Functional and molecular characterization of a peptide transporter in the rat PC12 neuroendocrine cell line

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Abstract **We have studied functional properties of peptide transport in the pheochromocytoma neuroendocrine cell line from rat. The neutral peptide D-Phe-L-Ala (resistant to hydrolysis) is a good substrate for uptake into these cells. Transport is substantially inhibited by diethylpyrocarbonate pretreatment and is stimulated by external acidification. It is sodium-independent and, unexpectedly, insensitive to membrane potential. Peptide uptake is inhibited by a wide variety of other di- and tripeptides but not by amino acids. The neuropeptide kyotorphin (opioid dipeptide (L-Tyr-L-Arg)) inhibits uptake of labelled peptide and trans-stimulates efflux showing that it is a transported substrate. These findings are discussed in relation to the molecular basis and physiological role of this transport system.** **© 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Peptide transport; Peptide transporter-1; Kyotorphin; Neuroendocrine cell; PC12 cell

1. Introduction

It is well recognized that small peptides are absorbed into epithelial cells [1] by one of the two members (PepT1 or PepT2) of the H^+/oligopeptide transporter family (see [2] for review). Peptide transport in neurons or endocrine cells is less well studied. For example, it is not clear how peptide breakdown fragments produced from biologically active small neuropeptides in the central nervous system are removed from the interstitial fluid.

In a preliminary study, we reported peptide transport activity in neuroendocrine cells [3]. In the present study, we have systematically examined whether the biologically active kyotorphin (L-Tyr-L-Arg), an endogenous opioid [4], also interacts with the peptide transporter (PepT) in neuroendocrine cells as it does in brain synaptosomes [5]. A rat pheochromocytoma cell line (PC12) has therefore been used as a model to explore and screen for endogenous PepTs.

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Abbreviations: DEPC, diethylpyrocarbonate; PC12, pheochromocytoma cell line; Kyotorphin, opioid dipeptide (Tyr-Arg); PepT, peptide transporter

2. Materials and methods

2.1. Chemicals

Hydrolysis resistant D-[^{3}H]Phe-L-Ala was custom-synthesized commercially (Zeneca, Northwick, Cheshire, UK). Kyotorphin was bought from Bachem Ltd. (UK). All other chemicals were of high analytical grade purchased from Sigma (UK) unless otherwise stated.

2.2. Cell culture

PC12 cells (passage 30–49) were maintained in 75 cm^2 flasks in complete RPMI 1640 (Gibco Life Technologies, UK) medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 4 mM glutamine and 175 μg/ml gentamicin at 37°C in a 5% CO_2 and 95% air environment as described previously [6].

2.3. Flux studies

Prior to transport experiments, adherent cells were detached from culture flask by 1 min treatment with an EDTA/trypsin (containing 0.05% w/v porcine trypsin and 0.02% w/v EDTA) solution at 37°C. Cells were resuspended and washed/centrifuged (1200 rpm) twice for 5 min in excess PBS (without Mg^{2+} and Ca^{2+}) at room temperature. Cells were then resuspended in complete medium (see above) and cell number and viability was assessed by trypan blue exclusion. Cells were plated and grown in 12-well plates at initial density of 500 000 cells/well in 2 ml complete medium. Fresh medium was added 1 day prior to the flux experiments.

2.3.1. Influx experiments. Transport experiments were carried out on cells that had become fully confluent (at 4 days in culture). Prior to uptake experiments, cells were washed and incubated in RPMI 1640 medium (4 mM glutamine but with serum absent) for 1 h at 37°C in a 5% CO_2 and 95% air environment. Following this step, cells were equilibrated (5–10 min, 37°C) in HEPES-buffered Krebs (pH 7.4). The Krebs composition was (in mM): 120 NaCl, 4.5 KCl, 1 $MgSO_4$, 1.25 $CaCl_2$, 10 glucose buffered with either 25 HEPES or 29 MES.

The initial rate of uptake of the hydrolysis-resistant peptide [^{3}H]D-Phe-L-Ala (420 nM, specific activity 10 Ci/mmol) was determined at 37°C in the absence or presence of potential competing substrates. Uptake was rapidly stopped by quick aspiration and cells were washed three times in 3 ml ice-cold Krebs (pH 5.5 or 7.4) to remove remaining extracellular radioactivity and the cells dissolved in 1 ml (1 M) NaOH. Aliquots were then taken for scintillation counting. All experiments were performed at pH 5.5 (MES-buffered Krebs) unless otherwise stated. Non-specific uptake (i.e. 0 s) was subtracted from all data points, this was typically less than 10% of the mediated uptake.

2.3.2. Efflux experiments. Cells were preloaded with [^{3}H]D-Phe-L-Ala (420 nM, specific activity 10 Ci/mmol) for 30 s at pH 5.5 (37°C) and then washed three times in 3 ml Krebs (pH 5.5) at room temperature to remove any remaining extracellular radioactivity. The efflux of labelled dipeptide was initiated by the addition of isotope-free solutions with or without unlabelled peptide substrate. The reaction was rapidly stopped at different time points by quick aspiration. The cell radioactivity in each well was determined as described above.

2.3.3. Diethylpyrocarbonate (DEPC) pretreatment. Immediately before the uptake experiment, DEPC was made up fresh in Krebs from a working stock (100 mM in 50% ethanol) kept at 4°C. Cells in each well were treated with 1 ml of DEPC (1 mM) for 10 min at room temperature on a slow speed shaker plate. In control cells, 0.1% ethanol was added as a vehicle for DEPC. After pretreatments, cells

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Table 1
Rat primers used for RT-PCR in cultured PC12 cells

Rat gene	Oligonucleotide sequence	Nucleotide number	Product size (bp)
PepT1			
	Sense: 5′-GCGAGGTGGTCTTCTCTGTC-3′	1838–1858	444
	Anti-sense: 5′-TCTCCAGGCAAGGACTCTGT-3′	2261–2281	
PepT2			
	Sense: 5′-ACCTTGTCATTTACCGGCTG-3′	1239–1259	260
	Anti-sense: 5′-AAAAGAGCTGACGGACTCCA-3′	1478–1498	
PHT1			
	Sense: 5′-TGGAGCATTAATTTGGGAGC-3′	653–673	381
	Anti-sense: 5′-CACAATCTTGACCAGGGCTT-3′	1013–1033	
PHT2 (accession number AB026665)			
	Sense: 5′-AATCTGGGTGCCATTCTGTC-3′	728–748	379
	Anti-sense: 5′-CACAAGGGTCACCATCACAG-3′	1086–1106	
GAPDH			
	Sense: 5′-GGTGATGCTGGTGCTGAGTA-3′	264–283	304
	Anti-sense: 5′-CCACAGTCTTCTGAGTGGCA-3′	570–589	

were washed once in 2 ml of Krebs (pH 5.5) at room temperature and immediately placed in a water bath (37°C) for the transport experiment.

2.3.4. Protein determination. Protein concentration of cells in each well was determined using the BCA Protein Assay kit with bovine serum albumin as a standard (Pierce, Rockford, IL, USA).

2.4. mRNA analysis

mRNA analysis was carried out using RT-PCR as described by [7] using rat specific primers for PepT1, PepT2, PHT 1, PHT 2 and GAPDH as summarized in Table 1. RNA samples were treated with DNase I prior to RT-PCR to remove any contaminating DNA. One microgram RNA was reverse transcribed into cDNA using an oligo(dT)$_{12-18}$ primer. The reverse transcription reaction was sequentially incubated at 25°C for 10 min, at 42°C for 50 min and at 70°C for 15 min, and then cooled on ice. The synthesized cDNA was used for PCR amplification. The PCR conditions were: 94°C for 1 min, 60°C for 1 min and 72°C for 2 min; then 30 cycles for PepT1, 40 cycles for PepT2, PHT1 and PHT2, and 25 cycles for GAPDH of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min; followed by a 10 min final extension at 72°C. To check for DNA contamination, reactions were run with or without RNA in the absence of the reverse transcriptase and revealed no amplified product (data not shown). PCR products were separated on a 2% agarose gel. Gels were stained with ethidium bromide.

2.5. Statistical analysis

The effects of inhibitors of peptide influx was determined by a *t*-test. Statistical significance was taken at the 5% level. The (*n*) number represents the number of wells examined in each experimental condition. For efflux experiments, the time course was fitted by a mono-exponential fit using Sigma Plot and statistical analysis of the rate constants for efflux was determined.

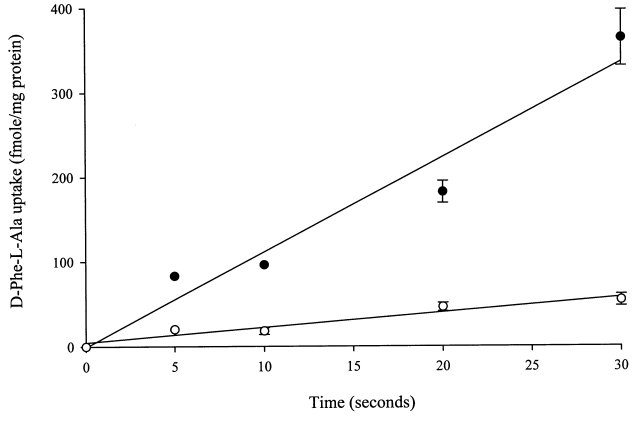

Fig. 1. Time course of D-[^{3}H]Phe-L-Ala (420 nM) influx into cultured PC12 cells. Uptake was determined at pH 5.5 and at 37°C in cells that had (open circles) or had not (closed circles) been subject to pretreatment with DEPC (1 mM). The error bars show mean ± S.E.M. for $n = 3$–4 for each data point.

3. Results

Fig. 1 shows the time course of uptake of labelled dipeptide into PC12 cells over a period of 30 s at 37°C. The effects of pre-exposure of the cells to DEPC on the time course of peptide influx is also shown. It is apparent that the rate of entry is linear over time for up to 30 s and that DEPC pretreatment strongly inhibits influx. In the presence of DEPC, the rate was reduced to approximately 20% of that in the control.

Fig. 2 shows that D-Phe-L-Ala uptake is stimulated by external acidification (the rate of influx at pH 5.5 is some four-fold greater than at pH 7.4). This effect was not observed for the DEPC-insensitive component. At each external pH, there is no significant difference in the rate of peptide entry when the cells are exposed to high KCl (isotonically replaced by

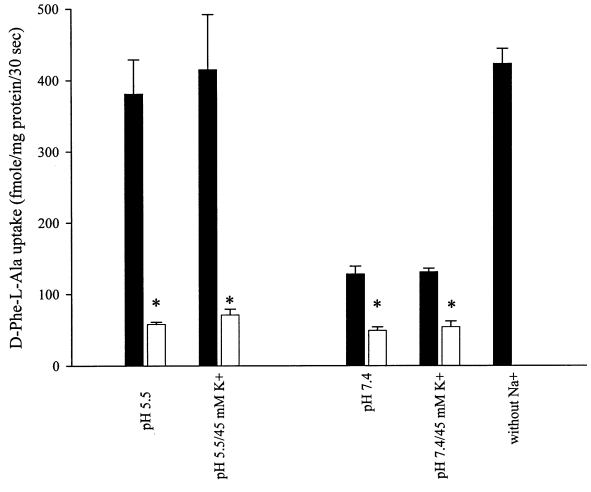

Fig. 2. Influx over 30 s of D-[^{3}H]Phe-L-Ala (420 nM) into cultured PC12 cells at pH 5.5 and 7.4 in the presence or absence of 45 mM K^+. Open bars show influx into cells pretreated with DEPC (1 mM) and closed bars show influx in untreated control cells. This figure also shows influx over 30 s of D-[^{3}H]Phe-L-Ala (420 nM) into cultured PC12 cells at pH 5.5 in the presence (far left closed bar) or absence (choline substitution; far right closed bar) of Na^+. The data are shown as mean ± S.E.M. for $n = 3$ where $^*P < 0.05$

NaCl). This suggests that transport of peptide is not influenced by membrane depolarization. As also shown in Fig. 2, peptide transport is not altered by sodium removal (isotonically replaced by choline). This suggests that peptide transport is sodium independent.

Fig. 3 shows that the stimulation of transport by external acidification is near maximal at pH 6.5.

Fig. 4 shows the inhibitory effects of a number of competing substrates on labelled dipeptide influx. Both di- and tripeptides at 1 mM are effective inhibitors (Fig. 4a). The amino acid sequence within a given peptide appears to influence quantitatively the ability of that unlabelled peptide to inhibit influx. For example, at equimolar concentration Gly-Tyr is less potent than Tyr-Gly. In addition to natural di- and tripeptides, a peptide space mimic (4-amino methylbenzoic acid [8]) is also an effective inhibitor. A number of naturally occurring compounds are also able to inhibit peptide influx (Fig. 4b). Antibiotics (ampicillin and cephapirin), bioactive peptides (formylmethionylarginylphenylalanyl amide and thyrotropin releasing hormone (β-Glu-His-Pro amide)) and kyotorphin (an opioid dipeptide) are all effective inhibitors. Strikingly, at equimolar concentration, the amino acid constituents of the kyotorphin molecule (Tyr+Arg) were ineffective.

The DEPC-sensitive component of peptide influx was inhibited in a concentration-dependent way by kyotorphin. The concentration of peptide giving half maximal inhibition was 0.35 ± 0.08 mM (mean ± S.E.M., $n = 3$–7) (Fig. 5). In contrast, there was minimal inhibition of the DEPC-insensitive flux by kyotorphin.

Because of the inhibitory effect of this biologically active peptide (which is known to be synthesized in adrenal medulla) [4], we wished to discover whether this molecule was itself a substrate for the PepT. Fig. 6 shows the results of an efflux assay. Addition of unlabelled kyotorphin trans-stimulated the exit of labelled peptide from preloaded PC12 cells. The rate constant for peptide efflux is 8.7 ± 1.9 in the absence and $61.7 \pm 22 \times 10^{-3}$ s^{-1} ($n = 3$) in the presence of 5 mM kyotorphin. This is proof that kyotorphin is a translocated substrate. Figure 6b shows that kyotorphin but not its constituent amino acids stimulates labelled peptide efflux in a concentration-dependent manner with a K_t (0.95 ± 0.37 mM, mean ± S.E.M., $n = 3$) similar to the concentration of kyotorphin giving half maximal inhibition of DEPC-sensitive D-Phe-L-Ala influx (Fig. 5).

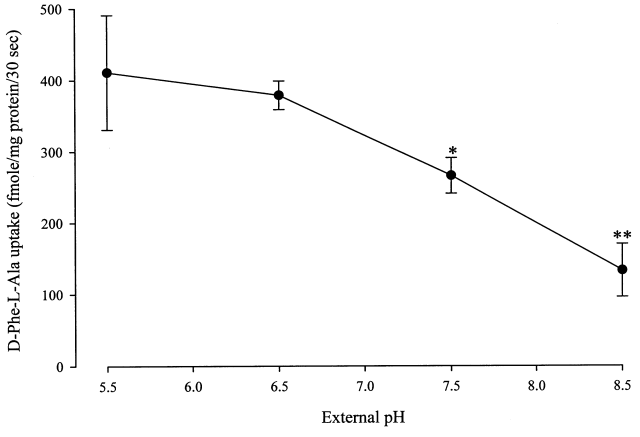

Fig. 3. Influx over 30 s of D-[^{3}H]Phe-L-Ala (420 nM) into cultured PC12 cells at different external pH. Data are shown as mean ± S.E.M. for $n = 3$ where $^*P < 0.03$ and $^{**}P < 0.01$ compared with influx at pH 5.5.

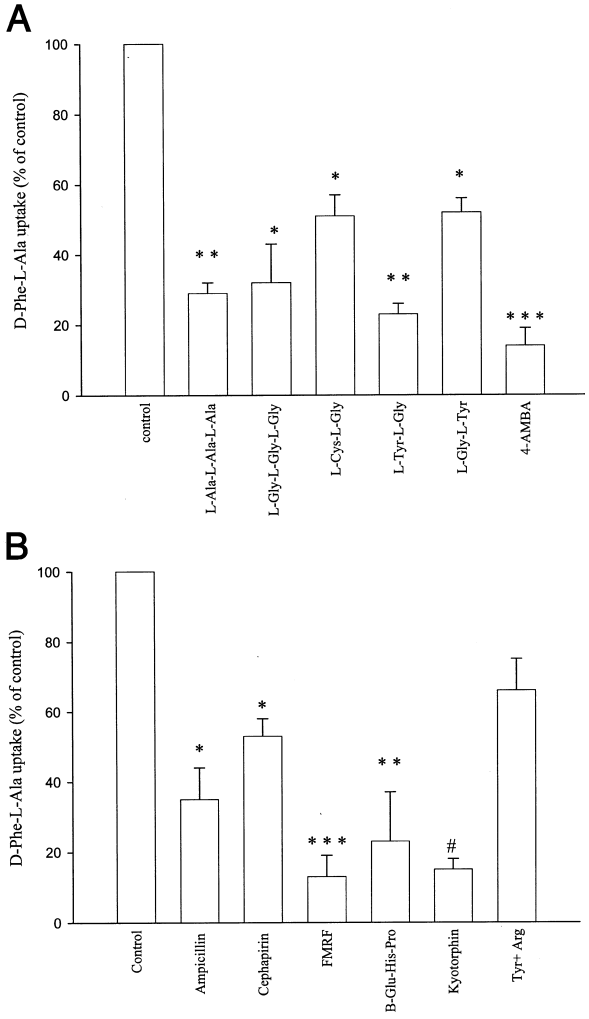

Fig. 4. A: 30 s uptake (normalized to control) of DEPC-insensitive D-[^{3}H]Phe-L-Ala (420 nM) flux into cultured PC12 cells at pH 5.5 in the presence or absence of di- and tripeptides (all at 1 mM). The data are shown as mean ± S.E.M. for $n = 3$–6 for each inhibitor tested with $^*P < 0.05$, $^{**}P < 0.02$ and $^{***}P < 0.01$ compared with control sample (4-AMBA, 4-amino methylbenzoic acid). B: 30 s uptake (normalized to control) of DEPC-insensitive D-[^{3}H]Phe-L-Ala (420 nM) flux into cultured PC12 cells at pH 5.5 in the presence or absence of biologically active peptides (all at 1 mM with the exception of kyotorphin and its constituent amino acids (Tyr+Arg), both of which were used at 5 mM). (FMRF, formylmethionylarginylphenylalanyl amide.) The data are shown as mean ± S.E.M. for $n = 3$ for each inhibitor tested where $^*P < 0.05$, $^{**}P < 0.02$ and $^{***}P < 0.01$ compared with influx in control cells. The influx in the presence of kyotorphin was significantly lower ($^{\#}P < 0.01$) compared with that in the presence of its constituent amino acids Tyr+Arg.

Fig. 7 shows that PepT1 but not PepT2, PHT1 or PHT2 mRNA is detected with the predicted product size (444 bp) by RT-PCR. Immunocytochemistry however, using an anti-rat C-terminal PepT1 antibody [9] revealed no immunoreactivity in PC12 cells (data not shown).

4. Discussion

These functional studies suggest that there is a PepT in PC12 cells. This transporter, however, has some features

that are unexpected, specifically the finding that peptide transport is electroneutral (see Fig. 8). This is in contrast to the well-studied electrogenic properties of currently known PepTs [10]. In other respects, however, the system we describe here has obvious similarities to PepT1 and PepT2. It is stimulated by external acidification and this stimulation is abolished (data not shown) by a proton ionophore (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone); it is sodium-independent and DEPC-sensitive (a well-known histidine modifier that effectively inhibits epithelial peptide transport [11]). Moreover, small peptides (di- and tripeptides) appear to be substrates, whereas amino acids are not. The high micromolar substrate affinity suggests greater similarity to PepT1 than PepT2.

In addition to PepT1 and PepT2, a novel transporter was recently identified [12]. This transporter (PHT1) was unusual in that in addition to peptides it also translocated the amino acid histidine. Our transporter is not PHT1 since we have measured labelled histidine influx into PC12 cells in the presence or absence of 5 mM unlabelled kyotorphin. No inhibition of amino acid influx was observed (80 ± 17 in the absence compared to 77 ± 7 fmol/mg protein/30 s in the presence of 5 mM unlabelled kyotorphin (mean ± S.E.M., $n = 3$)). Moreover, neither PHT1 nor PHT2 mRNAs are expressed in PC12 cells.

A further member of the PepT family is PepT3 (human)/cI-1 (mouse macrophage). This molecule has been identified through in silico genomics (accession number AB026665) but not characterized functionally. The expression of this transporter is induced by cAMP. However, again preliminary experiments suggest that PepT3 is not the system we describe since there was no change in the rate of peptide influx following pharmacological stimulation. For example, after 1 h pretreatment with 500 μM CPT-cAMP, a membrane permeable analogue of cAMP, the rate of D-Phe-L-Ala influx was 568 ± 64 compared to 593 ± 36 fmol/mg protein/30 s in the absence of stimulation (mean ± S.E.M., $n = 3$). The most likely explanation for our functional findings is that PC12 cells express a variant of PepT1 which lacks the C-terminal 18 amino

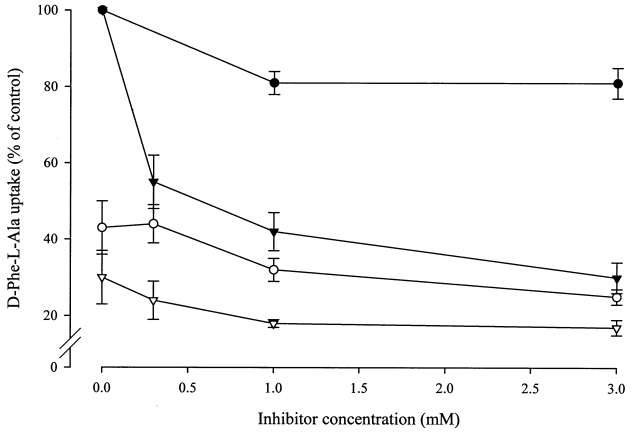

Fig. 5. Influx over 30 s of D-[^{3}H]Phe-L-Ala (420 nM) into cultured PC12 cells in the presence of different concentrations of substrates either unlabelled kyotorphin (triangles) or its constituent amino acids Tyr+Arg (circles). Filled symbols represent uptake in the absence of, and open symbols represent uptake following, pretreatment with DEPC (1 mM). The data are shown as mean ± S.E.M. for $n = 3$–6 for each data point. The rate of uptake in control samples was 335 ± 35 fmol/mg protein/30 s.

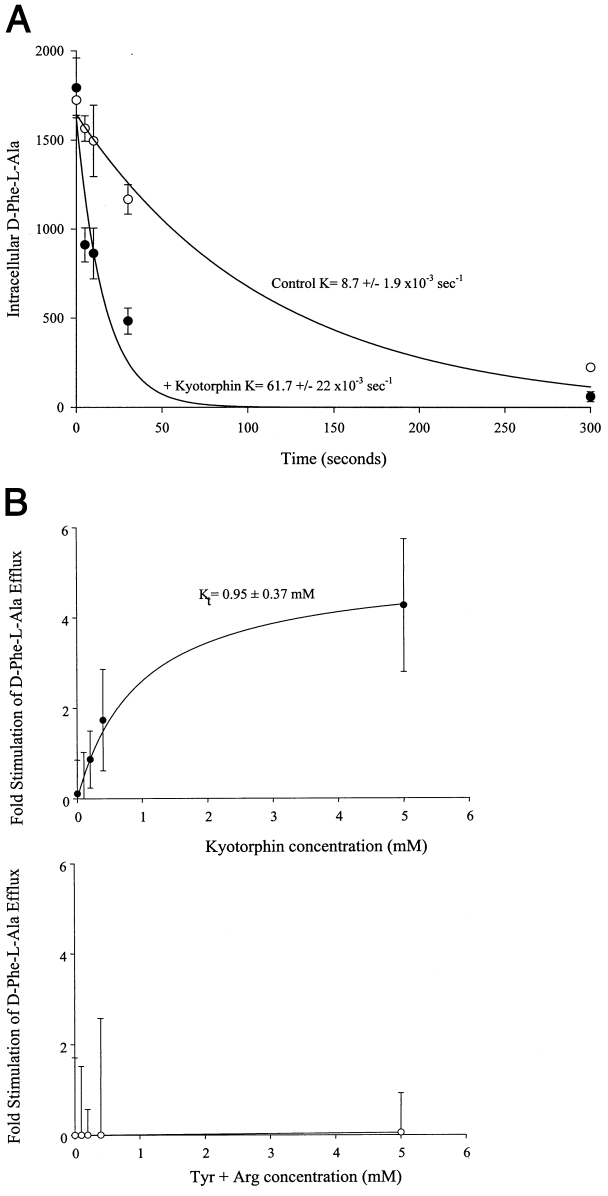

Fig. 6. A: D-[^{3}H]Phe-L-Ala (fmol/mg protein) remaining in PC12 cells preloaded with labelled peptide (420 nM) following efflux into a medium with (filled symbols) or without (open symbols) 1 mM kyotorphin. The data are shown as mean ± S.E.M. for $n = 3$ for each data point. Lines of best fit for a monoexponential efflux have been generated (Sigma Plot) and the rate constants describing these lines are shown. B: Stimulation of labelled D-Phe-L-Ala efflux by increasing concentration of kyotorphin (top plot) but not its constituent amino acids (bottom plot).

acids and which also has a catalytic cycle that is electroneutral. This proposal needs further experimental analysis.

Functionally, why might PepTs be expressed in neuroendocrine cells? It is interesting that the endogenous PepT found in this cell line is able to translocate the biologically active neuropeptide kyotorphin. This peptide is synthesized [4] in the adrenal medulla and up until now its neural release has been assumed to be by exocytosis. An alternative explanation for the mechanism of kyotorphin release is by reversed uptake (cf. Fig. 6). Additionally and alternatively, PepT expression in adrenal medulla may be required for neuropeptide uptake and metabolism as elsewhere in both the central [5] and peripheral

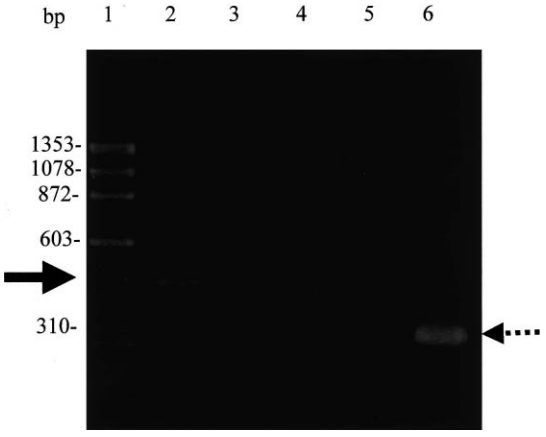

Fig. 7. Ethidium bromide-stained agarose gel showing RT-PCR products amplified from cultured rat PC12 cells. Molecular weight markers are shown on the left (lane 1) followed by mRNA products for rat PepT1, PepT2, PHT1, PHT2 and GAPDH (lanes 2,3,4,5 and 6, respectively). The expected band size of GAPDH was 304 bp (dotted arrow) and that for PepT1 was 444 bp (thick arrow).

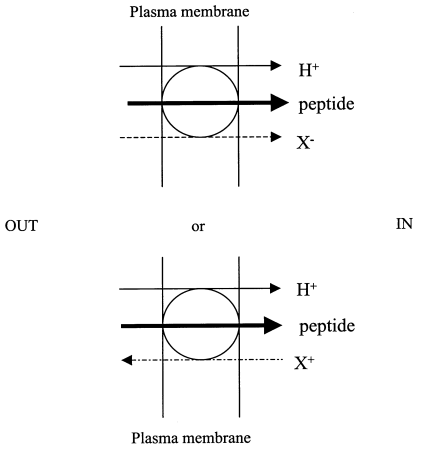

Fig. 8. Schematic diagram of peptide transport in PC12 cells. In the upper panel, cotransport of proton and peptide is accompanied by symport with an anion (X^-); in the lower panel, cotransport of proton and peptide is accompanied by antiport of cation (X^+).

[13] nervous systems. The neuropeptide nerve growth factor (NGF) is known to stimulate differentiation of PC12 cells towards a sympathetic neuronal phenotype. Initial experiments with NGF (50 ng/ml) showed that such differentiation had no detectable effect on the functional properties of this PepT.

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