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IN VIVO METABOLISM OF BRAIN NATRIURETIC PEPTIDE IN THE RAT INVOLVES ENDOPEPTIDASE 24.11 AND ANGIOTENSIN CONVERTING ENZYME

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The metabolism of brain natriuretic peptide (BNP) was studied in rats infused with ^{125}I-BNP. During the infusion, the intact peptide was progressively converted to labelled degradative products, separated into nine peaks of radioactivity on HPLC, and accounting for ~ 70 % of total plasma radioactivity at the plateau phase. After stopping the infusion, intact BNP disappeared with a half-life of 1.23 ± 0.35 min whereas the labelled fragments accounted for progressively greater proportion of total activity. The degradation of BNP was significantly reduced by phosphoramidon ($t_{1/2}$, 11.28 ± 0.49 min) and captopril ($t_{1/2}$, 6.99 ± 0.34 min). A maximal effect was observed when both protease inhibitors were given simultaneously ($t_{1/2}$, 15.3 ± 0.48 min). When ^{125}I-BNP was incubated *in vitro* with purified endopeptidase 24.11 (E-24.11) and angiotensin-converting enzyme (ACE), there was a time-dependent disappearance of the intact peptide associated with the generation of six labelled fragments, corresponding to fragments found *in vivo*. In serum the peptide was rapidly degraded with a half-life of 4.6 ± 0.1 min, and the pattern of labelled fragments was similar to that observed during *in vitro* incubation with ACE. Captopril significantly reduced the rate of degradation of BNP in serum. The results allow to associate two define enzyme activities, namely E-24.11 and ACE, with the metabolism of BNP *in vivo*. They also indicate that, despite a close homology between ANP and BNP, the two peptides undergo different pathways of clearance.

Brain natriuretic peptide (BNP) was first isolated and sequenced in 1988 from porcine brain (1). The peptide isolated by Sudoh and his colleagues showed a remarkable structure homology with atrial natriuretic peptide (ANP) which is also present in brain. The two hormones display however a distinct and non-overlapping distribution pattern in the central nervous system (2). Recent works provided evidence that, as ANP, BNP is synthesized by peripheral tissue, namely mammalian cardiac atria (3), and is secreted in circulation (4). Although the molecular forms of BNP in mammalian atria seem to differ among species, they all share a 17-amino acid residue disulfide loop.

BNP elicits a pharmacological spectrum very similar to ANP. Intravenous injection of synthetic porcine BNP into anaesthetized rats produced an increase in water and electrolytes

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Abbreviations used in this paper: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; TFA, trifluoroacetic acid; IodoGen, 1,3,4,6-Tetrachloro-3a,6a-diphenylglycouril; E-24.11, endopeptidase E.C. 3.4.24.11.; ACE, peptidyl dipeptidase A, E.C.3.4.15.1 (angiotensin-converting enzyme).

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excretion into urine, and a significant decrease in mean blood pressure, in a manner comparable to the effects induced by ANP at the same dose (1). The great similarities in structure and physiological effects between ANP and BNP led us to investigate whether the two peptides undergo similar metabolic pathways. Therefore, we have now infused ^{125}I-labelled BNP into rats, and studied the effects of protease inhibitors on the degradation rate of the peptide. Our data demonstrate that BNP has a longer half-life *in vivo* compared to ANP, and that the breakdown of the peptide involves at least two distinct peptidases, namely endopeptidase 24.11 (E-24.11) and angiotensin converting enzyme (ACE).

MATERIALS AND METHODS

Materials. Purified rabbit lung ACE (batch n° 66F-9610; activity, 3 units/mg protein) and phosphoramidon were obtained from Sigma (St Louis, MO); Captopril was a gift from the Squibb Institute for Medical Research (Princeton, N.J.). IodoGen was obtained from Pierce (Rockford, IL) and porcine BNP from Cambridge Research Biochemicals (Cambridge, U.K.).

Radioiodination. Porcine BNP(1-26) (1 nmol) was labelled with 18.5 MBeq ^{125}Iodine by means of IodoGen as described previously (5). The specific activity of the labelled peptide was ~ 5.5 MBeq/nmol of peptide.

Experimental procedures. Two types of experiments were carried out. First, *in vivo* metabolism was studied in rats during and after infusion of ^{125}I-BNP either alone or in presence of enzyme inhibitors, phosphoramidon and/or captopril. Second, metabolism of ^{125}I-BNP was studied *in vitro* during incubation with serum or with pure enzymes, E-24.11 and ACE.

^{125}I-BNP infusions in rat. Male Wistar rats (240-300 g) were anaesthetized with ether and the left femoral and right carotid arteries were cannulated. After 10 min infusion of 0.15 M NaCl either alone or together with the enzyme inhibitor to be studied, 0.17-0.25 MBeq ^{125}I-BNP was added to the infusion and given during 10 min. In a first test, four rats were infused with ^{125}I-BNP without addition of enzyme inhibitors. In a second test, rats were infused with ^{125}I-BNP and 10^{-4} M phosphoramidon (3 nmol/min per kg; n = 3) or 10^{-5} M captopril (0.3 nmol/min per kg; n = 3). Finally, three rats were infused with ^{125}I-BNP together with phosphoramidon and captopril. Blood samples of 0.6 ml were taken over a period varying from 2.5 to 25 min, depending on the enzyme inhibitor infused. Plasma was made 4 % in acetic acid and the peptidic material was extracted on a SepPak C_{18} cartridge (Waters, Millford, MA). The samples were stored at -20 °C before HPLC fractionation.

In vitro incubation of ^{125}I-BNP. ^{125}I-labelled BNP (1.5 KBeq) was incubated for 1 h at 37 °C either with purified E-24.11 (6) (1 μg protein.ml^{-1}) in 200 μl 0.05 M Tris-HCl buffer pH 7.6 containing 0.1 % Triton X-100, or with a commercial preparation of rabbit lung ACE (20 μg protein.ml^{-1}) in 200 μl Tris HCl pH 7.6 containing 200 mM NaCl. Reaction was stopped by the addition of 20 μl 10 % TFA. ^{125}I-BNP (1.5 KBeq) was also incubated at 37 °C with 100 μl rat serum alone or with 10^{-6} M phosphoramidon and/or 10^{-6} M captopril. The incubation was allowed for 1, 2, 4 or 6 min, and the reaction was stopped by addition of an equal volume of 20 % TFA. After centrifugation, the supernatant was fractionated by HPLC.

HPLC separation of labelled peptidic fragments. Radiolabelled BNP-related peptides were separated by reverse-phase HPLC on a Waters μBondapak C_{18} column. Elution was carried out at room temperature using a linear gradient from 5 to 40 % acetonitrile in 0.08 % TFA over 50 min. Fractions of 1 ml were collected and counted for radioactivity in a gamma-counter (LKB). The columns were calibrated with the ^{125}I-labelled standards Tyr, Arg-Tyr and BNP(1-26).

Statistical methods. Difference between means was evaluated by Student's *t*-test. Linear regression analysis was performed by the method of least squares. Results were expressed as mean ± SEM.

RESULTS

Metabolism of ^{125}I-BNP in vivo. Seven min after beginning the infusion of ^{125}I-BNP, the plasma concentration of intact peptide reached a plateau level. When the infusion was

stopped, intact labelled BNP disappeared rapidly, with a calculated mean half-life of 1.23 ± 0.35 min. HPLC separation of plasma taken during the plateau phase of ^{125}I-BNP infusion revealed a major peak of radioactivity eluting in the position of standard intact BNP and accounting for ~ 30 % of the total radioactivity. Nine radioactive peaks eluted in front of intact labelled BNP (Fig. 1A). On basis of their retention time, peaks 1 and 2 corresponded to ^{125}I-Tyr and ^{125}I-Arg-Tyr, respectively. These nine peaks of BNP-derived radioactivity were also found in plasma samples collected 2 min after stopping the infusion. However, peaks 2, 3 and 4 increased relative to total radioactivity, whereas the peak of intact ^{125}I-BNP was proportionnaly reduced.

When ACE activity was inhibited by infusion of captopril, the plasma concentration of intact ^{125}I-BNP at the plateau level was significantly increased, and after stopping the infusion, the half-life of the intact peptide was significantly prolonged from 1.23 to 6.99 ± 0.34 min (Fig. 2). Also, the pattern of circulating radioactivity was modified since peak 2, corresponding to Arg-Tyr, and peak 3 were no longer present, whereas the generation of peaks 4 to 9 was not inhibited by captopril (Fig. 1B). After stopping the infusion, there was a progressive accumu-

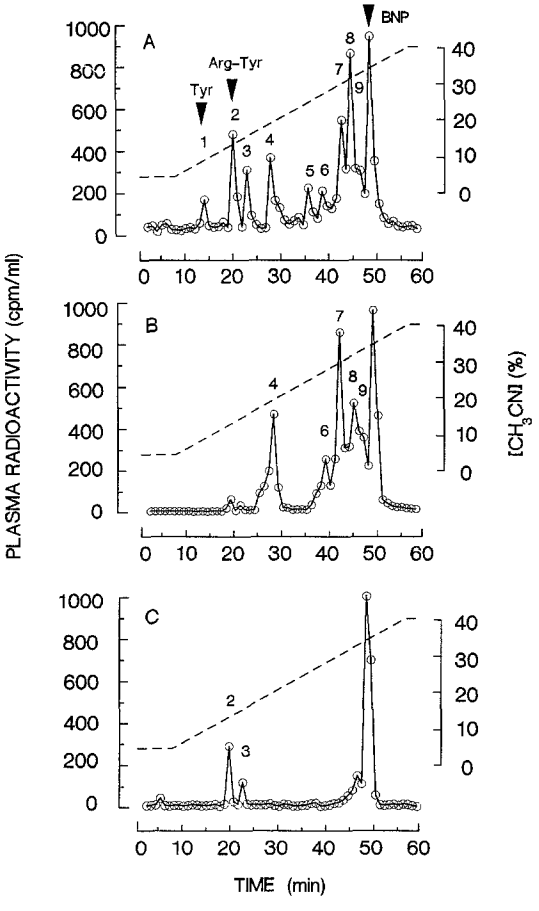

Figure 1. HPLC analysis of plasma radioactivity collected during infusion of ^{125}I-BNP. The peptide was infused for 10 min (A) alone, (B) with captopril or (C) with phosphoramidon. Plasma radioactivity was extracted on SepPak C_{18} cartridges and fractionated by HPLC using a linear gradient of acetonitrile (---). Arrows indicate the elution position of monoiodinated standards.

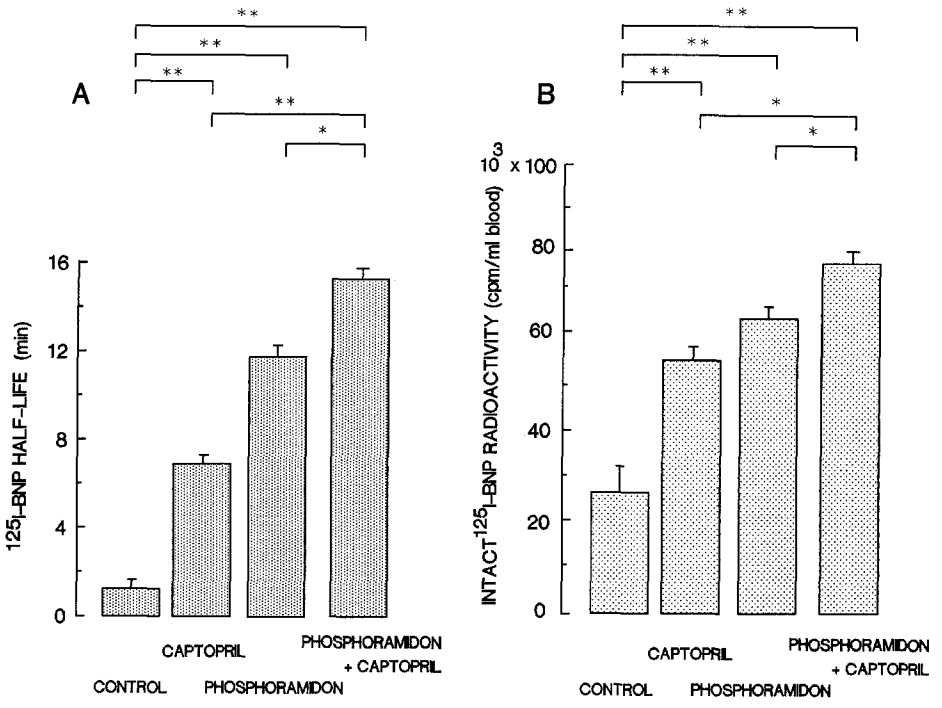

Figure 2. Effect of phosphoramidon and captopril on the plasma half-life (A) and concentration (B) of infused ^{125}I-BNP. The labelled peptide was infused in absence of inhibitor, with captopril, phosphoramidon, and a combination of the two inhibitors. Blood samples were collected at timed intervals during and after stopping the infusion. The level of circulating intact peptide was obtained by HPLC analysis of plasma radioactivity. Values (mean ± SEM) are significantly different with (*) $p = 0.05$ or (**) $p = 0.001$.

lation of peak 4 which was the only labelled degradation product detected in plasma collected 15 min after the end of infusion.

Infusion of phosphoramidon, a specific E-24.11 inhibitor, together with ^{125}I-BNP led to a 10-fold increase of the disappearance rate of the intact peptide ($t_{1/2}$, 11.8 ± 0.49 min) (Fig. 2). HPLC fractionation of plasma taken during the infusion, revealed that ~ 80 % of radioactivity was carried by intact BNP, and the remaining 20 % coeluted with peak 2 and 3 (Fig. 1C). Peaks 4 to 9, detected when the peptide was given alone or with ACE, were no longer present in detectable amounts during phosphoramidon infusion.

A maximal prevention of BNP degradation was observed when captopril and phosphoramidon were infused simultaneously. The half-life of ^{125}I-BNP significantly increased, reaching 15.3 ± 0.48 min, while the plasma concentration of intact peptide was increased ~ 3-fold as compared to the values obtained in absence of inhibitor (Fig. 2). Intact labelled BNP accounted now for almost 100 % of circulating radioactivity since no labelled degradation fragment could be detected by HPLC separation of plasma collected during the infusion.

In vitro incubation of ^{125}I-BNP. Upon incubation with purified E-24.11, ^{125}I-BNP was converted into several radioactive fragments that were separated by HPLC (Fig. 3A). Four peaks of radioactivity were generated, which corresponded to the peaks 6 to 9 found during infusion of ^{125}I-BNP in rats (Fig. 1A) . The pattern of peaks observed *in vitro* with E-24.11 resembled that observed *in vivo* in presence of captopril and was characterized by the absence

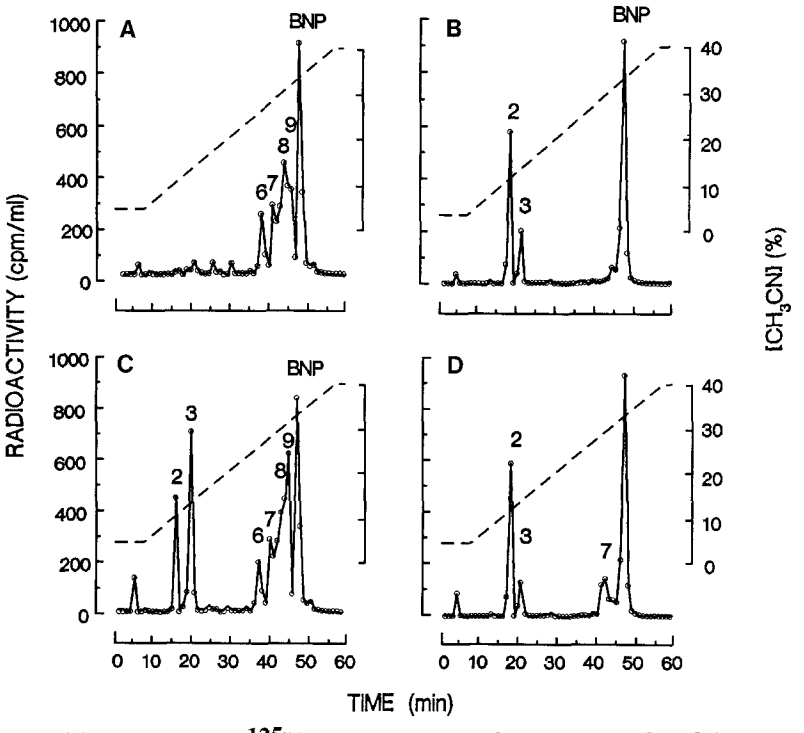

Figure 3. HPLC analysis of ^{125}I-BNP degradation fragments produced in vitro upon incubation with purified enzymes or in serum. ^{125}I-BNP was incubated for 1 h at 37 °C with (A) E-24.11, (B), ACE, or (C) a combination of the two enzymes. Incubation experiments performed in rat serum (D) were stopped after 5 min. The reaction mixtures were separated by HPLC.

of small C-terminal labelled fragments, namely peaks 2 and 3. In contrast, these latter peaks were the only radioactive peaks produced by incubation of labelled BNP with purified ACE (Fig. 3B): the first and major peak corresponded, on basis of coelution with a standard, to the labelled dipeptide Arg-Tyr whereas the other peak (peak 3) could correspond to the C-terminal tripeptide Arg-Arg-Tyr, but was not formally identified. The hydrolysis of the antepenultimate bond by ACE is not surprising since the enzyme has already been shown to have a tripeptidyl carboxypeptidase activity on other substrates (7). This pattern of radioactive forms produced with ACE was identical to that found *in vivo* during infusion of phosphoramidon and ^{125}I-BNP. Incubation of ^{125}I-BNP with a mixture of the two purified enzymes led to the generation of peaks 2, 3, 6, 7, 8 and 9 (Fig 3C). Thus, six from the nine fragments present in rat circulation could be attributed to cleavage by either ACE or E-24.11. In contrast, none of these two enzymes could generate fragments compatible with the peaks 4 and 5 observed upon infusion of ^{125}I-BNP *in vivo* (Fig. 1).

When ^{125}I-BNP was incubated in serum, the intact peptide disappeared rapidly with a half-life of 4.6 ± 0.1 min. Interestingly, the labelled degradation fragments separated by HPLC corresponded to peaks 2 and 3, also observed with ACE. In addition, a small amount of peak 7 was also detectable (Fig. 4D). Addition of captopril significantly increased the peptide half-life from 4.6 to 8.3 ± 0.3 min,whereas phosphoramidon had no significant effect ($t_{1/2}$, 5.6 ± 0.2 min). In presence of a mixture of the two inhibitors, the peptide half-life in serum reached 9.9 ± 0.4 min.

DISCUSSION

The principal finding of this study is that, after infusion, ^{125}I-BNP is rapidly degraded ($t_{1/2}$, 1.23 ± 0.35 min), giving rise to two labelled C-terminal degradation fragments that were also found during *in vitro* incubation of the peptide with serum or purified angiotensin-converting enzyme. In addition, four other labelled fragments were found that can be produced by incubation of ^{125}I-BNP with endopeptidase 24.11 (E-24.11). Although circulating BNP degradation was decreased by a factor of ten in presence of phosphoramidon, several labelled fragments (peaks 2 and 3 on HPLC) were still generated, suggesting that, in addition to E-24.11, other enzyme activities take part in the metabolism of BNP. The idea that a dipeptidyl carboxypeptidase activity could be involved was supported by the fact that one labelled fragment identified during phosphoramidon infusion, corresponded to the C-terminal dipeptide Arg-Tyr (peak 2). Our *in vitro* and *in vivo* results demonstrate that angiotensin-converting enzyme could play a physiological role in the inactivation of BNP. First, incubation of ^{125}I-BNP with purified ACE led to the generation of two radiolabelled fragments corresponding to peaks 2 and 3 detected during infusion of ^{125}I-BNP alone or with phosphoramidon. Second, infusion of captopril led to a 6-fold increase of the peptide half-life and abolished the generation of peaks 2 and 3.

On basis of the remarkable structure homology between ANP and BNP, it could be assumed that both peptides share a common metabolic pathway. Our results suggest that this assumption is only partially justified. Indeed, ANP is not hydrolysed by ACE despite the presence of an antepenultimate aromatic residue (Phe-Arg-Tyr) which, in other substrates, has been shown to enhance the enzyme affinity. It is likely that differences in amino acids are responsible for tertiary structural modifications that can regulate the recognition of ACE specific cleavage site and thereby influence the efficiency of the enzyme. The fact that limited primary structure differences between BNP and ANP may play an important role in site specific recognition for enzymatic hydrolysis is further illustrated by the recent work of Vogt-Schaden and colleagues (8). Incubating porcine BNP-26 with a membrane preparation from kidney cortex, the peptide was converted into several fragments. Characterization of these BNP-derived peptides allowed the deduction of 5 cleavage sites, all located inside the disulfide-linked loop. Involvement of endopeptidase 24.11 was confirmed by the effect of phosphoramidon. However, the cleavage sites found in BNP were principally different from those reported for ANP (9,10). Moreover, no cleavage was found between Cys and Phe whereas this peptidic bond has been shown to constitute the initial and only physiologically relevant site at which E-24.11 cleaves ANP (5). In the present study, the infusion rate of ^{125}I-BNP was low in order to maintain the level of circulating peptide in the physiological range. As a consequence, the amount of BNP-derived material obtained was not sufficient to allow identification by amino acid analysis. However, in the light of the results obtained by Vogt-Schaden et al., it is likely that the labelled fragments fractionated into peaks 6 to 9 correspond to BNP-derived peptides exhibiting single or multiple amino acid deletions inside the disulfide-linked loop.

It is commonly admitted that ANP is almost stable in plasma. To determine whether conversion of BNP to shorter fragments was due to the action of serum enzymes, we performed incubation experiments in rat serum. In contrast to ANP, BNP was efficiently degraded by serum enzyme activities producing a pattern of fragments similar to that generated by purified ACE. The presence of captopril significantly increased the peptide resistance to serum degradation. These observations indicate that not only membrane-bound but also circulating ACE activities could be of physiological importance in the clearance of the intact peptide.

Although endopeptidase activity is also detectable in normal plasma (11,12,13), its circulating levels are probably too low to play a role in the inactivation of circulating BNP.

The present results provide a first insight into the enzymatic mechanisms involved in the degradation of circulating BNP. Despite a close structure homology, BNP and ANP undergo distinct metabolic pathways. BNP is rapidly cleared from circulation by the combined action of E-24.11 and ACE whereas ANP clearance is not affected by ACE. The fact that E-24.11 is involved in the metabolism of BNP indicates that the observed potentiation of the diuretic and natriuretic effects of ANP following administration of E-24.11 inhibitors (14,15,16,17), may reflect the protection of both ANP and BNP from proteolytic degradation. The evidence that BNP is hydrolysed *in vivo* by at least two distinct peptidases, together with our results demonstrating the involvement of both E-24.11 and a kallikrein-like peptidase in the breakdown of ANP (5) reinforce the idea that the use of a combination of protease inhibitors to protect the two peptides from degradation should open new perspectives in the therapeutical approach of cardiovascular diseases.

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REFERENCES

1. Sudoh, T., Kangawa, K., Minamino, N. and Matsuo, H. (1988) Nature 332, 78-81.
2. Ueda, S., Minamino, N., Sudoh, T., Kangawa, K. and Matsuo, H. (1988) Biochem. Biophys. Res. Commun. 155, 733-739.
3. Kambayashi, Y., Nakao, K., Mukoyama, M., Saito, Y., Ogawa, Y., Shiono, S., Inouye, K., Yoshida, N. and Imura, H. (1990) FEBS Lett. 259, 341-345.
4. Togashi, K., Hirata, Y., Ando, K., Takei, Y., Kawakami, M. and Marumo, F. (1989) FEBS Lett. 250, 235-237.
5. Vanneste, Y., Pauwels, S., Lambotte, L., Michel, A., Dimaline, R. and Deschodt-Lanckman, M. (1990) Biochem. J. 269, 801-806.
6. Deschodt-Lanckman, M., Pauwels, S., Najdovski, T., Dimaline, R. and Dockray, G.J. (1988) Gastroenterology 94, 712-721.
7. Cascieri, M.A., Bull, H.G., Mumford, R.A., Patchett, A.A., Thornberry, N.A. and Liang T. (1984) Molec. Pharmacol. 25, 287-293.
8. Vogt-Schaden, M., Gagelmann, M., Hock, D., Herbst, F. and Forssmann, W.G. (1989) Biochem. Biophys. Res. Commun. 161, 1177-1183.
9. Stephenson, S.L. and Kenny, A.J. (1987) Biochem. J. 243, 183-187.
10. Vanneste, Y., Michel, A., Dimaline, R., Najdovski, T. and Deschodt-Lanckman, M. (1988) Biochem. J. 254, 531-537.
11. Almenoff, J., Teirstein, A., Thornton, J.C. and Orlowski, M. (1984) J. Lab. Clin. Med. 103, 420-431.
12. Johnson, A.R., Coalson, J.J., Ashton, J., Larumbide, M. and Erdös, E.G. (1985) Am. Rev. Respir. Dis. 132, 1262-1267.
13. Deschodt-Lanckman, M., Michaux, F., De Prez, E., Abramowicz, D., Vanherweghem, J.L. and Goldman, M. (1989) Life Sci. 45, 133-141.
14. Olins, G.M., Krieter, P.A., Trapani, A.J., Spear, K.L. and Bovy, P.R. (1989) Mol. and Cell. Endocrinology 61, 201-208.
15. Sybertz, E.J., Chiu, P.J.S., Vemulapalli, S., Pitts, B., Foster, C.J., Watkins, R.W., Barnett, A. and Haslanger, M.F. (1989) J. Pharmacol. and Exp. Ther. 250, 624-631.
16. Gros, C., Souque, A., Schwartz, J.C., Duchier, J., Cournot, A., Baumer, P. and Lecomte, J.M. (1989) Proc. Natl. Acad. Sci. 86, 7580-7584.
17. Northridge, D.B., Jardine, A.G., Alabaster, C.T., Barclay, P.L., Connell, J.M.C., Dargie, H.J., Dilly, S.G., Findlay, I.N., Lever, A.F. and Samuels, G.M.R. (1989) The Lancet II (8663), 591-593.