# ENKEPHALIN EVOKES BIOCHEMICAL CORRELATES OF OPIATE TOLERANCE AND DEPENDENCE IN NEUROBLASTOMA X GLIOMA HYBRID CELLS

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## 1. Introduction

Since opiates are not normally occurring in animals, opiate receptors must be made to recognize endogenous agents that resemble opiates [1]. Some of these compounds have been isolated [2–5] and characterized as two pentapeptides named leucine- and methionine-enkephalin [6], for which structural similarity to opiates has been suggested [7,8]. The endogenous ligands of the opiate receptors compete with opiates for the same receptors [2,4] and cause analgesia if applied properly [9]. In the presence of prostaglandin $E_1$ ($PGE_1$) the neuroblastoma-glioma hybrid cells 108CC15 [11] increase their intracellular concentration of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) [12]. This increase is inhibited by acetylcholine [13], noradrenalin [14], morphine [15–17] and enkephalin [10].

Besides these short-term actions of opiates on the hybrid cells, also long-term effects were observed which could be correlated with morphine tolerance, dependence and withdrawal phenomena: Hybrid cells that had been preincubated with morphine for many hours showed an increased response to $PGE_1$ [18,19]. In the present communication we report that also enkephalins can cause such long-term effects. In addition we present evidence that the enkephalins are inactivated by incubation with the hybrid cells.

## 2. Materials and methods

Leucine-enkephalin (1-enk) and methionine-enkephalin (m-enk) were synthesized as described [10]. $PGE_1$ was a gift of Dr J. Pike, The Upjohn Co., Kalamazoo. The neuroblastoma X glioma clonal hybrid cells 108CC15 were grown in plastic Petri dishes as described [12,17]. $2.5 \times 10^5$ viable (exclusion of nigrosin) cells were seeded. Four days later 200 μl of a 0.1 mM solution (in distilled water) of enkephalin was added. In some cases this addition was repeated 3 h later. Control plates received water only. These preincubations were ended after various times (0 to 15 h) within the range of 4 h. At the end of the preincubation period, the medium was removed and the cell layer was washed with 5 ml of incubation medium (37°C) [20] before the main incubation (10 min, 37°C) in 5 ml of this medium was started by the addition of 15 μl $PGE_1$ in ethanol (96%) (final concentration 0.3 μM). In some cases also 1 μM 1-enk was present during this period. After the main incubation, the cellular content of cyclic AMP was determined as described [10].

## 3. Results

Hybrid cells preincubated with 1 μM 1-enk for 1 to 3 h show a strongly enhanced response to $PGE_1$ during the main incubation (fig.1, curves 1a and b, 0 to 3 h). This result is analogous to that obtained with morphine [18]. If the preincubation period exceeds 3 hours, the increased response to $PGE_1$ is gradually lost again (fig.1, curve Ia). This observation contrasts with that previously made [18] when morphine was present during the preincubation. Under these conditions a plateau of the enhanced response to $PGE_1$ was reached after 10 h and was maintained at least up to 62 h. The declining effect of 1-enk suggested that the peptide 1-enk might be broken down in the culture


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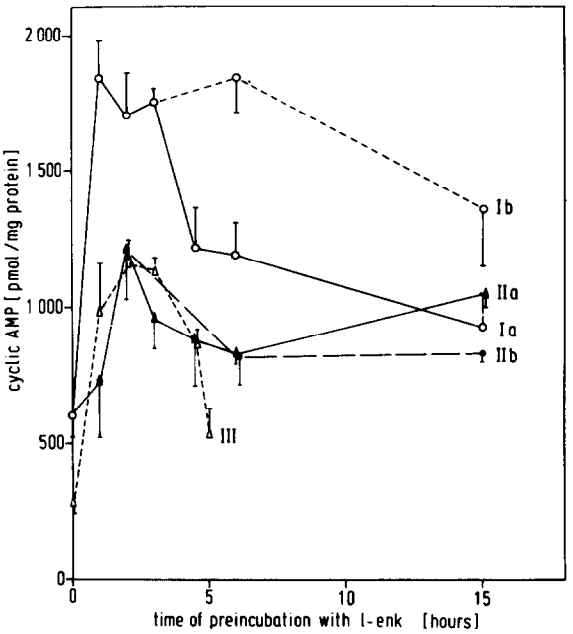


Fig.1. Effect of the preincubation with 1-enk on the response to $PGE_1$ of the hybrid cells 108CC15. 1.8 × $10^6$ viable hybrid cells (viability 91.8%) were preincubated at 37°C with 1-enk for various lengths of time. To this end 1-enk (200 μl 0.1 mM) (curves Ia, Ib and III) or 200 μl of water (curves IIa and IIb) were added once (at 0 h; curves Ia, IIa and III) or twice (at 0 and 3 h; curves Ib and IIb) to the cultures containing 20 ml of growth medium. After the preincubation, the medium was replaced by incubation medium [30] and the main (challenge) incubation in the presence of 0.3 μM $PGE_1$ (10 min, 37°C) was carried out before the intracellular concentration of cyclic AMP was determined. The main incubation corresponding to curve III also contained 1 μM 1-enk. Cells preincubated as those of curve Ib but challenged with $PGE_1$ + 1 μM 1-enk after 5 h of preincubation contained 1200 ± 130 pmol cyclic AMP per mg protein. All points are mean values ± s.d. obtained from 3 parallel incubations.

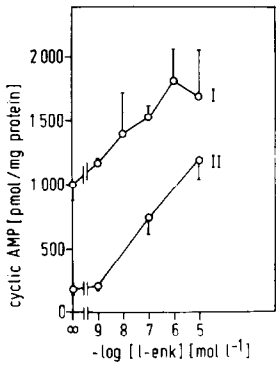


Fig.2. Effect of the concentration of 1-enk during the pre-incubation (2.5 h) on the increase in the level of cyclic AMP caused by $PGE_1$ during the main incubation (10 min, 37°C). Main incubation in the presence of 0.3 μM $PGE_1$ (curve I) or 0.3 μM $PGE_1$ + 0.1 μM 1-enk (curve II). 1.1 × $10^6$ viable cells, viability 96.8%, other details as in fig.1.

during preincubation. In order to test this hypothesis, 1-enk was added again after 3 h of preincubation. Indeed, the enhanced response to $PGE_1$ could now be observed up to more than 6 hours (fig.1, curve Ib). Control cells receiving at 0 hours (fig.1, curve IIb) or 0 and 3 hours (fig.1, curve IIb) only water instead of the solution of 1-enk for the preincubation showed much less pronounced changes in their response to $PGE_1$. After all times of preincubation with 1-enk the response to $PGE_1$ during the challenge incubation with $PGE_1$ could be attenuated by 1-enk (fig.1, curve III).

The preincubation with m-enk afforded results essentially identical to those obtained with 1-enk.

The development of the increased response to $PGE_1$ in the main incubation is dependent on the concentration of 1-enk during the preincubation period. If the cells are preincubated with 1-enk for 2.5 h, the maximal effect is observed at 1 μM 1-enk. (fig.2, curve I). A further increase in the concentration of 1-enk does not cause any further changes. Irrespective of the concentration of 1-enk used in the pre-incubation the action of $PGE_1$ can be inhibited if 1-enk is present during the main incubation (fig.2, curve II). The stronger the effect of the preincubation with 1-enk the smaller is the inhibitory effect of 1-enk in the main incubation (compare I and II in fig.2).

## 4. Discussion

In response to $PGE_1$ hybrid cells preincubated for several hours with enkephalins, adrenergic or cholinergic agonists show increased accumulation of intracellular cyclic AMP. If present during a 10 min incubation, all these compounds including morphine [15,16] and the enkephalins [10] prevent the $PGE_1$ induced increase in the intracellular level of cyclic AMP, which might be associated with the analgesic action of the drugs [21–23] and the pleasant sensation they evoke. On the other hand then, an increase in the level of

cyclic AMP above normal should be correlated with unpleasant sensations in men and animals. As pointed out previously [11,18 19], from these effects and the long-term actions of morphine or enkephalin a model can be developed for opiate tolerance, dependence and withdrawal phenomena. In fact, agents known to increase the intracellular concentration of cyclic AMP (cyclic AMP and its derivatives, inhibitors of cyclic AMP phosphodiesterase activity) elicit quasi abstinence syndroms in animals if applied properly [24,25].

If in vitro the enkephalins cause biochemical correlates of opiate tolerance and withdrawal, the question arises why animals and men do not become addicted to their own endogenous ligands of the opiate receptors. The answer likely to be correct is that such peptides are rapidly inactivated before they can evoke long-term actions. This is what one would expect if the enkephalins are neurotransmitters or constituents of neurotransmitters. Removal of the opiate from hybrid cells 'addicted' to morphine is followed by the complete loss of this phenomenon within 3 h [17]. A reversal of the state of 'addiction' caused by 1-enk is found even without washing it away from the cells (fig.1, curve Ia). The most straight forward explanation is that the peptide is inactivated in the culture. This view is supported by the fact that a timely second administration of 1-enk prolongs the state of 'addiction' of the cells (fig.1, curve Ib). Thus, the hybrid cells may not only be a good model system for studying the molecular mechanism of opiate action but also for studying the breakdown of enkephalins and similar peptides. The finding presented demonstrates that the analogy between opiates and enkephalins not only holds for their short-term but also extends to their long-term actions. Except for the degradation of the peptides, the molecular mechanism for the actions of opiates might also reflect those for the actions of peptides with opiate-like effects.

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## References

[1] Collier, H. O. J. (1973) Proc. 5th Int. Congr. Pharmacol. San Francisco 1972, Vol. 1, pp. 65–76, Karger, Basel.
[2] Terenius, L. and Wahlström, A. (1974) Acta pharmacol. toxicol, 35, Suppl., 55; (1975) Acta physiol. scand. 94, 74–81; (1975) Life Sci. 16, 1759–1764.
[3] Hughes, J. (1975) Brain Res. 88, 295–308.
[4] Pasternak, G. W., Goodman, R. and Snyder, S. H. (1975) Life Sci. 16, 1765–1769.
[5] Teschemacher, H., Opheim, K. E., Cox, B. M. and Goldstein, A. (1975) Life Sci. 16, 1771–1776.
[6] Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. and Morris, H. R. (1975) Nature 258, 577–579.
[7] Bradbury, A. F., Smyth, G. D. and Snell, C. R. (1976) Nature 260, 165–166.
[8] Horn, A. S. and Rodgers, J. R. (1975) Nature 260, 795–797.
[9] Belherzci, J. D., Grant, W., Garsky, V., Sarautakis, D., Wise, C. D. and Stein, L. (1976) Nature 260, 625–626.
[10] Brandt, M., Gullis, R. J., Fischer, K., Buchen, C., Hamprecht, B., Moroder, L. and Wünsch, E. (1976) Nature, in the press.
[11] Hamprecht, B. (1974) in 25. Kolloquium der Gesellschaft für Biologische Chemie (L. Jaenicke, ed.), pp. 391–423, Springer, Berlin; (1976) Angew. Chem. 88, 211–223 and Angew. Chem. Intern. Ed. Engl. 15, 194–206; (1976) Intern. Rev. Cytol., in the press.
[12] Hamprecht, B. and Schultz, J. (1973) Hoppe-Seyler's Z. physiol. Chem. 354, 1633–1641.
[13] Traber, J., Fischer, K., Buchen, C. and Hamprecht, B. (1975) Nature 255, 558–560.
[14] Traber, J., Reiser, G., Fischer, K. and Hamprecht, B. (1975) FEBS Lett. 52, 327–332.
[15] Traber, J., Fischer, K., Latzin, S. and Hamprecht, B. (1975) Nature 253, 120–122.
[16] Sharma, S. K., Klee, W. A. and Nirenberg, M. (1975) Proc. Natl. Acad. Sci. USA 72, 3092–3096.
[17] Traber, J., Gullis, R. and Hamprecht, B. (1975) Proc. 10th FEBS Meeting 41, 165–177.
[18] Traber, J., Gullis, R. and Hamprecht, B. (1975) Life Sci. 16, 1863–1868.
[19] Klee, W. A., Sharma, S. K. and Nirenberg, M. (1975) Life Sci. 16, 1869–1874.
[20] Traber, J., Fischer, K., Latzin, S. and Hamprecht, B. (1974) Proc. 9th Int. Cong. Collegium internationale Neuropsychopharmacologicum, 956–969.
[21] Ho, I. K., Loh, H. H. and Way, E. L. (1972) Nature 238, 397–398.
[22] Ho, I. K., Loh, H. H. and Way, E. L. (1973) J. Pharmacol. Exp. Ther. 185, 336–346.
[23] Collier, H. O. H. and Roy, A. C. (1974) Prostaglandins 7, 361–376.
[24] Collier, H. O. J., Francis, D. L., Henderson, G. and Schneider, C. (1974) Nature 249, 471–473.
[25] Collier, H. O. J. and Francis, D. L. (1975) Nature 255, 159–162.