Increment of Activated Serine/Threonine Protein Phosphatase in Brain Membrane Fraction Synchronized with Antinociceptive Effect of Morphine in Mice

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We have examined the involvement of serine/threonine protein phosphatase (PP) sensitive to okadaic acid (OA) in the antinociceptive effect of morphine in mice. The present study was performed to elucidate subcellular distribution and activity of OA-sensitive PPs in the brain when mice exposed to morphine. Subcutaneous administration of morphine (5 mg/kg) produced the antinociceptive effect with the maximum 30 min after its administration, evaluated by tail-pinch test. The antinociception was accompanied by an increment of activity in OA-sensitive PPs in the membrane fraction prepared from the whole brain of mice treated with morphine: The temporal profile of the morphine-induced increment of OA-sensitive PP activity was consistent with that of antinociceptive effects of morphine. The morphine-induced increase in OA-sensitive PP activity was dependent on the dose and attenuated by the concurrent administration of naloxone (1 mg/kg). To identify the subtype of OA-sensitive PPs in morphine-enhanced activity, we examined the level of PP2A and PP5, OA-sensitive PPs, in the subcellular fraction prepared from the whole brain of mice receiving morphine. Western blot revealed that morphine elicited the significant increase in the level of PP5, but not PP2A, in the membrane fraction, with the same peak time for the increment of PP5 as the antinociception. No significant change was observed in the level of OA-sensitive PPs in the cytosolic fraction at any examined time after morphine. These results suggest that the translocation of PP5 to the membrane fraction is, at least in part, involved in the antinociceptive effect of morphine in mice.

Key words antinociception; morphine; serine/threonine protein phosphatase; protein phosphatase 2A; protein phosphatase 5

Opioid analgesics exhibit antinociceptive effect through inhibition of the ascending transmission of nociceptive information from the spinal cord dorsal horn and stimulation of pain control circuits that descend from midbrain (e.g. the periaqueductal gray) to the spinal cord dorsal horn.1) The opioid receptors are coupled, via GTP-binding protein, to inhibition of adenyl cyclase activity, activation of receptor-operated K+ currents, and suppression of voltage-gated Ca2+ currents. These are tenable but still unproven mechanisms for explaining blockade of pain transmission by opioids.

The phosphorylation state of functional protein molecules, catalyzed by protein kinases, determines their physiological activity in many cellular events. As for the opioid action, prolonged exposure to opioids, which can cause opioid tolerance and dependence, may activate the mitogen-activated kinases and the phospholipase C-mediated cascade, e.g. protein kinase C (PKC), as the coupling second messenger systems.2) For example, opioids are suggested to be capable of activating PKC which phosphorylates the opioid receptors, as evidenced by enhanced translocation of the enzyme to the cell membrane.3,4) and subsequently the opioid receptors are down-regulated or desensitized for a short term. Thus, although serine (Ser)/threonine (Thr) protein kinases involved in the effect of opioids are recognized, the critical contribution of Ser/Thr protein phosphatase (PP) has more recently been appreciated. Indeed, we examined the involvement of PP sensitive to okadaic acid (OA), a potent inhibitor for PP, in antinociceptive effect of morphine, an opioid analgesic.5) The results suggested that morphine exerted the stimulation of PP2A and PP5, PP subfamilies sensitive to OA, in the whole-cell fraction of the periaqueductal gray and the spinal cord to elicit the antinociceptive effect in mice. That has implications for the possibility that OA-sensitive PP may be a novel effector in intracellular second messenger systems for opioid receptor, but the detailed mechanisms remain unclear.

In the present study, we examined subcellular distribution and activity of PPs in the brain when mice exposed to morphine. That temporal profile was compared to that of the antinociceptive effect of the opioid. We report here that the activated OA-sensitive PP was increased in the membrane fraction by μ-opioid receptor stimuli and that its kinetics was similar to that of its antinociceptive effect.

MATERIALS AND METHODS

Subjects Male ICR mice (SLC, Hamamatsu, Japan) weighing 20—30 g were used. They were housed 5 to a cage in an air-conditioned (23—24 °C, 60% humidity) and light-controlled (lights on from 7:00 to 19:00 h) room. All procedures were approved by Animal Research Committee of Wakayama Medical University in accordance with Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and The Guidelines for Animal Experiments in Wakayama Medical University (approval number 64).

Analgesic Test The antinociceptive effect was evaluated by the tail-pinch method as we reported previously.5) In brief, a flattened clip (approximately 6-mm wide) was placed at the base of the tail. The pressure produced by the clip on the tail was adjusted to approximately 500 g. The nociceptive response was indicated by the time (latency) required for the mouse to respond to this pressure by vocalizing or biting at the clip. The clip was never applied for longer than 15 s. The percentage of maximum possible effect (%MPE) was...
calculated using the formula; %MPE=100×(each latency—baseline latency)/(15—baseline latency). Mice were given subcutaneous administration of either 5 mg/kg morphine hydrochloride (Takeda, Osaka, Japan) or saline.

Preparation of Subcellular Fraction Mice were killed by decapitation before (0 min indicated in Figs. 1, 3, 4) and 15 min, 30 min, 60 min or 120 min after morphine administration. In Fig. 2, the mice were killed 30 min after saline (MOR 0 mg/kg), morphine and/or naxalone administration. The whole brain was quickly removed and homogenized using a motor-driven glass-Teflon homogenizer in ice-cold buffer A containing 20 mM Tris–HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupeptin, 0.1 mg/ml aprotonin and 0.32 M sucrose. The homogenate was centrifuged at 10000 g for 10 min and resultant supernatant was recentrifuged at 100000 g for 30 min at 4 °C, yielding the supernatant (S1 fraction) and pellets. The pellets were washed with buffer B (buffer A without sucrose) and homogenized in ice-cold lysis buffer: 50 mM Tris (pH 7.0), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM diethiothreitol, 0.1% Triton X-100, 0.1 mM p-aminobenzamide, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF. After incubating for 45 min, the soluble fractions were obtained by ultracentrifugation at 100000 g for 30 min and then were retained as the total cell membrane fractions, referred to as membrane fraction hereafter. The S1 fraction was subject to precipitation with acetone: 10 volume of cold acetone was added to the S1 and left alone on ice for 10 min, resulting in the precipitate. The precipitate was dissolved in the cold lysis buffer and retained as cytosolic fraction. The protein concentration of subcellular fractions was evaluated by Bradford method (Coomassie Protein Assay Kit, Pierce, Rockford, IL, U.S.A.) and adjusted to 5 μg/μl. Western blotting was performed in the prepared fraction to confirm whether employed preparation of subcellular fraction worked. We found that there was immunopositivity to 5′-nucleotidase, a marker for membrane fraction and to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a marker for cytosol fraction (data not shown).

Phosphatase Activity The subcellular fractions were subjected to PP activity assay. To remove endogenous phosphate, the sample for subcellular fraction was passed once through Micro Bio-Spin Chromatography Columns (BIO-RAD, Tokyo, Japan) as described by the manufacturer's supplied instruction for use. Phosphatase assays were performed using the molybdate: malachite green: phosphate complex as substrate, following the manufacturer's instruction (Serine/Threonine Phosphatase Assay System, Promega, Madison, WI, U.S.A.). In brief, the tissue sample was incubated at 37 °C for 30 min in 50 μl reaction buffer including 5 nmol synthetic phosphopeptide: 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% β-mercaptoethanol and 0.1 mg/ml bovine serum albumin (BSA). Reactions were terminated by adding the molybdate dye buffer and incubated at room temperature for 30 min. Absorbance was determined at 600 nm. The liberated free phosphate was detected neither in the reaction mix lacking the synthetic phosphopeptide nor in the reaction mix without the tissue sample (data not shown). This indicates that detected free phosphate did not originate from autolysate of added synthetic phosphopeptide and was not liberated from endogenous phosphopeptide in the tissue sample. Therefore, we regarded the free phosphate detected after incubation as being liberated from the synthetic phosphopeptide that resulted from dephosphorylation by PP. OA-sensitive PP activity was determined as the difference in PP activity in the absence of and in the presence of OA (Sigma-Aldrich, Tokyo, Japan). The employed concentration was 100 nM at which OA reportedly completely inhibited both activities of PP2A and PP5, but had the lower affinity to other PPs, PP1.6) In addition, manufacturer’s instruction in the phosphatase assay kit employed said that the supplied phosphopeptide was a poor substrate for PP1 because of its more stringent structural requirements. Therefore, the observed subfamilies of PPs were possibly PP2A and PP5, rather than PP1.

Western Blot The subcellular fractions were subjected to Western blot. The following sample buffer in equal proportion to the prepared subcellular fraction was added, followed by boiling for 7 min: 0.5 M Tris, 10% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 1% bromophenol blue. Proteins in the sample were separated by size on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes in blotting buffer: 25 mM Tris, 192 mM glycine, 0.05% SDS and 20% methanol. The transferred membranes were blocked in phosphate-buffered saline (PBS) containing 5% non-fat dried milk at room temperature for 2 h. The membrane was incubated in primary antibody diluted in PBS containing 5% BSA at 4 °C overnight: anti-PP2A catalytic α subunit antibody and anti-PP5/PPT antibody (BD Bioscience, Tokyo, Japan), followed by 2 h incubation at room temperature in secondary antibody, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (ZYMED LABORATORIES, San Francisco, CA, U.S.A.) diluted in PBS containing 5% non-fat dried milk and 0.05% Tween 20. Chemiluminescence of the antigen–antibody peroxidase complex was performed with LumiGLO Reagent and Peroxide (Cell Signaling Technology, Beverly, MA, U.S.A.), and detected by Chemiluiminator (ATTO Technology, Tokyo, Japan) and analyzed by NIH image (NIH, Bethesda, MD, U.S.A.). The densities in bands immunoreactivity to 5′-tubulin. The ratio of PPs to β-tubulin was calculated as the percentage of control (0 min).

Data Analysis All values are expressed as mean±S.E.M. Statistical significance was assessed using two-way ANOVA for multiple group comparisons, followed by Dunnett test. Statistical significance was set at the level of p<0.05. All statistical tests were performed using Instat 2.01 (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS Subcutaneous administration of morphine prolonged latency of nociceptive response to a noxious pressure stimulus in mice. The maximal effect of 5 mg/kg morphine was observed 30 min after its administration (Fig. 1). The activity of OA-sensitive PPs was altered in subcellular fraction prepared from the whole brain of mice treated with morphine. Morphine augmented the activity of OA-sensitive PPs in the membrane fraction with the time-course similar to that of antinociceptive effect of morphine (Fig. 1). On the contrary,
the cytosolic fraction had the significantly less activity at the time point when the antinociceptive effect of morphine was maximal (Fig. 1). The morphine-induced increment of OA-sensitive PPs in the membrane fraction was in a dose-dependent manner and suppressed by concurrent administration of naloxone (1 mg/kg subcutaneously (s.c.)) (Fig. 2a). The same characteristic was also observed in the morphine-induced reduction of OA-sensitive PP activity in the cytosolic fraction (Fig. 2b).

To test whether OA-sensitive PPs were redistributed by morphine stimuli in the brain cell, we examined the influence of 5 mg/kg morphine administration on the content of PP2A and PP5 in the subcellular fraction of the whole brain. In both membrane fraction and cytosol fraction in saline group (SAL), there was no significant change in PP2A and PP5 at any time examined, compared to before saline administration (0 min). The protein level of PP5 in the membrane fraction reached a peak for morphine-induced increment of PP5 30 min after morphine administration, which was significantly more than that in saline-treated mice (Fig. 3). The level of PP2A was significantly changed neither in the membrane fraction nor in the cytosolic fraction in morphine-treated mice at any examined time after its administration, compared to that in saline-treated animals (Fig. 4).

DISCUSSION

Phosphorylation of some protein molecules on the membrane or in the membrane periphery may result in the pro-
found effect on the intracellular signal transduction of opioid receptors, which underlies the expression of antinociceptive effect of opioids. Our previous study revealed that inhibition of PP2A and PP5 in the spinal and supraspinal tissue suppressed the antinociceptive effect of morphine in mice.\(^5\) That result indicates that the OA-sensitive PP plays a functional role in the expression of morphine analgesia, whereas that does not provide any information on intracellular molecular mechanisms as a novel effector for opioid receptors. In the present study, we assessed activation of OA-sensitive PPs by measuring its distribution and activity in the subcellular fraction, which is referred to as translocation, as recently reported as evidence of OA-sensitive PP activation.\(^7,8\) Our present results clearly demonstrate that morphine induces an increase in the level of membrane PP5, accompanied with an increment of activity of OA-sensitive PPs in the membrane fraction in a naloxone-reversible manner. The temporal profiles of both membrane PP5 level and OA-sensitive PP activity were consistent with that in the antinociceptive effect of morphine. On the contrary, the cytosolic activity of OA-sensitive PPs exhibited a U-shaped time-course in mice treated with morphine, symmetrical to the membrane activity. The results include that the translocated PP5 contributes, at least in part, to the enhanced activity of OA-sensitive PPs in the membrane fraction.

The \(\mu\)-opioid receptors are distributed throughout the brain tissue, although ubiquitously to some extent.\(^1\) Therefore, it is appropriate to use the whole brain to evaluate the effect of morphine on subcellular localization and activity of PPs in the brain. In addition, the amount of the PAG and lumbar spinal cord derived from a mouse was not enough to fractionate the tissue to subcellular compartment. We could need to pool the PAG and spinal cord from a number of mice for preparation of subcellular fraction. The future studies will be needed to elucidate subcellular activity and distribution of PPs in the discrete brain region, especially, the related region to antinociceptive effects of morphine.

Morphine administration did not significantly change the level of cytosolic PP5, in contrast to the cytosolic activity of OA sensitive PP. Our present studies have no direct answer to the discrepancy, but the possible answers are proposed as follows. One is based on the fact that PP5 is primarily localized to the cytoplasm\(^9,10\) or the nucleus\(^6\) in untreated cells, suggesting that the cytoplasmic pool of PP5 is larger than the plasma membrane pool. This leads us to assume that even though just slight ratio of PP5 in the cytosolic fraction is translocated to the cell membrane, it could result in the significantly larger change in the level of PP5 in the membrane fraction. This hypothesis may also explain the fact that the change in OA sensitive PP activity in cytosolic fraction was smaller than that in membrane fraction (Figs. 1, 2). In addition to the difference of the magnitude of subcellular pool, another possibility is that stimulation of \(\mu\) opioid receptor translocates another OA-sensitive PP as well as PP5 from cytosolic to membrane fraction, leading to the significant increase in OA-sensitive PP activity. Our previous study revealed, in fact, that suppression of PP2A and PP5 expression by antisense-oligodeoxynucleotide against respective PP resulted in the significant, but partial, attenuation of antinociceptive effect of morphine in mice, while intracerebroventricular injection of OA produced greater inhibition of its effect.\(^11\) That fact may offer the involvement of another OA-sensitive PP in morphine antinociception. PP4 might be a candidate for another OA-sensitive PP,\(^12\) although it is unclear what role PP4 plays in antinociceptive effect of morphine.

In contrast to PP5, PP2A was not redistributed in subcellular fraction of the mouse brain by morphine administration. The result does not seem to give an explanation for our previous study,\(^5\) if translocation of PP2A to the plasma membrane is essential for the antinociceptive effect of morphine. There are some reports suggesting that the physiological stimuli are sufficient to induce PP2A translocation to the plasma membrane, which triggers physiological response *in vivo*.\(^7,8\) On the other hand, there are other reports that PP2A which originally exists in the cell membrane peripheral, but not one recruited from the cytoplasm, associate with some membrane molecules, such as ion channels. That association may activate PP2A to modulate those physiological function.\(^13,14\) These reports enable us to propose that PP2A itself, but not its translocation in the subcellular fraction, is required for antinociceptive effect of morphine. Further studies are needed to elucidate what functional membrane molecules could associate with PP2A in response to \(\mu\) opioid receptor stimulation to contribute to the antinociceptive effect of morphine.

In conclusion, we report here that administration of morphine elicited an increase in the level of PP5 and the OA-sensitive PP activity in the membrane fraction of whole brain, which was synchronized with the antinociceptive effect of morphine in mice. Our results are the first evidence that the translocation of PP5 to the membrane fraction is involved in the antinociceptive effect of morphine in mice.

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**REFERENCES**