether the AS-ODN administered i.t. may affect the expression of encoded protein involved in the spinal pain pathway. Although this possibility cannot be excluded, its contribution seems to be negligible, particularly in the case with $G\alpha_{i1}$ or $G\alpha_{i2}$-AS-ODN, because this antisense treatment did not produce any significant changes in the nociceptive responses by agonists for BK B_2, NK1, and H1 receptors, which are coupled to $G\alpha_{q/11}$ (unpublished data). Furthermore, we observed preliminary findings that the NK1 receptor antisense treatment completely blocked the peripheral SP responses but not the nociceptive responses by i.t. injected SP, suggesting that the AS-ODN may predominantly distribute into the DRG rather than into SP-responsive neurons in the spinal cord (Ueda, 1999). Indeed, we observed that fluorescein isothiocyanate-labeled NK1 receptor AS-ODN administered i.t. was predominantly distributed to DRG neurons over the spinal dorsal horn (Ueda, 1999), where SP (i.t.)-responsive sites exist (Dubuisson and Dennis, 1977). In addition, Kyo-induced flexor responses were supposed to be mediated through PLC and $InsP_3$ receptor, because they were abolished by local applications of some pharmacological agents, including PLC inhibitor and $InsP_3$ receptor antagonist (Fig. 4, A and C). The important issue is the finding that thapsigargin did not block the Kyo responses. Because it was proved that the dose (i.pl.) of thapsigargin used here is sufficient to deplete Ca^{2+} from the intracellular stores from the experiments using compound 48/80, a His releaser from mast cells (Fig. 5), $InsP_3$ produced in nerve endings likely uses a mechanism other than intracellular Ca^{2+} stores. Here, we propose that Ca^{2+} influx may be involved in this mechanism, because EGTA, a membrane-impermeable Ca^{2+} chelating agent completely blocked the Kyo responses (Fig. 6A). This hypothesis is consistent with a recent report that $InsP_3$ gates Ca^{2+} influx into nerve endings in experiments with resealed vesicles of presynaptic plasma membrane preparations (Ueda et al., 1988). In these studies $G\alpha_{i1}$-coupled Kyo receptor was found to gate Ca^{2+} influx through an $InsP_3$ formation, and there was no significant $InsP_3$-mediated Ca^{2+} mobilizing effect in permeabilized synaptosomes, suggesting that $InsP_3$-mediated Ca^{2+} mobilization from intrasynaptosomal Ca^{2+} stores is negligible. We propose a hypothetical scheme, as shown in Fig. 8. In this scheme, Kyo activates its receptor, Kyo receptor, followed by activations of $G\alpha_i$ and PLC. $InsP_3$ thus generated

activates the $InsP_3$ receptor in the presynaptic plasmalemma to gate Ca^{2+} influx, because the thapsigargin-treatment blocks the His release from mast cells but has no effect on His- (Kyo)-mediated actions on nociceptor endings. We are speculating that the slow increase in $InsP_3$ production through $G\alpha_i$ (Ueda et al., 1995a,b) may preferentially contribute to the neuropeptide (SP) release through Ca^{2+} influx, as well as previously reported in nociceptin/orphanin FQ-induced responses (Inoue et al., 1998a), compared with the case through $G\alpha_{q/11}$. There are accumulating reports supporting that PTX-sensitive G proteins mediate PLC activation (Dickenson et al., 1995; Dorn et al., 1997; Jiang et al., 1996; Zhu and Birnbaumer, 1996). On the other hand, His gates Ca^{2+} influx more strongly, possibly due to the much higher intrinsic activity of $G\alpha_q$ to stimulate PLC than that of $G\alpha_i$ (Ueda et al., 1995a,b), thereby directly leads to a production of action potential in the same scheme.

Another issue to be discussed is the identification of nociceptive sensory neurons attacked by Kyo. We recently reported that Kyo-induced nociception was abolished by the i.pl. injection with NK1 antagonists, by the local pretreatment with capsaicin to deplete SP from nociceptor endings, or in mice with targeted disruption of the tachykinin 1 gene (Inoue et al., 1999). All of these findings suggest that Kyo induced nociception through a SP release from SP-containing neurons. Furthermore, we confirmed this view with the present data that i.t. injection of NK1 antagonist abolished the Kyo responses (Fig. 7).

Now it remains to clarify the physiological significance of such an extremely sensitive nociceptive action of Kyo, particularly the relationship to the source of this dipeptide. Kyo and its specific synthetase have been originally discovered from the brain, but they widely, but unevenly, exist throughout the brain (Ueda et al., 1987, 1988) and peripheral tissues, particularly in the adrenal gland (unpublished data). Indeed, Kyo synthetase was recently found in adrenal gland (Kawabata et al., 1996). Taken into consideration that extremely low doses of Kyo have potent actions in this test, circulatory Kyo may be a candidate for this mechanism as well as neuronally released Kyo.

In summary, we demonstrated that in vivo signaling of Kyo-induced nociception includes Ca^{2+} influx through PLC and plasmalemmal $InsP_3$ receptor activation.

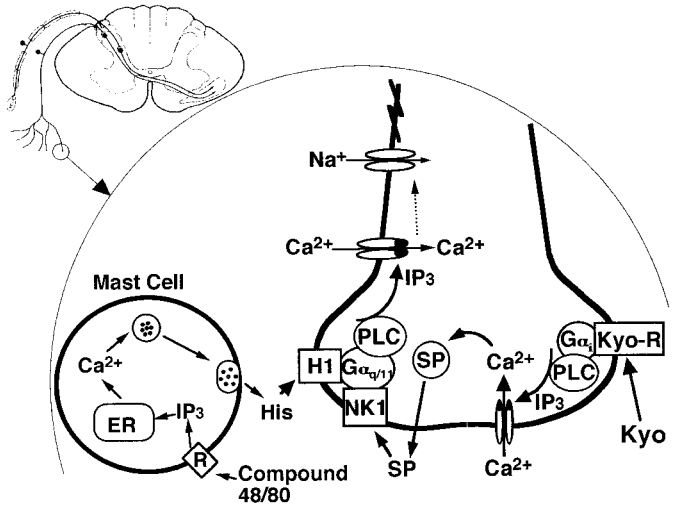


Fig. 8. Schematic model of in vivo signaling in nociceptor endings. The model represents the site of action of Kyo through its receptor, Kyo-R. In the model the nociceptor ending represents SP-containing polymodal C-fiber. It is not yet determined that H1-type His receptor colocalizes with Kyo-R on the same nociceptor ending; however, we have preliminary data showing that His-induced nociception was blocked by i.t. injection of NK1 antagonist (Inoue et al., unpublished data). Details are given in the text. IP_3, $InsP_3$; H1, type 1 His receptor; NK1, neurokinin 1 receptor; R, unidentified receptor; ER, endoplasmic reticulum.

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