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Selectivity of Various Opioid Peptides Towards Delta-, Kappa; and MU-Opioid Receptors Mediating Presynaptic Inhibition of Neurotransmitter Release in the Brain

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Abstract—The selectivity of a series of opioid peptides towards the μ-, δ- and κ-opioid receptors mediating differential inhibition of electrically-induced neurotransmitter release from rat brain slices was studied, viz. cortical [^{3}H]noradrenaline release (inhibited via μ-receptors), striatal [^{3}H]dopamine release (inhibited via κ-receptors) and striatal [^{14}C] acetylcholine release (inhibited via δ-receptors).

The highest affinity pD2 7.4) and selectivity towards μ-receptors was exhibited by Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAGO), whereas [D-Pen^2, D-Pen^5]enkephalin (DPDPE) was found to be the most selective δ-receptor agonist (pD2 7.3). Also the hexapeptides [D-Ser^2]Leu-enkephalin-Thr (DSLET) and [D-Thr^2]Leu-enkephalin-Thr (DTLET) showed a relatively high selectivity and, in addition, a high affinity (pD2 8.2-8.4) for δ-opioid receptors.

Both dynorphin(1-13) and dynorphin(1-8) exhibited a high affinity for κ-receptors (pD2 resp. 8.3 and 8.0), but the latter was far less selective. Both of the dynorphin A-related peptides showed affinity to μ-receptors (pD2 6.7-6.8), but dynorphin(1-8), in contrast to dynorphin(1-13), also displayed a high affinity to δ-receptors (pD2 7.6).

Introduction

At least three pharmacologically different opioid receptor subtypes are known to exist in the central nervous system (CNS), viz. δ-, κ- and μ-receptors (5, 7, 12, 18). Although the functional implications of opioid receptor multiplicity still remain far from clear, studies on presynaptic modulation of neurotransmitter release from brain slices have shown that activation of different opioid receptor subtypes may have different functional consequences. Thus, dynorphin and other agonists with κ-opioid receptor selectivity, such as bremazocine and ethylketocyclazocine, were found to inhibit striatal dopamine (DA) release, with little or no effect on acetylcholine (ACh) release. On the other hand, Leu-enkephalin and [D-Ala^2, D-Leu^5] -enkephalin (DADLE) appear to inhibit the release of ACh from rat striatal slices through activation of δ-opioid receptors (10, 13). Finally, μ- but not δ- or κ- receptor activation results in an

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inhibition of noradrenaline (NA) release from rat cortical or hippocampal slices as well as synaptosomes (10, 11, 13, 16).

In the present study we have examined the selectivity of a series of opioid peptides towards the δ-, κ- and μ-opioid receptors mediating differential presynaptic inhbition of neurotransmitter release. The peptides investigated include Leu- and Met-enkephalin, dynorphin(1-8) and (1-13) and a number of synthetic enkephalin analogues.

Methods and Materials

Preparation and incubation of brain slices

Male Wistar rats (140-180g body weight) were decapitated and the brains were rapidly removed. Neocortical and/or neostriatal slices (approx. 0.3 × 0.3 × 2mm) were prepared, labelled and superfused essentially as described previously (10, 13, 15). In short, neocortical slices (about 100mg fresh tissue weight) were incubated for 15min in 2.5ml Krebs-Ringer-bicarbonate medium containing 0.1μM [^{3}H]NA to label nonadrenergic nerve terminals selectively. Similarly, striatal slices were incubated in medium containing 0.1μM [^{3}H]DA and 1μM [^{14}C]choline, resulting in a selective labelling of dopaminergic and cholinergic nerve terminals, respectively.

Superfusion of brain slices and addition of drugs

After labelling the slices were transferred to each of 24 chambers (volume 0.2ml; 3-4mg of tissue per chamber) of a superfusion apparatus and subsequently superfused (0.25ml/min) with medium (gassed with 95% O_2–5% CO_2) at 37°C. After 40min of superfusion (i.e. t = 30min) the superfusate was collected in 10min samples.

In experiments examining the effect of one concentration (1μM) of each of the peptides the slices were exposed at t = 50min to electrical stimulation (biphasic block pulses, 1pps, 2ms duration) for 10min to induce calcium-dependent release of the radiolabelled neurotransmitters. For [^{3}H]NA release from neocortical slices a current of 15mA was used and for [^{3}H]DA and [^{14}C]ACh release from striatal slices 30mA. The opioid peptides were added to the superfusion medium 20min before electrical stimulation of the slices and remained present until the end of the experiment. In each experiment quadruplicate observations were made. At the end of the experiment remaining tissue radioactivity was extracted with 0.1M HCl.

The potencies of various opioid peptides to inhibit transmitter release were determined using a cumulative dose-response technique, developed previously for studying the pharmacological characteristics of presynaptic receptors in superfused brain slices and described in detail elsewhere (4). In brief, cortical slices labelled with [^{3}H]NA or striatal slices labelled with [^{3}H]DA and [^{14}C]choline were exposed continuously to electrical field stimulation (biphasic blockpulses, 1pps, 2ms duration) from 40min after beginning superfusion (t = 40min). In experiments with cortical slices a current of 15mA was used and the superfusion medium routinely contained 3μM of the NA uptake inhibitor desipramine. In experiments with striatal slices the stimulation current was 30mA and the superfusion medium contained 3μM of the DA uptake inhibitor nomifensine. Increasing concentrations of the opioid peptides were added cumulatively to the medium with 10-min intervals, from t = 50 to t = 80min. In each experiment the inhibitory effect of each of the peptides was determined in duplicate, by comparing the electrically-evoked release of radiolabelled transmitter in its presence with the release found in control superfusion chambers not exposed to the peptide.

Calculation of the release data

The radioactivity in the superfusion samples and tissue extracts was determined by liquid scintillation counting. The flux of radio-activity during each 10-min collection period was expressed as the fraction of the amount of radioactivity present in the tissue at the beginning of the respective collection period (fractional rate constant). To calculate the electrically-evoked release of the radiolabelled neurotransmitter, the spontaneous efflux of radioactivity was subtracted from the total overflow of radioactivity during stimulation and the 10min following. The electrically-evoked release of [^{3}H]NA from cortical slices amounted to 3-4.5% of total tissue content and for striatal

[^{3}H]DA and [^{14}C]ACh release the figures were 2-3% and 8-10%, respectively.

A detailed description of the calculation of the data obtained in the experiments using the cumulative dose-response technique has been published elsewhere (4). In these experiments the spontaneous efflux of radioactivity was determined separately in chambers in which the slices were not exposed to electrical stimulation. The apparent affinities of the peptides, as derived from the log concentration – effect curves, have been expressed as pD2-values (pD2 = −log EC50).

The data were analyzed by two-way analysis of variance,, followed by Duncan's multiple range test, using SPSS/PC+V2.0 (SPSS, Inc.). Significance was defined at the 0.01 level.

Radiochemicals and drugs

1-[7-^{3}H]noradrenaline (37 Ci/mmol), [7,8-^{3}H] dopamine [47 Ci/mmol) and [methyl-^{14}C]choline (50 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, UK). All of the opioid peptides used were purchased from Bachem (Switzerland), except [D-Pen^2, D-Pen^5] enkephaline and dynorphin(1-13), which were kindly donated by, respectively, Dr. V.J. Hruby (Univ. Arizona, Tucson, USA) and Organon (Oss, The Netherlands). Desipramine was a gift from Ciba-Geigy and nomifensine from Hoechst-Pharma.

Results

In this study we first determined the effect of a concentration of 1 μM of each of the peptides on neurotransmitter release induced by a 10 min exposure to electrical field stimulation; these data are summarized in Tables 1 and 2. Then the compounds causing about 40% inhibition of release or more were further tested using a cumulative dose-response technique (see Methods) and examining the concentration range of 1 nM to 1 μM (for the effects on [^{3}H]DA and [^{14}C]ACh release from striatal slices) or 10 nM to 10 μM (for the effects on [^{3}H]NA from cortical slices). As an example the Figure shows the concentration-effect curves of the inhibitory effects of dynorphin(1-13), dynorphin(1-8) and DSLET on the release of [^{3}H]DA and [^{14}C]ACh from striatal slices. From

Table 1 Inhibitory effects of various opioid peptides on the electrically-evoked release of [^{3}H]DA and [^{14}C]ACh from rat striatal slices and their apparent affinities for the opioid receptors (κ and δ, respectively) involved. The inhibitory effects of 1 μM concentrations of the peptides were determined in experiments, in which the slices were exposed to 10 min of electrical stimulation (1 pps, 2 ms, 30 mA) and are the means ± s.e.m. of 12-24 values obtained in 3-6 separate experiments. The apparent affinities (pD2-values) were determined in experiments using a cumulative dose-response technique (see Methods and Figure) and are the means of 6-10 values obtained in 3-5 separate experiments; in all cases the s.e.m. values were 0.1-0.15.

Opioid peptide	*[^{3}H]DA release (striatum) % inhibition at 1 μM*	*pD2*	*[^{14}C]ACh release (striatum) % inhibition at 1 μM*	*pD2*
Met-Enkephalin	11 ± 3.3*	–	49 ± 3.0*	7.4
Leu-Enkephalin	13 ± 3.8*	–	52 ± 2.6*	7.3
[Des-Tyr^1]Leu-Enk.	4 ± 3.2^0	–	9 ± 3.8^0	–
Dynorphin(1-8)	53 ± 3.7*	8.0	54 ± 2.6*	7.6
Dynorphin(1-13)	62 ± 3.6*	8.3	13 ± 3.3*	–
DADLE	8 ± 2.4*	–	65 ± 1.6*	7.9
DSLET	13 ± 3.9*	–	62 ± 2.1*	8.2
DTLET	3 ± 3.6^0	–	64 ± 1.7*	8.4
DPDPE	6 ± 2.5^0	–	50 ± 1.4*	7.3
DAGO	6 ± 3.5^0	–	15 ± 2.9*	–

* different from control at the 0.01 level
* different from control at the 0.05 level
^{0} not different from control

such curves the pD2-values were derived, that are summarized in the tables for the various peptides examined.

Striatal [^{3}H]DA release

The electrically-evoked release of [^{3}H]DA from striatal slices was strongly inhibited only by dynorphin(1-13) and dynorphin(1-8) (Table 1). The maximal inhibition caused by the dynorphin fragments was 50-60%; the EC50 of dynorphin(1-13) was about 5 nM and that of dynorphin(1-8) 10 nM. At a concentration of 1 μM Leu- and Met-enkephalin as well as the enkephalin analogue DSLET also slightly (by 10-13%) inhibited [^{3}H]DA release, but DADLE, DTLET, DPDPE and DAGO had no effect.

Striatal [^{14}C]ACh release

Leu- and Met-enkephalin, DADLE, DSLET, DTLET and DPDPE as well as dynorphin(1-8) all strongly inhibited the release of [^{14}C]ACh from striatal slices (Table 1). The maximal inhibition caused by these peptides was 50-65% and the EC50's varied between 5 and 100 nM. [Des-Tyr^1] Leu-enkephalin did not significantly affect [^{14}C] ACh release, whereas DAGO and dynorphin (1-13) at a concentration of 1 μM caused only a slight inhibition (by 13-15%).

Cortical [^{3}H]NA release

Leu- and Met-enkephalin, DADLE, DAGO, DSLET and DTLET all strongly inhibited the release of [^{3}H]NA from cortical slices (Table 2). The maximal inhibitory effects of these peptides varied between 40 and 75% and the EC50's between 30 and 300 nM. [^{3}H]NA release was hardly affected by DPDPE. On the other hand, dynorphin(1-13) as well as the fragment (1-8) appeared to inhibit [^{3}H]NA release at least as effectively as Leu- and Met-enkephalin.

Discussion

It has been well established by now that the inhibition of [^{3}H]DA release and that of [^{14}C]ACh release from rat striatal slices by opioids are mediated exclusively by κ- and δ-receptors, respectively, whereas [^{3}H]NA release from rat cortical slices is inhibited by opioids only if they display μ-receptor activity (10, 13, 16, 17). In the present study we have used these selective functional paradigms of C.N.S. opioid receptor sub-

Table 2 Inhibitory effects of various opioid peptides on the electrically-evoked release of [^{3}H]NA from rat cortical slices and their apparent affinities for the μ-opioid receptors involved. The inhibitory effects of 1 μM concentrations of the peptides were determined in experiments, in which the slices were exposed to 10 min of electrical stimulation (1 pps, 2 ms, 15 mA) and are the means ± s.e.m. of 12-24 values obtained in 3-6 separate experiments. The apparent affinities (pD2-values) were determined in experiments using a cumulative dose-response technique (see Methods) and are the means of 6-10 values obtained in 3-5 separate experiments; in all cases the s.e.m. values were approximately 0.1.

Opioid peptide	*[^{3}H]NA release (cortex) % inhibition at 1 μM*	*pD2*
Met-Enkephalin	45 ± 3.0*	6.7
Leu-Enkephalin	43 ± 3.2*	6.5
Dynorphin(1-8)	41 ± 3.4*	6.8
Dynorphin(1-13)	56 ± 2.7*	6.7
[D-Ala^2,D-Leu^5]enkephalin DADLE	69 ± 1.8*	7.1
[D-Ser^2]Leu-enkephalin-Thr (DSLET)	59 ± 3.8*	6.7
[D-Thr^2]Leu-enkephalin-Thr (DTLET)	70 ± 2.4*	6.6
[D-Pen^2,D-Pen^5]enkephalin (DPDPE)	7 ± 2.2*	–
Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAGO)	75 ± 1.8*	7.4

* different from control at the 0.01 level
\# different from control at the 0.05 level

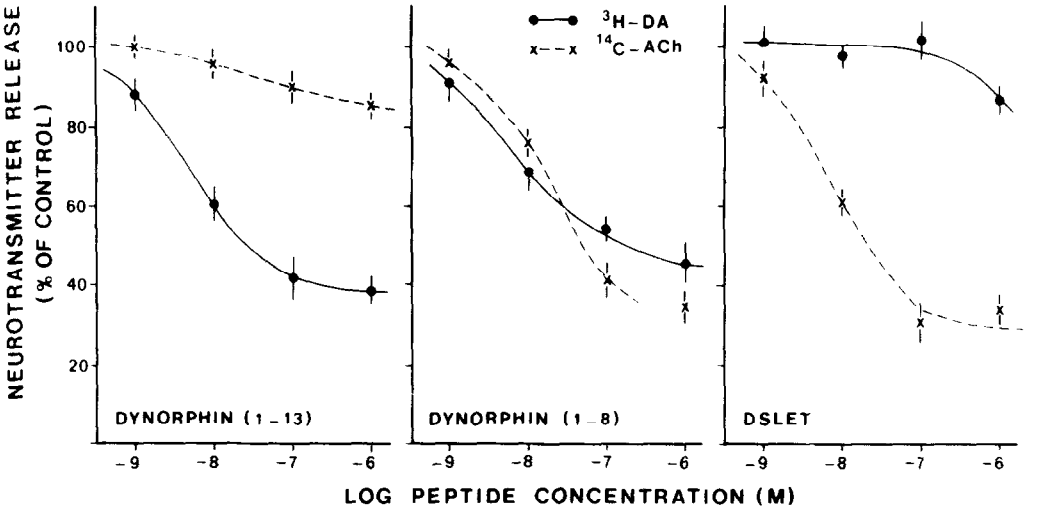

Figure Inhibitory effects of dynorphin(1-13), dynorphin(1-8) and DSLET on the electrically-evoked release of [^{3}H]DA and [^{14}C]ACh from rat striatal slices. These experiments were carried out using a cumulative dose-response technique in which the slices were exposed continuously to electrical stimulation (1 pps, 2 ms, 30 mA) (see Methods). The data are means ± s.e.m. of 6 determinations in 3 separate experiments.

types to examine the affinities and selectivities of a number of opioid peptides for these receptors.

At a concentration of 1 μM Met- and Leu-enkephalin nor any of their synthetic analogues affected striatal [^{3}H]DA release to an appreciable extent, confirming their very low affinity to κ-opioid receptors (3, 5, 12). All of the enkephalins strongly inhibited both cortical [^{3}H]NA release and striatal [^{14}C]ACh release, except DAGO, that only slightly inhibited [^{14}C]ACh release and DPDPE, that did not affect [^{3}H]NA release. DAGO appeared to display both the highest selectivity and affinity (pD2 7.4) towards μ-opioid receptors, whereas DPDPE was found to be the most selective δ-opioid receptor agonist, in agreement with data obtained with bioassays using peripheral tissue preparations and data from ligand-receptor binding studies using brain membrane preparations (3, 5, 9, 12). However, the hexapeptides DSLET and DTLET also showed an appreciable selectivity towards δ-opioid receptors: the affinity of DSLET for these receptors was 30-40 times and that of DTLET 60-80 times greater than for μ-receptors. Moreover, these hexapeptides (pD2 8.2-8.4) appeared to be about 10 times more potent than DPDPE as agonists on the δ-opioid receptors mediating inhibition of striatal ACh release. The degree of δ-selectivity found for DSLET and DTLET in the present study was higher than reported by other investigators (3, 9) on the basis of ligand-receptor binding (roughly 10-20 times higher affinity for the δ- than for the μ-binding sites in brain membranes), but lower than the selectivity for opioid receptors in peripheral tissue preparations reported by the same investigators (170-330 times higher apparent affinity for the δ-receptors in the mouse van deferens than for the μ-receptors in the guinea-pig ileum).

Met- and Leu-enkephalin as well as DADLE are far less selective towards δ-opioid receptors than the other enkephalin analogues examined, in agreement with other studies (3, 12); the ratio between the apparent affinities for δ- and μ-receptors, respectively, appeared to be 7-8. [Des-Tyr^1] Leu-enkephalin did not significantly affect striatal [^{14}C]ACh release, indicating that the Tyr^1-residue is essential for the opioid receptor agonist activity of Leu-enkephalin.

Both dynorphin(1-13) and dynorphin(1-8) have been suggested to be endogenous ligands for κ-opioid receptors (1, 2). Using bioassays with peripheral tissue preparations or ligand-receptor-binding techniques others have shown that dynorphin(1-13) exhibits a high selectivity for κ-opioid receptors, but that dynorphin(1-8) is far less selective (2, 5, 6, 12, 14). In the present study dynorphin(1-8), like dynorphin(1-13), was found

to cause a strong inhibition of striatal [^{3}H]DA release, but with an about two times lower apparent affinity for the κ-receptors involved. Both of the dynorphin A-related peptides also strongly inhibited cortical [^{3}H]NA release, with similar apparent affinities for the μ-receptors involved. This study has shown that dynorphin(1-13) has a 40-50 times greater affinity for κ- than for μ-opioid receptors. Dynorphin(1-13) had very little effect (at a conc. of 1 μM) on striatal ACh release, indicating that its affinity for δ-receptors is yet considerably lower than that for μ-receptors. In agreement with other studies mentioned above, dynorphin(1-8) appears to be much less selective than dynorphin(1-13). In fact, the apparent affinity of dynorphin(1-8) for the κ-receptors studied here was found to be only about three times higher than that for the δ-receptors and 15-20 times higher than that for the μ-receptors. In this context it is interesting to note that yet a smaller fragment, i.e. dynorphin(1-5), which in fact is Leu-enkephalin, lacks affinity for κ-receptors, but retains the same apparent affinities as dynorphin(1-8) for δ- and μ-receptors.

Finally it should be noted here, that the present study was carried out without using peptidase inhibitors to prevent breakdown of opioid peptides (2, 8). Therefore, the estimated apparent affinities of some of the peptides, viz. Met- and Leu-enkephalin and dynorphin(1-8), which are known to be liable to degradation by peptidases may have been underestimated in this study. However, it is unlikely that in the presence of peptidase inhibitors the *ratio's* between the affinities for the respective opioid receptor subtypes, i.e. the estimated degree of selectivity of these peptides, would be much different from those found in the present study.

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