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Characterisation of the expression and post-translational processing of the preprotachykinin-I gene and the regulated release of tachykinins by the RINm5F cell-line

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Normal transcription and postranslational processing of the preprotachykinin (PPT)-I gene and regulated release of substance P and neurokinin A by the rat pancreatic endocrine cell-line, RINm5F, has been demonstrated, using radioimmunoassays (RIAs), reversed-phase (rp)HPLC and Northern blot analysis. This is the first stable cell-line found to express the PPT-I gene and provides an opportunity for investigating PPT-I gene expression and tachykinin biosynthesis. RIN5mF cells are a model for the pancreatic beta-cell, which is not known to exhibit PPT-I gene expression which may, therefore, be a feature of the transformed state of these cells. These data may imply that the tachykinins are important in pancreatic islet embryogenesis.

Preprotachykinin-I; Tachykinin; RINm5F cell; Post-translational processing; Regulated release

1. INTRODUCTION

The PPT-I gene codes for the tachykinin peptide, substance P, which has been the most extensively studied of neuropeptides and is involved in the regulation of many physiological processes [1]. However, substance P is only part of the mammalian tachykinin signal. Three other tachykinin peptides, neurokinin A and the neurokinin A-derived, neuropeptide K and neuropeptide c also arise from the PPT-I gene [2]. In addition there is a second tachykinin gene, the PPT-II gene which codes for a single tachykinin, neurokinin B [3,4]. The PPT-I and PPT-II genes are differentially expressed and seem, therefore, to be under different regulatory control [5]. The biosynthesis of the PPT-I gene involves differential RNA splicing and differential precursor processing [6]. Pharmacological studies show that the tachykinin products of PPT-I gene expression have variable potencies and may interact in novel ways [7]. Therefore the differential biosynthesis of these peptides will influence the nature of the tachykinin signal. The availability of a stable cell line expressing the PPT-I gene should be valuable in studying factors regulating PPT-I gene expression and the mechanisms of differential biosynthetic processing.

This paper reports that the rat insulinoma cell-line, RINm5F, [8] expresses the preprotachykinin (PPT)-I gene. This is the first report of a stable cell-line expressing the PPT-I gene. The results indicate that the biosynthetic products of PPT-I gene expression in the RINm5F cell are similar to those observed in examples of normal tissues in which the PPT-I gene is expressed. Therefore this cell-line should serve as a good model for further studies. Additionally, this study extends the biochemical characterisation of the RINm5F cell-line. This cell-line is a tumor cell-line which possesses many of the differentiated characteristics of the pancreatic β-cell for which it is a widely-used model [8]. Although the pancreatic β cell does not normally express the PPT-I gene our finding may relate to an ontogenetic characteristic of the pancreatic β cell.

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Abbreviations: PPT, preprotachykinin; SP, substance P; NKA, neurokinin A; NKB, neurokinin B; IR, immunoreactivity; RIA, radioimmunoassay; rpHPLC, reversed-phase high-pressure liquid chromatography; EDTA, ethylene diaminetreta-acetic acid; 8-Br-cAMP, 8-bromo-cyclic adenosine monophosphate; HEPES, *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid.

2. MATERIALS AND METHODS

2.1. *Materials*

Peptides were supplied by Peninsula Laboratories (St Helens, UK) and by Bachem Biochemica GmbH (Heidelberg, Germany). The PPT-C-flanking peptide fragment used as RIA standard was supplied by Cambridge Research Biochemicals (Cambridge, UK). HPLC solvents were obtained from Merck GmbH (Darmstadt, Germany). Chemicals for RNA extraction and analysis were obtained from Paesel and Lorei GmbH (Frankfurt, Germany) and Fluka Chemika-Biochemika

GmbH (Neu-Ulm, Germany). 8-Bromo-cyclic adenosine monophosphate (8-Br-cAMP) and RNasin and Proteinase K were obtained from Boehringer Mannheim Biochemika GmbH (Mannheim, Germany). Bestatin and phosphoramidon were obtained from Serva GmbH (Heidelberg, Germany). The nylon transfer filters (Hybond N), *Bam*H1 endonuclease and the Klenow fragment of DNA polymerase 1 were obtained from Amersham-Buchler (Braunschweig, Germany). The XOMAT X-ray film was obtained from Eastman Kodak Co (Zurich, Switzerland). Sep-pak cartridges containing octadecylsilica (C-18) were obtained from Waters Associates (Millford, USA).

2.2. *RINm5F cell-line*

This cell line is derived from a radiation-induced insulin-producing rat tumor [8], which retains many of the differentiated functions of pancreatic β cells. The cells were grown in plastic Petri dishes under the conditions described by Praz and colleagues [9].

2.3. *Extraction of cells and tissue for analysis of peptides*

After decanting the culture medium cells were detached using a rubber policeman and extracted in boiling 1 M acetic acid, containing 0.2% dithiothreitol, for the subsequent measurement of neurokinin A and substance P. Cells were extracted in boiling water for the subsequent assay of the PPT C-flanking peptide. For quantitation of the cellular concentration a measured portion of each sample was taken before extraction for DNA measurement [10]. For comparison with normal rat tissues, similar extracts of rat small intestine were prepared as previously described [11]. Before RIA and HPLC analysis, samples were partially purified and concentrated using solid-phase extraction on SEP-PAK C-18 cartridges as previousy described [11].

2.4. *Radioimmunoassays (RIAs)*

Specific and sensitive RIAs for three different end products of PPT-I biosynthesis, substance P(SP), neurokinin A(NKA) and the C-flanking peptides of PPT (CPPT), were performed as described previously. The SP RIA antiserum(rabbit), SP3, is specific for SP showing no cross-reaction with any other mammalian peptide including NKA, neurokinin B(NKB) and C-PPT [12]. The NKA RIA antiserum (rabbit), $\alpha\beta 4$, exhibited, in RIA, the following variable crossreactivity with the NKA-related peptides; NKB (30%), neuropeptides K (100%) and γ (160%), and no cross-reaction with SP and C-PPT [13]. The C-PPT RIA employed the antiserum (guinea pig), GP4, which fully cross-reacts with the isolated human and rat PPT C-flanking peptides and exhibits no cross-reaction with the tachykinin peptides [11].

2.5. *High-pressure liquid chromatography*

The rpHPLC analysis was performed as previously described [13]. Each of the partially purified cell tissue extracts was injected onto a 125×4 mm LiChro CART column of C-18 LiChrospher (100-RP-18,5 μm), preequilibrated with 50 mM potassium phosphate buffer, pH 2.5. The column was calibrated with standard synthetic peptides as previously described. After sample injection the column was eluted with a linear gradient of solvent B (acetonitrile/water (70:30) containing potassium phosphate buffer, final concentration 37 mM, pH 2.5). The gradient conditions used are indicated in Fig. 1. Each of the fractions was evaporated and, following addition of 1 ml water, and then assayed at dilution by RIA.

2.6. *Release studies*

These experiments were carried out using plated cells according to the procedure described by Jeng and colleagues [14] with certain modifications. The peptidase inhibitors phosphoramidon (4×10^{-4} M) and bestatin (10^{-4} M) were always included in the incubation buffer which was Krebs-Ringer bicarbonate buffer containing 0.07% bovine serum albumin and 10 mM HEPES buffer, pH 7.4. The incubation buffer was collected before and after 15 min incubation with either 50 mM potassium chloride (replacing the equivalent amount of sodium chloride) or 8-Br-cAMP (2 mM). Before RIA the samples of collected medium were concentrated by solid phase extraction using Sep-pak (C-18) cartridges as for the tissue extractions.

2.7. *RNA extraction and Northern blotting*

Total RNA was extracted from cultured cells and from frozen tissue samples using a procedure based on a protocol by Cathala and colleagues [15]. The following rat tissues were selected as control tissues for the RNA analysis; corpus striatum and trigeminal ganglia which are known to be rich sources of PPT-I mRNA [5] and cerebral cortex (without olfactory lobes) and liver which appear devoid of PPT-I mRNA. The brain dissections were performed according to Glowinski and Iverson [16]. Lysis of both cells and tissue was achieved by addition of 5 M guanidinium isothiocyanate containing 50 mM Tris-HCl buffer, pH 7.5, 10 mM EDTA and 8% β-mercaptoethanol. Tissue samples were then homogenized (Ultra Turrax). Nucleic acids were precipitated at 4°C overnight in the presence of 7 vols. 4 M LiCl. Following sedimentation by centrifugation (10,000×g) the samples were washed with 3 M LiCl, resedimented by centrifugation and resuspended in 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% SDS and 100 μg/ml Proteinase K. Following 1 hour incubation at 42°C, the reaction was terminated by phenol/chloroform extraction and the aqueous phase precipitated by addition of 1/10 vol. 3 M sodium acetate pH 5.2 and 2.5 vol. 100% ethanol. The precipitates were collected by centrifugation, resuspended in 20 mM Tris-HCl buffer, pH 7.4, 60 mM NaCl, 7 mM $MgCl_2$, 1 mM dithiothreitol, 250 U/ml RNasin, 100 U/ml DNase and incubated at 37°C for 30 min. The reaction mixture was extracted twice with phenol/chloroform and the final aqueous phase precipitated with ethanol. Following recentrifugation, the pellet was resuspended in 75% ethanol and again centrifuged. The final RNA pellet was dissolved in diethylpyrocarbonate treated water and stored at −30°C before further analysis.

Electrophoresis of the RNA samples was performed using a 1.2% agarose gel containing 2.2 M formaldehyde. The RNA was then transferred to nylon filters (Hybond N) (overnight capillary blot) using 3.6 M NaCl, 0.2 M sodium phosphate, pH 7.7, 20 mM EDTA. Following baking, the membrane was stained in 0.5 M sodium acetate pH 5.2 containing 0.05% Methylene blue which allowed identification of the positions of 28S and 18S rRNA. The membranes were prehybridized for 3 h in 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 5×Denhardt's (0.1% each of ficoll, polyvinylpyrrolidone and bovine serum albumin (fraction V)), 1 μg/μl sonicated salmon sperm DNA. The filter was hybridized overnight with the ^{32}P-labelled PPT-I cDNA (see section 2.8) contained in prehybridization solution containing only 1×Denhardt's. Prehybridization and hybridization temperature was 42°C. The blot was washed in 2×SSC at room temperature, for 15 min in 2×SSC containing 1% sodium dodecylsulphate at 42°C, for 10 min in 0.1×SSC at room temperature and for 15 min in 0.1×SSC at 42°C. The washed filter was exposed to X-ray film (XOMAT) at −80°C in the presence of an intensifying screen.

2.8. *Radiolabelling of PPT-I cDNA*

The β-PPT cDNA insert (containing exons 1–6 and 90 nucleotides of exon 7 of the PPT 1 gene) was excised from the plasmid pG1β-PPT [17] (a gift from Dr. J.E. Krause) using restriction endonuclease *Bam*H1, 50 ng β-PPT and incubated with Klenow fragment of DNA-polymerase 1 in the presence of random sequence hexanucleotides, [α-^{32}P]dCTP (Amersham-Buchler, Germany) and the three other, unlabeled deoxyribonucleotides. Unincorporated nucleotides were removed by gel filtration. The specific activity of the β-PPT probe: $5–10\times10^6$ cpm/10 μl (10 ng).

3. RESULTS AND DISCUSSION

Similar concentrations of tachykinin (substance P and neurokinin A)-immunoreactivities were measured in RINm5F cell acidic extracts (Table I). Concentrations are expressed in terms of micrograms cell DNA.

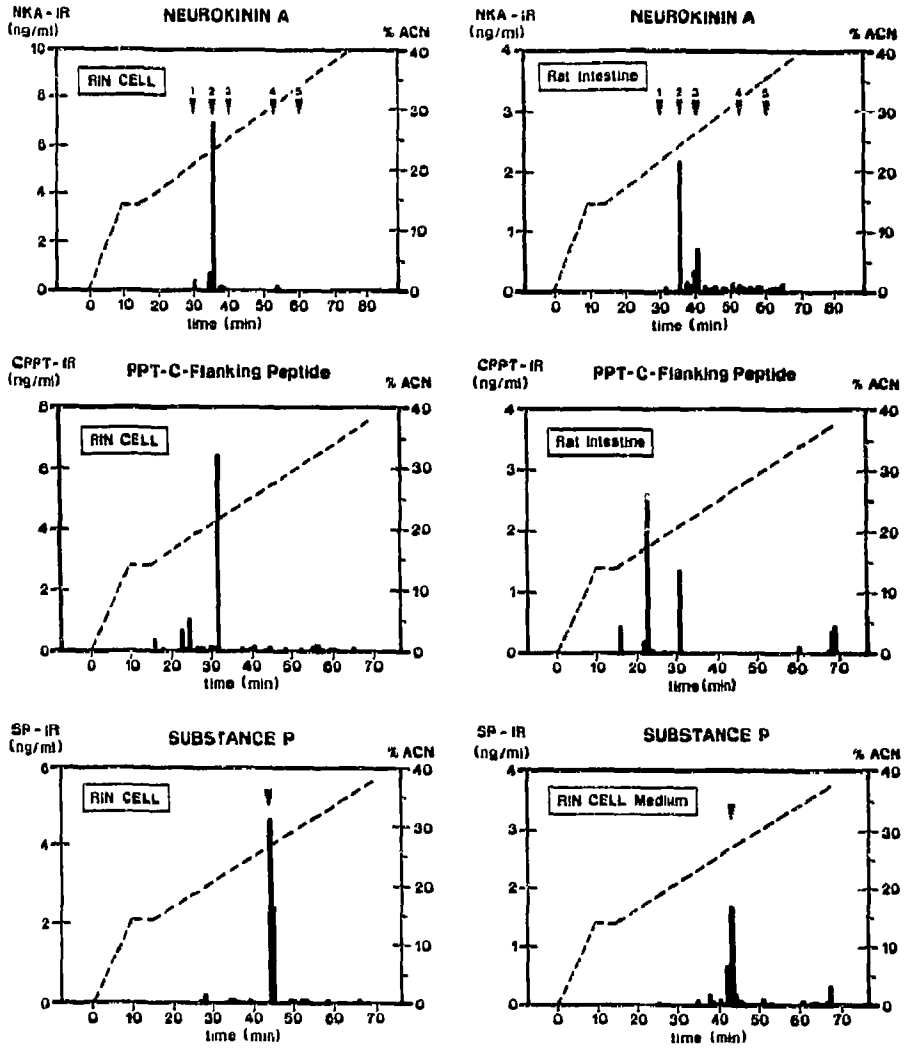

Fig. 1. HPLC fractionation of SP-, NKA-, PPT-I-C-flanking peptide-IRs extracted from RINm5F cells, from rat small intestine (data for SP-IR not shown) and from an extract of medium (containing 50 mM KCl) collected following incubation with RINm5F cells (data for SP-IR only shown). The dashed line in each profile indicates the gradient of acetonitrile (ACN) with which the column was eluted. The arrows in the NKA-IR profiles indicate the elution times, under these conditions, of the following synthetic peptides: neuropeptide gamma; (1), neurokinin A(4–10), neuropeptide K (4) and neurokinin B (5). The arrow in the SP-IR profiles indicates the elution time, under these conditions, of synthetic substance P.

Similarly expressed concentrations of the PPT C-flanking peptide measured in RINm5F cell water extracts are also shown and are of the same magnitude as the tachykinin concentrations. The similar molar concentrations of substance P- and the PPT-C-flanking peptide-IRs have also been measured in extracts of normal rat tissue [11]. The concentration of NKA-IR relative to these other PPT-I-derived immunoreactivities appears, normally, to be tissue-dependent presumably because of tissue-variable expression of the alpha form of PPT [2] which does not contain the transcript of the NKA-encoding, exon 6 of the PPT-I gene. Since the amounts of all three PPT-I-derived peptides measured in the RIN cell extracts are not significantly different, the RINm5F cells appear to express little of α-PPT.

The rpHPLC analysis reveals that RINm5F cells contain similar molecular forms of NKA-, SP- and PPT-C-flanking immunoreactivities as in rat intestine (Fig. 1), however with some interesting differences. SP-IR elutes, in each case, as a single peak with an elution time identical to the synthetic standard peptide (data for small

Table I

Concentrations (pmol/mg DNA) of substance P-IR (SP), neurokinin A-IR (NKA) and the PPT-I-C flanking peptide-IR (C-PPT) in RINm5F cells. Values expressed means ± S.E.M. n = number of extraction samples.

SP (n = 9)	NKA (n = 9)	C-PPT (n = 5)
33.9 ± 3.7	30.8 ± 5.0	28.9 ± 4.7

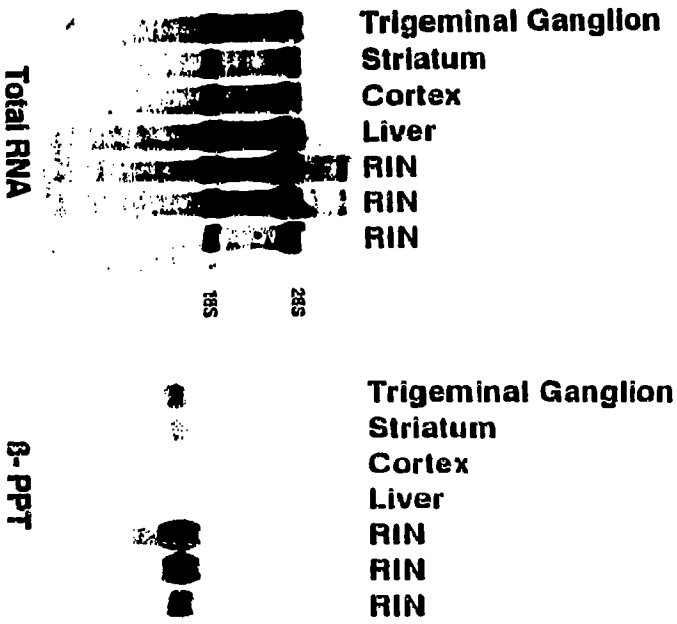

Fig. 2. A Northern blot of total RNA extracted from the trigeminal ganglion (5 μg), corpus striatum (striatum) (3.5 μg), cerebral cortex (cortex) (6 μg), and liver (10 μg) of rat, and from RINm5F cells (12, 6 and 3 μg).

intestine not shown). NKA-IR elutes, in each case, as one major peak with an elution time corresponding to that of the NKA standard. However in extracts of rat intestine a significant amount of NKA-IR elutes at a position corresponding to neurokinin A (4–10) and this is not detected on analysis of RIN cell extracts. Also no significant NKA-IR extracted from the RIN cell elutes with the elution times of neuropeptides K and γ. The PPT C-flanking-IR from the RIN cell elutes as a single major peak unlike PPT C-flanking-IR from the rat small intestine which, as previously shown [11], resolves on rpHPLC as two major peaks. The second of these two intestinal PPT-I C-flanking-immunoreactive peaks has an elution time (31 min) identical to that of the single major RIN cell PPT-I C-flanking-IR peak and has been previously characterised as corresponding to the rat homologue of the structurally characterised bovine PPT-I C-flanking peptide [11].

Table II

Concentrations (pmol/ml) of SP-IR (SP) and NKA-IR (NKA) measured in control and 'stimulation' media collected following incubation with plated RINm5F cells. See text for contents of control medium. The 'stimulation' media consisted of control medium containing either 50 mM KCl (included in place of the equivalent amount of NaCl of the control medium) (in Experiment 1) or 2 mM 8-Br-cAMP (Experiment 2). Values shown as mean ± S.D.

		SP	NKA
Experiment 1			
Control	(n=5)	201 ± 56	582 ± 110
50 mM KCl	(n=9)	994 ± 461	1,571 ± 554
Experiment 2			
Control	(n=2)	143 ± 16	130 ± 22
2 mM 8-Br-cAMP	(n=2)	408 ± 56	420 ± 35

The combined rpHPLC and RIA analysis indicates that the post-translational processing of the PPT I gene is similar to what occurs in an example of a normal rat tissue which expresses the PPT-I gene. However the data also indicates that some interesting detailed differences do exist between the RIN cell and rat intestine in the pattern of post-translational processing of PPT-I and these require further investigations. Furthermore, Northern blot analysis indicates that the RINm5F cell appears to transcribe the PPT-I gene in a similar manner to normal rat tissue. Fig. 2 shows the results of the analysis of similar amounts of total RNA extracted from RINm5F cells and the corpus striatum, cerebral cortex, trigeminal ganglion and liver of the rat. With RNA from the RIN cell, corpus striatum and trigeminal ganglion, a single hybridisation signal of identical mobility was detected with the ^{32}P-labelled β-PPT cDNA, whereas no signal was detected with cerebral cortex [5] and liver RNA, which are tissues known not to express the PPT-I gene. The position of the β-PPT hybridisation signal in relation to the ribosomal RNA bands (revealed following total RNA staining with Bromophenol blue) corresponds approximately to the predicted position of PPT mRNAs [6].

The regulated release of tachykinins from RINm5F cells was determined by RIA of extracted incubation buffer before and following the inclusion of raised pottasium concentrations (50 mM) or 8-Br-cAMP. An increase of both SP-IR and NKA-IR was detected in the incubation buffer in the presence of raised pottasium and 8-Br-cAMP (Table II) compared to samples of control media collected immediately prior to exposure to the test reagent. Therefore membrane depolarisation by raised extracellular potassium and stimulation of the regulated secretory pathway with 8-Br-cAMP causes tachykinin release from RINm5F cells. In Fig. 1 the result of rpHPLC of SP-IR in medium collected following stimulation with elevated pottasium concentrations is shown and reveals a single peak of IR corresponding to authentic substance P.

This study presents the RINm5F cell line as a model for the investigation of PPT-I gene expression and the mechanisms of post-translational processing and regulated release of the tachykinins. The results of this study also extend the biochemical characterisation of the RINm5F cell-line which is a well-used model for the pancreatic β cell. The expression of the PPT-I gene by the RINm5F cell appears to involve normal endocrine biosynthetic processes and regulated secretion of the tachykinins and is further evidence of the differentiated state of this cell-line. The RINm5F cell-line also synthesises somatostatin [8] in addition to insulin, and other pancreatic islet tumor cells also exhibit pluripo-

tential for islet peptide hormone synthesis [18,19]. It is suggested that such tumors cells represent a stage in islet embryogenesis [18], which appears to involve a pluripotential progenitor cell [20]. Although the pancreatic beta; cell appears normally not to express the PPT-I gene, it is possible, that the PPT-I gene is transiently expressed during embryogenesis of the β cell. There are examples of neuroendocrine genes being transiently 'switched on' during development [21–23] or under specific physiological conditions [23,24]. Such physiological phenotypic plasticity is often revealed in the neoplastic state [25]. The tyrosine hydroxylase gene, which is normally considered a marker for catecholaminergic cells is expressed by pancreatic β cells when they undergo physiologically-induced hyperplasia and also during islet cell embryogenesis [23]. Furthermore, embryological studies have indicated that precursor cells of pancreatic islet cells are phenotypically similar to those of catecholaminergic cells [20]. Interestingly, sympathetic catecholaminergic cells have been shown to express the PPT-I gene [24].

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