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ENKEPHALINS AFFECT HIPPOCAMPAL MEMBRANE PHOSPHORYLATION

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Slices of rat brain hippocampus were incubated with methionine-enkephalin, leucine-enkephalin, [Des-Tyr^1] methionine-enkephalin or etorphin. After incubation the endogenous phosphorylation of proteins was measured using crude mitochondrial fractions prepared from the incubated slices. Methionine- and leucine-enkephalin specifically enhanced the radiophosphate incorporation into one protein band with an apparent molecular weight of 50K. The effect could also be detected in a fraction containing purified synaptosomal plasma membranes. The effect was time- and dose-dependent and could be mimicked by etorphin and blocked by naloxone. If methionine-enkephalin was added directly to a membrane fraction a marked inhibition of incorporation of phosphate into the 50K band was observed. It is suggested that opiate-receptor activation underlies the effects of enkephalin on hippocampal phosphoprotein metabolism.

[Des-Tyr^1] methionine-enkephalin Opiate receptor Enkephalins Hippocampus Slices Phosphoproteins

1. Introduction

Hippocampal slices have the property that, under appropriate in vitro conditions, they display chemical and electrical characteristics comparable to those found in intact animals (Yamamoto and McIlwain, 1966; Andersen et al., 1969, 1971).

Recently it was shown that repetitive stimulation of the hippocampal slice induces specific changes in some synaptic phosphoproteins (Browning et al., 1967a). Phosphorylation of proteins may affect their tertiary and quaternary structure, and can modify their functional properties. With respect to phosphorylation of membrane proteins, changes in membrane permeability in both nervous and non-nervous tissue have been reported (Walton et al., 1975; Greengard, 1976). There is evidence that phosphoproteins may be involved in the efficacy of the neurotransmission process (Weller and Rodnight, 1970; Greengard, 1976).

In view of the effectiveness of methionine-enkephalin to alter synaptic events in the hippocampus in vivo (Segal, 1977; Zieglgänsberger et al., 1978; Fry et al., 1979; Elazar et al., 1979) we studied the effect of this opioid neuropeptide on endogenous phosphorylation of hippocampal membranes in the intact slice. Because changes in membrane phosphorylation produced by in vivo or in vitro treatment with drugs are reflected in a subsequent assay of endogenous phosphorylation (Zwiers et al., 1976; Holmes et al., 1977; Browning et al., 1979b), it was assumed that if methionine-enkephalin would affect hippocampal phosphoproteins in the intact slice, this influence could then be detected in a subsequent in vitro endogenous phosphorylation assay. Indeed, a specific effect of methionine-enkephalin was found on the phosphorylation

of a membrane protein band with an estimated molecular weight of 50 000 daltons.

2. Materials and methods

2.1. Preparation of slices

Slices were cut, perpendicular to the long axis of the hippocampus, from hippocampi of male Wistar rats (110-120 g) (Lynch et al., 1975). The skull of the animal was opened after decapitation, the brain was removed and the hippocampi dissected out within 3 min after death. Immediately after dissection the tissue was placed in a Krebs-Ringer buffer (KRB; 124 mM NaCl, 5 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 0.75 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4) at 29° C. This buffer was previously oxygenated with a CO_2-O_2 gas mixture.

2.2. Incubation with test substances

Four slices were pooled per test tube and preincubated for 60 min in 1 ml KRB in a waterbath at 29° C. Ten min before incubation with the test substances the buffer was removed and replaced by 1 ml fresh KRB, containing bacitracin (30 μg/ml) as a peptidase inhibitor (Besbuquois et al., 1974). Test substances were added, dissolved in 100 μl KRB and the test tubes were placed in a shaking waterbath at 36° C. During preincubation and incubation, a CO_2-O_2 mixture (5%-95%) was constantly bubbled through the KRB thus providing a constant O_2 supply, a constant pH and gentle movement of the slices. The duration of the incubation was varied from 30 to 90 min.

2.3. Preparation of membrane fractions

2.3.1. Crude mitochondrial fraction (CM)

Unless specified otherwise, all further treatment was carried out at 0-4° C. At the end of the incubation period the KRB was removed and the slices were washed twice with 5 ml of an icecold isotonic sucrose solution (0.32 M). The slices were then homogenized by seven strokes of a Potter-Elvejehm glass homogeniser with a teflon pestle (clearance 0.125 mm) in 200 μl of the same sucrose solution. The homogenate was spun down (10 min, 1000 × *g*) to remove cell debris and nuclei and a crude mitochondrial fraction prepared according to Whittaker et al. (1964): the 1000 × *g* supernatant was centrifuged at 10 000 × *g* for 20 min. The resulting pellet, containing mitochondria, myelin, synaptosomes and plasma membranes, was resuspended in an acetate buffer (50 mM Na-acetate, 10 mM Mg-acetate, pH 6.5) yielding a protein content of 1.0-2.0 μg/μl. This crude mitochondrial fraction (CM) was used in the assay for endogenous phosphorylation. Protein pattern and phosphorylation profile are shown in fig. 1.

2.3.2. Naloxone-treated CM fraction

After resuspending the CM pellet in 0.32 M sucrose, this suspension was incubated for 20 min at 30° C, with or without naloxone (10 μM). The suspension thus treated was spun down at 10 000 × *g* for 20 min and the pellet subjected to osmotic shock in a hypotonic buffer (1 mM Tris; 50 μM $CaCl_2$, pH = 8.0) for 15 min at 4° C, and subsequently centrifuged for 20 min at 10 000 × *g*. The resulting pellet was resuspended in acetate buffer and dialysed against a large volume of the same buffer.

This dialysed fraction was used to establish the in vitro peptide effect on endogenous phosphorylation.

2.3.3. Synaptosomal plasma membrane fraction (SPM)

The CM pellet, obtained from 12 slices per incubation was osmotically shocked. This shocked material was used to prepare synaptosomal plasma membranes (SPM) using discontinuous sucrose gradient centrifugation essentially as described by De Robertis and Rodriquez de Lores Arnaiz (1969). The material floating on the 1.0 M sucrose layer

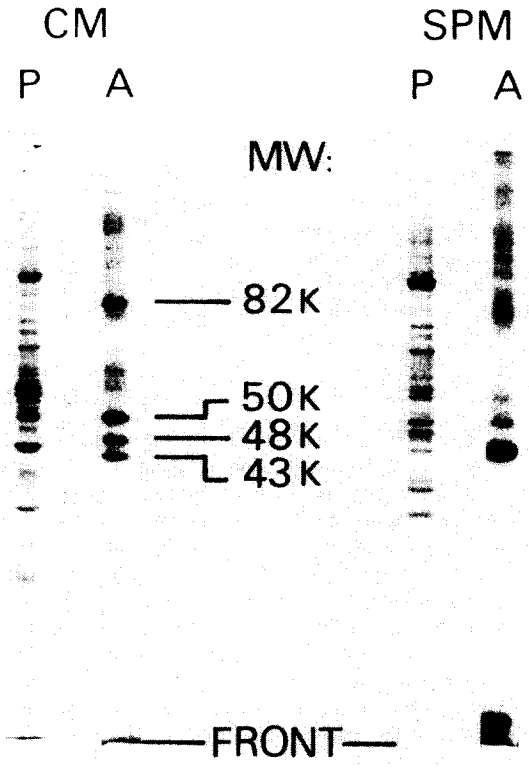

Fig. 1. Protein pattern (P) and autoradiogram (A) of a crude mitochondrial fraction (CM) and a synaptosomal plasma membrane fraction (SPM). Indicated are the position of the tracking dye front (FRONT) and the estimated molecular weights of four protein bands. The band with molecular weight = 43 000 is absent in the SPM autoradiogram.

was collected and washed in acetate buffer and was used as the SPM fraction (± 1 μg protein/μl). Protein pattern and phosphoprotein profile are shown in fig. 1.

2.4. Assay of endogenous phosphorylation

Endogenous protein phosphorylation was measured essentially as described previously under conditions allowing maximal phosphorylation of the various proteins within seconds (Zwiers et al., 1976; Wiegant et al., 1978).

The reaction mixture for this assay contained 10-30 μg protein, 2 μCi [γ-^{32}P]ATP with a final ATP concentration of 7.5 μM in a total volume of 25 μl. After a preincubation of 20 μl of the protein suspension during 5 min at 30°C, the reaction was started by adding the labelled and unlabeled ATP, dissolved in 5 μl acetate buffer. The reaction mixture was vortexed for 15 sec and the enzymatic reaction was then terminated by adding 12.5 μl of a 'stop' solution resulting in (final concentration): 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromphenol blue, 5% 2-mercaptoethanol.

In experiments on the effects of drugs on endogenous phosphorylation of crude SPM, these drugs were added 15 sec prior to the addition of the [γ-^{32}P]ATP.

2.5. Quantitative and qualitative analysis

The membrane proteins, solubilised with sodium dodecylsulphate (SDS) were separated according to their molecular weight after application of 20-30 μl of the reaction mixture to 11% polyacrylamide slab gels, as described by Zwiers et al. (1976).

Electrophoresis was carried out at room temperature using 40 mA per gel, until the bromphenol blue front had run as far as one cm from the bottom of the gel (approximately 2.5 h). The proteins were stained and fixed with a mixture of acetic acid, water and methanol (10 : 40 : 50 vol%) containing 0.1% Fast Green. After overnight destaining in the same solution without Fast Green and subsequent drying of the gel, quantitative autoradiography was performed to visualize radioactive material in the gel (fig. 1) (Zwiers et al., 1976). Kodak Royal X-O-Mat film was used, the exposure time varying from 2-5 days. Densitometric scanning of the autoradiograms was performed with the aid of a linear gel scanner (slid witdth 0.1 mm) combined with a Zeiss PMQ 11 spectrophotometer (wave length 700 nm; Wiegant et al., 1978). In order to quantitate ^{32}P-radioactivity, peak heights above background were measured as described by Ueda et al. (1973). Comparisons were made only between samples on one gel. Reference membrane samples were

included on each gel and the exposure time allowed a linear relationship between radioactivity and grain density per band. This quantitation procedure was verified by counting 1 mm gel cuts for radioactivity by liquid scintillation counting.

2.6. Chemicals

[γ-^{32}P]ATP (specific activity >2000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, U.K. Marker proteins used on the slab gels for determination of molecular weights were purchased from Pharmacia, Uppsala, Sweden (phosphorylase b 92 500; albumin 67 000; ovalbumin 43 000; carbonic anhydrase 30 000; trypsin inhibitor 20 100; α-lactalbumin 14 400).

Synthetic methionine-enkephalin, [Des-Tyr^1] methionine-enkephalin and leucine-enkephalin were kindly donated by Dr. H.M. Greven (Organon Int. b.v., Oss, The Netherlands). Naloxone was purchased from ENDO, New York, etorphin from Reckitt and Colman, Hull, U.K.

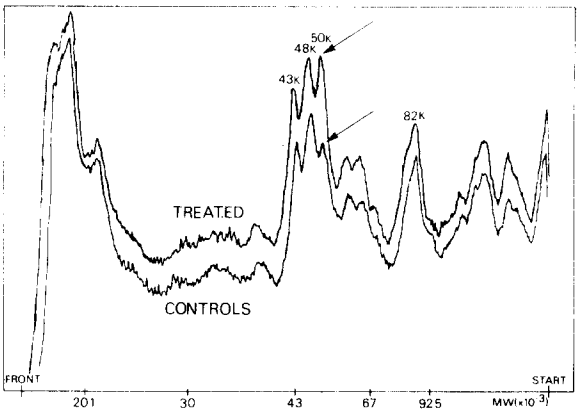

Fig. 2. Effect of treatment of intact hippocampal slices with 10^{-5} M methionine-enkephalin. A scan of the resulting autoradiogram was made after protein separation and autoradiography (see section *2.5.*). In order to allow comparison two such scans (*lower*: control slices; *upper:* treated slices) are combined in this picture. Overlap of the scans was avoided by adjusting the recorder along the y-axis before scanning the second autoradiogram. Numbers on the x-axis indicate the molecular weights of marker proteins, M.W.s of the important peaks are also indicated. Arrows indicate the peak of the 50K protein band.

3. Results

3.1. Effects of the enkephalins

Slices of rat hippocampus were incubated with 10^{-5} M methionine-enkephalin for 60 min. When the endogenous phosphorylation of the crude mitochondrial membrane fraction prepared from these slices was determined, a consistent increase in phosphorylation of one protein band (estimated MW 50K) was found. Other phosphorylated proteins visible in the autoradiogram, showed only small and variable changes (MW 82K, 48K) or no change at all (MW 43K) (fig. 2). In order to study the time dependence of the enhanced phosphorylation of the 50K band we measured the endogenous phosphorylation of slices incubated with 10^{-5} M methionine-enkephalin for 30, 60 and 90 min. After 30 and 90 min no difference from control slices could be detected (fig. 3A). It was decided after these experiments to focus our attention on the 50K band and to use 60 min as standard incubation time. In a subsequent experiment we measured radiophosphate incorporation after incubating slices for 60 min and varying concentrations of the peptide in the medium: 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} M. As can be seen from fig. 3B, there was a clear dose-dependent effect of methionine-enkephalin on incorporation into the 50K band with an optimum at 10^{-6} M. At 10^{-10} M methionine-enkephalin no effect could be detected.

In view of the low yield of purified synaptosomal plasma membranes, in most experiments the crude mitochondrial fraction was used to assay endogenous phosphorylation. However, in two separate experiments we have demonstrated that the effect of methionine-enkephalin could also be detected in a

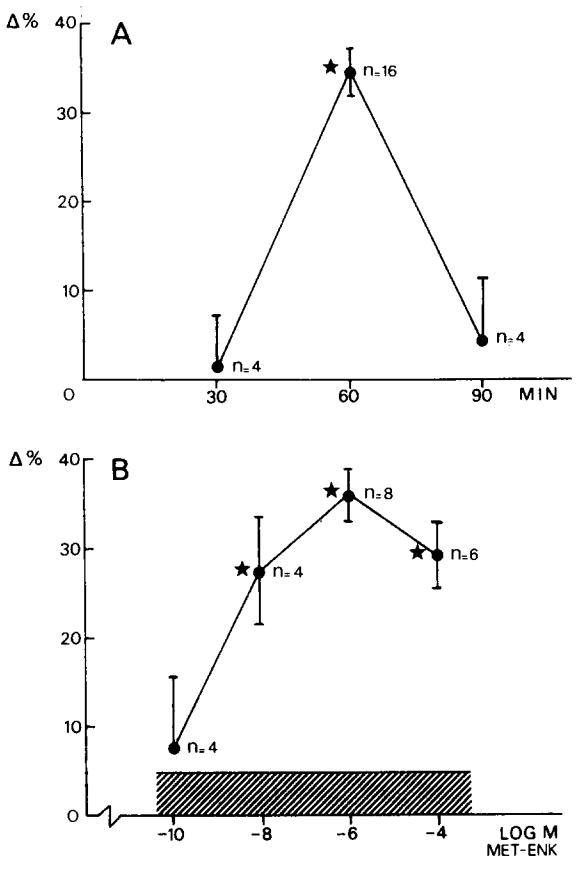

Fig. 3. Time-response curve (A) and dose-response curve (B) of the increased ^{32}P-phosphate incorporation into the 50 000 dalton protein band. Indicated are: the number (n) of determinations per point, the standard error of the mean (S.E.M., bars), the S.E.M. of the controls (shaded area) and, by means of an asterisk, whether or not a given value differs significantly from its control. Data are presented as relative difference from controls. A. Increase of ^{32}P-phosphate incorporation into the 50K band as a function of incubation time. Incubations were performed with 10^{-5} M methionine-enkephalin. B. Increase of ^{32}P-phosphate incorporation into the 50K band as a function of peptide concentration. Duration of incubation was 60 min.

purified SPM fraction (table 1B).

Incubation with 10^{-5} M leucine-enkephalin for 60 min resulted in a significant, though somewhat smaller increase of the post hoc phosphate incorporation into the 50K band (table 1A).

3.2. Simultaneous incubation of methionine-enkephalin with naloxone

In order to determine whether the effect of methionine-enkephalin on phosphate incorporation into membrane proteins was mediated by opiate receptor(s) or by some other mechanism, we tested whether or not naloxone, a specific opiate antagonist (Blumberg and Dayton, 1975) could prevent the peptide effect.

Incubation of slices with naloxone (10^{-4} M) alone slightly enhanced radiophosphate incorporation into the 50K band. However, simultaneous incubation of naloxone (10^{-4} M) and methionine-enkephalin (10^{-6} M) did not stimulate subsequent in vitro phosphorylation of the protein band 50K (table 1A). Thus it seems likely that the influence of methionine-enkephalin on hippocampal protein phosphorylation was mediated by opiate receptors.

3.3. Incubation with etorphin and [Des-Tyr^1] methionine-enkephalin

To substantiate the possibility that methionine-enkephalin affects hippocampal membrane protein phosphorylation via opiate receptors, similar experiments were carried out using [Des-Tyr^1] methionine-enkephalin and etorphin (both 10^{-5} M). It has been shown by others that [Des-Tyr^1] methionine-enkephalin has no affinity for the opiate receptor as a result of the absence of the N-terminal tyrosine (Frederickson, 1977). Etorphin was used as a highly potent opiate receptor agonist (Høllt, 1978).

Indeed, incubation of hippocampal slices for 30, 60 and 90 min with 10^{-5} M [Des-Tyr^1] methionine-enkephalin was without effect on post hoc phosphorylation of the 50K protein band (table 1A). Furthermore, incubation with etorphin (10^{-5} M) resulted in a 40% increase in phosphorylation of the 50K protein band after 60 min (table 1A).

TABLE 1

Endogenous phosphorylation of the 50K protein band in crude mitochondrial fractions (A) and synaptosomal plasma membrane fractions (B) after treatment of intact hippocampal slices.

Substance [1]	Conc. (M)	% [2]	[3]	n
(A)				
Met-enkephalin	10^{-5}	+28	$P < 0.01$	18
Leu-enkephalin	10^{-5}	+16	$P < 0.05$	4
Naloxone	10^{-4}	+8	n.s.	3
Naloxone + Met-enkephalin	$10^{-4}/10^{-6}$	+7	n.s.	4
[Des-Tyr[1]]Met-enk.	10^{-5}	+1	n.s.	4
Etorphin	10^{-5}	+40	$P < 0.05$	4
(B)				
Met-enkephalin	10^{-5}	+41	$P < 0.05$	6

[1] After 60 min preincubation (29°C), slices were incubated with the given substances during 60 min at 36°C.
[2] Percentage increase compared to controls.
[3] Significance tested with Student *t*-test, two-tailed; n.s. = not significant; n = number of incubations.

3.4. Methionine-enkephalin added directly to the endogenous phosphorylation assay

A shocked and dialysed CM fraction prepared from rat hippocampi was incubated with [γ-^{32}P]ATP in the presence of methionine-enkephalin to try and show that the effect of methionine-enkephalin on hippocampal phosphoproteins was due to a direct effect of the peptide on the phosphorylation process. This CM fraction was obtained as described in *2.3.1.* Methionine-enkephalin, added prior to the ATP in the endogenous phosphorylation assay inhibited the incorporation of radiophosphate into the 50K protein band at 10^{-4} M (—40%) but not at 10^{-6} M. If, however, the membranes were pretreated prior to lysis with naloxone (10^{-5} M) for 20 min at 30°C, a marked inhibition of phosphate incorporation into the 50K protein band was also found at lower peptide concentrations (table 2).

4. Discussion

In the present study we examined the influence of methionine-enkephalin on membrane protein phosphorylation in slices from rat hippocampi incubated under conditions

TABLE 2

Endogenous phosphorylation of the 50K protein band in a shocked and dialyzed CM fraction, subjected to naloxone treatment during membrane preparation.

	Untreated [1]		Naloxone-treated [1]	
Met-enk. 10^{-4} M [2]	—44%	$P < 0.05$ [3]	—45%	$P < 0.02$
Met-enk. 10^{-6} M	—1%	n.s.	—30%	$P < 0.05$

[1] Treatment consisted of incubation with water ('untreated') or 10 μM naloxone (naloxone-treated) of the crude mitochondrial pellet prior to lysis. Results given as percentage decrease compared to controls; n = 4.
[2] Methionine-enkephalin was added 10 sec prior to addition of the [γ-^{32}P]ATP.
[3] Significance tested with Student *t*-test, two-tailed; n.s. = not significant.

known to preserve the electrical and chemical characteristics of intact hippocampus tissue (Yamamoto and McIlwain, 1966; Andersen et al., 1969, 1971; Browning et al., 1979a,b; Bär et al., 1979). Specific changes in brain membrane phosphorylation, produced by in vivo treatment of rats or by in vitro manipulation of rat brain slices, were preserved in post hoc in vitro phosphorylation of membrane proteins, despite possible variability arising from post mortem changes in protein kinase or protein phosphatase activity (Forn and Greengard, 1978). Reports of such changes concern the effects of behavioral experiences (Ehrlich et al., 1978; Holmes et al., 1977), of post-tetanic potentiation of hippocampal slices (Browning et al., 1967a,b; Bär et al., 1980), and of in vivo treatment of rats with $ACTH_{1-24}$ (Zwiers et al., 1977).

It is reported in the present study that incubation of hippocampal slices with methionine-enkephalin resulted in changes in protein phosphorylation as detected in a post hoc in vitro endogenous phosphorylation assay (table 1, fig. 2). The same effect could be found whether the membranes were obtained from crude mitochondrial fractions or from synaptosomes (table 1A,B). The effect of methionine-enkephalin was largely confined to one protein band with an apparent molecular weight of 50 000 dalton. This protein band differs in molecular weight from those responsive to post-tetanic stimulation of hippocampal slices (Browning et al., 1979a,b; 27K, 40K and 53K) and the SPM-proteins sensitive to $ACTH_{1-24}$ (Zwiers et al., 1976; 48K, 20K, 18K, 17K and 15K). The recently isolated and purified SPM protein band B50 with an apparent molecular weight of 48K (Zwiers et al., 1979, 1980) is also one of the major hippocampal phosphoprotein bands but was not responsive under the present conditions (figs. 1,2).

The relatively long latency period for the methionine-enkephalin-induced effect might imply that the effect was the result of an indirect influence on membrane phosphorylation (see below). However, this long latency might be caused partly by the particular experimental design, as with $ACTH_{1-24}$ the optimal effect on endogenous phosphorylation of rat brain SPM was also found to occur 30-60 min after its in vivo administration whereas the effect was instantaneous in broken cell preparations (Zwiers et al., 1977, 1978). Furthermore, some of the effects of neuropeptides on behavioral (De Wied, 1974) and neurophysiological (Urban and De Wied, 1975; Wouters and Van den Bercken, 1979) parameters have similar long latency periods.

The effect of methionine-enkephalin on hippocampal protein phosphorylation reported here was transient as 30 min before or after the occurrence of the enhancement no differences from control could be detected (fig. 3A). The enhancement of the incorporation of radiophosphate was brought about by relatively low concentrations of methionine-enkephalin in the incubation medium (10^{-8}-10^{-6} M). Although bacitracin was added to the incubation mixture as a precaution against peptide breakdown (Besbuquois et al., 1974; Miller et al., 1977), identical data were obtained in the absence of this protease inhibitor when 10^{-5} M methionine-enkephalin was used (Bär, unpublished).

Structure-activity studies revealed that leucine-enkephalin also affected phosphorylation of the 50K band. However [Des-Tyr^1]-methionine-enkephalin was without effect (table 1A). The latter peptide lacks the N-terminal tyrosine residue which was shown to be crucial for opiate receptor binding and opiate-like activity (Frederickson, 1977). Taken together with the findings that naloxone prevented, and etorphin mimicked the methionine-enkephalin-induced effect (table 1A), the data strongly suggest an opiate receptor mediation of enkephalin action on hippocampal membrane phosphoproteins.

Other investigators have indicated that the enkephalins may affect hippocampal processes. Segal (1976) found that leucine-enkephalin antagonized cellular responses to glutamate or aspartate, but potentiated some cellular responses to other amino acids. Elazar

et al. (1979) injected leucine-enkephalin into the hippocampus where it produced long-lasting epileptiform changes in the EEG of the treated cats. Fry et al. (1979) reported inhibitory and excitatory responses of hippocampal neurones after microelectrophoretic application of opioids. Only part of the responses could be blocked by naloxone. Rigter et al. (1977) reported on antiamnesic effects of enkephalin and De Wied et al. (1978) on retardation of extinction of conditioned avoidance behavior. The hippocampus is thought to play an essential role in the regulation of these behaviors (Van Wimersma Greidanus and De Wied, 1976). Both behavioral effects of enkephalin were brought about independently from opiate receptors as treatment with naloxone did not reverse the behavioral effect of enkephalin.

According to Fry et al. (1979) naloxone-irreversible effects tend to prevail in areas with a low density of opiate receptors. The hippocampus is known to contain relatively few opiate receptors (Pert et al., 1975; Atweh and Kuhar, 1977). Yet the effects reported here on hippocampal phosphoproteins are most likely brought about by opiate receptor activation.

The mechanism by which opiate receptor activation alters hippocampal phosphoproteins is not clear. The endogenous phosphorylation of membrane proteins as studied in the present experiments is the resultant of the activity of (i) the protein kinase(s), (ii) protein phosphatase(s), and (iii) the availability of substrate phosphorylatable amino acid residues and ATP (Rodnight et al., 1975).

In view of the identical protein patterns which were obtained from treated and untreated slices and the specificity of the enhancement it may be assumed that effects on protein degradation or availability of ATP are unlikely. To test the possibility that the activity of the protein kinases/phosphatases was altered by the addition of the peptide, the endogenous phosphorylation of a membrane fraction obtained from rat hippocampi was studied in the presence of methionine-enkephalin. The membranes were prepared under conditions which displace endogenous opioid-ligands e.g. pretreatment of the crude mitochondrial fraction with naloxone prior to osmotic shock as first reported by Ehrlich et al. (1979). Indeed it could be demonstrated that small amounts of methionine-enkephalin added to the in vitro assay were able to rapidly decrease the incorporation of radiophosphate into the 50K band (table 2). Previously it was shown that under these short incubation conditions the activity of protein kinase predominated over that of the protein phosphatase (Rodnight et al., 1975; Wiegant et al., 1978; Zwiers et al., 1978; DeLorenzo and Greengard, 1973). This observation is in agreement with the inhibition of membrane protein kinase(s) by β-endorphin as reported by Ehrlich et al. (1979). As also concluded by these authors, opiate receptor activation may involve the inhibition of (certain) protein kinase(s) and may therefore result in alterations of the phosphorylation of certain membrane phosphoproteins (Ehrlich et al., 1979; O'Callaghan et al., 1979). This direct effect of methionine-enkephalin on membrane protein kinase activity can account for the changes seen after incubation of the slice with the peptide. As inhibition of the protein kinase in the slice may have led to the presence of a relatively dephosphorylated substrate protein in the subsequent post hoc in vitro assay and as there would thus be more sites available one could expect an enhancement of endogenous phosphorylation under these conditions (see also Browning et al., 1979b; Zwiers et al., 1977).

The present study at least supports the possibility that neuropeptides may exert their modulatory role in neuronal activity partly through mechanisms that involve (de)phosphorylation of specific membrane proteins.

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