Saturable [D-Ala^2, D-Leu^5]-enkephalin transport into cholinergic synaptic vesicles

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Incubation of cholinergic synaptic vesicles purified from the electric organ of *Torpedo ocellata* with radiolabeled [D-Ala^2, D-Leu^5]-enkephalin results in its accumulation in the vesicles. Kinetic analysis of the initial rates of uptake reveals temperature-dependent saturation kinetics which are best fitted by a single transport system ($K_T = 12 \pm 2$ μM and $V_{max} = 0.85 \pm 0.24$ pmol/mg protein per min). The specific rates and extents of [^{3}H]-[D-Ala^2, D-Leu^5]-enkephalin uptake, like those of [^{3}H]acetylcholine uptake, are highest in the purified synaptic vesicles fraction. These findings suggest that *Torpedo* cholinergic synaptic vesicles contain an opioid peptide transporter. The physiological significance of this transporter is discussed in view of the recent observation that *Torpedo* nerve terminals contain an endogenous enkephalin-like peptide and presynaptic opiate receptors.

Enkephalin uptake *Synaptic vesicle* *Acetylcholine* Torpedo

1. INTRODUCTION

The recent findings that the cholinergic nerve terminals of the *Torpedo* electric organ contain an endogenous enkephalin-like peptide and that acetylcholine (ACh) release from these neurons is inhibited by morphine in a naloxone reversible fashion [1] suggest that the efficacy of cholinergic transmission in *Torpedo* is modulated by endogenous opiates.

In mammalian brain, enkephalin is derived from a propeptide which is synthesized in the cell body and packaged into vesicles. The propeptide is then processed by proteolytic cleavage during axoplasmic transport [2]. Recently, it has been shown that isolated brain nerve terminals (synaptosomes) and synaptic vesicles contain specific enkephalin transporters [3,4]. This suggests that in mammalian brain enkephalinergic neurons also contain a recycling mechanism by which the released peptides may be replenished. The intraterminal compartmentation and metabolism of the *Torpedo* enkephalin-like peptide and its interaction with the life cycle of ACh have not yet been determined.

This work investigated the possibility that *Torpedo* cholinergic synaptic vesicles take up and accumulate opioid peptides. The results obtained indicate that incubation of the vesicles with the stable enkephalin analog [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin results in its uptake, via a saturable transporter, into the vesicles. This vesicular transporter may function together with the ACh carriers [5,6], in replenishing the vesicular contents following presynaptic stimulation.

2. EXPERIMENTAL

2.1. *Purification of* Torpedo *electric organ synaptic vesicles*

Torpedo electric organs were excised from live *T. ocellata* during the spring and stored at -70°C till use (up to 9 months). Synaptic vesicles were purified from frozen electric organs by differential and density gradient centrifugation and by permeation chromatography on a controlled pore glass beads column as described for freshly excised

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tissue [7], except that the frozen electric organs were crushed with a pestle and mortar prior to thawing and homogenization. The purified synaptic vesicles (fraction SV of [7]) were stored in liquid nitrogen for up to a month without any detectable decrease in their ACh content.

2.2. *Measurement of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin uptake into synaptic vesicles*

Prior to the experiment the vesicles were incubated for 16 h at 25°C in 0.8 M glycine, 50 mM Hepes (pH 7.4). This resulted in depletion of their endogenous neurotransmitter store. The vesicles were then washed by centrifugation (250000 × *g* for 1 h) and resuspended (0.2–0.3 mg protein/ml) in the above buffer. Uptake was initiated by a 1:1 dilution of the vesicles with 0.8 M glycine, 50 mM Hepes (pH 7.4) which contained [D-Ala^2,D-Leu^5]-enkephalin at the indicated concentration and [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin (2 μCi/ml). The reaction mixture also contained captopril (10 μM) and puromycin (50 μM) which inhibit endogenous peptidase activities [8]. The amount of radioactivity taken up by the vesicles was determined at the indicated time intervals by placing aliquots (20 μl) of the reaction mixture on 0.45 μm Millipore filters (HAWP) and rapidly washing (3 × 2.5 ml) with ice cold buffer. The amount of radioactivity retained on the filter was then measured by scintillation spectrometry.

Thin-layer chromatography (TLC) of the opioid peptides was performed as described by Altstein et al. [8]. Protein was determined as in [9].

3. RESULTS AND DISCUSSION

Incubation of purified *Torpedo* synaptic vesicles with [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin (2 μM) at 25°C resulted in the accumulation of radiolabel in the vesicles (fig.1). Uptake was linear for up to 15 min (0.14 ± 0.04 pmol/mg protein per min; n = 11) and then leveled off to a plateau (2.9 ± 0.6 pmol/mg protein per min; n = 8). When vesicles preloaded with [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin were subjected to a 100-fold dilution with water, about half the vesicle-associated radioactivity was lost within 1 min of the dilution (51 ± 9% of the total; n = 3), whilst only a small fraction (11 ± 7% of the total; n = 3) was released upon a similar dilution with isotonic buffer (fig.2).

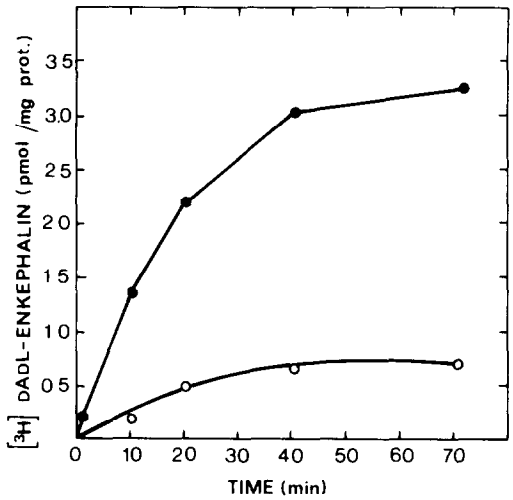

Fig.1. Time course of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin uptake ([^{3}H]-DADL-enkephalin) by cholinergic synaptic vesicles purified from the *Torpedo* electric organ. Uptake was measured at 2 μM by filtration at 25°C (●) and 4°C (○) as described in section 2.

After 10 min of the addition of water there was a small increment in the amount of radioactivity lost from the vesicles (56 ± 9% of the total; n = 2), whereas an increase in the loss of radioactivity (35 ± 3% of the total; n = 2) was observed with the isotonic buffer. Addition of Triton X-100 (0.1%) to vesicles preloaded with [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin resulted in the rapid loss of virtually all the vesicle-associated radioactivity (fig.2). These findings suggest that the accumulated radiolabel is indeed transported into the vesicles. Analysis by TLC [8] of the material taken up by the vesicles, revealed that virtually all vesicle bound radioactivity (>90%) cochromatographed with a [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin standard (not shown).

Examination of the dependence of the initial rate of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin uptake (determined from the slope of the uptake curve during the first 15 min) on the ligand concentration revealed that the rate of uptake saturated (fig.2) and that within the concentration range investigated (2–30 μM) the results were best fitted with a single transport system with K_T = 12 ± 2 μM and V_{max} = 0.85 ± 0.24 pmol/mg protein per min (n = 3). Examination of the temperature dependence of the initial rate of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin (2 μM) influx revealed that it was much slower (6-fold) at 4°C than at 25°C (fig.1).

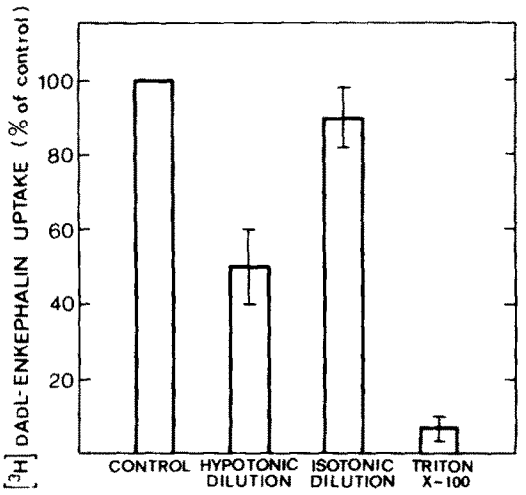

Fig.2. The effect of hypotonic and isotonic dilution and of Triton X-100 on the accumulation of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin ([^{3}H]-DADL-enkephalin) within *Torpedo* synaptic vesicles. Uptake was performed at 25°C using 2 μM of the opioid peptide until a plateau was reached (t = 60 min), after which the vesicles were diluted 1:100 with either water, isotonic buffer (0.8 M glycine, 50 mM Hepes, pH 7.4), or isotonic buffer which contained Triton X-100 (0.1%, w/v). The radioactivity retained within the vesicles 1 min after the dilution was determined by filtration as described in section 2. Results presented are the mean ± SD of 3 experiments. Control (100% = 2.8 ± 0.7 pmol/mg protein; n = 3) corresponds to the amount of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin taken up by the vesicles prior to the dilution. Results presented are the mean ± SD of 3 experiments.

These findings suggest that [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin is taken up into the vesicles by facilitated diffusion via a saturable transport system.

The extent of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin uptake, unlike its rate, did not saturate but rather increased linearly with the external ligand concentration (2–300 μM). Calculation of the intravesicular concentration of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin from the amount of opioid peptide taken up by the vesicles (e.g., 2.9 ± 0.6 pmol/mg protein at 2 μM; n = 8) and from the known vesicular volume (3.8 μl/mg protein) [6], yielded a value which was 2.5-fold lower than that of the external medium. This suggests that either the extent of uptake is coupled to a thermodynamically limiting factor (e.g., $\Delta\psi$ or ΔpH) or that *Torpedo* synaptic vesicles are heterogeneous and contain subpopulations which differ in their ability to accumulate [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin. The possibility that the opioid peptide is taken up by vesicles which differ completely from those which accumulate ACh is unlikely in view of the purity of the synaptic vesicle fraction [7], and the finding that the rates and extents of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin uptake, like those of [^{3}H]ACh [6], are highest in the purified synaptic vesicle fraction (e.g., at 2 μM [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin, the specific rates and extents of uptake by the crude vesicular fraction P_3 [7] are, respectively, 6- and 10-fold lower than those of the purified vesicles).

The opiate receptor agonist morphine (10 μM), and antagonist naloxone (10 μM) had virtually no effect on either the rate (94 ± 15 and 120 ± 14% of control respectively; n = 2) or the extent (96 ± 20 and 102 ± 10% of control respectively; n = 2) of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin (2 μM) uptake. This suggests that the ligand recognition site of the vesicular transport system differs from that of the *Torpedo* presynaptic opiate receptor [10].

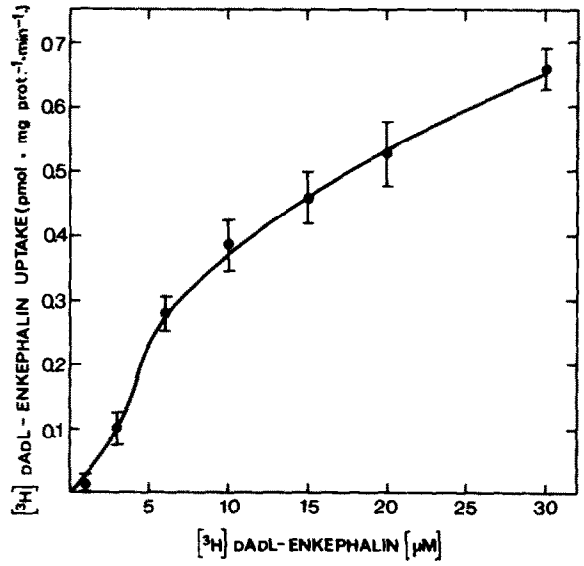

Fig.3. The dependence of the initial rate of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin uptake ([^{3}H]-DADL-enkephalin) by *Torpedo* synaptic vesicles on ligand concentration. Initial rates were calculated from the slopes of the linear phase (0–15 min) of uptake which was performed as described in section 2. Results presented are the mean ± SD of 3 experiments.

The physiological role of the vesicular opioid transport system is not yet known. Preliminary studies indicate that ATP enhances the rate of [^{3}H]-[D-Ala^2,D-Leu^5]-enkephalin uptake and that incubation of the vesicles with [^{3}H]Leu-enkephalin in the absence of peptidase inhibitors results in the uptake of both the intact peptide and its breakdown products (in preparation). Thus, it is likely that the vesicular opioid peptide transport system, like the ACh carrier [5,6,11], is involved in reloading the vesicles following stimulation. However, the possibility that its function is to release endogenous vesicular peptides cannot be ruled out. Further studies of the uptake of intact and fragmented enkephalin into synaptic vesicles and isolated synaptosomes will undoubtedly further our understanding of the life cycle of opioid peptides in the *Torpedo* cholinergic nerve terminal.

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