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DIFFERENTIAL MODIFICATION OF OPIATE RECEPTOR ACTIVITY BY ARYLSULFATASE TREATMENT

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Abstract—Treatment with the enzyme arylsulfatase *in vivo* selectively attenuated the effect of analgesia induced by morphine, β-endorphin or ethylketocyclazocine but not that induced by Sandoz FK33824 or D-ala^2-D-leu^5-enkephalin. The effect on morphine analgesia was indicated both by an increased morphine ED_{50} in the presence of a fixed dose of naloxone and by a decreased naloxone ED_{50} in the presence of a fixed dose of morphine. Arylsulfatase treatment *in vivo* also selectively affected *in vitro* ligand binding; B_{max} values of the low affinity binding site of dihydromorphine, naloxone, D-ala^2-D-leu^5-enkephalin, D-ala^2-met^5-enkephalinamide and ethylketocyclazocine were decreased significantly while the B_{max} values of the high affinity sites as well as the K_D values of both the high and low affinity sites were affected little or not at all. The data suggest that the change induced by the enzyme may have been due to the alteration of certain constituents of the low affinity opiate binding site.

It has been generally accepted that separate receptors for the opioids morphine (μ) and enkephalin (δ) exist in brain [1]. These two receptors seem to differ in some properties, though both agonists may produce analgesia. Of possible relevance to this, studies of the chemical nature of opiate binding sites have revealed the importance of both proteins and lipids in receptor binding constitution, though the evidence generally does not distinguish between μ and δ subtypes. Certain membrane-bound acidic lipids, such as phosphatidylserine (PS) or cerebroside sulfate (CS) have been implicated in opiate receptor function, and structure–activity relationship studies indicate that lipids are involved more in alkaloid (μ) than in peptide (δ) binding [2]. Law *et al.* [3] presented evidence showing that the number of alkaloid binding sites *in vitro* was decreased after enzymatic treatment of synaptic membrane with arylsulfatase A, which hydrolyzed cerebroside sulfate (CS) to cerebroside (C), but whether this binding site was related to opiate analgesia is unclear. In this study, we show that *in vivo* treatment with arylsulfatase (a) diminished only morphine-, β-endorphin- and ethylketocyclazocine (EKC)-induced analgesia, and not that of enkephalin peptides, and (b) decreased the detectable B_{max} of the low but not the high affinity binding sites of dihydromorphine, naloxone, D-ala^2-D-leu^5-enkephalin, D-ala^2-met^5-enkephalinamide and ethylketocyclazocine. The K_D values of either high or low affinity sites for all ligands tested were little altered by arylsulfatase treatment.

MATERIALS AND METHODS

[^{3}H]D-ala^2-D-leu^5-Enkephalin (31 Ci/mmole), [^{3}H]D-ala^2-met^5-enkephalinamide (30 Ci/mmole) and [^{3}H]ethylketocyclazocine (15 Ci/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). [^{3}H]Dihydromorphine (65–73 Ci/mmole) and [^{3}H]naloxone (40 Ci/mmole) were from Amersham (Arlington Heights, IL). Naloxone hydrochloride was a gift from the Endo Co. (Garden City, NY), morphine sulfate was purchased from the Mallinckrodt Co. (St. Louis, MO), and D-ala^2-D-leu^5-enkephalin was a gift from the Burroughs Wellcome Co. (Research Triangle Park, NC). Dihydromorphine was a gift from NIDA, Sandoz FK33824 from the Sandoz Co. (Basel, Switzerland) and ethylketocyclazocine methyl sulfonate from Dr. E. Iwamoto (University of Kentucky). Arylsulfate sulfohydrolase was purchased from the Sigma Chemical Co. (catalogue no. S-8629, St. Louis, MO).

Male ICR mice (25 ± 2 g), from Simonsen Laboratories (Gilroy, CA), were kept on water and food *ad lib*. Each animal was used only once. Antinociception was measured by the modified tail-flick method of Tulunay and Takemori [4], and ED_{50} values and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon [5].

Another analgesic test was also used as described by Nott [6]; in this test, the mouse tail was immersed in a 52° water bath. The elapsed time until the animal moved its tail from the water was measured. Each animal was tested for its base latency first; then the control group (N = 10) received 200 or 500 μg bovine serum albumin (BSA) intracerebraventricularly (i.c.v.) 30 min before drug, and the experimental group (N = 10) received arylsulfatase (200 or 500 μg) instead of BSA. Morphine and EKC analgesias were tested 30 min, and D-ala^2-D-leu^5-enkephalin (DADLE) 15 min, after the injection. Peptides and arylsulfatase were injected i.c.v. in a volume of 4 μl saline per unanesthetized mouse. Morphine sulfate or naloxone hydrochloride was given either s.c. or i.c.v. as indicated. Tail-flick latency was measured before, and 30 min after, drug treatment.

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Table 1. Effect of arylsulfatase treatment on morphine-, β-endorphin-, D-ala^2-D-leu^5-enkephalin- and Sandoz FK33824-induced analgesia in naive animals

Drugs*	Arylsulfatase	ED_{50} (μg/mouse, i.c.v.)
Morphine	−	2.75 (1.62–4.68)†
	+	8.00‡ (5.10–12.6)
β-Endorphin	−	0.154 (0.097–0.245)
	+	0.315‡ (0.216–0.460)
D-ala^2-D-leu^5-Enkephalin	−	0.39 (0.24–0.63)
	+	0.34 (0.21–0.56)
Sandoz FK33824	−	0.0051 (0.0034–0.0076)
	+	0.0060 (0.0042–0.0086)

* All drugs were given i.c.v. 30 min before test.
† Numbers in parentheses are 95% confidence limits.
‡ $P < 0.05$.

For *in vitro* binding assay, animals were injected i.c.v. with arylsulfatase in a volume of 4 μl/mouse. After 30 min, the animals were killed by dislocation, and the brains (minus cerebellum and medulla) were pooled. Crude synaptosomal (P_2) fractions were obtained according to the methods of Gray and Whittaker [7] as modified by the method of Terenius [8]. P_2 pellets were resuspended in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (25 mM)–sucrose (0.32 M) buffer, pH 7.7.

Samples for *in vitro* binding assay were incubated in a 37° shaking incubator for 30 min in a final volume of 2 ml containing 25 mM HEPES, 1.4 mg protein and various concentrations of ^{3}H-labeled ligand. Afterwards, the samples were kept on ice for 1 hr and filtered on Whatman GF/B filters under vacuum. The filters were then washed with 2 × 5 ml of ice-cold HEPES buffer, 5 mM, pH 7.7, and counted in the presence of 10 ml of Liquiscint (National Diagnostics) in a Beckman LS-100C Scintillation counter 24 hr later.

All the binding assays were carried out in triplicate, and the variability of the samples was usually less than 15% of the mean. The saturation experiments were performed with 14–24 concentrations of the tritiated ligand, and nonsaturable binding was determined as the binding remaining after exposure to a 1 μM concentration of the non-radiolabeled form of the ligand.

Scatchard plots of receptor binding were analyzed by computer fitting [9–13] assuming two independent binding sites for every ligand. The mathematical equation used is as follows:

$$B = B_1 + B_2 = \frac{B_{max1} \cdot L}{K_{D1} + L} + \frac{B_{max2} \cdot L}{K_{D2} + L}$$

where 1 = high affinity, 2 = low affinity, L = free ligand concentration, B = amount bound (total binding − non-specific binding), and K_D = affinity constant.

The concentration of ligand used for Scatchard analysis ranged from below the K_D value of the high affinity site to above the K_D value of the low affinity site as suggested by Molinoff *et al.* [14].

Protein was determined by the method described by Lowry *et al.* [15] using bovine serum albumin as standard.

RESULTS

As seen in Table 1, arylsulfatase (200 μg/mouse, i.c.v.) treatment *in vivo* significantly attenuated the analgesia induced by morphine or β-endorphin although the enzyme treatment alone did not alter tail-flick latency (data not shown). The ED_{50} was shifted from 2.75 (1.62–4.68) to 8.00 (5.10–12.6) μg/mouse for morphine and 0.154 (0.097–0.245) to 0.315 (0.216–0.460) μg/mouse for β-endorphin. However, the ED_{50} for D-ala^2-D-leu^5-enkephalin or Sandoz FK33824 remained unchanged. The effect of arysulfatase on naloxone antagonism of morphine- and Sandoz FK33824-induced analgesia was also measured. It was found (Table 2) that arylsulfatase significantly decreased the effectiveness of morphine in the presence of naloxone, while the effect of Sandoz FK33824 was not changed. Furthermore, the ED_{50} dose of naloxone s.c. needed to antagonize a given morphine dose was also decreased significantly from 43 (29–65) to 19 (13–28) μm/kg.

To determine whether the effect of arylsulfatase on morphine-induced analgesia was the same with another method of measuring analgesia, we used the 52° water immersion test [6]. As can be seen in Table 3, arylsulfatase, 200 or 500 μg, significantly affected morphine- and ethylketocyclazocine-induced analgesia, but not that of DADLE. The latencies were reduced significantly for morphine ($P < 0.01$) and ethylketocyclazocine ($P < 0.05$) induced analgesia after arylsulfatase treatment.

Opiate receptor binding *in vitro* was determined on crude synaptosomal fractions obtained from the animals that had been pretreated with saline (control) or arylsulfatase (treated) *in vivo*. It is known that the Scatchard plot for opiate receptor binding is often curvilinear, which has been interpreted as due to a multiplicity of binding sites [16]. Therefore, in the analysis of our experimental data, we assumed two independent binding sites for each ligand [16]. Initially, we estimated the K_D values and the B_{max} values using linear regression analysis; however, this procedure may result in erroneous estimates [2, 17]. The K_D and B_{max} values may be over- or underestimated depending on the situation. When comparing one set of control data with a set of experimental (in our case, control vs arylsulfatase-treated), the

Table 2. Effect of arylsulfatase treatment on naloxone antagonism of morphine- and Sandoz FK33824-induced analgesia

Treatment*	Without sulfatase	With sulfatase§
Naloxone ED_{50} (μg/kg, s.c.) + morphine (16 mg/kg, s.c.)	43 (29–65)†	19‡ (13–28)
Morphine ED_{50} (μg/mouse i.c.v.) + naloxone (125 μg/kg, s.c.)	6.2 (3.9–9.9)	16.2‡ (10.8–24.3)
Morphine ED_{50} (mg/kg, s.c.) + naloxone (125 μg/kg, s.c.)	28 (21–38)	105‡ (72–152)
Sandoz FK33824 ED_{50} (mg/kg, s.c.) + naloxone (125 μg/kg, s.c.)	6.0 (5.0–7.7)	7.4 (5.6–9.8)

* All drugs were given 30 min before test.
† Numbers in parentheses are 95% confidence limits.
‡ Significantly different from the control group (without sulfatase).
§ 200 μg/mouse i.c.v.

interpretation can be incorrect. Therefore, our data were analyzed by means of computer fitting; the program utilized is basically an iterative search for the parameters (B_{max_1}, K_{D_1}, B_{max_2}, and K_{D_2}) that best fit the experimental data. This kind of program has been used previously in the resolution of receptor subtypes [14, 18]. Curvilinear Scatchard plots (Figs. 1–5) were usually found for dihydromorphine (DHM), naloxone, ethylketocyclazocine (EKC), D-ala^2-D-leu^5-enkephalin (DADLE) and D-ala^2-met^5-enkephalinamde (DAMA). The K_D values of the high affinity sites for DHM, naloxone, ethylketocyclazocine, D-ala^2-D-leu^5-enkephalin and D-ala^2-met^5-enkephalinamide were 1.05, 1.6, 0.59, 0.95 and 3.4 nM respectively; ethylketocyclazocine had the largest detectable binding capacity, 45 fmoles/mg and 581 fmoles/mg for the high and low affinity respectively. Naloxone also had a large binding capacity (70 and 470 fmoles/mg). The other ligands bound with approximately half of the naloxone capacity, D-ala^2-D-leu^5-enkephalin (22 and 222 fmoles/mg), DHM (18.5 and 195 fmoles/mg) and D-ala^2-met^5-enkephalinamide (55 and 203 fmoles/mg).

Arylsulfatase treatment *in vivo* did not change the general characteristics of the Scatchard plots (Figs. 1–5); two binding sites for DHM, naloxone, ethylketocyclazocine, D-ala^2-D-leu^5-enkephalin, and D-ala^2-met^5-enkephalinamide were still observed. The K_D values for the high and low affinity sites as well as the B_{max} values for the high affinity sites for all ligands were affected little or not at all by the enzyme treatment. However, the B_{max} values of the low affinity sites (Site II) for all ligands were altered significantly (Table 4). The B_{max} values of the low affinity binding sites for DADLE and DAMA seem to have been affected more than those of the other ligands; after 500 μg enzyme treatment, the B_{max} values for these two ligands were reduced to 31 and 13% of the control, respectively, although the analgesia induced by DADLE was not affected significantly. Among all the ligands tested, the binding for naloxone was least affected, even after 500 μg enzyme treatment. The B_{max} was only reduced to 71% of the control. The values for binding of DHM and EKC were also affected.

DISCUSSION

Brain opiate receptors are thought to contain both protein and lipids as integral components, but their exact chemical nature is not known [19–23]. Fur-

Table 3. Effect of arylsulfatase (AS) treatment on opiate-induced analgesia (52° water test)*

		Analgesic latencies (sec ± S.E.)			
		BSA (200 μg)	AS (200 μg)	BSA (500 μg)	AS (500 μg)
Morphine (8 μg, i.c.v.)	−	2.07 ± 0.18	1.85 ± 0.18	2.3 ± 0.23	2.2 ± 0.16
	+	8.46 ± 1.4†	3.62 ± 0.6‡	11.2 ± 0.43†	5.2 ± 0.86‡§
D-ala^2-D-leu^5-Enkephalin (0.6 μg, i.c.v.)	−			1.74 ± 0.13	1.62 ± 0.16
	+			7.71 ± 1.47†	6.55 ± 1.04†
Ethylketocyclazocine (16 μg, i.c.v.)	−			1.3 ± 0.11	1.62 ± 0.13
	+			4.92 ± 0.62†	3.06 ± 0.37§‖

* Statistical significance was determined by analysis of variance followed by the Student–Newman–Keuls multiple comparison test. The analgesic latencies were measured as described in Materials and Methods.
† $P < 0.01$, comparing before and after opiate treatment.
‡ $P < 0.01$, comparing the effect of AS with the BSA control.
§ $P < 0.05$, comparing before and after opiate treatment.
‖ $P < 0.05$, comparing the effect of AS with the BSA control.

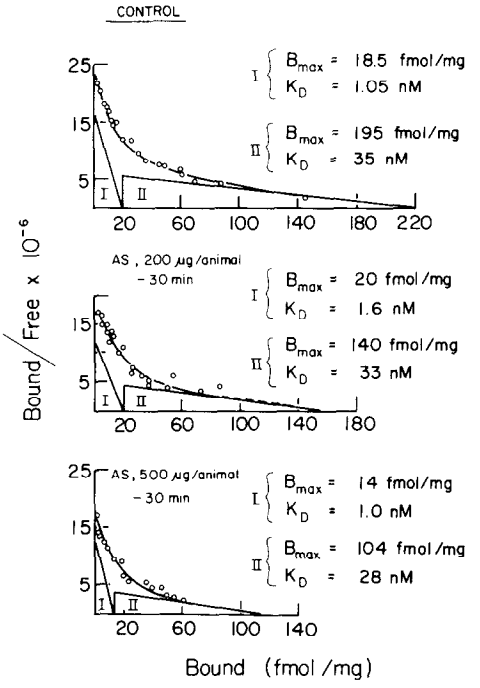

Fig. 1. Scatchard analysis of [^{3}H]dihydromorphine binding. The experiment was carried out using the P_2 fraction obtained from brains of mice that had or had not been pretreated *in vivo* with different amounts of the enzyme arylsulfatase (AS). Each experiment was performed in triplicate, and at least fourteen concentrations were studied ranging from below the K_D value of the high affinity site to above the K_D value of the low affinity site. Y-axis (ligand bound/free) $\times 10^{-6}$; X-axis, fmoles bound/mg protein. The B_{max} and K_D were determined by computer fitting.

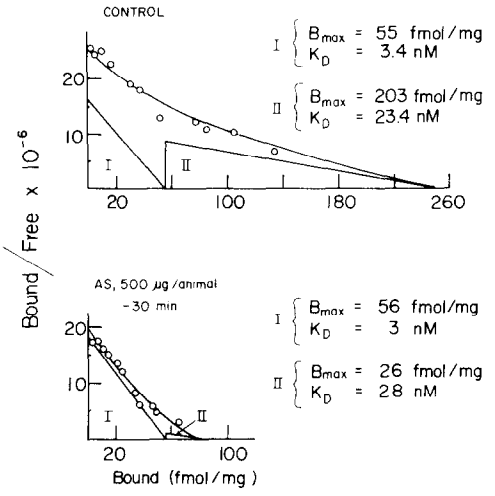

Fig. 3. Scatchard analysis of [^{3}H]D-ala^2-met^5-enkephalinamide binding. The experimental design was the same as described in the legend of Fig. 1.

thermore, most of the evidence has been obtained by using enzymatic treatment *in vitro* and performing binding afterwards; thus, it is difficult to extrapolate the *in vitro* binding to *in vivo* pharmacological effects which, in this case, was analgesia. In addition, it is not clear whether the pharmacologically defined μ and δ receptors share the same properties in terms of protein and lipid requirements.

In this study, we have demonstrated an *in vivo* effect of arylsulfatase, a lipolytic agent, on opioid-induced analgesia and, furthermore, have

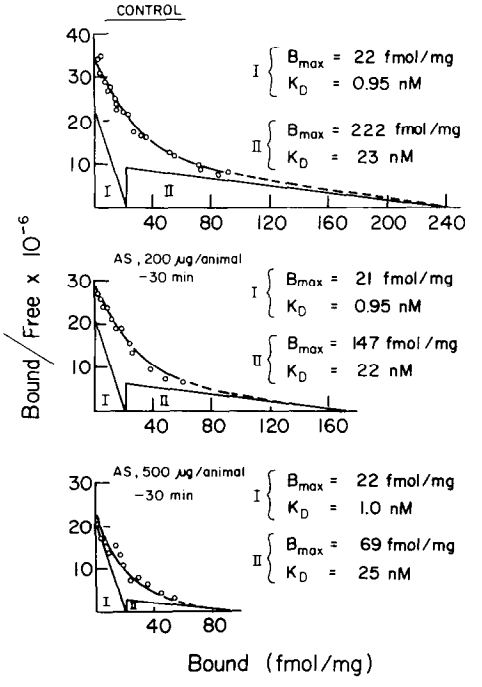

Fig. 2. Scatchard analysis of [^{3}H]D-ala^2-D-leu^5-enkephalin binding. The experimental design was the same as described in the legend of Fig. 1.

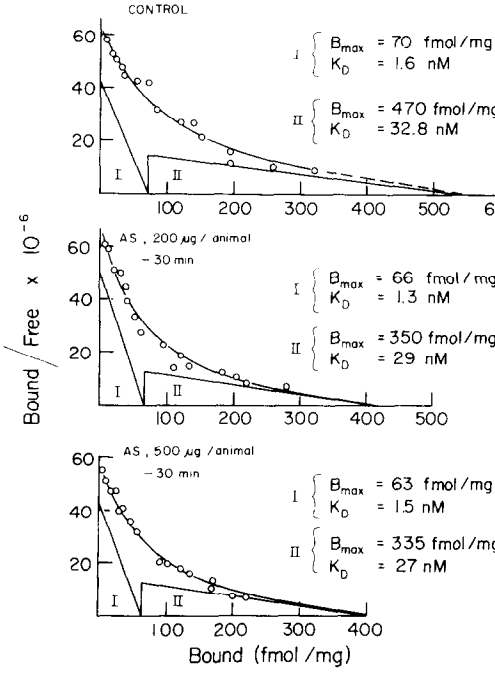

Fig. 4. Scatchard analysis of [^{3}H]naloxone binding. The experimental design was the same as described in the legend of Fig. 1.

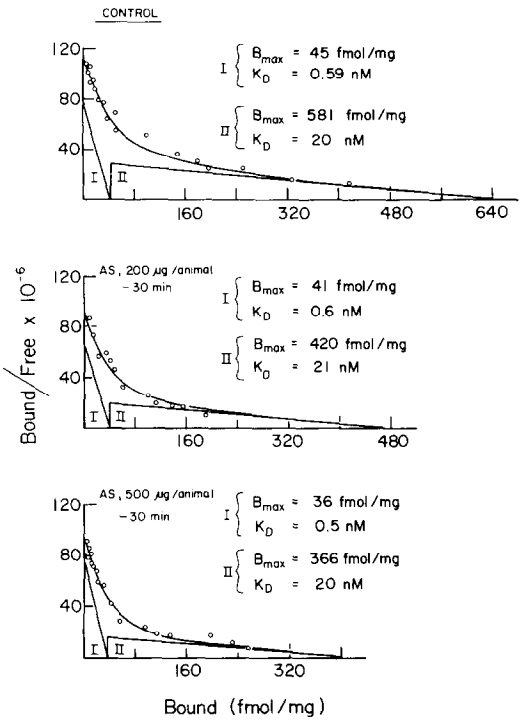

Fig. 5. Scatchard analysis of [^{3}H]ethylketocyclazocine binding. The experimental design is the same as described in the legend of Fig. 1.

shown that only sensitivity to alkaloids and β-endorphin was affected. In addition, when the effect of arylsulfatase on naloxone antagonism of morphine- or Sandoz FK33824-induced analgesia was evaluated, it was found again that arylsulfatase treatment had significant effects on antagonism of morphine, but not of FK33824. The data reveal that the effect of morphine was weakened significantly by such treatment, as indicated, by a decreased naloxone ED_{50} in the presence of morphine and an increased morphine ED_{50} in the presence of naloxone (Table 2).

To be sure that the effect of arylsulfatase on morphine-induced analgesia was not due to the tail-flick method, we also used a 52° water bath method [6] to test antinociception. The results obtained with both analgesic tests are comparable, revealing that arylsulfatase only affected alkaloid analgesia and not that of DADLE. Ethylketocyclazocine-induced analgesia was also reduced by arylsulfatase treatment. Whether this pharmacological effect occurs via the μ (morphine-like) receptor or the κ receptor is not known.

The arylsulfatase effect seemed to be due to an enzymatic reaction, since the effect was lost when the enzyme preparation was boiled to denature the protein (data not shown). In addition, 200 or 500 μg of bovine serum albumin injected either before or concomitantly with morphine had no effect on the baseline latencies or the opiate analgesia, further indicating that a nonspecific protein effect was not involved. However, we cannot conclude positively that the action of sulfatase was to release sulfate from cerebroside sulfate or other components containing sulfate, since sulfate release *in vivo* cannot be determined.

Nevertheless, we can conclude from these data that it is possible to alter morphine, ethylketocyclazocine- or β-endorphin-induced analgesia selectively without affecting that of D-ala^2-D-leu^5-enkephalin or Sandoz FK33824. Since the effect was selective, it is not likely to have been due to general membrane perturbation; the simplest interpretation of these data is that the enzyme destroyed or altered some component of the morphine or β-endorphin receptor critical to analgesia. An alternative explanation is that the enkephalin receptor is located in an area outside of the region reached by i.c.v. administration of the enzyme and was unaffected for this reason only. However, our data suggest that the enzyme, or its effect, was present in all parts of the brain because (a) when both morphine and naloxone were administered s.c., the enzyme treatment still significantly enhanced the morphine ED_{50}, and (b) binding studies, which indicated a detectable loss of low affinity receptors, were carried out on whole brain tissue.

The fact that K_D values for both the high and low affinity sites were not altered by the enzyme treatment suggests that the enzyme did not affect the recognition site of the receptor; of course, a very large increase in K_D (decrease in affinity) would have appeared simply as a decrease in B_{max}, since these sites would not be detectable in our binding assay. It is also interesting to point out that, since only the B_{max} values of the low affinity sites decreased, the high and low affinity sites must be different in chemical nature.

A striking result of our studies is that, even though the low affinity B_{max} values for all the ligands tested decreased after enzyme treatment, only morphine- and EKC-induced analgesias were affected. This suggests that the low affinity sites of these alkaloids are more important for analgesia than is the low affinity

Table 4. Effect of arylsulfatase on B_{max} values of low affinity binding sites

Arylsulfatase (μg/animal)	B_{max} values (fmoles/mg protein)				
	DHM	Naloxone	DADLE	DAMA	EKC
Control	195	470	222	203	581
200	140 (72)*	350 (74)	147 (66)		420 (72)
500	104 (53)	335 (71)	69 (31)	26 (13)	366 (63)

* Numbers in parentheses equal percents of control.

site of DADLE. It is possible that just the high affinity site of D-ala^2-D-leu^5-enkephalin mediates its analgesic effect or, alternatively, that the intrinsic activity of the low affinity site for DADLE is so high that, even after it has been decreased drastically by the enzyme, it is still able to induce analgesia through the remaining receptors. This latter possibility implies the presence of low affinity spare receptors for the analgesic effect of D-ala^2-D-leu^5-enkephalin. On the other hand, the synthetic peptide Sandoz FK 33824 which has been proposed as the μ ligand [24] did not suffer any loss of analgesic activity after arylsulfatase treatment. This observation might also result from a greater efficacy at the receptor level. We have also studied Sandoz FK 33824 binding (results not shown), and the enzyme effect was consistent with a decrease of the low affinity receptors.

Unlike the agonist binding, the binding of the antagonist naloxone was not very sensitive to the enzyme treatment, suggesting that the enzymatic reaction changed something in the receptor which was more important to agonist than to antagonist binding. It is possible that the agonist binding required certain conformational changes in the receptor whereas the antagonist binding did not; in that case, the arylsulfatase treatment would have resulted in one particular conformation which was less preferred by agonist binding. When we compared the displacement of naloxone by morphine, we observed that, although naloxone binding could still be displaced completely after the enzyme treatment, the IC_{50} of morphine had increased 3-fold for both high and low affinities, indicating that morphine was less effective in displacing naloxone from the receptor after enzyme treatment (Table 5). Furthermore, it is difficult to establish definitively the homogeneity of the low affinity site to all opiates; however, we have shown that this binding site was the one affected by the enzyme treatment. In any case, since it was the low affinity but not the high affinity binding site that was altered by the enzyme treatment, our results point to different chemical properties of the high and low affinity sites.

In summary, we have presented data which show that the treatment of arylsulfatase *in vivo* can selectively attenuate morphine, ethylketocyclazocine- and β-endorphin-induced analgesia but not that of synthetic pentapeptides. The change may be due to the destruction or alteration of certain components responsible for opiate binding. The fact that a decrease in binding of DADLE did not coincide with a decrease in analgesia may reflect the intrinsic activity of this agonist to the receptor, a factor which should always be considered when correlating biochemical and pharmacological data. The enzyme effect was not due to a non-specific destruction of the membrane, because the enzyme effect was ligand selective, and denatured enzyme or BSA would not substitute for it. The effect of arylsulfatase treatment on the low affinity binding site seems to have been an alteration of a certain component(s) and not a complete destruction, because the antagonist naloxone binding was less affected by the enzyme treatment. The fact that the high affinity site was not affected suggests a difference in the chemical natures of the high and the low binding sites. The possibility of a common, low affinity site exists for all the opiates.

Table 5. IC_{50} of morphine in displacing [^{3}H]naloxone binding to mouse P_2 fractions with or without arylsulfatase treatment*

Naloxone (nM)	Morphine IC_{50} (nM) Control	AS-treated
0.5	4.4	15.4
10.0	340.0	944.0

* The arylsulfatase (AS) treatment and receptor bindings were the same as described in Materials and Methods.

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