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Chromogranin A can act as a reversible processing enzyme inhibitor

Evidence from the inhibition of the IRCM-serine protease 1 cleavage of pro-enkephalin and ACTH at pairs of basic amino acids

N.G. Seidah, G.N. Hendy*, J. Hamelin, J. Paquin, C. Lazure, K.M. Metters+, J. Rossier+ and M. Chrétien

*Laboratories of Biochemical and Molecular Neuroendocrinology, Clinical Research Institute of Montreal (Affiliated with the University of Montreal), 110 Pine Avenue West, Montreal, Quebec H2W 1R7, *Departments of Medicine, Royal Victoria Hospital and McGill University, Montreal, Quebec H3A 1A1, Canada* and *+Laboratoire de Physiologie Nerveuse, CNRS, 91190 Gif-sur-Yvette, France*

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Bovine parathyroid chromogranin A inhibits the cleavage of Z-Ala-Lys-Arg-AMC by either trypsin or IRCM-serine protease 1 (IRCM-SP1), a putative novel processing enzyme originally isolated from porcine pituitary anterior and neurointermediate lobes. On larger substrates, chromogranin A is a reversible competitive inhibitor of the cleavage at pairs of basic amino acids by IRCM-SP1. The substrates tested included pituitary ACTH and adrenal medulla pro-enkephalin-derived peptides such as the 8.6 kDa synenkephalin-containing precursor and peptide B. Chromogranin A is itself selectively processed by IRCM-SP1, and ACTH was shown to compete for such cleavage. These data suggest that chromogranins as a class of acidic proteins could participate in the tissue-specific processing of pro-hormones.

Chromogranin; Processing enzyme; Enzyme inhibitor; Pro-enkephalin; IRCM-serine protease 1; Pro-opiomelanocortin

1. INTRODUCTION

Recently, a novel enzyme called IRCM-serine protease 1 (IRCM-SP1) was isolated and characterized from porcine pituitary anterior and neurointermediate lobes [1–3], and from rat heart atria and ventricles [4]. The enzyme is a homodimer and has a subunit structure consisting of two polypeptide chains linked together by a disulfide bridge(s) typical of regulatory serine proteases [5]. The cleavage specificity of this protease was studied using a variety of peptides including tripeptide fluorogenic substrates and larger natural peptide substrates derived from pro-opiomelanocortin (POMC) [3,4] or pro-atrial natriuretic factor (Pro-ANF) [4]. These data demonstrated the remarkable ability of IRCM-SP1 to cleave selectively all these substrates at specific basic amino acids, including both single and pairs of basic residues, known to be cleaved in vivo [3,4]. The fact that this enzyme in vitro cleaves all the bonds known to be cleaved in vivo in various pro-hormone precursors suggests that there must be an additional mechanism for tissue-specific differential processing of pro-hormones, which is known to occur for a number of precursors [6]. This process may be due to a kinetic phenomenon reflecting differential contact time with active enzyme and/or differing enzyme:substrate ratios

Correspondence address: M. Chrétien, Laboratories of Biochemical and Molecular Neuroendocrinology, Clinical Research Institute of Montreal, 110 Pine Ave West, Montreal, Quebec H2W 1R7, Canada

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[3,4]. Another possibility is the tissue-specific presence of varying amounts of a general processing enzyme modulator or reversible inhibitor. Such a putative factor(s) would interact with either prohormones and/or their processing enzyme(s) thereby selectively modulating the cleavage sensitivity of these precursors, thus regulating post-translational hormone biosynthesis.

Chromogranins A, B and C represent a family of closely related, but distinct, acidic proteins found in secretory granules derived from endocrine and neuroendocrine cells [7–9]. The cDNA sequence coding for chromogranin A, the most abundant member of the family, has recently been reported [10,11]. Although the chromogranins have been shown to bind Ca^{2+} [12], their exact physiological role has not yet been defined. Their possible participation in pro-hormone vesicular packaging and/or sorting has been proposed [7–11], but not proven. The present work demonstrates that chromogranin A is a reversible competitive inhibitor of IRCM-SP1 cleavage at pairs of basic amino acids in a number of prohormone substrates derived from either POMC or pro-enkephalin.

2. MATERIALS AND METHODS

The substrates used included Z-Ala-Lys-Arg-AMC (Enzyme System Products), human ACTH 1–39 (purified in this laboratory from human pituitaries [3]), and bovine pro-enkephalin-derived peptides such as peptide B (Peninsula Labs) and the 8.6 kDa enkephalin precursor of structure synenkephalin-Lys-Lys-Tyr-Gly-Gly-Phe-Met which was purified from adrenal medulla as described [13]. Bovine parathyroid chromogranin A (also known as parathyroid secretory protein 1) was purified as in [14].

The ^{125}I labeling of ACTH 1–39 and chromogranin A was done by the lactoperoxidase method followed by HPLC purification of the ^{125}I-labeled peptides [3]. All HPLC purifications were done on a Vydac protein C_4 column (0.46 × 25 cm) eluted with a linear gradient of heptafluorobutyric acid (0.13%, HFBA)/CH_3CN as described [3]. For analysis of ^{125}I-labeled peptides an on-line radioactivity flow detector (Flo-One, Radiomatic Instruments) was connected at the exit of the C_4 column [3].

The proteases used included TPCK-trypsin (Worthington), porcine anterior pituitary IRCM-SP1 [1–3] and human IRCM-SP1 (Seidah et al., in preparation). All incubations were done at room temperature in a final concentration of buffer containing 200 mM Bes, 2 mM EDTA, pH 8.5. One unit of enzyme activity (U) is defined as the amount of protease necessary to release 1 pmol AMC/s from the Z-Ala-Lys-Arg-AMC substrate at 42 μM in a buffer containing 100 mM Bes and 1 mM EDTA at pH 8.5.

Initial rate assays on the release of fluorescent AMC from Z-Ala-Lys-Arg-AMC (ranging from 10 to 175 μM) by either trypsin (45.03 U) or porcine IRCM-SP1 (41.82 U) were done as described [2] in a final volume of 500 μl. The effect of chromogranin A (4 and/or 8 μM) was also studied.

30 min digestions of ^{125}I-ACTH 1–39 were done in duplicate with human IRCM-SP1 (4.86 U) with various concentrations of unlabeled ACTH 1–39 (300, 500 and 700 μM in 50 μl), maintaining a constant specific activity (43000 cpm/nmol) either in the absence (control) or presence of 40 μM chromogranin A. Here, analysis of the reaction products by HPLC/radioactivity detection revealed the release of ACTH 18–39 (CLIP) and 1–17 as reported [3]. The integration of the CLIP peak allowed the calculation of the velocity of the reaction, taking into account that the tyrosines at positions 2 and 23 of ACTH are labeled in a ratio of 60:40 [3].

Amino acid analysis of the reaction products of peptide B obtained following digestion by IRCM-SP1 and HPLC purification defined the exact cleavage as being C-terminal to the Lys-Arg↓ bond producing the heptapeptide Tyr-Gly-Gly-Phe-Met-Arg-Phe (see fig.1). Therefore, peptide B (50, 100, 200, 300 μM in 50 μl) was digested with human IRCM-SP1 (5.56 U) for 30 min, the reaction products purified by HPLC and then quantitated by UV absorption at 230 nm, relative to a standard heptapeptide. This permitted the calculation of the velocity of the release of this heptapeptide from peptide B, and the influence of chromogranin A (25 and 15 μM) on this reaction.

The digestion of the 8.6 kDa pro-enkephalin precursor was performed in a final volume of 25 μl (final concentration 2 μM) in the Bes/EDTA (pH 8.5) buffer with 3.7 U human IRCM-SP1 with (0.2 and 4 μM) or without chromogranin A. At 0, 10,

20 and 30 min intervals, 5 μl aliquots were diluted with 2 vols glacial acetic acid and boiled for 20 min. After evaporation, the released Met-enkephalin (Tyr-Gly-Gly-Phe-Met) was measured by radioimmunoassay as in [15].

The digestion of 2 nmol ^{125}I-chromogranin A (500000 cpm/nmol) with human IRCM-SP1 (4.2 U) was done for 30 and 60 min at room temperature in a final volume of 50 μl (i.e. 40 μM chromogranin A), in either the absence or presence of 700 μM ACTH 1–39. A control incubation of chromogranin A in buffer was also made. Analysis of the digestion products was done on SDS-PAGE using 10% acrylamide separating gel, under non-reducing conditions, as described [2]. The separated bands were stained with Coomassie brilliant blue, the gel was then dried and autoradiography was performed as in [2].

3. RESULTS

When chromogranin A was initially tested for its possible effect on the IRCM-SP1 digestion of ACTH, it was found that concentrations below 10 μM had no appreciable effect. However, at 30 μM chromogranin A, a clear inhibitory effect was observed on the previously reported [3] conversion of ACTH into ACTH 1–17 and CLIP. Also, it was found that the addition of Ca^{2+} (up to 5 mM) did not appreciably affect the inhibitory potency of chromogranin A. In order to study systematically such an inhibition, and to test its generality, it was decided to compare a number of different substrates in terms of the susceptibility of their IRCM-SP1 cleavage to chromogranin A inhibition.

In fig.1 is shown the effect of 25 μM chromogranin A on the human IRCM-SP1 cleavage of pro-enkephalin peptide B [16]. The amino acid composition of the cleavage products clearly showed that IRCM-SP1 cleaves the 31-amino-acid peptide B C-terminal to Lys-Arg↓ to generate the heptapeptide Tyr-Gly-Gly-Phe-Met-Arg-Phe and the N-terminal 1–24 segment. In brain, the heptapeptide has been reported to represent a major product of pro-enkephalin processing [17], implying a similar cleavage of peptide B in vivo. As seen in fig.1B, 25 μM chromogranin A is sufficient to inhibit more than 80% of this 30 min cleavage reaction.

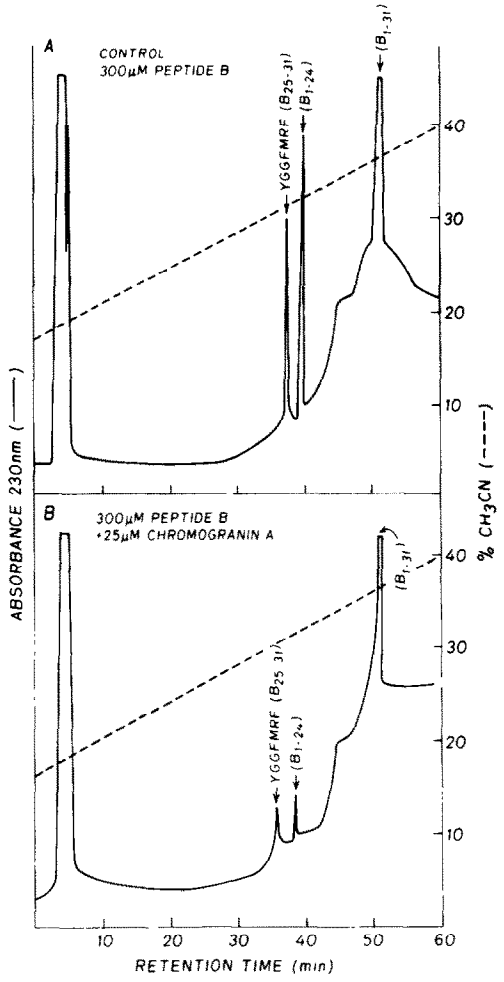

Fig.1. Reverse-phase HPLC of a 30 min human IRCM-SP1 digest of 300 μM of the 31-amino-acid bovine pro-enkephalin peptide B (sequence FAEPLPSEEEGESY-SKEVPEMEKRYGGFMRF) (B_{1-31}) in the absence (A, control) or presence of 25 μM chromogranin A (B). The dashed line represents the CH_3CN gradient. The identity of the released heptapeptide YGGFMRF and the N-terminal segment B_{1-24} is based on the amino acid composition of the material eluting under these peaks. Under these conditions, chromogranin A inhibits by more than 80% the Lys-Arg↓ cleavage of peptide B.

The chromogranin A inhibition was also tested on either the porcine IRCM-SP1 or trypsin cleavage of the fluorogenic substrate Z-Ala-Lys-Arg-AMC to release AMC. As shown in fig.2A

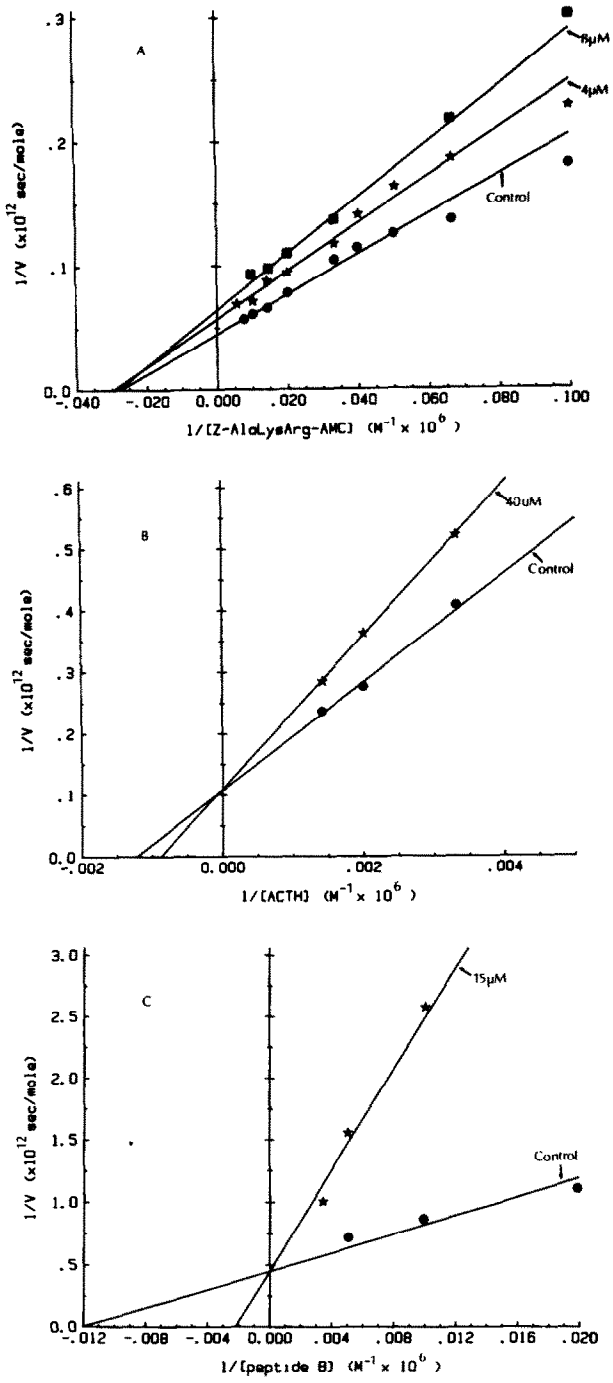

Fig.2. Double-reciprocal plot of the kinetic data obtained for the digestion of Z-Ala-Lys-Arg-AMC (A), human ACTH (B) and bovine pro-enkepohalin peptide B (C). The concentrations of chromogranin A are indicated in each graph. The kinetic parameters for each study are reported in table 1.

and table 1, such an inhibition was found to be non-competitive, whereby increasing concentrations of chromogranin A (4 and 8 μM) seem to decrease the maximum velocity of the reaction (V_{max}) but have little effect on the K_m. Interestingly, a similar inhibition pattern was observed on the trypsin cleavage of this substrate (table 1). This suggests that the inhibitory effect of chromogranin A is not selective for IRCM-SP1, and that the latter can interact with the catalytic chain of IRCM-SP1. As controls, we have tested whether other acidic proteins could also affect these cleavage reactions. However, our results demonstrated that neither human serum albumin (up to 10 μM) nor hen ovalbumin (up to 12 μM) had any detectable effect on either trypsin or IRCM-SP1 cleavage of Z-Ala-Lys-Arg-AMC (not shown).

When the chromogranin A inhibition of either ACTH or peptide B was studied kinetically, it was found that in both cases the inhibition was of a competitive type (fig.2B,C and table 1), i.e. addition of chromogranin A to the reaction medium caused an increase in K_m but did not appreciably affect the V_{max}. This would mean that chromogranin A and either ACTH or peptide B compete for the same active site of IRCM-SP1. Notably, the IRCM-SP1 cleavage of peptide B was found to be more sensitive than that of ACTH to chromogranin A inhibition (see fig.2B,C and table 1). Since binding of chromogranins to prohormones has been suggested [18], these data might reflect a differential affinity of chromogranin A for either ACTH or peptide B.

Since chromogranin A is found in high concentrations in chromaffin cells of the adrenal medulla [7–9] it was of interest to find out whether it could also inhibit the IRCM-SP1 cleavage of the 8.6 kDa pro-enkephalin precursor isolated from chromaffin granules [13]. IRCM-SP1 cleaves the 8.6 kDa precursor at a single Lys-Lys↓ bond releasing Met-enkephalin and synenkephalin-Lys-Lys↓ (Metters et al., in preparation). When such a reaction was performed in the presence of as little as 0.4 μM chromogranin A a high degree of inhibition was observed over at least 30 min (fig.3). These data show that the IRCM-SP1 cleavage of the 8.6 kDa pro-enkephalin precursor is exquisitely sensitive to the presence of chromogranin A even at a mole ratio of 8.6 kDa precursor to chromogranin A of 5:1.

Table 1

Kinetic parameters for the hydrolysis of Z-Ala-Lys-Arg-AMC by either porcine IRCM-SP1 or trypsin, and of ACTH and peptide B by human IRCM-SP1

	[Chromogranin A] (M) ($\times 10^6$)	K_m (M) ($\times 10^6$)	V_{max} ($mol \cdot U^{-1} \cdot s^{-1}$) ($\times 10^{12}$)	V_{max}/K_m ($mol \cdot U^{-1} \cdot s^{-1} \cdot M^{-1}$) ($\times 10^9$)
Trypsin				
Z-Ala-Lys-Arg-AMC	0	30.5 ± 1.6	0.40 ± 0.01	12.98
	8	27.2 ± 2.8	0.30 ± 0.01	11.17
IRCM-SP1				
Z-Ala-Lys-Arg-AMC	0	35.8 ± 3.2	0.53 ± 0.02	14.93
	4	33.7 ± 3.3	0.42 ± 0.02	12.46
	8	35.1 ± 1.7	0.37 ± 0.01	10.61
Human ACTH 1–39	0	817 ± 166	1.92 ± 0.24	2.35
	40	1136 ± 117	1.88 ± 0.13	1.65
Bovine peptide B	0	83 ± 24	0.40 ± 0.05	4.84
	15	455 ± 26	0.40 ± 0.01	0.88

Values were obtained by fitting the data with a Kinfit program [20] and expressed with the standard deviation of the fit

Since the above data suggest that chromogranin A is a competitive inhibitor of IRCM-SP1 cleavage of ACTH, peptide B, and possibly the pro-enkephalin 8.6 kDa precursor, it could itself serve as a substrate for this enzyme. Indeed, sequence data demonstrate that 8 pairs of basic amino acids are found in chromogranin A [10,11], potentially representing cleavage sites for IRCM-SP1. As shown in fig.4, IRCM-SP1 can cleave chromogranin A into smaller fragments, as yet uncharacterized, migrating with an apparent molecular mass on SDS-PAGE (under non-reducing conditions) of 68 kDa (major), 59 kDa and 55 kDa. Also shown is the inhibition of the chromogranin A cleavage by ACTH (see fig.3, lanes 3,6). These data clearly confirm that chromogranin A is a competitive inhibitor of ACTH cleavage by IRCM-SP1. However, the presence of more than one cleavage site on chromogranin A is expected to complicate the interpretation of the kinetic inhibition data. Indeed, for the 8.6 kDa precursor, the plot of the reciprocal of the velocity vs inhibitor concentration is not linear (not shown), indicating a complex mechanism of interaction of chromogranin A and, possibly its cleavage products, with the substrate and enzyme.

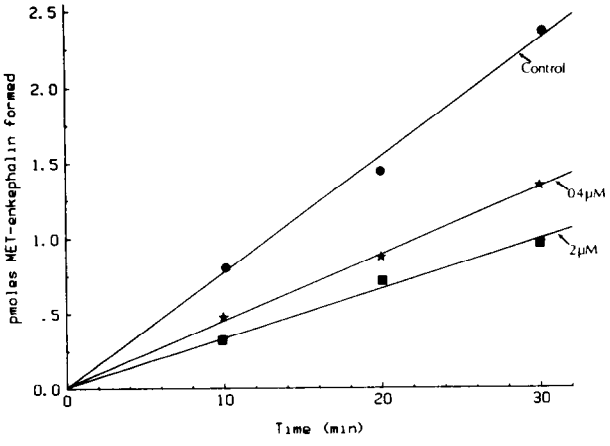

Fig.3. Time study of the IRCM-SP1 release of the C-terminal Met-enkephalin from the 8.6 kDa pro-enkephalin precursor involving a Lys-Lys↓ cleavage, in the absence (control) or presence of 0.4 and 2 μM chromogranin A.

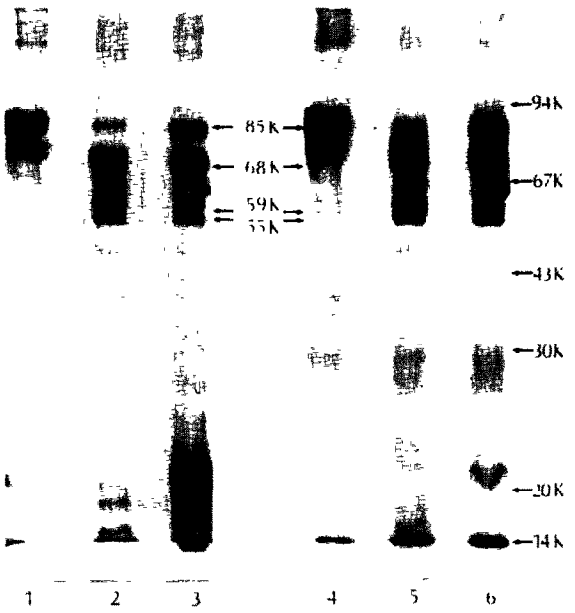

Fig.4. SDS-PAGE analysis (under non-reducing conditions) of a 30 min IRCM-SP1 digest of chromogranin A/^{125}I-chromogranin A mixture in the absence (lanes 2,5) and presence (lanes 3,6) of ACTH. Lanes 1–3 represent the Coomassie blue coloration of the gel, and lanes 4–6 the corresponding autoradiogram. Lanes 1 and 4 show the migration of the control chromogranin A, which in this system migrates as an 85 kDa band. The large band seen at the bottom of lane 3 is the position of unlabeled ACTH in this system. Although not shown, a similar but less apparent inhibition is seen after 1 h reaction time, possibly due to extensive digestion of ACTH and chromogranin A on that time scale.

4. DISCUSSION

The hypothesis that IRCM-SP1 represents a serious candidate for a pro-hormone processing enzyme at both pairs and single basic residues [1–4] is now reinforced since we showed it to cleave precursors related to pro-enkephalin at sites expected from in vivo derived products. However, as previously noted [2–4], since IRCM-SP1 cleaves all the bonds known to be processed in vivo, tissue-specific processing of pro-hormones [6,17] has to involve another factor(s) controlling the velocity of cleavage. This work suggests that chromogranin A, and possibly the other members of this family, can participate in the biosynthetic pathway of pro-hormones leading to the final peptide forms that are secreted by a given tissue. The extent of this participation would depend on the relative concentration of chromogranin with respect to pro-hormones within the secretory granules and its binding affinity to the pro-hormone structure. For example, since ACTH is not appreciably cleaved in the anterior lobe of the pituitary but mostly cleaved in the intermediate lobe [6], one would expect that there are different amounts of chromogranins in the corticotrophs of the two lobes. However, exact quantitative data on the amounts of chromogranins between these different cells are not yet available [18]. Furthermore, in the adrenal medulla one finds predominantly large molecular mass pro-enkephalin fragments [17,19], as opposed to brain where mostly enkephalins are found [16]. Since the chromogranin content within chromaffin granules is very high, possibly in the 0.1–10 mM range [7–9,18], and certainly higher than in brain, this could explain the difference in the extent of pro-enkephalin processing between the adrenals and hypothalamus. Clearly, more work will be necessary to confirm fully the chromogranins' potential inhibitory action on processing enzymes. However, the data presented here suggest an important role for chromogranin A as a regulator of post-translational processing of pro-hormones.

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REFERENCES

[1] Cromlish, J.A., Seidah, N.G. and Chrétien, M. (1985) Neuropeptides 5, 493–496.

[2] Cromlish, J.A., Seidah, N.G. and Chrétien, M. (1986) J. Biol. Chem. 261, 10850–10858.
[3] Cromlish, J.A., Seidah, N.G. and Chrétien, M. (1986) J. Biol. Chem. 261, 10859–10870.
[4] Seidah, N.G., Cromlish, J.A., Hamelin, J., Thibault, G. and Chrétien, M. (1986) Bioscience Reports 6, 835–844.
[5] Neurath, H. (1984) Science 224, 350–357.
[6] Lazure, C., Seidah, N.G., Pelaprat, D. and Chrétien, M. (1983) Can. J. Biochem. Cell Biol. 61, 501–515.
[7] Winkler, H. and Westhead, E. (1980) Neuroscience 5, 1803–1823.
[8] Cohn, D.V., Zangerle, R., Fisher-Colbrie, R., Chu, L.L.H., Elting, J.J., Hamilton, J.W. and Winkler, H. (1982) Proc. Natl. Acad. Sci. USA 79, 6056–6059.
[9] Rosa, P., Hille, A., Lee, R.W.H., Zannini, A., De Camilli, P. and Hutner, W.B. (1985) J. Cell Biol. 101, 1999–2011.
[10] Benedum, U.M., Baeuerle, P.A., Konecki, D.S., Frank, R., Powell, J., Mallet, J. and Hutner, W.B. (1986) EMBO J. 5, 1495–1502.
[11] Iacangelo, A., Affolter, H.-U., Eiden, L.E., Herbert, E. and Grimes, M. (1986) Nature 323, 82–86.
[12] Reiffen, F.U. and Gratzl, M. (1986) FEBS Lett. 195, 327–330.
[13] Metters, K.M. and Rossier, J. (1986) J. Neurochem., in press.
[14] Cohn, D.V., Morrissey, J.J., Hamilton, J.W., Shofstall, R.E., Smardo, F.L. and Chu, L.L.H. (1981) Biochemistry 20, 4135–4140.
[15] Patey, G., Cupo, A., Mazarguil, H., Morgat, J.-L. and Rossier, J. (1985) Neuroscience 15, 1035–1044.
[16] Udenfriend, S. and Kilpatrick, D.L. (1983) Arch. Biochem. Biophys. 221, 309–323.
[17] Liston, D., Patey, G., Rossier, J., Verbanck, P. and Vanderhaegen, J.J. (1984) Science 225, 734–737.
[18] Rindi, G., Buffa, R., Sessa, F., Tortora, O. and Solcia, E. (1986) Histochemistry 85, 19–28.
[19] Patey, G. and Rossier, J. (1986) Ann. Endocrinol. 47, 71–87.
[20] Knack, I. and Rohm, K.H. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1119–1130.