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# SHORT COMMUNICATIONS

## Effects of (D-alanine$^2$, methionine$^5$) enkephalinamide on the release of acetylcholine and noradrenaline from brain slices and isolated nerve terminals

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The pentapeptides leucine and methionine enkephalin are endogenous ligands for the opiate receptors and have opiate-like pharmacological actions which are blocked by opiate antagonists such as naloxone.

It has been suggested that these peptides function as neuromodulators of synaptic transmission [2–4]. They depress the electrically evoked contractions of the guinea pig ileum and mouse vas deferens. The output of acetylcholine from the myenteric plexus of the guinea pig ileum is inhibited by the enkephalins and also the release of noradrenaline from rat occipital cortex stimulated electrically or by 20 mM KCl [6, 7]. A similar effect has been demonstrated on the release of acetylcholine from slices prepared from the rat hippocampus and on the release of dopamine from corpus striatum slices [8, 18]. These results would imply that opiates act to inhibit the functional release of some neurotransmitters in the central nervous system. The purpose of this investigation was to try and clarify the effects of opiates on the release of acetylcholine and noradrenaline using *in vitro* systems.

The peptides are subject to extremely rapid inactivation which occurs principally by the cleavage of the tyrosine–glycine bond. Therefore the stable analogue (D-alanine$^2$, methionine$^5$) enkephalinamide (subsequently referred to as (D-ala$^2$)met.enk.), in which this bond is protected was used [1]. Slices and isolated nerve terminals prepared from various brain regions were used in an attempt to distinguish any pre-synaptic and post-synaptic actions of the opioid on the release of transmitters.

### *Materials and methods*

*Preparation and incubation of slices.* Slices of tissue (0.15–0.25 mm) were prepared from whole brain (without cerebellum), cerebral cortex and corpus striatum using a McIlwain chopper, similar to the method described by Richter and Marchbanks [9]. 0.15 g of tissue was suspended in 3 ml of the normal incubation medium that contained 135 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM glucose, 10 mM $Na_2HPO$–$KH_2PO_4$ buffer pH 7.4 and 2.6 mM $CaCl_2$. After equilibrating on ice for 10 min 0.5 $\mu$Ci/ml of [$^{14}$C]choline (59 mCi/mmole) was added and the suspension incubated for a total of 60 min at 37° in an atmosphere of 95% $O_2$ and 5% $CO_2$.

In some experiments slices were preincubated with [$^3$H]noradrenalin (10 Ci/mmole) (0.5 $\mu$Ci/ml). 1 $\mu$M ascorbic acid was added as an antioxidant but no other additions were made. At the end of incubation the slices were washed twice with 250 ml of cold incubation medium from which the calcium had been omitted but which contained 0.1 M eserine sulphate and placed into 25 ml Erlenmeyer flasks containing 3 ml of the same medium. Additions of 1 $\mu$M (D-ala$^2$)met.enk., with or without 1 $\mu$M naloxone were made and the slices pre-incubated for 10–15 min at room temperature. After this period the medium was replaced by 3 ml of the depolarizing or normal media with additions or omissions as indicated in the results. Where there was an increase in KCl concentration, the osmolarity was kept constant by a corresponding decrease in NaCl. At the end of a 30 min incubation at 37°, the contents of the vessels were centrifuged to separate the incubation medium containing the released ACh or noradrenaline. Results of release experiments are expressed as dpm released per mg of tissue protein.

*Preparation and incubation of synaptosomes.* The method used in the preparation of the nerve terminals was similar to that of Gray and Whittaker [10]. The synaptosome ($P_2B$) fraction was removed from the gradient and gradually diluted with an equal volume of the normal incubation medium (see below). The synaptosomes were then sedimented by centrifugation at 11,000 rpm for 20 min and resuspended in incubation medium to give a final concentration of 1–2 mg protein/ml.

The incubation medium contained 10 mM $Na_2HPO_4$–$KH_2PO_4$ buffer pH 7.4, 3 mM KCl, 180 mM NaCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM glucose. After equilibrating on ice for 10 min, the suspension was divided into portions as indicated in the results. Where uptake and synthesis were to be measured additions of 1 $\mu$M (D-ala$^2$) met.enk. and/or 1 $\mu$M naloxone were added. All fractions were incubated at 37° for 30 min in the presence of [$^{14}$C]choline (0.1 $\mu$Ci/ml). Following this incubation, the suspensions were sedimented by centrifugation at 3000 rpm for 5 min in a MSE bench centrifuge.

In studies of release, the synaptosomes were resuspended in normal medium and sub-divided into equal fractions. After a further centrifugation, the washed synaptosomes were suspended in either a normal medium or a depolarizing medium both containing eserine, (D-ala$^2$)met.enk. and/or naloxone as indicated, and incubated at 37° for 30 min.

In some experiments synaptosomes prepared from the cerebral cortex were preloaded with [$^3$H]noradrenaline, (10 Ci/mmole), 0.1 $\mu$Ci/ml and 1 $\mu$M ascorbic acid.

*Extraction and isolation of acetylcholine and choline.* The supernatants containing the released [$^{14}$C]-ACh, were transferred to tubes kept on ice containing 5 $\mu$l of [$^3$H]acetylcholine, internal standard (approximately 20–30,000 dpm) and 0.02 ml of 5% (2/v) trichloroacetic acid. Synaptosomal pellets were washed twice by resuspending in cold normal medium containing eserine but without calcium and centrifuging at 10,000 rpm. After discarding the supernatants, the pellets were resuspended in normal medium containing 0.1 ml of 5% (w/v) trichloroacetic acid, and 5 $\mu$l of the internal standard. Acetylcholine and choline were extracted as described by Marchbanks and Israel [11], and were separated using thin layer chromatography. The recovery of [$^3$H]acetylcholine was between 50 and 80 per cent and all results were corrected for the individual recoveries determined from the internal standard. Results for pellets and media were expressed as dpm/mg protein; for synaptosomal media this calculation was based on the mean concentration of synaptosomal suspension. In slice prep-

Abbreviations used: (D-ala$^2$)met.enk., D-alanine$^2$, methionine$^5$ enkephalinamide. ACh, acetylcholine. Ch, choline.

arations individual protein determinations from each group of homogenised slices were used.

*Measurement of choline uptake and acetylcholine synthesis.* After precipitation of the synaptosomes by centrifugation at 10,000 rpm in a Janetzki TH11 high speed microcentrifuge, the pellet was resuspended in normal medium. 0.05 ml of the suspension was removed and the radioactivity estimated, using a scintillation fluid containing 0.5% (w/v) 2,5-diphenyloxazole (PPO) + 0.2% (w/v) 1–4 di [2-(5-phenyloxazolyl)] benzene in toluene: Triton X-100; 0.1 M $NH_4OH$ (2:1:0:2). The total $^{14}C$ radioactivity in the pellet was used as a measure of the uptake of choline into the synaptosomes. After removal of an aliquot for protein determination, the remainder of the sample was used in the separation of [$^{14}C$]acetylcholine and [$^{14}C$]choline.

*Protein measurements.* Protein was determined by the method of Lowry *et al* [17] using bovine plasma albumin as the standard.

*Materials.* All radiolabelled compounds were from the Radiochemical Centre, Amersham, Buckinghamshire, U.K. (D-alanine$^2$, methionine$^5$) enkephalinamide was supplied by Bachem. Inc. (Fine Chemicals) acetyl(methyl-$^3H$) choline was prepared by incubating [$^3H$]choline with acetic anhydride [12]. Naloxone hydrochloride was kindly donated by Endo Laboratories, Belgium.

## Results

*Effect of (D-ala$^2$)met.enkephalinamide on the depolarisation evoked noradrenaline release.* In order to determine the specificity of the enkephalins with regard to their effect on the release of acetylcholine and also to establish a satisfactory experimental method which could be used in studying that release, the inhibition of noradrenaline release, as reported by Taube *et al.* [6] and Arbella and Langer [7] was investigated. Cerebral cortex slices were preloaded with [$^3H$]noradrenaline (0.5 $\mu$Ci/ml) in the normal medium containing $Ca^{2+}$ and 1 $\mu$M ascorbic acid. The slices were washed twice with 250 ml of ice cold medium containing ascorbic acid but not $Ca^{2+}$. They were then subdivided into equal fractions and pre-incubated for 15 min in 3 ml of the same medium with additions of 1 $\mu$M (D-ala$^2$)met.enk. with or without the addition of 1 $\mu$M naloxone as indicated. After preincubation this medium was replaced by 3 ml of the depolarising or normal media also with additions or omissions as indicated in Table 1. Measurement of the $^3H$ radioactivity released into the supernatant during an incubation period of 20 min at 37° showed that an increase in potassium concentration was effective in evoking a 181 per cent increase in the release of noradrenaline, when compared to the non-depolarising condition. The evoked release required the presence of $Ca^{2+}$.

(D-ala$^2$)met.enkephalin inhibited the release of [$^3H$]noradrenaline under depolarising conditions but did not affect release when $Ca^{2+}$ was absent. Under non-depolarising conditions there was no change in the release brought about by the opioid. The addition of naloxone to non-depolarised slices (in the presence of (D-ala$^2$)met.enk.) did not affect the release of noradrenaline but it did reverse the inhibition of the depolarization evoked release caused by (D-ala$^2$)met.enkephalin. Experiments in which dimethylimipramine (1 $\mu$M) was included to prevent uptake of the amine showed no significant difference to the results. Neither the opioid nor naloxone alone or in combination had any significant effect on the release of noradrenaline from synaptosomes prepared from the cerebral cortex.

*Effect of (D-ala$^2$)met.enkephalinamide on the $K^+$ stimulated release of [$^{14}C$]acetylcholine released from slices.* Tissue stores of acetylcholine were labelled by preincubation with [$^{14}C$]choline as described in Materials and Methods. Slices from the corpus striatum showed that an increase in KCl concentration elicited an increase in release of total $^{14}C$ radioactivity when compared to that released under the non-depolarising conditions. This release was dependent on $Ca^{2+}$ (data not shown), and was inhibited in the presence of (D-ala$^2$)met.enk. However, when the radioactivity was chromatographically separated into acetylcholine and choline there was no significant inhibition of [$^{14}C$]acetylcholine released by potassium depolarisation. However there was a significant decrease in the amount of [$^{14}C$]choline released. This decrement is reversed when the medium contains 1 $\mu$M naloxone. Similar experiments were carried out with cerebral cortex slices, but although there was a calcium-dependent release of [$^{14}C$]-ACh into the medium we were not able to discern an effect (in seven experiments) by (D-ala$^2$)met.enk. or naloxone.

It is possible that the opioids affect choline transport or the synthesis of acetylcholine. In order to investigate these possibilities the synaptosome preparation (being more manipulable for uptake studies) was used. The uptake of [$^{14}C$]choline, its conversion to [$^{14}C$]-ACh and its release on depolarizing stimuli were measured as described in the methods section with results as shown in Table 3. In synaptosomes prepared from both cerebral cortex and whole brain (without cerebellum) [$^{14}C$]acetylcholine was synthesised from [$^{14}C$]choline transported into the terminals. Depolarizing concentrations of KCl caused a significant increase in [$^{14}C$]-ACh release into the medium which was dependent on the presence of calcium ions. However (D-ala$^2$)met.enk. did not affect uptake of [$^{14}C$]choline or the

Table 1. The effect of (D-ala$^2$)met$^5$ enkephalinamide on the release of radioactivity from cerebral cortex slices and synaptosomes after pre-loading with [$^3H$]noradrenaline

| Condition | Amount of $^3H$ radioactivity released (dpm/mg protein) Slices | Synaptosomes |
|---|---|---|
| 3 mM $K^+$ | 6479 ± 610 (4) | 24,839 ± 1745 (3) |
| 50 mM $K^+$ | 11,767 ± 946 (4) | 61,437 ± 6898 (3) |
| 50 mM $K^+$–$Ca^{2+}$ | 5210 ± 1059 (4)* | 38,915 ± 662 (3)* |
| 3 mM $K^+$(D-ala$^2$)met.enk. | 6649 ± 810 (4) | 31,217 ± 2699 (3) |
| 50 mM $K^+$(D-ala$^2$)met.enk. | 7907 ± 666 (4)* | 64,075 ± 13092 (3) |
| 50 mM $K^+$–$Ca^{2+}$(D-ala$^2$)met.enk. | 7630 ± 782 (3) | 47,155 ± 5325 (3) |
| 3 mM $K^+$ + (D-ala$^2$)met.enk. + naloxone | 7644 ± 783 (4) | 30,894 ± 6051 (3) |
| 50 mM $K^+$ (D-ala$^2$)met.enk. + naloxone | 11,214 ± 1473 (4) | 44,396 ± 3062 (3) |
| 50 mM $K^+$–$Ca^{2+}$ + (D-ala$^2$)met.enk. + naloxone | 9303 ± 517 (3) | 42,432 ± 871 (2) |

Tissue was preincubated for 60 min with [$^3H$]noradrenaline (0.5 $\mu$Ci/ml) followed by a 30 min release period with additions as shown. Results are the means ± S.E.M. (or range) of the number of determination given in parentheses.

* Indicates a significant difference with $P < 0.05$ from the depolarised control (50 mM $K^+$) condition using Student's *t* test.

Table 2. Release of total radioactivity and that due to chromatographically separated [$^{14}$C]choline and [$^{14}$C]-ACh from corpus striatum slices

| | Control | | + (D-ala$^2$)met.enk. | | + (D-ala$^2$)met.enk. + Naloxone | |
|---|---|---|---|---|---|---|
| | 3 mM KCl | 50 mM KCl | 3 mM KCl | 50 mM KCl | 3 mM KCl | 50 mM KCl |
| Total radioactivity (dpm/mg) | 2366 ± 273 (4) | 5636 ± 1519 (5) | 2060 ± 464 (5) | 2263 ± 952 (5)† | 2605 ± 394 (5) | 4985 ± 872 (4) |
| [$^{14}$C]choline (dpm/mg) | 921 ± 308 (4) | 873 ± 185 (4) | 653 ± 178 (4) | 321 ± 136 (4)† | 576 ± 178 (4) | 831 ± 105 (4) |
| [$^{14}$C]-ACh (dpm/mg) | 888 ± 235 (4) | 2475 ± 639 (4)* | 945 ± 327 (4) | 2240 ± 760 (4)* | 1002 ± 258 (4) | 1674 ± 382 (4)* |

Results are expressed as means ± S.E.M. of the number of observations given in parentheses. Slices prepared from the corpus striatum were preincubated with [$^{14}$C]choline (0.5 μCi/ml) for 60 min at 37°. Following a 30 min release period in the presence of (D-ala$^2$)met.enk., the total radioactivity released into the medium was measured and then separated chromatographically. The recovery of [$^{14}$C]acetylcholine was corrected for an internal standard and was 53% ± 0.06(7). (D-ala$^2$)met. enk. showed no significant effect on this recovery.

* Indicates a significant difference with $P < 0.05$ from the non-depolarised control condition using a Student's $t$ test.

† Indicates a significant difference with $P < 0.05$ from the depolarised control condition using a Student's $t$ test.

Table 3. Summary of the effect of (D-ala$^2$)met.$^5$enkephalinamide on the synaptosomal cholinergic system

| Condition | [$^{14}$C]Choline uptake (dpm/mg) | [$^{14}$C]-ACh synthesis (dpm/mg) | [$^{14}$C]-ACh release (dpm/mg) 3 mM $K^+$ | 50 mM $K^+$ | 50 mM $K^+$–$Ca^{2+}$ |
|---|---|---|---|---|---|
| Cerebral cortex synaptosomes | | | | | |
| Control (dpm/mg) | 4406 ± 1915 (3) | 1627 ± 495 (3) | 1129 ± 305 (5) | 2145 ± 450 (5)* | 1106 ± 483 (5) |
| + (D-ala$^2$)met.enk. | 4493 ± 2031 (4) | 1853 ± 328 (3) | 1142 ± 427 (4) | 2975 ± 285 (5)* | 614 (1) |
| + Naloxone | 4267 ± 1535 (4) | 2356 ± 608 (5) | — | — | — |
| + (D-ala$^2$)met.enk. + naloxone | 4418 ± 1884 (4) | 2512 ± 438 (3) | — | — | — |
| Whole brain synaptosomes | | | | | |
| Control (dpm/mg) | 9350 ± 1349 (3) | 9921 ± 1994 (4) | 1402 ± 303 (4) | 2417 ± 394 (4)* | 1529 ± 964 (4) |
| ( + D-ala$^2$)met.enk | 10,546 ± 655 (3) | 9928 ± 3419 (4) | 687 ± 167 (4) | 2238 ± 773 (4) | 2329 ± 1091 (4) |
| + Naloxone | 10,204 ± 224 (3) | 9857 ± 1998 (4) | 966 ± 411 (4) | 2082 ± 589 (4) | 1053 ± 155 (3) |
| ( + D-ala$^2$)met.enk. + naloxone | 9960 ± 251 (3) | 10,419 ± 2810 (4) | 895 ± 163 (4) | 2800 ± 523 (3)* | 1214 ± 617 (4) |

Synaptosomes were incubated with [$^{14}$C]choline (0.1 μCi/ml) for 30 min followed by 30 min release period in the presence of either (D-ala$^2$)met.enk. and/or naloxone. The radioactivity within the tissue and that released into the medium was chromatographically separated by thin layer chromatography. Results are expressed as mean ± S.E.M. of the number of observations given in parentheses.

* Indicates a significant difference from the 3 mM $K^+$ condition of $P < 0.05$ using a Student's $t$ test.

synthesis and release of [$^{14}$C]-ACh in synaptosomes from either preparation. Naloxone was similarly without affect.

*Discussion*

(D-ala$^2$)met.enk. diminished the release of [$^3$H]noradrenaline evoked by a high extracellular concentration of potassium and this effect was antagonised by naloxone. This is in agreement with previous studies [6, 7] and in addition we have demonstrated that this effect is dependent on the presence of $Ca^{2+}$ indicating that the opioid acts as an inhibitory modulator of the functional release of the transmitter. Neither the opioid nor naloxone alone or in combination affected the release of [$^3$H]noradrenaline from isolated nerve terminals prepared from the cerebral cortex. This suggests that enkephalin does not affect the release by a presynaptic mechanism and that the integrity of the whole cell is necessary for the inhibitory action to occur. Since brain slices contain many intact neurons, the action of the opiates could be on a noradrenergic cell sensitive to opiates or on an intermediate cell that utilises some other transmitter.

It is well known that the electrically evoked contractions of the myenteric plexus longitudinal muscle preparation can be inhibited by morphine [13] and this test has been used as a criterion in the isolation of the enkephalins. Morphine has also been shown to inhibit the acetylcholine release in the myenteric plexus longitudinal muscle [14] and similar findings using enkephalin have been demonstrated [5]. Subramanian *et al.* [8] have reported that enkephalin diminishes the potassium evoked release of acetylcholine from slices prepared from the rat hippocampus. When the total $^{14}$C radioactivity released from slices prepared from the corpus striatum was examined our results seemed to confirm these findings. However a different picture emerged when [$^{14}$C]acetylcholine and [$^{14}$C]choline were chromatographically separated. An analysis of our data (Table 2) showed that if the radioactivity is measured without separation then there does appear to be an inhibitory effect of (D-ala$^2$)met.$^5$enk. on the stimulated release from tissue pre-labelled with [$^{14}$C]choline. However when the radioactivity is separated it can be seen that this decrement appears mainly in the unesterified [$^{14}$C]choline. However this decrement would not account for the decrease in total radioactivity release brought about by (D-ala$^2$)met.enk. Some metabolic product of choline whose efflux from the slices is increased by depolarization must also be affected by (D-ala$^2$)met.enk. The discrepancy in the results would arise in that Subramanian did not separate acetylcholine and choline but assumed that the radioactivity released after preloading with choline is only due to acetylcholine. As our results have indicated it is possible that varying contaminations by unchanged radioactive choline or its metabolites might have influenced their results.

In so far as the actions of enkephalin can be analogous to those of morphine in the central nervous system our results support Jhamandas *et al.* [15] and also Richter and Marchbanks [9], in that only unphysiologically high concentrations of morphine and its analgesically inactive isomer blocked the stimulated release of acetylcholine from cerebral cortex slices. This inhibition of the evoked release of the transmitter is therefore probably unrelated to the mechanism of action of morphine-like compounds. We conclude that the opioid (at least as represented by (D-ala$^2$)met.enk. does not have a general effect in modulating central cholinergic transmission, although its action on choline metabolism during depolarisation remains unclear.

In summary; 50 mM KCl elicited a $Ca^{2+}$-dependent release of [$^3$H]noradrenaline from preloaded slices prepared from the cerebral cortex of the guinea pig brain. The $K^+$-stimulated release was inhibited by the action of the stable enkephalin analogue (D-alanine$^2$, methionine$^5$) enkephalinamide when $Ca^{2+}$ was present. This inhibition was prevented by naloxone. The peptide failed to prevent the evoked release of [$^3$H]noradrenaline from cerebral cortex synaptosomes. Acetylcholine from slices and isolated nerve terminals prepared from various brain regions was labelled by preincubation with [$^{14}$C]choline. Although 50 mM KCl elicited a $Ca^{2+}$-dependent release of [$^{14}$C]-ACh the opioids showed no effect on the release of [$^{14}$C]-ACh from synaptosomes, or slices from cerebral cortex or whole brain. Nor was [$^{14}$C]choline uptake or ACh synthesis affected. In the case of corpus striatum slices depolarization evoked release of total unseparated radioactivity was inhibited by (D-alanine$^2$, methionine$^5$) enkephalinamide. However when the released radioactivity was separated into [$^{14}$C]acetylcholine and [$^{14}$C]choline it was found that the inhibitory effect of the opioid was not on the release of [$^{14}$C]acetylcholine but was mainly due to inhibition of the efflux of [$^{14}$C]choline or a metabolic product. Naloxone reversed this effect.

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