Morphine enhances the phosphorylation of a 58 kDa protein in mouse brain membranes

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Morphine and [D-Ala²,D-Leu⁵]enkephalinamide enhance the phosphorylation of a 58 kDa protein in mouse brain synaptosomal membranes. The enhancement of phosphorylation was inhibited by naloxone, an antagonist of morphine. The phosphorylated 58 kDa protein was retained on wheat-germ-agglutinin-agarose and morphinone-Affi-Gel 401 columns and biospecifically eluted out from the columns with *N*-acetyl-D-glucosamine and naloxone respectively. These results suggest a strong possibility that the opiate-binding protein undergoes phosphorylation by endogenous protein kinase. Since the molecular mass of a μ -type opioid receptor in mouse brain is suggested to be 58 kDa, coincident with those of rat brain and neuroblastoma $\times$ glioma hybrid cells, it is conceivable that the phosphorylated 58 kDa protein is a μ -type receptor.

INTRODUCTION

Receptors in mammalian plasma membranes for hormones and growth factors have been demonstrated to be phosphorylated by endogenous protein kinases in response to ligand binding, and this receptor phosphorylation is believed to play an important role in the regulation of signal transduction (Cohen, 1982; Nestler *et al.*, 1983; Nishizuka, 1986).

Recently considerable progress in the identification (Klee *et al.*, 1982; Newman & Barnard, 1984; Howard *et al.*, 1985) and purification of opioid receptors (Bidlack *et al.*, 1981; Gioannini *et al.*, 1985; Maneckjee *et al.*, 1985; Cho *et al.*, 1986) has been made and the μ -type receptor has been isolated as a 58 kDa glycoprotein (Cho *et al.*, 1986; Ueda *et al.*, 1987). In the present paper, we report that morphine enhances the phosphorylation of a 58 kDa protein in crude synaptosomal membranes (P_2 membranes) from mouse brain and that the phosphorylation is inhibited by naloxone, an antagonist of morphine.

MATERIALS AND METHODS

Chemicals

[γ -³²P]ATP (sp. radioactivity 3000 Ci/mmol) and [¹²⁵I]- β -endorphin (human) (sp. radioactivity 2000 Ci/mmol) were purchased from Amersham International. [³H]-Naloxone (sp. radioactivity 44.4 Ci/mmol) and Aquasol-2 were purchased from New England Nuclear (Boston, MA, U.S.A.). Disuccinimidyl suberate (DSS) was obtained from Pierce Co (Rockford, IL, U.S.A.). [D-Ala²,D-Leu⁵]enkephalinamide, electrophoresis chemicals and premixed molecular-mass standards were from Sigma (St. Louis, MO, U.S.A.). Naloxone was a gift from Sankyo Co. (Tokyo, Japan). Wheat-germ agglutinin-(WGA)-agarose gel was from Hohnen Seiyu Co. (Tokyo, Japan). X-ray films (XAR-5) were from Kodak (Rochester, NY, U.S.A.) and intensifying screens (Cronex Lighting Plus) were from du Pont (Wilmington,

DE, U.S.A.). Preparation of morphinone-Affi-Gel 401 (morphinone-agarose) was carried out by the reaction of morphinone with the thiol group of Affi-Gel 401 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) in 0.1 M-sodium phosphate buffer, pH 7.4 (K. Nagamatsu, K. Suzuki & T. Terao, unpublished work; details will be provided by K.N. on request). All other reagents were of the highest grade available.

Phosphorylation of crude P_2 membranes

Crude P_2 membranes were prepared from mouse brain without cerebellum. Aliquots of membranes were pre-incubated for 15 min at 30 °C in a reaction mixture (100 μ l) containing the membranes (1 mg of protein/ml), 50 mM-Tris/HCl (pH 7.4), 0.1 mM-phenylmethanesulphonyl fluoride (PMSF), 1 μ M-leupeptin, 10 μ M-soybean trypsin inhibitor and 10 mM-MgCl₂ in the presence or absence of an opioid. The reaction was initiated by the addition of [γ -³²P]ATP (final concn. 15 μ M), and incubation was carried out at 30 °C for an appropriate time. The reaction was terminated by boiling the mixture for 1 min after the addition of 10 μ l of 10% SDS solution containing 10 mM-dithiothreitol (DTT).

Affinity chromatography of opiate receptors

P_2 membranes (95 mg of protein) were extracted with 20 ml of 10 mM-3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate (CHAPS) in 50 mM-Tris/HCl, pH 7.4, containing 0.1 mM-PMSF (Zukin & Maneckjee, 1986). The solubilized sample was applied to a morphinone-agarose column (1 cm $\times$ 10 cm) that had been pre-equilibrated with 50 mM-Tris/HCl, pH 7.4. The eluate was collected in 7 ml fractions and the A_{280} was monitored. The column was washed extensively with 50 mM-Tris/HCl, pH 7.4, containing 1 mM-CHAPS and 0.1 mM-DTT. When the A_{280} had reached nearly zero, proteins non-specifically bound to the column were removed with 50 mM-Tris/HCl, pH 7.4, containing 100 mM-

Abbreviations used: DSS, disuccinimidyl suberate; WGA, wheat-germ agglutinin; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate; GlcNAc, *N*-acetylglucosamine; PAGE, polyacrylamide-gel electrophoresis

KCl, 1 mM-K₂EDTA, 1 mM-CHAPS and 0.1 mM-DTT. Elution of opiate receptor was then carried out with 10 μ M-naloxone in 50 mM-Tris/HCl, pH 7.4, containing 100 mM-NaCl, 1 mM-CHAPS and 0.1 mM-DTT. For the assay of ligand-binding activity, the fractions were dialysed against 2 litres of 50 mM-Tris/HCl buffer, pH 7.4, containing 0.1 mM-DDT and 0.1 mM-PMSF for 24 h at 4 °C with three changes of the buffer to remove naloxone.

For the isolation of phosphorylated opiate receptors, the phosphorylation reaction was performed in the presence of 10 μ M-morphine and quenched under non-denaturation conditions by the addition of 2 ml of 50 mM-Tris/HCl, pH 7.4, containing 1 mM-K₂EDTA, 50 mM-KF and 5 mM-potassium pyrophosphate (Buffer A). The phosphorylated P₂ membranes (1 mg of protein) were collected by centrifugation (20000 g, 15 min) and extracted with 0.5 ml of 10 mM-CHAPS in Buffer A containing 10 μ M-soybean trypsin inhibitor at 4 °C for 30 min. The extract was centrifuged at 100000 g for 30 min at 4 °C. The supernatant was diluted 5-fold with Buffer A and applied to a WGA-agarose (0.5 cm $\times$ 2 cm) or morphinone-agarose (0.5 cm $\times$ 4 cm) column that had been pre-equilibrated with Buffer A. Procedures for column washing and elution of non-specifically bound proteins were as described above. Specifically absorbed proteins were eluted with 0.5 M-N-acetyl-D-glucosamine (GlcNAc) or 10 μ M-naloxone in Buffer A from the WGA or morphinone column respectively. The fractions containing ³²P radioactivity were pooled for SDS/polyacrylamide-gel electrophoresis (SDS/PAGE).

Opiate-receptor binding assay

Assay of the receptors was performed by the poly(ethylene glycol) method (Cho & Zukin, 1983). Briefly, aliquots (100 μ l) of the dialysed fractions were incubated with 2 nM-[³H]naloxone in 50 mM-Tris/HCl, pH 7.4, in the presence or absence of 10 μ M unlabelled naloxone for 30 min at 25 °C. A 40 μ l portion of 1% bovine γ -globulin solution was added to each tube, followed by 1 ml of 23% (w/v) poly(ethylene glycol) in 50 mM-Tris/HCl, pH 7.4. The reaction mixtures were incubated for an additional 10 min at 4 °C and then filtered through Whatman GF/B filters to remove the free ligand. The filters were rapidly washed twice with 5 ml of 7% poly(ethylene glycol) in 50 mM-Tris/HCl, pH 7.4, transferred to vials containing 10 ml of Aquasol-2 and the radioactivity was measured. Specific binding was defined as the binding that was displaced by 10 μ M unlabelled naloxone.

Affinity-labelling of opioid receptors

Cross-linking of ¹²⁵I- β -endorphin to opioid receptors was carried out by a slight modification of the procedure of Howard *et al.* (1985). A 1 ml portion of P₂ membranes (2 mg of proteins/ml) in 50 mM-potassium phosphate buffer, pH 7.4, was incubated for 20 min at 30 °C with 2 nM-¹²⁵I- β -endorphin in the presence or absence of 1 μ M-naloxone. The incubation mixtures were diluted 2-fold with ice-cold 50 mM-potassium phosphate, pH 7.4, and centrifuged at 20000 g for 15 min at 4 °C. The membrane pellets were resuspended in 1 ml of 50 mM-potassium phosphate, pH 7.4. The cross-linking was initiated by the addition of DSS (freshly prepared in dimethyl sulphoxide) to a final concentration of 1 mM. After 15 min at 0 °C, the reaction was quenched by the

addition of 4 ml of 50 mM-Tris/HCl, pH 7.4, and the quenched mixtures were centrifuged at 20000 g for 15 min at 4 °C. The membrane pellets were subjected to SDS/PAGE.

Phosphoamino acid analysis

The partially purified opiate receptors from the morphinone-agarose column were preincubated with 10 μ M-morphine for 15 min at 30 °C in 50 mM-Tris/HCl, pH 7.4, containing 0.1 mM-PMSF, 1 μ M-leupeptin, 20 mM-MgCl₂ and 5 mM-MnCl₂. The reaction was started by the addition of 15 μ M-[γ -³²P]ATP (final reaction volume 100 μ l), and incubation was carried out for 5 min at 30 °C. The reaction was terminated by the addition of 10 μ l of 10% (w/v) SDS solution containing 10 mM-DTT, and the mixture was boiled for 1 min. The sample was subjected to SDS/PAGE and the gel was stained with Coomassie Brilliant Blue R-250. The gel was dried and autoradiographed overnight. The position of the phosphorylated 58 kDa protein on the dried gel was located by aligning it with its corresponding autoradiogram. The ³²P-labelled 58 kDa protein band was excised and rehydrated in a minimal volume of 1% SDS by boiling for 30 min. The swollen gel piece was homogenized and extracted with 0.5% SDS. Approx. 75% of the ³²P-labelled protein was recovered from the gel. A portion (20 μ g) of bovine serum albumin was added as a carrier, and the sample was dialysed against ethanol to remove SDS. The sample was dried and suspended in 6 M-HCl for partial acid hydrolysis at 105 °C for 2 h in a tube sealed under vacuum. HCl was removed by evaporation. The hydrolysate was dissolved in a marker mixture containing phosphoserine, phosphothreonine and phosphotyrosine and analysed on a cellulose thin-layer plate by electrophoresis at pH 3.5 (pyridine/acetic acid/water, 1:10:289, by vol.) for 25 min at 1 kV. The markers were detected by spraying with ninhydrin; [³²P]-phosphoamino acids were located by autoradiography.

SDS/PAGE

This was performed by the method of Laemmli (1970) using 9% (w/v) polyacrylamide slab gels (1 mm $\times$ 120 mm $\times$ 135 mm). Upon completion of the electrophoresis, gels were stained with Coomassie Brilliant Blue R-250, dried, and autoradiographed at -70 °C for 2-4 days. Radioactive protein bands were excised and ³²P was quantified on a liquid-scintillation spectrometer (Aloka LSC 701).

Protein determination

Proteins were determined by the procedure of Lowry *et al.* (1951) or by Protein Assay Kit (Bio-Rad), with bovine serum albumin as the standard.

RESULTS

Phosphorylation of P₂ membranes

Fig. 1 is an autoradiogram of the phosphorylated P₂ membranes after SDS/PAGE. When P₂ membranes were incubated with [γ -³²P]ATP, various proteins with different molecular masses were phosphorylated (lane a). The addition of morphine (10 μ M) to the reaction mixture enhanced the incorporation of ³²P into a band with a molecular mass of 58 kDa (lane b). However, the co-addition of naloxone (10 μ M) and morphine abolished the enhanced phosphorylation of the 58 kDa protein

Fig. 1. Morphine- or enkephalinamide-enhanced phosphorylation of P_2 -membrane proteins

Aliquots of membranes were preincubated in a reaction mixture containing 50 mM-Tris/HCl, pH 7.4, 0.1 mM-PMSF, 1 μ M-leupeptin, 10 μ M-soybean trypsin inhibitor and 10 mM-MgCl₂ in the presence or absence of opioid. The reaction was initiated by the addition of [γ -³²P]ATP (final concn. 15 μ M) and incubation was carried out at 30 °C for 30 s. Lane a, control; lane b, morphine (10 μ M); lane c, morphine (10 μ M) + naloxone (10 μ M); lane d, enkephalinamide (10 μ M); lane e, enkephalinamide (10 μ M) + naloxone (10 μ M); lane f, naloxone (10 μ M)

Fig. 2. Effect of morphine on the phosphorylation of the 58 kDa protein

Membranes were first preincubated for 15 min at 30 °C in the presence of indicated concentrations of morphine and then incubated with [γ -³²P]ATP at 30 °C for 1 min as described in Fig. 1. The phosphorylated samples were subjected to SDS/PAGE and the 58 kDa protein band was excised from the gel. Radioactivity was measured as described in the text. Values are means $\pm$ S.E.M. (bars) for three experiments.

(lane c). Naloxone alone (10 μ M) did not enhance the phosphorylation of this protein (lane f). To confirm that this phenomenon was related to ligand binding to opiate receptors in P_2 membranes, we investigated the effect of [D-Ala²,D-Leu⁵]enkephalinamide, an agonist of μ and δ opiate receptors (but less potent with respect to the former), on the reaction. Results shown in Fig. 1 indicate that [D-Ala²,D-Leu⁵]enkephalinamide similarly enhanced the phosphorylation of the 58 kDa protein (lane d) and that the reaction was also abolished by naloxone (lane e), suggesting that the enhancement of phosphorylation was mediated by the binding of the opiate agonist to their specific binding sites in the mouse brain membranes.

Fig. 2 illustrates the dose-response relationship noted for the morphine-enhanced phosphorylation of the 58 kDa protein. The enhancement was apparent at a concentration lower than 10⁻⁹ M, and the incorporation reached a plateau at 10⁻⁶–10⁻⁵ M. The ED₅₀ value was taken to be the concentration of morphine that produced half-maximal phosphorylation. The apparent ED₅₀ was 1 $\times$ 10⁻⁸ M.

Kinetic analysis of phosphorylation of the 58 kDa protein

Fig. 3 shows the time course of the morphine-enhanced incorporation of ³²P into the 58 kDa protein. Membranes were first preincubated for 15 min at 30 °C in the presence or absence of 10 μ M-morphine and then incubated with [γ -³²P]ATP for the indicated time. Autoradiograms of phosphorylated protein were obtained after SDS/PAGE as described above. The band corresponding to the ³²P-labelled 58 kDa protein was excised and radioactivity was measured. The reaction occurred rapidly, and an approximately 2-fold increase in ³²P incorporation was observed at 2 min in the membranes with morphine in comparison with those without the drug. Longer incubation times tended to decrease the incorporation of ³²P into this protein. After 10 min, ³²P incorporation into the protein diminished to 78 % of the maximal amount. This suggests the presence of endogenous protein phosphatase activity in the P_2 -membrane fraction.

Effects of bivalent cations

The effects of bivalent cations on the phosphorylation reaction were studied. The membranes preincubated with morphine were subsequently incubated with [γ -³²P]ATP in the presence of bivalent cations. Results are shown in Table 1. Mg²⁺ dose-dependently stimulated the incorporation of ³²P into the 58 kDa protein. Mn²⁺ was also effective. However, essentially no effect on the reaction was observed in the presence of 1 mM-Ca²⁺.

Isolation of opiate receptors

Treatment of brain-cell membranes from various mammalian species with CHAPS resulted in solubilization of opiate-binding sites (Zukin & Maneckjee, 1986). We therefore treated the mouse brain P_2 membranes with CHAPS to solubilize opiate receptors, purification of the receptors being accomplished by affinity chromatography using a morphinone-agarose column. Opiate ligand binding to the solubilized preparation is inhibited by CHAPS at concentrations in excess of 2 mM (Simonds *et al.*, 1980). Thus solubilized brain cell membranes were diluted 5-fold before applying to the affinity column. A typical elution pattern from the morphinone column is shown in Fig. 4. The opiate-binding activity was

Fig. 3. Time course of ^{32}P incorporation into the 58 kDa protein

Membranes were first preincubated for 15 min at 30 °C in the presence (●) or absence (○) of 10 μM -morphine, and then incubated with [γ - ^{32}P]ATP for the indicated time as described in Fig. 1. The phosphorylated samples were subjected to SDS/PAGE, and the 58 kDa protein band was excised from the gel. Radioactivity was measured as described in the text. Values are means $\pm$ S.E.M. for three experiments.

measured by the specific binding of [^3H]naloxone to the receptors in the eluted fractions from the column. Naloxone was removed by dialysis against 50 mM-Tris/HCl, pH 7.4, before the assay. Fractions with the binding activity were pooled, concentrated by use of an Amicon PM-10 membrane. About 30% of the binding activity was recovered in naloxone/NaCl (fractions 47–51 in Fig. 4). A single pass through the morphinone column resulted in a 3000–4000-fold purification of active opiate receptors. SDS/PAGE analysis of the concentrated receptor solution showed several protein bands, including a 58 kDa protein, when silver-stained or examined by autoradiography after ^{125}I -iodination (results not shown).

Affinity chromatography of the phosphorylated 58 kDa protein on WGA-agarose or morphine-agarose

Opiate receptors are glycoproteins that bind to WGA-agarose columns (Gioannini *et al.*, 1982). To obtain evidence that the phosphorylated 58 kDa protein has affinity for WGA-agarose, the phosphorylated P_2 membranes were extracted with 10 mM-CHAPS and the extract was applied to a WGA-agarose column. Proteins specifically retained on the column were eluted from the column with 0.5 M-GlcNAc. The amount of ^{32}P specifically eluted from the column with the haptenic sugar was 0.8% of the total radioactivity applied to the column. SDS/PAGE analysis of the GlcNAc eluate revealed radioactive bands with molecular masses of 58 kDa, 53 kDa and 116 kDa (Fig. 5).

We next investigated the binding of the phosphorylated 58 kDa protein to a morphinone-agarose column. Pro-

Table 1. Bivalent-cation requirement for the morphine-stimulated phosphorylation of the 58 kDa mouse brain P_2 -membrane protein

Aliquots of membranes were preincubated in a reaction mixture containing 50 mM-Tris/HCl, pH 7.4, 0.1 mM-PMSF, 1 μM -leupeptin, 10 μM -soybean trypsin inhibitor and bivalent cation in the presence or absence of 10 μM -morphine. The reaction was initiated by the addition of 15 μM -[γ - ^{32}P]ATP and incubation was carried out at 30 °C for 2 min. The phosphorylated samples were subjected to SDS/PAGE and the 58 kDa protein band was excised from the gel. Radioactivity was measured as described in the text. Results are means $\pm$ S.E.M. for three experiments carried out in duplicate.

Addition compound	Concn. (mM)	^{32}P incorporation (c.p.m.)	
		Without morphine	With morphine
Control		377 $\pm$ 32	393 $\pm$ 49
MgCl_2	1	680 $\pm$ 83	816 $\pm$ 41
	5	923 $\pm$ 101	1443 $\pm$ 93
	20	1287 $\pm$ 192	1921 $\pm$ 186
MnCl_2	1	858 $\pm$ 51	1215 $\pm$ 94
	5	1202 $\pm$ 72	1455 $\pm$ 57
CaCl_2	1	327 $\pm$ 14	364 $\pm$ 63

Fig. 4. Profile of opiate receptors eluted from the morphinone-agarose column

P_2 membranes were solubilized with 10 mM-CHAPS and applied to a morphinone-agarose column (1 cm $\times$ 10 cm) which had been pre-equilibrated with 50 mM-Tris/HCl, pH 7.4. The column was washed with 50 mM-Tris/HCl, pH 7.4, containing 1 mM-CHAPS and 0.1 mM-DTT and then with 50 mM-Tris/HCl, pH 7.4, containing 100 mM-KCl, 1 mM- K_2EDTA , 1 mM-CHAPS and 0.1 mM-DTT. Opiate receptors were eluted with 10 μM -naloxone in 50 mM-Tris/HCl, pH 7.4, containing 100 mM-NaCl, 1 mM-CHAPS and 0.1 mM-DTT. Buffers were changed where indicated. Each fraction was dialysed against 50 mM-Tris/HCl buffer, pH 7.4, containing 0.1 mM-DTT and 0.1 mM-PMSF. [^3H]Naloxone binding to the dialysed material in each fraction was assayed by the poly(ethylene glycol) method (see the text).

teins retained on the column were specifically eluted with naloxone. The amount of ^{32}P specifically eluted from the morphinone-agarose column with naloxone was 0.4–0.5% of the total radioactivity applied to the column. SDS/

Fig. 5. Autoradiograms of the eluate from WGA-agarose after SDS/PAGE

Phosphorylation of P_2 membranes was performed in the presence of $15 \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $10 \mu\text{M}$ -morphine. The phosphorylated membranes were washed with Buffer A by centrifugation. The CHAPS (10 mM) extract of the phosphorylated membranes was applied to a WGA-agarose column ($0.5 \text{ cm} \times 2 \text{ cm}$). Unadsorbed materials were washed out with Buffer A and the absorbed materials were eluted with 0.5 M -GlcNAc in Buffer A. SDS/PAGE of the eluate was carried out after dialysis against 50 mM -Tris/HCl, pH 7.4. The gel was dried and subjected to autoradiography (Kodak XAR-5 film with intensifying screens). Lane a, CHAPS extract; lane b, radioactive proteins eluted from the WGA-agarose column with GlcNAc.

Fig. 6. Autoradiograms of the eluate from the morphinone-agarose column after SDS/PAGE

Phosphorylation of P_2 membranes was performed in the presence of $15 \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $10 \mu\text{M}$ -morphine. The phosphorylated membranes were washed with Buffer A by centrifugation. The CHAPS (10 mM) extract of the phosphorylated membranes was applied to a morphinone-agarose column ($0.5 \text{ cm} \times 4 \text{ cm}$). Conditions for column chromatography were the same as those shown in Fig. 4. A sample eluted from the morphinone-agarose column with $10 \mu\text{M}$ -naloxone in Buffer A was subjected to SDS/PAGE. The gel was dried and autoradiographed (Kodak XAR-5 film with intensifying screens).

PAGE analysis of the eluate revealed two distinct radioactive bands with molecular masses of 58 and 53 kDa (Fig. 6).

These results suggest that the phosphorylated 58 kDa protein is one of the opioid receptor proteins, most probably of the μ -type. Howard *et al.* (1985) reported that the δ -receptor has a molecular mass of 53 kDa. The phosphorylated 53 kDa protein shown in Figs. 5 and 6 might, therefore, be the δ opiate receptor. However, we cannot rule out the possibilities that this protein is a proteolytic product of the 58 kDa protein or a derivative of 58 kDa protein carrying less sugar content.

The phosphorylated 116 kDa protein is detected only by use of the WGA column and is not isolated by the morphinone column. This protein might therefore be a glycoprotein lacking an affinity for opioids.

Affinity cross-linking of ^{125}I - β -endorphin

To determine the molecular masses of opioid-binding proteins in the mouse P_2 membranes, affinity labelling was carried out by cross-linking of ^{125}I - β -endorphin (human) to its receptors by the method of Howard *et al.*

Fig. 7. Affinity cross-linking of ^{125}I - β -endorphin to mouse brain P_2 membranes

A 1 ml portion of P_2 membranes (2 mg of protein) in 50 mM -potassium phosphate, pH 7.4, was incubated for 20 min at 30°C with 2 nM - ^{125}I - β -endorphin in the presence or absence of $1 \mu\text{M}$ -naloxone. The membranes were collected by centrifugation and resuspended in 1 ml of 50 mM -potassium phosphate, pH 7.4. Cross-linking was carried out by the addition of 1 mM -DSS to the membrane suspension for 15 min at 0°C . The reaction was terminated by the addition of an excess of 50 mM -Tris/HCl buffer, pH 7.4. Membrane pellets were collected by centrifugation ($20000 g$ for 15 min at 4°C) and solubilized in SDS/PAGE sample buffer (Laemmli, 1970). After SDS/PAGE, the gel was dried and subjected to autoradiography (Kodak XAR-5 film with intensifying screens). Lane a, without DSS; lane b, with DSS; lane c, with DSS and $1 \mu\text{M}$ -naloxone. The positions of the molecular-mass standards are shown.

Fig. 8. Phosphoamino acid analysis of the phosphorylated 58 kDa protein

Phosphorylation of partially purified morphine receptors by the morphinone-agarose column was performed in the presence of 15 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 μ M-morphine, 20 mM- MgCl_2 and 5 mM- MnCl_2 . The phosphorylated 58 kDa protein was separated by SDS/PAGE and the gel was dried and autoradiographed. The ^{32}P -labelled 58 kDa protein band was excised from the dried gel, swollen in 1% SDS solution and the radiolabelled protein was extracted from the swollen gel piece. The extract was dialysed against ethanol to remove SDS. Ethanol was evaporated and ^{32}P -labelled 58 kDa protein was hydrolysed in 6 M-HCl. The hydrolysate was analysed by cellulose-thin-layer-plate electrophoresis. Phosphoamino acids were detected by ninhydrin, and ^{32}P phosphoamino acids were detected by autoradiography. Ellipses indicate the positions of the standards. Abbreviations: P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; P_i , inorganic phosphate.

(1985). The specific cross-linking of this ligand revealed the presence of two bands at 63 and 38 kDa after SDS/PAGE and autoradiography (Fig. 7). These two bands seem to be opioid-binding proteins, since they were eliminated when the labelling was carried out in the presence of naloxone. These results are consistent with the fact that the phosphorylated 58 kDa protein is an opiate receptor, since the presence of the 63 kDa protein band is accounted for by the cross-linking of the 58 kDa protein with ^{125}I - β -endorphin (molecular mass about 4 kDa). The identity of the cross-linked 38 kDa protein is unknown at present.

Phosphoamino acid analysis

Phosphoamino acid analysis was attempted with the hydrolysate of the phosphorylated 58 kDa protein from morphine-stimulated opiate receptor. Fig. 8 shows autoradiography of the phosphorylated amino acids separated by cellulose thin-layer electrophoresis. In addition to P_i , the major phosphorylated component co-migrated with phosphoserine.

DISCUSSION

Pharmacological, physiological and biochemical lines of evidence have suggested the existence of at least three types of opioid receptors, termed ' μ ', ' δ ', and ' κ ', on the plasma membranes of target cells (Paterson *et al.*, 1984). Recent efforts to purify opioid receptors have revealed that the μ -type receptor of rat brain has a molecular mass of 58–60 kDa (Newman & Barnard, 1984; Cho *et al.*, 1986; Ueda *et al.*, 1987). We also confirmed the molecular mass of one of the receptor proteins for β -endorphin in the P_2 membranes from mouse brain to be 58 kDa by affinity labelling with ^{125}I - β -endorphin as a probe (Fig. 7). Since β -endorphin has a high affinity for μ - and δ -type

receptors (Kosterlitz *et al.*, 1983), it is probable that one of the opiate receptors of mouse brain is of molecular mass 58 kDa.

Here we present lines of evidence that the P_2 membranes from mouse brain contain morphine-enhanced protein kinase activity which incorporates ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into the 58 kDa protein (Fig. 1). The result of affinity chromatography on a morphinone-agarose column suggests that the phosphorylated 58 kDa protein has the ability to bind to morphine (Fig. 5), since morphinone itself is an agonist of morphine (Nagamatsu *et al.*, 1982). We assume that this protein is an opiate receptor, most probably of the μ -type, in mouse brain. However, we cannot rule out the possibility that the present protein is the δ -type receptor, which was shown to have a molecular mass of 58 kDa in neuroblastoma $\times$ glioma hybrid cells (Klee *et al.*, 1982), although other investigators have claimed that μ - and δ -type receptors could be distinguished on the basis of molecular mass (Howard *et al.*, 1986; Ueda *et al.*, 1987).

Phosphoamino acid analysis of the phosphorylated 58 kDa protein after acid hydrolysis indicated the presence of phosphoserine as a major phosphoamino acid (Fig. 8).

Involvement of cyclic AMP-dependent protein kinase on the signal transduction of morphine has been suggested (Bachrach *et al.*, 1979). However, neither cyclic AMP nor cyclic GMP influenced the phosphorylation of the 58 kDa protein in our experimental system. Fairly recently, Roy *et al.* (1986) reported that a purified μ -type receptor from bovine brain was associated with phosphatase activity. In our study the release of ^{32}P from the phosphorylated 58 kDa protein was rapid. Therefore it is conceivable that the 58 kDa protein is also associated with a protein phosphatase in mouse brain membranes.

Isolation of phosphorylated membrane-bound neurotransmitter receptors have been demonstrated for nicotinic acetylcholine receptor (Huganir & Greengard, 1983), muscarinic acetylcholine receptor (Kwatra & Hosey, 1986) and α -adrenergic receptor (Leeb-Lundberg *et al.*, 1987) by affinity chromatography.

Phosphorylation of muscarinic acetylcholine receptor has been suggested to result in the loss of ligand-binding sites (Burgoyne, 1983; Rosenbaum *et al.*, 1987). We investigated the effect of phosphorylation on the ligand-binding activity of opiate receptors. Incubation of mouse P_2 membranes under phosphorylating conditions led to about 42% decrease in the amount of specific $[\text{H}]\text{-naloxone}$ binding to the membrane-located opiate receptors (K. Nagamatsu, K. Suzuki & T. Terao, unpublished work). On the other hand, the phosphorylated opiate receptors seem to retain the ability to bind to their ligands, since the phosphorylated 58 kDa protein could be isolated by affinity chromatography on morphinone-agarose (Fig. 4). These results suggest that the phosphorylation of opiate receptors is implicated in the affinity to their ligands in mouse P_2 membranes. Further investigations using the purified receptors are necessary to confirm this point.

From these observations we assume that the phosphorylation of opiate receptors is implicated in the regulation of their functions.

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