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ScienceDirect
Pharmacology, Biochemistry and Behavior 85 (2006) 629–636
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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P-glycoprotein ATPase activating effect of opioid analgesics and their P-glycoprotein-dependent antinociception in mice

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Received 28 July 2006; received in revised form 6 October 2006; accepted 25 October 2006
Available online 28 November 2006

Abstract

It is well known that opioid analgesics exert central antinociceptive actions. However, in vivo and in vitro studies have shown that some opioid analgesics given systemically have limited access to the central nervous system because of the blood-brain barrier (BBB). P-glycoprotein (P-gp), an ATP-dependent drug efflux transporter, is one component of the BBB. In this report, we assessed the antinociceptive effect of morphine, fentanyl, and meperidine in P-gp deficient (*mdr*1a KO) mice, and compared these effects with those in wild type (WT) mice. The antinociceptive effects of morphine and fentanyl in *mdr*1a KO mice were significantly greater than those in WT mice. However, there was no clear difference in the antinociceptive effects of meperidine in the two genotypes. In addition, we determined the effect of opioid analgesics on P-gp ATPase activity, which is requisite for drug transport, using mouse brain capillary endothelial cells. In our observations, morphine and fentanyl, but not meperidine, significantly increased P-gp ATPase activity, and the drugs' concentration-response curves were bell-shaped, reaching a peak at a concentration of 1 μM. These results suggest that P-gp ATPase activity may be, at least in part, involved in the antinociceptive potencies of those opioid analgesics that are substrates for P-gp.

Keywords: Opioid analgesics; Antinociception; Blood-brain barrier; P-glycoprotein ATPase activity; *mdr*1a deficient mice

1. Introduction

Opioids are potent analgesics, employed for the treatment of severe acute and chronic pain. Binding to specific opioid receptors such as mu, delta and kappa in the central nervous system (CNS) is an important step for these drugs to exert their antinociceptive effects. It is known that the antinociceptive potencies of opioid analgesics such as morphine, fentanyl and meperidine are partly dependent on their receptor binding affinities. The greatest binding is found with mu-opioid receptor, while each opioid analgesics also have variable binding with delta- and kappa-opioid receptors. The rank order of the binding affinity to each receptors is morphine ≥ fentanyl > meperidine (Emmerson et al., 1996; Gourlay, 2005). The affinity of meperidine to mu-, delta- and kappa-opioid receptor is 200-(50-), 50-(30-) and 20-(10-) folds lower than that of morphine (or fentanyl), respectively, suggesting that meperidine exerts its antinociceptive effect particularly at higher doses than those of morphine or fentanyl (Emmerson et al., 1996; Gourlay, 2005).

In addition to the affinity to the receptor, the brain permeability of opioid analgesics will also affect the antinociceptive effects. The blood-brain barrier (BBB) limits the permeability of opioid analgesics (Henthorn et al., 1999; Bauer et al., 2005). It is known that drug permeability through the BBB is correlated with drug lipophilicity (Abbruscato et al., 1997; de Boer et al., 2003). However, some drugs that are known to be lipophilic show lower permeability into the brain than expected, suggesting that a drug efflux pump may be a constituent of the BBB (Tsuji et al., 1997; Fromm, 2004; Bauer et al., 2005).

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doi:10.1016/j.pbb.2006.10.018

One of the most important efflux pumps for opioid analgesics in the BBB is P-glycoprotein (P-gp), a multidrug efflux pump, which is a product of multidrug resistance gene (*MDR*1 in human) expressing at the luminal membrane of the brain capillary endothelial cells (BCECs) (Gottesman and Pastan, 1993; Idriss et al., 2000). In mouse, both *mdr*1a and *mdr*1b genes correspond to the human *MDR*1 gene, and the *mdr*1a isoform is the major isoform in brain capillaries (Regina et al., 1998). P-gp has 12 transmembrane domains contained in two homologous halves and there are two ATP-binding cassette domains in each of the halves that catalyze ATP hydrolysis (Idriss et al., 2000). The original function described for P-gp is drug efflux, which requires energy that is derived from the hydrolysis of ATP. Furthermore, P-gp substrates, such as verapamil or vinblastine, are known to stimulate its ATPase activity (Ambudkar et al., 1992; Sarkadi et al., 1992; He and Liu, 2002).

In vitro and in vivo studies reveal that P-gp functions may modulate opioid pharmacokinetics and pharmacodynamics. Overexpression of P-gp in cultured cells decreases the cellular uptake of opioid analgesics given extracellularly (Callaghan and Riordan, 1993; Wandel et al., 2002), and others report that the inhibitors of P-gp increase the brain uptake or potentiate the antinociceptive effect of opioid analgesics in mice and rats (Letrent et al., 1999; Rodriguez et al., 2004; Shimizu et al., 2004). These results suggest that the brain uptake and the antinociceptive effects of opioid analgesics are dependent on P-gp function. Although P-gp has a broad spectrum for substrates and interacts with a range of chemically diverse substrates, such as opioid analgesics, calcium antagonists, antiarrythmics and immunosuppressants, the structural requirements for efficient P-gp transport are poorly defined (Ecker and Chiba, 1995).

Among opioid analgesics, there are some differences in substrate specificity for P-gp. In this study, we focused on the differences in the effecting levels of opioid analgesics on P-gp function. For this purpose, we determined the antinociceptive effect of opioid analgesics in *mdr*1a knockout (KO) mice. In addition, we measured P-gp ATPase activity using the plasma membrane preparation of BCECs and examined the effects of morphine, fentanyl, and meperidine on this activity in order to make a comparison of their effect on P-gp ATPase activity. Then, we compared the P-gp dependency of their antinociceptive effect with their P-gp ATPase activating effect, using these in vitro and in vivo analyses.

2. Materials and methods

2.1. Animals

Male ICR mice (25–35 g) were obtained from SLC (Osaka, Japan). Male *mdr*1a KO mice and wild type (WT) mice were obtained from Taconic (Germantown, NY). The animals were housed at a temperature of 23–24 °C with a 12 h light–dark cycle (light on 8:00 am to 8:00 pm). Food and water were available ad libitum. The present study was conducted in accordance with the Declaration of Helsinki and with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society.

2.2. Opioid antinociception

The antinociceptive effects of morphine, fentanyl, and meperidine were measured by the tail-pinch method (Takagi et al., 1966), which elicits the mechanical noxious stimuli. 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 500 g. A 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 maximal possible effect (%MPE) was calculated using the following formula: $\%MPE = 100 \times (\text{each latency} - \text{baseline latency}) / (15 - \text{baseline latency})$. The clip was applied prior to and every 15 min following subcutaneous administration of morphine, fentanyl, or meperidine over a 120 min observation period. A time-action curve was constructed and the area under the curve (AUC) was calculated by %MPE versus time. The potentiating effect of opioid analgesics-induced antinociception in *mdr*1a KO mice was estimated by equianalgesic dose ratio, using dose response curve of AUC.

2.3. Morphine concentration

Morphine concentrations in the serum and brain were measured by high performance liquid chromatography-electrochemical detection (HPLC-ECD) method as described in our previous report with some modifications (Miyamoto et al., 1991). Briefly, blood and brain samples were collected 45 min after morphine administration (4 mg/kg, s.c.). Serum was separated by centrifugation (2000 ×*g* for 30 min at 4 °C). The brain was homogenized in 1 ml of pure water by sonication (20 s) at 20% of maximum power (SONIFIER 250; Branson, Kanagawa, Japan). The mixture of sample (serum 100 μl or brain homogenate) and 40% K_2HPO_4 solution (1 ml) was shaken with 5 ml of ethyl acetate for 20 min and then centrifuged at 2000 ×*g* for 5 min at 4 °C. The organic layer was collected, and the aqueous layer was re-extracted with 5 ml of ethyl acetate. Then, the morphine in the organic layer was extracted with 1 ml of 1 M acetic acid, and a 0.9 ml portion of the aqueous layer was lyophilized. The samples were dissolved in 200 μl of 0.01 M HCl, and 20 μl was analyzed by the HPLC-ECD system. The conditions of the HPLC-ECD system were as follows: column, Eicompak MA-ODS (Eicom, Kyoto, Japan); mobile phase, 0.1 M citric acetate buffer (pH 3.9)/methanol (82/18) containing 3 mg/l of EDTA and 150 mg/l of sodium octane sulfonate; flow rate, 1 ml/min; detector, ECD-100 (Eicom) 750 mV Ag/AgCl; temperature, 25 °C.

2.4. Isolation of mouse brain capillaries

Mouse brain capillaries were isolated from mouse cerebrum, as described previously with some modifications (Boado and Pardridge, 1991; Dallaire et al., 1991). Briefly, mouse brain

cortices (3 or 4 mice were used for one experiment) were stripped of the pial membrane and were gently homogenized in 3 volumes (v/w) of ice-cold physiological buffer (PB) composed of 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose and 15 mM HEPES, pH 7.4, with a tissue homogenizer run at 400 rpm for 20 stroke. Then the homogenate was centrifuged (5800 ×*g*) after adding dextran (15%). The resulting pellet was resuspended in PB and filtered through a 200 μm nylon mesh. The filtrate was passed over a column of 170–250 μm of glass beads (1.5 × 3 cm) and washed twice with 20 ml of PB. The capillaries adhering to the beads were collected by agitation for 15 min followed by centrifugation at 500 ×*g* for 10 min. All steps in the isolation procedure were carried out at 4 °C. The capillary preparation was resuspended in 1 ml of 0.1% (w/v) collagenase II in PBS and incubated at 37 °C for 30 min. The suspension was centrifuged at 1000 ×*g* for 10 min at 4 °C and the pellet was washed twice with phosphate buffered saline (PBS), and then BCECs were obtained (He and Liu, 2002).

2.5. Preparation of plasma membranes

Plasma membranes were prepared from BCECs as previously described (Garrigos et al., 1997). Isolated cells were suspended in hypotonic lysis buffer [10 mM Tris-HCl (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EGTA] and allowed to swell for 20 min at 4 °C. Swollen cells were disrupted by sonication for 10 s at 20% of maximum power (SONIFIER 250) and the resulting homogenate was centrifuged (1400 ×*g* for 10 min, 4 °C). The supernatant was then layed on a 46% sucrose cushion in lysis buffer and centrifuged (7000 ×*g* for 20 min, 4 °C). The layer at the sucrose interface was collected, diluted twice with lysis buffer and sedimented (13,500 ×*g* for 15 min, 4 °C). The pellet of total membranes was resuspended in lysis buffer supplemented with 100 mM NaCl at a total membrane protein concentration of about 0.5 or 1 mg/ml. The protein concentration was determined by the Coomassie (Bradford) Protein Assay Kit (PIERCE, Rockford), with bovine serum albumin as standard. We confirmed the expression of P-gp in our membrane preparation of BCEC by use of western blot analysis (data not shown).

2.6. Measurement of P-gp ATPase activity

The ATPase activity of the isolated BCEC membranes was estimated by measuring inorganic phosphate (Pi) liberation (He and Liu, 2002). Membrane suspensions (0.3 μg of membrane protein) were incubated at 37 °C for indicated periods in 0.05 ml of a reaction buffer containing: 50 mM Tris-HCl (pH 6.8), 2 mM DTT, 5 mM $MgCl_2$, 2 mM ouabain (to eliminate Na^+, K^+-ATPase activity), 5 mM sodium azide (to eliminate F_0F_1-ATPase activity), 2 mM EGTA (to eliminate Ca^{2+}-ATPase activity). The ATPase reaction was initiated by the addition of 0.5 mM MgATP. Pi was measured by the sensitive colorimetric reaction using BIOMOL GREEN Reagent according to manufacture's instructions (BIOMOL Research Laboratories Inc., PA). Activities were calculated from the absorbance read at 630 nm of the initial linear rate of Pi production.

2.7. Drugs

The drugs used and their suppliers were as follows: morphine hydrochloride (Takeda Chemical Industries Ltd., Osaka, Japan), fentanyl (Sankyo Co. Ltd., Tokyo, Japan), meperidine (Takeda Chemical Industries Ltd.), sodium orthovanadate (vanadate) (Wako Pure Chemicals, Osaka, Japan), ouabain (Sigma-Aldrich, St. Louis, MO), and sodium azide (Nacalai Tesque, Tokyo, Japan). For antinociceptive analysis, drugs were dissolved in saline and administered subcutaneously. For the measurement of P-gp ATPase activity, drugs except ouabain were dissolved in water. Ouabain was dissolved in dimethylsulfoxide.

2.8. Statistical analysis

Data are presented as the mean with S.E.M. The statistical significance was assessed with a one way analysis of variance followed by the unpaired Student's *t*-test. The differences were regarded as significant when P value was less than 0.05. To evaluate the potentiating effect of *mdr*1a deficient on antinociceptive effect of morphine or fentanyl, dose-response approximating curves were constructed using a computer program.

3. Results

3.1. Effects of the mdr1a deficiency on morphine antinociception and morphine content in the brain

In WT mice, administration of morphine 4 mg/kg produced an antinociceptive response that reached a peak of 68%MPE 30 min after morphine administration (Fig. 1A). On the other hand, morphine antinociception in *mdr*1a KO mice showed a maximal increase (100%MPE) 15 min after morphine administration, and the antinociceptive effect of morphine in *mdr*1a KO mice had not returned to control levels by 120 min after morphine administration (Fig. 1A). As determined by AUC, morphine was significantly more potent as an analgesic in

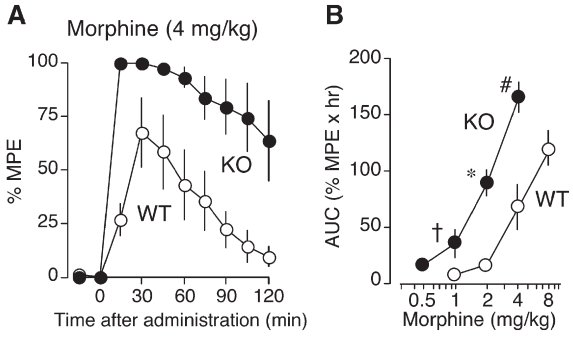

Fig. 1. Antinociceptive effects of morphine in *mdr*1a knock out (KO) and wild type (WT) mice. (A) Time course of antinociception. (B) Dose dependent morphine antinociception presented by area under the curve (AUC; %MPE × h). Antinociceptive effect was determined by tail-pinch method. Closed circle, *mdr*1a KO mice; Open circle, WT mice. Each point represents the mean with S.E.M. of 5 mice. # $p<0.05$, vs. WT (4 mg/kg of morphine). * $p<0.05$, vs. WT (2 mg/kg of morphine). † $p<0.05$, vs. WT (1 mg/kg of morphine).

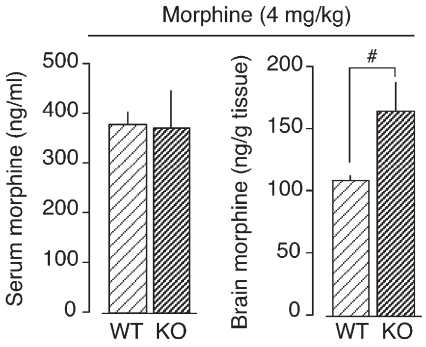

Fig. 2. Serum and brain concentrations of morphine in *mdr*1a KO mice. Morphine concentrations in serum and brain were determined by HPLC-ECD. WT, wild type mice; KO, *mdr*1a knock out mice. Each column represents the mean with S.E.M. of 4 or 6 mice. # $p<0.05$.

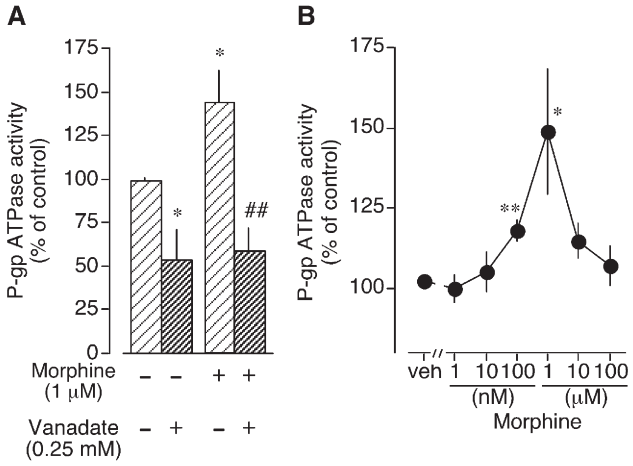

Fig. 4. Morphine-induced P-gp ATPase activation. (A) Effect of morphine (1 μM) and vanadate (0.25 mM) on the basal P-gp ATPase activity. Vanadate was added to the BCECs membrane fraction and incubated on ice for 30 min before the start of the P-gp ATPase reaction. 'control': basal P-gp ATPase activity without any added drugs. '−': added water instead of opioid analgesics and/or vanadate to the reaction mixture. Each column represents the mean with S.E.M. of 5–6 independent experiments. * $p<0.05$, vs. '−'. ## $p<0.01$, vs. morphine alone (1 μM). (B) Morphine concentration-dependent increment of P-gp ATPase activity. Each point represents the mean with S.E.M. of 5–8 independent experiments. 'veh': added water instead of morphine to the reaction mixture. * $p<0.05$, ** $p<0.01$, vs. 'veh'.

*mdr*1a KO mice than in WT mice (Fig. 1B). The morphine dose-response curve in *mdr*1a KO mice was shifted 2.8-fold to the left of that in WT mice.

The amount of morphine in serum and brain 45 min after administration of morphine (4 mg/kg, s.c.) was determined by HPLC-ECD. As shown in Fig. 2, the amount of morphine in the brain was significantly higher in the *mdr*1a KO mice than in WT mice, although there were no differences in the serum morphine levels.

3.2. Estimation of basal P-gp ATPase activity

In order to quantify the P-gp ATPase activity, we used plasma membrane preparations from the BCECs, as He and Liu (2002) described previously. P-gp ATPase activities were increased in a protein- and time-dependent manner (Fig. 3A and B). For the subsequent experiments, we decided to evaluate the effects of opioid analgesics on P-gp ATPase activities by using 0.3 μg of membrane protein and 60 min incubation periods. In these experiments, three ionic pump inhibitors, ouabain (an inhibitor of Na^+/K^+-ATPase), sodium azide (an inhibitor of F_0F_1-ATPase), and EGTA (an inhibitor of Ca^{2+}-ATPase) were added to the reaction mixture. The remaining ATPase activity could therefore be attributed to P-gp (Fig. 3C).

It is known that P-gp ATPase is activated in the resting condition without any added drugs (Ambudkar et al., 1999; Garrigues et al., 2002; Al-Shawi and Omote, 2005); this intrinsic basal P-gp ATPase activity in our study was 1.7 nmol of the released Pi levels (0.3 μg of membrane protein with 60 min incubation) (Fig. 3).

3.3. Effects of morphine on the basal P-gp ATPase activity

Morphine 1 μM significantly increased the P-gp ATPase activity (Fig. 4A). Vanadate, which inhibits ATPase enzymes by forming a tenaciously-bound complex with MgADP at the catalytic sites that resemble the pentacovalent phosphorus of the chemical transition state (Tombline et al., 2004), decreased both

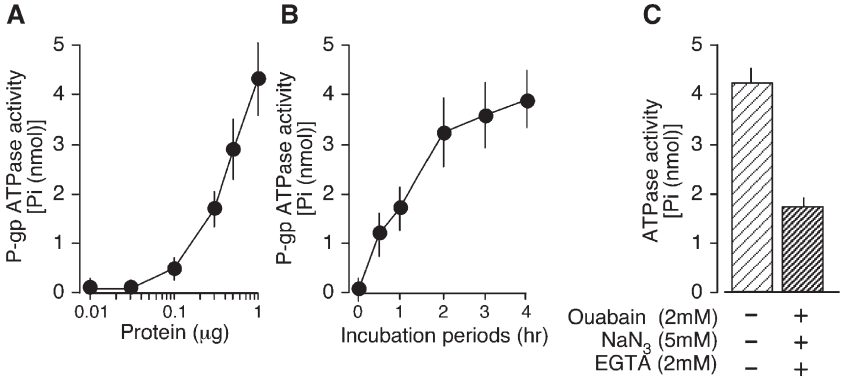

Fig. 3. Estimation of basal P-gp ATPase activity. (A) Protein concentration-dependent changes in basal P-gp ATPase activity after 60 min incubation with three ionic pump inhibitors (Ouabain, NaN_3, EGTA). (B) Time dependent changes in basal P-gp ATPase activity after incubation with three ionic pump inhibitors. (C) ATPase activity measured in the presence or absence of three ionic pump inhibitors after 60 min incubation. Each point or column represents the mean and vertical bar indicates the S.E.M. of 4–5 independent experiments.

the basal and morphine-stimulated P-gp ATPase activity. Furthermore, the concentration-response curve of morphine-induced P-gp ATPase activity had a bell-shape; the maximum activity was obtained at 1 μM of morphine and the activity decreased at morphine concentrations over 10 μM (Fig. 4B).

3.4. Effects of the mdr1a deficiency on the antinociceptive effects of fentanyl and meperidine

The antinociceptive effect of fentanyl (0.04 mg/kg, s.c.) in *mdr*1a KO mice was greater than that in WT mice (Fig. 5A). The dose-response curve in *mdr*1a KO mice showed a 2.2-fold leftward shift (Fig. 5B). On the other hand, the time course of meperidine-induced antinociception was similar in both genotypes, though slightly greater in *mdr*1a KO mice than that in WT mice (Fig. 5C). When determined by AUC, the antinociceptive effect of meperidine did not show any difference between the genotypes at 40 mg/kg, whereas it was slightly, but significantly, greater in *mdr*1a KO mice than that in WT mice at 20 mg/kg, (Fig. 5D). This indicates that meperidine did not show as potent a P-gp dependent antinociceptive effect as was the case with morphine and fentanyl.

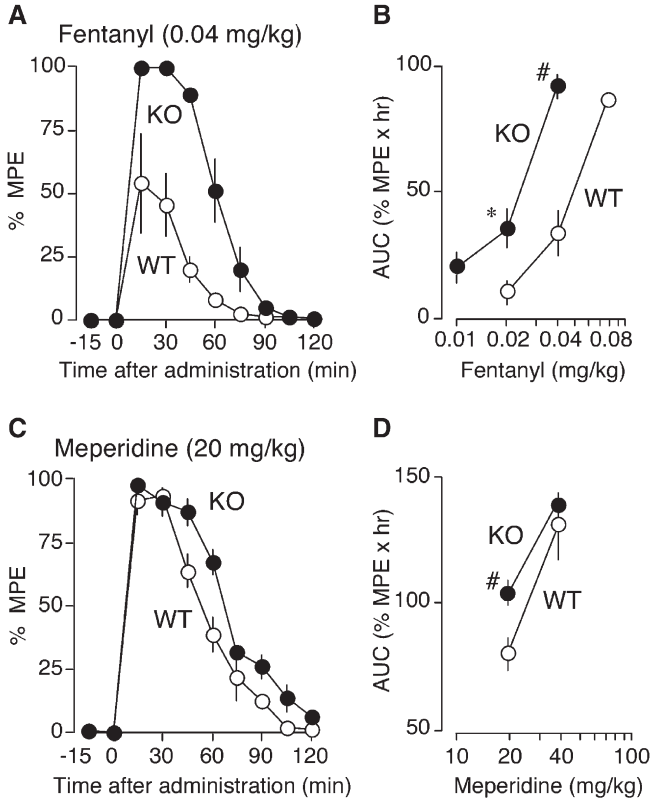

Fig. 5. Antinociceptive effects of fentanyl and meperidine in *mdr*1a knock out (KO) and wild type (WT) mice. (A) Time-course of fentanyl (0.04 mg/kg, s.c.) antinociception in *mdr*1a KO and WT mice. Each point represents the mean with S.E.M. of 3 (*mdr*1a KO) and 4 (WT) mice. (B) Dose-response curve of fentanyl antinociception presented by area under the curve (AUC; %MPE × h). Closed circle, *mdr*1a KO mice; Open circle, WT mice. Each point represents the mean with S.E.M. of 3 or 4 mice. # $p<0.05$, vs. WT (0.04 mg/kg of fentanyl). * $p<0.05$, vs. WT (0.02 mg/kg of fentanyl). (C) Time-course of meperidine (20 mg/kg, s.c.) antinociception in *mdr*1a KO and WT mice. Each point represents the mean with S.E.M. of 4 (*mdr*1a KO) and 5 (WT) mice. (D) Dose-response curve of meperidine antinociception presented by AUC. Closed circle, *mdr*1a KO mice; Open circle, WT mice. Each point represents the mean with S.E.M. of 3–5 mice. # $p<0.05$, vs. WT (20 mg/kg of meperidine).

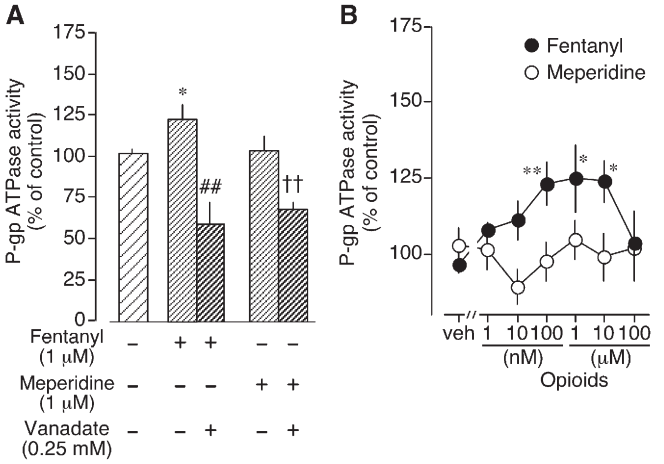

Fig. 6. Fentanyl- or meperidine-induced P-gp ATPase activity. (A) Effect of fentanyl (1 μM) and meperidine (1 μM) on the P-gp ATPase activity and its inhibition by vanadate (0.25 mM). Treatment with vanadate is same as that described in Fig. 4A. 'control': basal P-gp ATPase activity without any added drugs. '−': added water instead of opioid analgesics and/or vanadate to the reaction mixture. Each column represents the mean with S.E.M. of 5–6 independent experiments. * $p<0.05$, vs. '−'. ## $p<0.01$, vs. fentanyl (1 μM). †† $p<0.01$, vs. meperidine (1 μM). (B) Concentration-dependent changes in P-gp ATPase activity after administration of fentanyl, but not meperidine. Closed circle, fentanyl; Open circle, meperidine. 'veh': added water instead of opioid analgesics to the reaction mixture. Each point represents the mean with S.E.M. of 5–8 independent experiments. * $p<0.05$, ** $p<0.01$, vs. 'veh'.

3.5. Effects of fentanyl and meperidine on the basal P-gp ATPase activity

The P-gp ATPase activity was significantly increased by 1 μM of fentanyl, but not by meperidine. Furthermore, vanadate (0.25 mM) suppressed the P-gp ATPase activity in the presence of both fentanyl and meperidine (Fig. 6A). The concentration-response curve of fentanyl-induced P-gp ATPase activity showed a bell-shaped curve (peak at 1 μM) similar to that shown by morphine. On the other hand, meperidine (1 nM to 100 μM) did not activate the P-gp ATPase (Fig. 6B).

4. Discussion

In this research, we demonstrated that morphine and fentanyl, but not meperidine, activated P-gp ATPase, in vitro. In the in vivo study, morphine and fentanyl were more potent analgesics in *mdr*1a KO mice than in WT mice. Although there are many reports that show that the pharmacokinetics and pharmacodynamics of these drugs are influenced by inhibition or induction of P-gp (Letrent et al., 1999; Thompson et al., 2000; Zong and Pollack, 2000; Shimizu et al., 2004), this is the first report to determine the relationship between the P-gp ATPase activating effect of opioid analgesics and their P-gp dependent-antinociceptive effects.

As shown in Figs. 1 and 2, we demonstrated that morphine antinociception and morphine content in the brain in *mdr*1a KO mice was greater than those in WT mice, indicating that P-gp has a critical role in both morphine transport in the CNS and morphine antinociception. Furthermore, we also demonstrated that the antinociceptive effects of fentanyl were greater in the *mdr*1a KO mice than in WT mice (Fig. 5). These observations were consistent with previous reports, which indicated that the effect of morphine or fentanyl was enhanced by P-gp inhibitors such as cyclosporine and verapamil or by the deficient of *mdr*1a/1b or *mdr*1a in mice (Cirella et al., 1987; Thompson et al., 2000; King et al., 2001; Shimizu et al., 2004). Here, we confirmed P-gp dependent antinociception under mechanical noxious stimuli by use of the tail-pinch method, whereas Thompson et al. (2000) and King et al. (2001) demonstrated it under thermal noxious stimuli using hot-plate test and tail-flick test, respectively. In addition, it was reported that the brain uptake of morphine and fentanyl was increased in *mdr*1a KO mice when compared to WT mice (Dagenais et al., 2004). These investigators concluded that the 'P-gp effect' on brain uptake clearance of morphine was 1.24 when they compared the brain uptake clearance of *mdr*1a KO mice to that of WT mice. In this study, the 'P-gp effect' was 1.69 when we compared the ratio of morphine levels in serum and brain in both genotypes. On the other hand, in our study, the effect of the *mdr*1a deficiency on meperidine antinociception was not as clear as that seen with morphine or fentanyl. We found that high dose (40 mg/kg, s.c.) of meperidine did not produce differences in antinociception between the two genotypes, whereas low dose (20 mg/kg, s.c.) of meperidine produced significant difference (Fig. 5). Furthermore, Thompson et al. (2000) reported that the antinociceptive effect of meperidine against thermal noxious stimuli at a dose of 50 mg/kg was not influenced by *mdr*1a/1b deficiency. These findings suggest that the contribution of P-gp in meperidine antinociception or its transport into CNS is small, while whether a lower dose of meperidine produces even greater differences between the two genotypes or not is needed to be determined. We used mice deficient in only the *mdr*1a gene whereas Thompson et al. (2000) used mice deficient in both *mdr*1a and 1b genes, both of which are P-gp encoding genes in rodents. It is suitable for us to use just the *mdr*1a deficient mice to focus on the functions of P-gp in the BBB, because the *mdr*1a gene is specifically expressed in microvessels, whereas the *mdr*1b gene is present in the whole brain (Regina et al., 1998).

In contrast to the observations described above, comparatively little is known about the effect of opioid analgesics on the P-gp ATPase activity, which is an important function for P-gp to achieve its role in the BBB. P-gp uses the energy from ATP hydrolysis to export drugs from the BCECs. P-gp in plasma membranes of BCECs is known to show basal ATPase activity, which can be stimulated by several folds upon addition of substrates (Ambudkar et al., 1992). To determine the effect of opioid analgesics on P-gp ATPase activtiy, we used an enzymologic analysis using plasma membrane preparations of BCECs. All reactions were performed in the presence of ouabain, sodium azide and EGTA to inhibit ATPases other than P-gp as described previously (Fig. 3; He and Liu, 2002). As shown in Figs. 4 and 6, morphine and fentanyl significantly increased P-gp ATPase activity. Interestingly, the concentration-response curves were bell-shaped and peaked at 1 μM of opioid analgesics. That is, their P-gp ATPase activating effects were decreased at higher concentrations. This bell-shaped activating effect is similar to the results using verapamil, a prototypical P-gp substrate (Dong et al., 1996). The ATPase reaction consists of several transition steps, some of which are rate-limited by ATP binding, i.e., ADP release and release of drugs. Thus, it is possible that high-concentrations of drugs simply inhibit the ATPase reaction by stopping release of drug from the low-affinity release sites (Al-Shawi et al., 2003). Ambudkar et al. (1999) reported that the drug-stimulated ATPase activity reflects the nature of the interaction of P-gp with drug substrates, while the intrinsic basal activity in the absence of drugs is considered to be due to the transport of endogenous lipids or other unknown endogenous substrates. To clarify the opioid analgesics-induced ATPase activity, we used vanadate, a phosphate analog, which stabilizes the inhibited catalytic transition state of ATPase and inhibits many ATPases including P-gp (Tombline et al., 2004). Vanadate 0.25 mM suppressed the drug-stimulated ATPase activation completely, whereas the basal P-gp ATPase was partially suppressed (Figs. 4A and 6A). Similar results were obtained when we used 1 mM of vanadate (data not shown). Although it is possible that an unidentified ATPase that is insensitive to vanadate, ouabain, sodium azide and EGTA is present in these plasma membrane preparations of BCECs, it seems clear that morphine and fentanyl increased the P-gp ATPase activity in a vanadate-sensitive manner.

The fentanyl-induced activating effect of P-gp ATPase at a concentration of 1 μM (over 120% of control) was slightly lower than that of morphine (over 140% of control) (Figs. 4 and 6). Interestingly, this is consistent with the P-gp dependency of their antinociceptive effects obtained by the in vivo analysis, in which the antinociceptive effects of morphine and fentanyl in *mdr*1a KO mice were 2.8- and 2.2-fold more potent than those in WT mice, respectively. These findings suggest that the potency of P-gp-dependent antinociceptive effects of opioid analgesics can be correlated with their activating effect on P-gp ATPase. Surprisingly, despite the fact that the P-gp ATPase activating effect of both morphine and fentanyl peaked at the same concentration of 1 μM (Figs. 4B and 6A), the dose of fentanyl required for antinociceptive effect was 70-fold lower than that of morphine, when estimated by equianalgesic doses (the AUC of antinociception equal to 50; fentanyl 0.052 mg/kg, morphine 3.56 mg/kg) (WT mice in Figs. 1B and 5B). These discrepancies might be explained by the differences of their lipophilicity. We hypothesize that the high lipophilicity of fentanyl, which is 100-fold greater than that of morphine (Roy and Flynn, 1989), may cause its concentrations in the BCECs to reach the same level of morphine. Furthermore, the 'P-gp effect' of morphine and fentanyl are reported to be identical (Dagenais et al., 2004). Thus, the differences in antinociceptive effect of fentanyl and morphine might be due to some other factors such as opioid receptor binding affinity in the peripherally, rather than P-gp, in addition to the lipophilicity.

Interestingly, the substrate specificity of P-gp is still controversial. Callaghan and Riordan (1993) demonstrated that meperidine is a substrate of P-gp using an in vitro study,

whereas Dagenais et al. (2004) demonstrated the opposite results using an in vivo examination. Here, we showed that meperidine, in contrast to morphine and fentanyl, did not show any activating effect on P-gp ATPase (Fig. 6). This is similar to the results of antinociceptive studies in vivo, using these three opioid analgesics (Thompson et al., 2000), while the differences among the drugs are not clear (Figs. 5 and 6). Although a possible explanation for this phenomenon may be differences in the affinity for P-gp (Callaghan and Riordan, 1993), further studies, such as binding assays using in vivo samples, will be necessary to clarify the differences between morphine or fentanyl and meperidine in the P-gp ATPase activating effect.

In conclusion, this is the first study to show that morphine and fentanyl, but not meperidine, increased P-gp ATPase activity, in the BCECs. In agreement with this result, we confirmed that the antinociceptive effect of morphine and fentanyl in the *mdr*1a KO mice is greater than that in the WT mice as shown in previous report (Thompson et al., 2000). In the case of meperidine, the increment of antinociception in *mdr*1a KO mice was partial and small. These results suggest that the P-gp ATPase activating effects of opioid analgesics, which produce energy requisite for P-gp transport, may reflect their potency in P-gp dependent antinociceptive effects. Since P-gp is now recognized as one of the vital target factors for pharmacogenetic therapeutic strategies, which are important for individualized opioid therapy (Lotsch et al., 2004), further studies will be needed to elucidate the detailed properties of the effect of opioid analgesics on P-gp functions.

Acknowledgements

We are grateful to Dr. James H. Woods and Dr. Gail Winger for advice concerning the manuscript.

References

Abbruscato TJ, Thomas SA, Hruby VJ, Davis TP. Blood-brain barrier permeability and bioavailability of a highly potent and mu-selective opioid receptor antagonist, CTAP: comparison with morphine. J Pharmacol Exp Ther 1997;280:402–9.

Al-Shawi MK, Omote H. The remarkable transport mechanism of P-glycoprotein; a multidrug transporter. J Bioenerg Biomembranes 2005;37:489–96.

Al-Shawi MK, Polar MK, Omote H, Figler RA. Transition state analysis of the coupling of drug transport to ATP hydrolysis by P-glycoprotein. J Biol Chem 2003;278:52629–40.

Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM, Pastan I. Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. Proc Natl Acad Sci U S A 1992;89:8472–6.

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 1999;39:361–98.

Bauer B, Hartz AM, Fricker G, Miller DS. Modulation of p-glycoprotein transport function at the blood-brain barrier. Exp Biol Medicine (Maywood) 2005;230:118–27.

Boado RJ, Pardridge MM. A one-step procedure for isolation of poly(A)+mRNA from isolated brain capillaries and endothelial cells in culture. J Neurochem 1991;57:2136–9.

Callaghan R, Riordan JR. Synthetic and natural opiates interact with P-glycoprotein in multidrug-resistant cells. J Biol Chem 1993;268:16059–64.

Cirella VN, Pantuck CB, Lee YJ, Pantuck EJ. Effects of cyclosporine on anesthetic action. Anesth Analg 1987;66:703–6.

Dagenais C, Graff CL, Pollack GM. Variable modulation of opioid brain uptake by P-glycoprotein in mice. Biochem Pharmacol 2004;67:269–76.

Dallaire L, Tremblay L, Beliveau R. Purification and characterization of metabolically active capillaries of the blood-brain barrier. Biochem J 1991;276 (Pt 3):745–52.

de Boer AG, van der Sandt IC, Gaillard PJ. The role of drug transporters at the blood-brain barrier. Annu Rev Pharmacol Toxicol 2003;43:629–56.

Dong M, Penin F, Baggetto LG. Efficient purification and reconstitution of P-glycoprotein for functional and structural studies. J Biol Chem 1996;271: 28875–83.

Ecker G, Chiba P. Structure-activity-relationship studies on modulators of the multidrug transporter P-glycoprotein—an overview. Wien Klin Wochenschr 1995;107: 681–6.

Emmerson PJ, Clark MJ, Mansour A, Akil H, Woods JH, Medzihradsky F. Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the mu opioid receptor. J Pharmacol Exp Ther 1996;278:1121–7.

Fromm MF. Importance of P-glycoprotein at blood-tissue barriers. Trends Pharmacol Sci 2004;25:423–9.

Garrigos M, Mir LM, Orlowski S. Competitive and non-competitive inhibition of the multidrug-resistance-associated P-glycoprotein ATPase-further experimental evidence for a multisite model. Eur J Biochem 1997;244: 664–73.

Garrigues A, Escargueil AE, Orlowski S. The multidrug transporter, P-glycoprotein, actively mediates cholesterol redistribution in the cell membrane. Proc Natl Acad Sci U S A 2002;99:10347–52.

Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993;62:385–427.

Gourlay GK. Advances in opioid pharmacology. Support Care Cancer 2005;13: 153–9.

He L, Liu GQ. Interaction of multidrug resistance reversal agents with P-glycoprotein ATPase activity on blood-brain barrier. Acta Pharmacol Sin 2002;23:423–9.

Henthorn TK, Liu Y, Mahapatro M, Ng KY. Active transport of fentanyl by the blood-brain barrier. J Pharmacol Exp Ther 1999;289:1084–9.

Idriss HT, Hannun YA, Boulpaep E, Basavappa S. Regulation of volume-activated chloride channels by P-glycoprotein: phosphorylation has the final say! J Physiol 2000;524(Pt 3):629–36.

King M, Su W, Chang A, Zuckerman A, Pasternak GW. Transport of opioids from the brain to the periphery by P-glycoprotein: peripheral actions of central drugs. Nat Neurosci 2001;4:268–74.

Letrent SP, Pollack GM, Brouwer KR, Brouwer KL. Effects of a potent and specific P-glycoprotein inhibitor on the blood-brain barrier distribution and antinociceptive effect of morphine in the rat. Drug Metab Dispos 1999;27: 827–34.

Lotsch J, Skarke C, Liefhold J, Geisslinger G. Genetic predictors of the clinical response to opioid analgesics: clinical utility and future perspectives. Clin Pharmacokinet 2004;43:983–1013.

Miyamoto Y, Morita N, Kitabata Y, Yamanishi T, Kishioka S, Ozaki M, et al. Antinociceptive synergism between supraspinal and spinal sites after subcutaneous morphine evidenced by CNS morphine content. Brain Res 1991;552:136–40.

Regina A, Koman A, Piciotti M, El Hafny B, Center MS, Bergmann R, et al. Mrp1 multidrug resistance-associated protein and P-glycoprotein expression in rat brain microvessel endothelial cells. J Neurochem 1998;71:705–15.

Rodriguez M, Ortega I, Soengas I, Suarez E, Lukas JC, Calvo R. Effect of P-glycoprotein inhibition on methadone analgesia and brain distribution in the rat. J Pharm Pharmacol 2004;56:367–74.

Roy SD, Flynn GL. Transdermal delivery of narcotic analgesics: comparative permeabilities of narcotic analgesics through human cadaver skin. Pharm Res 1989;6:825–32.

Sarkadi B, Price EM, Boucher RC, Germann UA, Scarborough GA. Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J Biol Chem 1992;267:4854–8.

Shimizu N, Kishioka S, Maeda T, Fukazawa Y, Yamamoto C, Ozaki M, et al. Role of pharmacokinetic effects in the potentiation of morphine analgesia by L-type calcium channel blockers in mice. J Pharmacol Sci 2004;94:240–5.

Takagi H, Inukai T, Nakama M. A modification of Haffner's method for testing analgesics. Jpn J Pharmacol 1966;16:287–94.

Thompson SJ, Koszdin K, Bernards CM. Opiate-induced analgesia is increased and prolonged in mice lacking P-glycoprotein. Anesthesiology 2000;92:1392–9.

Tombline G, Bartholomew L, Gimi K, Tyndall GA, Senior AE. Synergy between conserved ABC signature Ser residues in P-glycoprotein catalysis. J Biol Chem 2004;279:5363–73.

Tsuji A, Sakata A, Tamai I. Tissue distribution of the multidrug-resistance gene product P-glycoprotein and its physiological function. Nippon Rinsho 1997;55: 1059–63.

Wandel C, Kim R, Wood M, Wood A. Interaction of morphine, fentanyl, sufentanil, alfentanil, and loperamide with the efflux drug transporter P-glycoprotein. Anesthesiology 2002;96:913–20.

Zong J, Pollack GM. Morphine antinociception is enhanced in mdr1a gene-deficient mice. Pharm Res 2000;17:749–53.