Irreversible photoactivation of an opiate receptor in the guinea pig ileum with two *p*-azidobenzoyl derivatives of [Leu⁵]-enkephalin

UKON NAGAI, YOSHIHISA KUDO, KAZUKI SATO, NORIKO TAKI and SHOJI SHIBATA*

Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machidashi, Tokyo 194, Japan, and *Department of Pharmacology, University of Hawaii, Honolulu, Hawaii, U.S.A.

ABSTRACT

Two new derivatives of [Leu⁵]-enkephalin, EKA and EKDA, were synthesized. They depressed the twitch contractile responses of the longitudinal muscle strip from guinea pig ileum to electrical stimulation dose-dependently with IC₅₀-values of 0.8×10^{-7} and 1.9×10^{-7} , respectively. The effect of the drugs in the dark was completely restored by perfusion with drug-free medium. However, the twitch response to the electrical stimulation recovered only partially (ca. 40%) even after a prolonged washing when the preparation was exposed to UV light during perfusion with the drugs. The specific nature of their action was confirmed by these two facts: (i) the effect was completely antagonized by naloxone in the dark, and (ii) the receptor was protected from irreversible activation by the presence of [Leu⁵]-enkephalin during the irradiation. This is the first demonstration of irreversible activation (not just binding) by photoaffinity labelling of the opiate receptors. These reagents seem to be useful for the study of opiate receptors.

KEY WORDS photoaffinity labelling / opiate receptor / irreversible agonist / enkephalin analogs

Since the discovery of endogenous opioid peptides (4, 5) convinced us of the presence of opiate receptors, isolation and characterization of the receptors have been the focus of interest in the field of molecular neuropharmacology. Photoaffinity labelling is known to be a promising approach to such a problem. Two photolabile derivatives of enkephalin showing specific binding to the receptors in the rat brain or neuroblastoma-glioma hybrid cells were reported (3, 6). For the latter case irreversible photoinactivation of the membrane was observed, preventing the binding of an opiate agonist (6). In the present study we also synthesized two new photosensitive enkephalin analogues and tested the biological activity using guinea pig ileum in the dark or under ultraviolet (UV) irradiation. We found the photoaffinity labelled receptor in this preparation to be irreversibly activated. The results are significant

in two respects: (i) this is the first demonstration of biological activity (not just binding) by photoaffinity labelling of the opiate receptor and (ii) the irreversible action is not inactivation but activation, contrary to the usual concept of covalent labelling of various receptors (7). These reagents seem to have considerable potential as pharmacological and biochemical tools for the study of opiate receptors.

The analogues synthesized are *N*-[Leu⁵]-enkephalyl-*N'*-*p*-azidobenzoyl ethylenediamine (EKA) and *N*-([Leu⁵]-enkephalyl-*N'*-dansyl-L-lysyl)-*N'*-*p*-azidobenzoyl ethylenediamine (EKDA). Coupling of Boc-[Leu⁵]-enkephalin (Boc = *tert*-butoxycarbonyl) and mono-*p*-azidobenzoyl ethylenediamine (Abz-Eda) with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole in dimethylformamide (DMF) produced the Boc-derivative of EKA, which was purified by chromatography with Sephadex LH-20 and

preparative layered chromatography on silica gel. Acidolysis of the product with 0.1N HCl in HCOOH produced EKA as the hydrochloride, which showed a single spot on silica gel TLC with three solvent systems, and had the following properties: m.p. 156–158° (dec); $R_F=0.20$ on silica gel TLC with CHCl_3 -MeOH (5:1); $\text{UV}=\lambda_{\text{max}}^{\text{EtOH}}$ 269 nm (ϵ , 21,100). The Boc-derivative of EKDA was synthesized similarly starting with *N*-(*N*^ε-dansyl-L-lysyl)-*N'*-*p*-azidobenzoyl-ethylenediamine (Lys(Dns)-Eda-Abz) as the amine component in the coupling reaction, and deprotected to give crude EKDA monohydrochloride, which was then purified by chromatography on silica gel using CHCl_3 -MeOH as eluent, and showed a single spot on TLC with three solvent systems. Its properties are as follows: m.p. 152–155° (dec), $R_F=0.40$ on silica gel TLC with CHCl_3 -MeOH (5:1), $\text{UV}:\lambda_{\text{max}}^{\text{EtOH}}$ 261 nm (ϵ , 27,000), 335 (4,000). Boc-[Leu⁵]-enkephalin was prepared from commercial [Leu⁵]-enkephalin (Leu-ENK; Protein Research Foundation) by reaction with di-*tert*-butyl dicarbonate. Abz-Eda was prepared from *N*-phthalyl-β-alanine in good yield by the route shown below.

Lys(Dns)-Eda-Abz was prepared from commercial *N*^α-Boc-L-Lysine (Kokusan Kagaku) by usual dansylation followed by coupling with Abz-Eda using DCC and *N*-hydroxysuccinimide in DMF and deprotection by acidolysis. The purity and structure of Abz-Eda and Lys(Dns)-Eda-Abz were confirmed by TLC and ¹H-NMR.

Both EKA and EKDA depressed the twitch

contractile responses of the longitudinal smooth muscle strip from guinea pig ileum to the electrical stimulation dose-dependently (10^{-8} – 10^{-6} M), and the IC_{50} (concentration of drug which inhibits contraction by 50%) of EKA and EKDA was 0.8×10^{-7} M and 1.9×10^{-7} M, respectively, and that of morphine was 2.2×10^{-8} M. The inhibitory effect of EKA (10^{-6} M) was completely antagonized by naloxone (10^{-6} M), a specific opiate antagonist (Fig. 2 A). The effect of EKA (10^{-6} M) in the dark was completely restored within 20 min by perfusion with drug-free medium (Fig. 2 B). However, the twitch response to the electrical stimulation recovered only partially (ca. 40%) even after washing for more than 1 hr when the preparation was exposed to UV light during perfusion with EKA (Fig. 2 C). UV irradiation itself in the drug-free medium had no effect on the twitch contractile

Table 1 *Inhibitory Effect of EKA on the Twitch Responses of Guinea Pig Ileum-Longitudinal Muscle with and without Ultraviolet Irradiation, and Recovery after Washing*

Treatment	N	Inhibition (%)	Recovery (%)
EKA (10^{-6} M) in the dark	4	76.5 ± 2.8	101.1 ± 4.3
EKA (10^{-6} M) + UV	4	85.2 ± 6.5	39.0 ± 9.8*
EKA (10^{-6} M) + UV + Leu-ENK (2×10^{-6} M)	3	77.8 ± 6.0	110.6 ± 4.8

Mean ± standard error. Percentage inhibition and recovery were calculated based upon the twitch responses after 60 min of perfusion with drug-containing medium and after 60 min of washing with drug-free medium, respectively. N: number of experiments. *: $P < 0.01$ (Student's *t*-test) compared with the recovery in the dark

H-Tyr-Gly-Gly-Phe-Leu-X

EKA:

EKDA:

Fig. 1 The structure of Leu-enkephalin derivatives. EKA: *N*-[Leu⁵]-enkephalyl-*N'*-*p*-azidobenzoyl-ethylenediamine, EKDA: *N*-([Leu⁵]-enkephalyl-*N*^ε-dansyl-L-lysyl)-*N'*-*p*-azidobenzoyl-ethylenediamine

Fig. 2 Effects of EKA on the twitch contractile responses of the guinea pig ileum longitudinal muscle strip. A longitudinal muscle strip of the guinea pig ileum was prepared according to the method described by Paton (8). The muscle strip (5–6 cm: resting length) was suspended in a small pyrex-glass organ bath (acting volume 5 ml; 1.0 mm thickness), through which oxygenated Krebs-Ringer solution was perfused at a constant rate of ca. 100 ml/hr to avoid degradation of the peptide by an endogenous peptidase and competition by the photolyzed product at the receptor site. The temperature of the perfusing medium was kept constant at 30°C by passing the medium through a thermostated water-jacket. One end of the muscle strip was connected to the electrode placed at the bottom of the organ bath, and the other end was attached to the isotonic transducer via cotton thread. The other electrode was placed at the upper end of the organ bath and field stimulation of the muscle strip was performed by an electric stimulator via the two electrodes (0.05 Hz, duration 1.0 msec). Drugs were applied by exchanging the perfusing medium with drug-containing Krebs-Ringer solution. Ultraviolet light was irradiated by two 15 W germicidal lamps (Mitsubishi GL-15) placed vertically at both sides of the organ bath at a distance of 10 cm each. A: EKA solution (10^{-6} M) was perfused in the dark followed by treatment with naloxone (10^{-6} M). B: EKA treatment (10^{-6} M) in the dark followed by perfusion with drug-free medium. C: EKA treatment (10^{-6} M) under UV-irradiation (hatched line) followed by perfusion with drug-free medium. D: EKA treatment (10^{-6} M) in the presence of Leu-ENK (2×10^{-6} M) under UV-irradiation (hatched line) followed by perfusion with drug-free medium. The gap in the recording indicates an interval of 10 min.

response of the smooth muscle strip. The depressed response after UV irradiation was not antagonized by naloxone (10^{-6} M), and the contractile response to exogenous acetylcholine (ACh; 10^{-7} M) was not altered before, during, or after the treatment (data not shown). Such irreversible depressant action by the drug (10^{-6}

M) was not observed when 2×10^{-6} M of Leu-ENK was present during the UV irradiation (Fig. 2D). The quantitative data of the experiments are summarized in Table 1. Similar results were also obtained with EKDA (data not shown here).

The effect of EKA in the dark was completely

antagonized by naloxone, and also the photoactivation of the receptor by the agent was protected by the presence of [Leu⁵]-enkephalin. Therefore EKA proved to be a specific agonist for the opiate receptor in the longitudinal muscle preparation of the guinea pig ileum, and the possibility that the irreversible activation might be caused by nonspecific covalent binding could also be eliminated. Since the effect of ACh was not altered after treatment with EKA under UV irradiation, the irreversible effect was not due to the myogenic abnormality. It was confirmed in the preliminary experiments that EKDA also shows similar irreversible photoactivation of the enkephalin receptor.

Although the long-lasting activation of the photolabelled receptor seems to be controversial to the classical receptor theory (7), a recent study by Caruso *et al.* demonstrated irreversible activation of the opiate receptor by chloroxymorphamine, a morphine derivative bearing an alkylating group (1). Moreover, similar irreversible photoactivation of a pancreatic secretagogue receptor with a photolabile analogue of cholecystokinin octapeptide was reported by Galardy *et al.* (2). Thus, the opiate receptor and some other endogenous peptide receptors may operate differently than the receptors of the classical neurotransmitters.

The photoaffinity derivatives of [Leu⁵]-enkephalin presented in this paper will be a powerful probe for pharmacological and biochemical studies of the opiate receptors. EKDA in particular will be useful as it contains a fluorescent group. Studies on the action of these derivatives on the receptors in the brain membrane are in progress.

Received for publication 3 February 1981

REFERENCES

1. CARUSO T. P., TAKEMORI A. E., LARSON D. L. and PORTOGHESE P. S. (1979) Chloroxymorphamine, an opiate receptor site-directed alkylating agent having narcotic agonist activity. *Science* **204**, 316-318
2. GALARDY R. E., HULL B. E. and JAMIESON J. D. (1980) Irreversible photoactivation of a pancreatic secretagogue receptor with cholecystokinin COOH-terminal octapeptides. *J. Biol. Chem.* **255**, 3148-3155
3. HAZUM E., CHANG K.-J., SHECHTER Y., WILKINSON S. and CUATRECASAS P. (1979) Fluorescent and photo-affinity enkephalin derivatives: preparation and interaction with opiate receptors. *Biochem. Biophys. Res. Commun.* **88**, 841-846
4. HUGHES J. (1975) Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. *Brain Res.* **88**, 295-308
5. HUGHES J., SMITH J. W., KOSTERLITZ H. W., FOTHERGIL L. A., MORGAN B. A. and MORRIS H. W. (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* **258**, 577-579
6. LEE T. T., WILLIAMS R. E. and FOX C. F. (1979) Photoaffinity inactivation of the enkephalin receptor. *J. Biol. Chem.* **254**, 11787-11790
7. PATON W. D. M. (1961) A theory of drug action based on the rate of drug-receptor combination. *Proc. Roy. Soc. London, Ser. B* **154**, 21-69
8. PATON W. D. M. (1975) Transmural and field stimulation of nerve-smooth muscle preparations. *Meth. Pharmacol.* **3**, 313-320