Contribution of blood and systemic circulation to the processing of pro-(atrial natriuretic factor)

Kishore K. MURTHY, Gaétan THIBAUT* and Marc CANTIN

Clinical Research Institute of Montreal, 110 Pine Ave West, Montreal, Quebec, Canada H2W 1R7

Atrial natriuretic factor-(Asn¹-Tyr¹²⁶)-peptide, the 13.6 kDa propeptide of atrial natriuretic factor (ANF), is stored in the secretory granules of atrial cardiocytes. ANF-(Ser⁹⁹-Tyr¹²⁶)-peptide, the 28-amino-acid species, is the circulating form of this hormone in the rat. As the site of maturation of the prohormone is still unknown, the present study was undertaken to understand the contribution of the circulation to the maturation process of pro-ANF. ¹²⁵I-ANF-(Asn¹-Tyr¹²⁶)-peptide was incubated with whole rat blood, plasma or serum for different time intervals, and the products were analysed. There was minimal activation of the propeptide in either whole blood or plasma. Incubation with serum, however, resulted in the formation of an 11 kDa and a 3 kDa peptide which corresponded respectively to the N-terminal and C-terminal parts of the propeptide. These results suggest that hydrolysis of the propeptide in serum is brought about by enzymes that may be stimulated during coagulation but which may not play a major role in the activation of pro-ANF in the circulation. Plasma analysis at different time intervals after prohormone injection indicated a non-specific hydrolysis of the pro-ANF molecule. The disappearance rate curves, obtained with radiolabelled pro-ANF, suggested the presence of two components with half-lives of 2.1 ± 0.4 min and 52.5 ± 8.4 min respectively. A metabolic clearance rate of 1.49 ± 0.22 ml/min and an initial distribution volume of 47.4 ± 8 ml were calculated. These results indicate that the maturation of pro-ANF to its active circulating form takes place before it is released into the circulation.

INTRODUCTION

Atrial natriuretic factor (ANF) is stored in specific granules of mammalian atrial cardiocytes [1–4] as a 126-amino-acid precursor [5–7]. The circulating form of this peptide in the rat has been identified as ANF-(Ser⁹⁹-Tyr¹²⁶)-peptide [8,9]. No propeptide has been reported in the circulation. However, the site of processing of the propeptide to its active 28-amino-acid form is still not known. Working with neonatal atrial myocytes in culture, Bloch *et al.* [10] and Glembotski & Gibson [11] found pro-ANF to be the secreted form, but Lang *et al.* [12] and Thibault *et al.* [13] demonstrated that isolated perfused rat hearts release the mature 3 kDa peptide into perfusates. No clear site of maturation or pathway of secretion has yet been assigned to ANF.

There have been some reports in the literature on the processing of ANF by rat serum [10,11,14]. However, no studies have yet been done on ANF processing in whole blood, a more physiological situation, and on the fate of pro-ANF released into the circulation. This investigation was undertaken to answer some of these questions. The contribution of blood to the activation process was examined by incubating the propeptide with whole blood, plasma and serum for different time intervals and analysing the products by molecular-sieve h.p.l.c. The half-life of this propeptide in the circulation and its processing *in vivo* were studied by injecting pro-ANF into the rat and analysing the components present in the circulation at various time points.

MATERIALS AND METHODS

Materials

ANF-(Ser⁹⁹-Tyr¹²⁶)-peptide was purchased from Institut Armand Frappier (Laval, Quebec, Canada). ANF-(Glu⁵⁴-Tyr¹²⁶)-peptide and the propeptide, ANF-(Asn¹-Tyr¹²⁶)-peptide, were purified from rat atria, as reported elsewhere [7,15]. The N-terminal fragment, ANF-(Asn¹-Arg⁹⁸)-peptide, was produced by incubation of pro-ANF with 'IRCM-serine protease #1' [16].

Methods

Blood collection. Blood was withdrawn from the abdominal aorta of male Sprague–Dawley rats (200–300 g) anaesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) and collected in prechilled Vacutainer tubes containing EDTA or heparin as anticoagulant. Plasma was separated by centrifugation at 3500 rev./min (r_{av} , 15 cm) for 20 min at 4 °C. To obtain serum, blood was collected in glass tubes and coagulated by incubation at 37 °C for 30 min. Serum was separated by centrifugation at 3500 rev./min for 20 min.

Iodination of pro-ANF. Pro-ANF was iodinated by the lactoperoxidase method and purified thereafter by h.p.l.c. [17]. The pro-ANF molecule contains two tyrosine residues, one at position 4 and the other at position 126. In order to verify to what extent each tyrosine residue was iodinated, two experiments were performed. Iodin-

Abbreviations used: ANF, atrial natriuretic factor; -CH₂Cl, chloromethane ('chloromethyl ketone').

* To whom correspondence and reprint requests should be sent.

ated pro-ANF was incubated with either 10 μ g of carboxypeptidase A or with 'IRCM-serine protease # 1', a serine proteinase that hydrolyses pro-ANF to ANF-(Ser¹⁰³-Tyr¹²⁶)-peptide with minor amounts of ANF-(Ser¹⁰²-Tyr¹²⁶)-peptide and ANF-(Ser⁹⁹-Tyr¹²⁶)-peptide [16]. The incubation media were thereafter analysed by molecular-sieve h.p.l.c. In both cases, 25 % of the radioactivity was present in the C-terminal portion, indicating that the iodination of tyrosine in position 4 was three times more efficient (probably aided by the secondary and tertiary structure of the pro-ANF molecule).

Degradation of pro-ANF in blood. Unlabelled ANF-(Asn¹-Tyr¹²⁶)-peptide (25 μ g/ml) or ¹²⁵I-ANF-(Asn¹-Tyr¹²⁶)-peptide (1 $\times$ 10⁶ c.p.m./ml) were incubated at 37 °C in whole blood [containing 10 mM-EDTA or 40 U.S.P. (U.S. Pharmacopeia) units of heparin/ml], plasma or serum for different periods of time. After incubation, portions of the mixture were acidified with an equal volume of 20 % (v/v) acetic acid kept on ice. Whole blood was first centrifuged for 5 min at 4 °C and the supernatant acidified.

In the case of the unlabelled material the acidified sample was extracted with a C₁₈ Sep-Pak cartridge [12]. The ethanol/acetic acid eluate was freeze-dried, reconstituted in molecular-sieve-h.p.l.c. buffer and fractionated on a Bio-Sil TSK column. The fractions eluted in the 11 kDa and 3 kDa regions were pooled separately and further analysed by reverse-phase h.p.l.c. on a C₁₈ μ Bondapak column (0.39 cm $\times$ 30 cm) eluted with a linear gradient (slope 0.5 %/min) of acetonitrile in 0.1 % trifluoroacetic acid.

Half-life determination. The rate of disappearance of pro-ANF from the circulation was essentially measured as reported previously [17]. In brief, ¹²⁵I-ANF-(Asn¹-Tyr¹²⁶)-peptide (10 $\times$ 10⁶ c.p.m.) or ANF-(Asn¹-Tyr¹²⁶)-peptide (25 μ g) was injected intrajugularly into male Sprague-Dawley rats (200–250 g) anaesthetized with sodium pentobarbital. Blood samples were collected from the left carotid artery at timed intervals from 5 s to 45 min, as indicated in Fig. 4 (below). Radioactivity in the blood was counted in a γ -radiation counter. The data were analysed by using a two-compartment model [18]. The plasma was separated by centrifugation, extracted with C₁₈ Sep-Pak cartridges [12] and analysed by molecular-sieve h.p.l.c. and by specific radioimmunoassays.

Radioimmunoassay. Two different antibodies were used, one directed towards the C-terminus of the ANF molecule [20], the other towards the N-terminus of the pro-ANF molecule [21]. Both radioimmunoassays were performed as described in the respective references cited.

Molecular-sieve h.p.l.c. This was conducted with a Bio-Sil TSK-125 column (0.75 cm $\times$ 60 cm), using a Varian 5060 liquid-chromatographic system. The peptides were eluted isocratically with 30 % (v/v) acetonitrile containing 0.1 % trifluoroacetic acid and 0.05 mM-NaCl. A flow rate of 0.5 ml/min and a collection time of 0.3 min/fraction were employed in the present study. The column was calibrated by using radioiodinated ANF peptides: ANF-(Ser⁹⁹-Tyr¹²⁶)-peptide (3 kDa); ANF-(Glu⁵⁴-

Tyr¹²⁶)-peptide (8 kDa); ANF-(Asn¹-Arg⁹⁸)-peptide (10.6 kDa); or ANF-(Asn¹-Tyr¹²⁶)-peptide (13.6 kDa).

RESULTS

Degradation of pro-ANF *in vitro*

Fig. 1 illustrates the elution profile of ¹²⁵I-ANF-(Asn¹-Tyr¹²⁶)-peptide incubated with whole blood containing 10 mM-EDTA as anticoagulant. In the control sample ($t = 0$), apart from the propeptide and free ¹²⁵I peaks, a third peak of higher M_r preceding that of pro-ANF was seen, indicating propeptide binding to a protein molecule. However, the proportion of this complex was small and remained constant throughout the incubation period. Upon incubation for 30 min, no major change occurred in the 13.6 kDa peak. However, a peak of radioactivity appeared in the 3 kDa region. It was much more pronounced after 60 min of incubation and was accompanied by a small shoulder in the 11 kDa region. However, even after this prolonged incubation, the major peak of radioactivity remained in the 13.6 kDa region, suggesting only a minor degradation of the propeptide molecule in whole blood in the presence of EDTA. There was also a slight increase in free ¹²⁵I after the 60 min incubation. This would indicate either the action of a blood carboxypeptidase on iodinated tyrosine at the C-terminus or the lability of bound iodine under the present reaction conditions.

The chromatographic profile of incubation of the propeptide with serum is shown in Fig. 2. After a 10 min incubation period, a peak appeared in the 3 kDa region, accompanied by a broadening of the 13.6 kDa peak, suggesting the presence of more than one peptide species in that region. After 30 min, however, almost all of the 13.6 kDa peak was converted into a 11 kDa peak, and there was a further increase in the 3 kDa peptide, indicating the presence of a pro-ANF-hydrolysing enzyme in serum.

To identify the two peptide species generated during incubation with serum, unlabelled ANF-(Asn¹-Tyr¹²⁶)-peptide (50 μ g) was incubated with serum for 120 min at 37 °C. The products were fractionated by molecular-sieve h.p.l.c. A study of immunoreactivity indicated that the fractions eluted in the 3 kDa region were recognized by the antibody directed to the C-terminus of the ANF molecule and the 11 kDa peptide by the antibody directed to the N-terminus. Each of these pools showed less than 1 % cross-reactivity with the other antibody. The results suggest that 13.6 kDa pro-ANF was converted into a 3 kDa C-terminal fragment and an 11 kDa N-terminal portion.

The above finding that three iodine atoms are found on tyrosine at position 4 for every one on tyrosine at position 126 and the observation that the C-terminal peptide ANF-(Ser⁹⁹-Tyr¹²⁶)-peptide is stable in whole blood, plasma [19] and serum [11] form the basis for the results shown in Fig. 3. The amount of 3 kDa peptide formed in serum at 30 min was taken as 100 %, and the rate of formation of this peptide in whole blood was calculated as a function of time. The rate of formation of the 3 kDa peptide in serum was at least 12 times higher than in whole blood (Fig. 3). Even after a 30 min incubation in whole blood, less than 50 % of the propeptide was converted into the circulating form, indicating it to be a very inefficient process.

Transformation of pro-ANF in two other situations

Fig. 1. Molecular-sieve h.p.l.c. of ^{125}I -ANF-(Asn¹-Tyr¹²⁶)-peptide incubated with whole blood

^{125}I -ANF-(Asn¹-Tyr¹²⁶)-peptide (1.2×10^6 c.p.m.) was incubated with 600 μl of whole blood containing 10 mM-EDTA at 37 °C for the indicated periods of time (t). The reaction was arrested by centrifugation and by diluting 250 μl of the supernatant with an equal volume of 20% (v/v) acetic acid. A portion (50 μl) of each sample was then analysed on a Bio-Sil TSK-125 column, as outlined in the Materials and methods section under 'molecular-sieve h.p.l.c.'. The arrows represent the elution position of marker ANF peptides whose molecular sizes are expressed in kDa. The total volume of the column was 42 ml.

was also investigated. Incubation of ^{125}I -ANF-(Asn¹-Tyr¹²⁶)-peptide with plasma containing 10 mM-EDTA as anticoagulant produced similar results. However, the proportion of the 11 kDa and the 3 kDa peptide was slightly higher in both the 30 min and 60 min incubations when compared with similar incubations with whole

Fig. 2. Molecular-sieve h.p.l.c. of ^{125}I -ANF-(Asn¹-Tyr¹²⁶)-peptide incubated with serum

The reaction conditions were the same as those outlined in the legend to Fig. 1, except that rat serum was used in place of whole blood.

blood, the formation of the 3 kDa peptide being about twice as high as that in the blood. Since the hydrolysis of pro-ANF appears to be dependent on the enzymes of coagulation, blood containing 10 mM-EDTA was incubated with labelled pro-ANF. At zero time 10 mM- CaCl_2 was then added to start coagulation and the rate of formation of the 3 kDa peptide examined (Fig. 3). A production rate intermediate between that of serum and plasma was observed.

In an attempt to identify an enzyme of the coagulation cascade which may be responsible for processing of pro-ANF, various inhibitors of coagulation were used.

EDTA is known to be a powerful chelator of bivalent

Fig. 3. Rate of production of 3 kDa peptide by whole blood, plasma and serum

The rate of production of the 3 kDa peptide was calculated assuming that pro-ANF is 3 times more iodinated in position 4 than in position 126 and that 100% conversion was obtained in serum at 30 min. Whole blood and plasma contained 10 mM-EDTA. In whole blood + CaCl_2 , 10 mM of CaCl_2 was added at zero time to start the coagulation process.

cations. To determine whether the difference in the hydrolysis characteristics of serum and whole blood or plasma was due to the inhibition of a cation-dependent enzyme, heparinized whole blood and plasma were used in the incubation studies. The elution profiles were identical with that obtained with EDTA as anticoagulant.

Heparin, a powerful anticoagulant, inhibits several factors in the coagulation cascade [22], as well as plasma kallikrein [23], through activation of antithrombin. To ascertain if any of these enzymes were involved in the hydrolysis of the 13.6 kDa peptide, iodinated ANF-(Asn¹-Tyr¹²⁶)-peptide was incubated for 30 min with serum containing 40 units of heparin/ml, and the products were analysed by molecular-sieve h.p.l.c. The elution pattern obtained was similar to that seen in the absence of heparin. To study the involvement of Ca^{+2} and other bivalent cations in the hydrolysis process, a 30 min incubation of the propeptide was carried out in serum containing 64 mmol of EDTA/ml. Even under these conditions, the 11 kDa peptide was the major species present, suggesting the activation of an enzyme during coagulation without inhibition by heparin or EDTA.

In order to further pinpoint a specific enzyme, the following experiment was conducted. To blood containing 10 mM-EDTA was added, at zero time, 10 mM- CaCl_2 , then, at 5 min, either 10 mM-EDTA, heparin (100 U.S.P./ml), Trasylol (aprotinin, 100 units/ml) or 10 μM -D-Phe-Phe-Arg- CH_2Cl to different portions. These last two agents are known to be potent inhibitors of serine proteinases and of plasma kallikrein respectively [24,25]. In one case, on addition of D-Phe-Phe-Arg- CH_2Cl , there was a slight decrease (30%) in the transformation of the pro-ANF, suggesting that plasma kallikrein may be in

Fig. 4. Disappearance rate curve of ^{125}I -ANF-(Asn¹-Tyr¹²⁶)-peptide after intravenous injection in normal rats

^{125}I -ANF-(Asn¹-Tyr¹²⁶)-peptide (10×10^6 c.p.m.) in heparinized saline was injected intrajugularly into the rat, and blood samples ($\sim 80 \mu\text{l}$) were collected through the carotid artery at timed intervals. The radioactivity in 50 μl of these blood samples was counted and the value represented as a percentage of the maximal radioactivity obtained (continuous). The two components of the curve are represented by broken lines. The fast component of the curve was calculated after subtracting the contribution of the slow component.

part implicated, although absence of coagulation was noted only in tubes containing EDTA and heparin.

The results indicate that hydrolysis of pro-ANF and production of the 3 kDa peptide appear to be in large part coagulation-dependent, and attempt to pinpoint a specific enzyme partially failed, suggesting that it may be due to several enzymes.

Degradation of pro-ANF *in vivo*

Experiments *in vivo* were conducted to study the rate of clearance of pro-ANF in the rat and to determine if its maturation could take place in the systemic circulation. Fig. 4 illustrates the rate of disappearance of iodinated pro-ANF from the circulation. The radioactivity in blood at different time intervals is presented as a percentage of the maximum obtained. From the disappearance curve, two components can be clearly distinguished. Half-lives of 2.1 ± 0.4 min and

Fig. 5. Molecular-sieve h.p.l.c. of rat plasma after intravenous injection of unlabelled ANF-(Asn¹-Tyr¹²⁶)-peptide

Unlabelled ANF-(Asn¹-Tyr¹²⁶)-peptide was injected intrajugularly into the rat, and blood samples (~500 µl) were collected through the carotid artery at timed intervals, the plasma separated, and a portion (200 µl) was loaded on a Bio-Sil TSK-125 column. The immunoreactive material in the individual fractions was estimated as outlined in the Materials and methods section under 'Radioimmunoassay'.

52.5 ± 8.4 min were calculated for the fast and slow components respectively. Similar half-life values were calculated by injecting unlabelled prohormone and monitoring the disappearance of N-terminal immunoreactivity (results not shown). An initial volume of distribution of 47.4 ± 8 ml and a metabolic clearance rate of 1.49 ± 0.22 ml/min were calculated for pro-ANF.

The various peptide fragments present in the circulation of the rat at different time points after the injection

of unlabelled pro-ANF are shown in Fig. 5. The plasma samples were subjected to molecular-sieve h.p.l.c. The immunoreactive material was analysed with two antibodies, one directed to the C-terminus and the other to the N-terminus of the pro-ANF molecule. No pro-ANF or the circulating C-terminal form could be detected in the control sample with the volume of plasma used. In the sample collected between 5 and 20 s after injection ($t = 5$), multiple peaks of both N- and C-terminal immunoreactive material were observed. The C-terminal immunoreactive peak in the 3 kDa region also showed partial N-terminal immunoreactivity. Since the cross-reactivities of the N-terminal fragment, ANF-(Asn¹-Arg⁹⁸)-peptide and the C-terminal fragment ANF-(Ser⁹⁹-Tyr¹²⁶)-peptide with the C-terminal and N-terminal antibodies respectively were negligible, the 3 kDa peak obtained would indicate the presence of more than one peptide species, suggesting non-specific hydrolysis of pro-ANF. In the sample collected at 2 min, two peaks were observed: one in the high M_r region and the other in the 3 kDa region. Both peaks had N- and C-terminal immunoreactivity. The broad peaks in both these regions would suggest the presence of multiple immunoreactive forms under each peak. A similar broad peak was also observed in the 10 min sample. These results indicate that injection of pro-ANF into the circulation results in its non-specific hydrolysis to give multiple immunoreactive forms and would therefore not contribute to its maturation into the active circulating form of the peptide.

DISCUSSION

In an effort to study the contribution of the circulation to the processing of pro-ANF, this peptide was incubated with whole blood, plasma and serum, and the products were analysed. The propeptide was hydrolysed in serum to give a 3 kDa peptide. However, both plasma and whole blood were found to be poor sources of this enzyme. The rate of conversion into the 3 kDa peptide in serum was about 12 times higher than in whole blood and about 5 times higher than in plasma. These results, which suggest that one or many enzymes present in serum are activated during the coagulation process are confirmed by the fact that addition of CaCl₂ to whole blood plus EDTA as anticoagulant caused rapid hydrolysis of pro-ANF. However, the physiological significance of such pro-ANF processing is not very clear, since its maturation in whole blood is minimal.

Similar results on the maturation of pro-ANF by serum have been reported previously [10,11]. Zisfein *et al.* [26] purified an enzyme from rat serum and showed it to be a serine proteinase having an M_r of 130 kDa. In a recent study, Gibson *et al.* [14] demonstrated that this enzymic cleavage in serum can be inhibited by D-Phe-Phe-Arg-CH₂Cl and D-Phe-Arg-CH₂Cl. They also determined that this conversion of pro-ANF into ANF could not be suppressed by these inhibitors in isolated perfused heart preparations. All these studies further support our contention that ANF processing in serum does not have any physiological significance. Similar conclusions were drawn by Michener *et al.* [27], who worked on the processing of atriopeptin prohormone.

Our studies *in vivo* in the rat indicated that the processing of injected pro-ANF in the system is non-specific. Clearance experiments established that the slow

component has a half-life of 52.5 min. Molecular-sieve h.p.l.c. also indicated the presence of one immunoreactive peak that was eluted just before the 13.6 kDa region, suggesting that the peptide binds to some plasma protein, thereby increasing its apparent M_r . This phenomenon probably enhances its resistance to both uptake and breakdown in the bloodstream. These studies also indicated the lack of specific maturation of pro-ANF to the circulating form upon release into the circulation.

In conclusion, ANF-(Asn¹-Tyr¹²⁶)-peptide is hydrolysed to different extents in whole blood, plasma and serum. Hydrolysis appears to be at its fastest in serum, suggesting the presence of enzymes activated during the coagulation process. However, the physiological significance of this hydrolysis is not clear, since only minimal activation is seen either in plasma or whole blood. Injection studies in the rat *in vivo* also suggest the lack of specific processing of the prohormone. Moreover, pro-ANF has never been detected in the blood collected directly from the coronary sinus of the heart [28,29] or in the effluent of perfused isolated rat heart [12,13]. This suggests that pro-ANF must be processed at a rate much higher than that which we have measured *in vitro* in the blood. In the light of these results, it could be hypothesized that maturation of ANF-(Asn¹-Tyr¹²⁶)-peptide, the form stored in atrial granules, into the circulating 28-amino-acid form, ANF-(Ser⁹⁹-Tyr¹²⁶), takes place before it is released into the circulation.

We thank Dr. J. Gutkowska for providing the C-terminal ANF antibody and Ms. C. Jutras for her excellent secretarial assistance. This work was supported in part by the Medical Research Council of Canada, the Canadian Heart Foundation, the National Research Council of Canada and the Ministère de la Science et de la Technologie du Québec via grants to the Multidisciplinary Research Group on Hypertension and by the Medical Research Council of Canada via Grant MA-9596 to G.T.

REFERENCES

- de Bold, A. J. (1982) *Can. J. Physiol. Pharmacol.* **60**, 324–330
- Garcia, R., Cantin, M., Thibault, G., Ong, H. & Genest, J. (1982) *Experientia* **38**, 1071–1073
- de Bold, A. J. & Salerno, T. A. (1983) *Can. J. Physiol. Pharmacol.* **61**, 127–130
- Nemeh, M. N. & Gilmore, J. P. (1983) *Circ. Res.* **53**, 420–423
- Kangawa, K., Tawaragi, Y., Oikawa, S., Mizuno, A., Sakuragawa, Y., Nakazato, H., Fukuda, A., Minamino, N. & Matsuo, H. (1984) *Nature (London)* **312**, 152–155
- Flynn, T. G., Davies, P. L., Kennedy, B. P., de Bold, M. C. & de Bold, A. J. (1985) *Science* **228**, 323–325
- Thibault, G., Garcia, R., Gutkowska, J., Bilodeau, J., Lazure, C., Seidah, N. G., Chrétien, M., Genest, J. & Cantin, M. (1987) *Biochem. J.* **241**, 265–272
- Thibault, G., Lazure, C., Schiffrin, E. L., Gutkowska, J., Chartier, L., Garcia, R., Seidah, N. G., Chrétien, M., Genest, J. & Cantin, M. (1985) *Biochem. Biophys. Res. Commun.* **130**, 981–986
- Schwartz, D., Geller, D. M., Manning, P. T., Siegel, N. R., Fok, K. F., Smith, C. E. & Needleman, P. (1985) *Science* **229**, 397–400
- Bloch, K. D., Scott, J. A., Zisfein, J. B., Fallon, J. T., Margolies, M. N., Seidman, C. E., Matsueda, G. R., Homcy, C. J., Graham, R. M. & Seidman, J. G. (1985) *Science* **230**, 1168–1171
- Glembotski, C. C. & Gibson, T. R. (1985) *Biochem. Biophys. Res. Commun.* **132**, 1008–1017
- Lang, R. E., Tholken, H., Ganten, D., Luft, F. C., Ruskoaho, H. & Unger, Th. (1985) *Nature (London)* **314**, 264–266
- Thibault, G., Garcia, R., Gutkowska, J., Lazure, C., Seidah, N. G., Chrétien, M., Genest, J. & Cantin, M. (1986) *Proc. Soc. Exp. Biol. Med.* **182**, 137–141
- Gibson, T. R., Shields, P. P. & Glembotski, C. C. (1987) *Endocrinology (Baltimore)* **120**, 764–772
- Thibault, G., Garcia, R., Cantin, M., Genest, J., Lazure, C., Seidah, N. G. & Chrétien, M. (1984) *FEBS Lett.* **167**, 352–356
- Seidah, N. G., Cromlish, J. A., Hamelin, J., Thibault, G. & Chrétien, M. (1986) *Biosci. Rep.* **6**, 835–844
- Murthy, K. K., Thibault, G., Schiffrin, E. L., Garcia, R., Chartier, L., Gutkowska, J., Genest, J. & Cantin, M. (1986) *Peptides* **7**, 241–246
- Oppenheimer, J. H. & Gurpide, E. (1979) in *Endocrinology (De Groot, L. J., ed.)*, vol. 3, pp. 2029–2036, Grune and Stratton, New York
- Murthy, K. K., Thibault, G., Garcia, R., Gutkowska, J., Genest, J. & Cantin, M. (1986) *Biochem. J.* **240**, 461–469
- Gutkowska, J., Thibault, G., Januszewicz, P., Cantin, M. & Genest, J. (1984) *Biochem. Biophys. Res. Commun.* **122**, 593–601
- Thibault, G., Murthy, K. K., Gutkowska, J., Seidah, N. G., Lazure, C., Chrétien, M. & Cantin, M. (1988) *Peptides* **9**, in the press
- Damus, P. S. & Rosenberg, R. D. (1976) *Methods Enzymol.* **45**, 653–669
- Lahiri, B., Bagdasarian, A., Mitchell, B., Talamo, R. C., Colman, R. W. & Rosenberg, R. D. (1976) *Arch. Biochem. Biophys.* **175**, 737–747
- Kettner, G. & Shaw, E. (1981) *Methods Enzymol.* **80**, 826–842
- Kassell, B. (1970) *Methods Enzymol.* **19**, 844–852
- Zisfein, J. B., Graham, R. M. & Homcy, C. J. (1986) *Clin. Res.* **34**, 634A
- Michener, M. L., Gierse, J. K., Seetharam, R., Fok, K. F., Olins, P. O., Mai, M. S. & Needleman, P. (1986) *Mol. Pharmacol.* **30**, 552–557
- Sugawara, A., Nakao, K., Mirii, N., Sakamoto, M., Suda, M., Shimokura, M., Kiso, Y., Kihara, M., Yamori, Y., Nishimura, K., Soneda, J., Ban, T. & Imura, H. (1985) *Biochem. Biophys. Res. Commun.* **129**, 439–446
- Yandle, T. G., Espiner, E. A., Nicholls, M. G. & Duff, H. (1986) *J. Clin. Endocrinol. Metab.* **63**, 72–79