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DEMONSTRATION OF CORTICOTROPIN-RELEASING FACTOR BINDING SITES ON HUMAN AND RAT ERYTHROCYTE MEMBRANES AND THEIR MODULATION BY CHRONIC ETHANOL TREATMENT IN RATS

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In a previous study we reported the presence of specific corticotropin-releasing factor (CRF) binding sites in peripheral tissues of the rat (Endocrinology, 116, 2152, 1985). Using ^{125}I-labeled rat or human CRF, specific CRF binding sites were identified on rat and human erythrocytes, but not on lymphocytes or platelets. Furthermore, identical CRF binding was observed in the presence of intact erythrocytes or lysed erythrocyte membranes. Maximal binding of ^{125}I-CRF occurred within 25 min at 4°C and was saturable. Scatchard analysis of CRF binding to erythrocyte membranes revealed the existence of a single class of binding site. Chronic exposure of rats to ethanol vapor, known to lower specific CRF binding to pituitary tissue by 35%, also decreased ^{125}I-rat CRF binding to erythrocyte membranes by approximately 45%, which was due to a decrease in the number of CRF binding sites. The parallel decrease of CRF binding to rat-erythrocyte and pituitary membranes following chronic ethanol treatment suggests that CRF binding to erythrocyte and pituitary membranes is modulated in a similar direction, which further suggests that the determination of CRF binding to erythrocytes may provide an important clinical tool to indirectly assess CRF-receptor levels in the pituitary gland and thereby enhance our understanding of ethanol-induced disorders of the hypothalamic-pituitary-adrenal axis in patients.

Corticotropin-releasing factor (CRF), a hypothalamic-regulatory peptide containing 41 amino acids, is known to be a potent stimulus for the release and synthesis of pro-opiomelanocortin (POMC)-derived peptides from the pituitary gland (1-8), such as ACTH, ß-endorphin (BE) and ß-LPH. Furthermore, CRF has extrahypophysiotropic effects, as evidenced by CRF-induced behavioral and electrophysiological changes after the intracerebroventricular administration of CRF (9-13), and has a wide extrahypothalamic distribution in the central nervous system (14-20). The existence of CRF-like activity in a variety of peripheral tissues and tumors, including ACTH-producing colonic carcinoma, ectopic ACTH-producing tumors, lung carcinoma, adrenal gland, gut, and pancreas, has been previously demonstrated (21-25). High affinity binding sites for CRF in the anterior and intermediate lobes of the pituitary gland have been identified and characterized (26-28). Binding of CRF to its receptors on pituitary-gland cells appears to initiate the physiological actions of CRF, which include stimulation of the adenylate cyclase-cAMP system. In a recent study, we demonstrated the presence of specific

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CRF-binding sites in a variety of peripheral tissues and reported that activation of specific CRF-binding sites in adrenal tissue stimulates the adenylate cyclase-cAMP system (29). These observations together with other findings suggest that CRF may have an important regulatory role in various peripheral tissues including the extra-adrenal peripheral autonomic nervous system (30). Since the existence of specific CRF binding sites in erythrocytes was not known, the objectives of the present study were to 1) identify specific CRF binding sites in human and rat erythrocytes, 2) define optimal CRF binding assay conditions and 3) establish whether or not changes in CRF binding to erythrocytes paralleled those in the pituitary gland.

METHODS

Materials: Rat CRF (rCRF), human CRF (hCRF), rat tyrosinated CRF (r[Tyr°]CRF), ovine CRF (oCRF), acetyl-human ß-endorphin (1-31; AchBE) and rat ß-endorphin (1-31; rBE) were purchased from Peninsula Laboratories (Belmont, CA) or Bachem (Torrance, CA).

Human Subjects: Normal male volunteers (30-40 yrs of age), who were on a regular diet and had fasted overnight, served as blood donors. At 0900 hrs 10 ml of venous blood was drawn into a heparinized Vacutainer tube containing 15 mg EDTA and 10 TIU aprotinin (Sigma Chemical Co; St. Louis, MO) and immediately placed on ice.

Animals: Adult male rats (200-250 g) of the Sprague-Dawley strain (NIH stock and Taconic Farms, Germantown, NY) were used in all experiments. Animals were maintained on a 12-h light-dark cycle, with food and water available *ad lib* . Following decapitation, trunk blood was collected into a heparinized Vacutainer tube at 4°C containing EDTA and aprotinin to achieve a final concentration of EDTA and aprotinin of 1.5 mg and 1 TIU per ml of blood, respectively.

CRF Binding Assay: All subsequent steps in the preparation of erythrocyte membranes, intact erythrocytes or isolation of lymphocytes and platelets for the CRF binding assay were performed at 4°C. The erythrocyte membrane preparation was similar to that described previously (31). Briefly, each blood sample was centrifuged at 900 x *g* for 10 min, the plasma was removed and each cell pellet was washed twice with 10 ml of phosphate-buffered saline (PBS), pH 7.4. Following each wash, the sample was recentrifuged at 900 x *g* for 10 min. The resulting pellet was resuspended in 30 ml of 5 mM phosphate buffer at pH 8.0 and recentrifuged. The lysed cell pellet was washed repeatedly in 5 mM phosphate buffer until all visible traces of hemoglobin were gone. The resulting membranes were resuspended in 10 mM MgCl2-25 mM Tris-HCl at pH 7.6 to provide approximately 3 mg/ml protein (32). In several experiments the erythrocyte, lymphocyte and platelet-rich fractions were isolated using Lymphocyte Separation Medium (Litton Bionetics, Inc., Charleston, SC). ^{125}I-labeled rat CRF or human CRF was prepared by a modification of the lactoperoxidase method of Thorell and Johansson (33) as described earlier (34). One hundred microliters of membrane suspension were incubated for various time intervals at either 4°C or 22°C with 60,000 - 70,000 cpm of ^{125}I-labeled rCRF or hCRF (specific activity of approximately 100 µCi/µg) with or without unlabeled rCRF or hCRF in a final volume of 0.5 ml of buffer (10 mM MgCl2/0.1% bovine serum albumin/25 mM Tris-HCl, pH 7.6). Incubation was terminated by adding 1 ml of chilled buffer to each reaction tube followed by centrifugation at 3200 rpm for 30 min. Pellets were washed with an additional 1 ml of buffer, recentrifuged and each pellet was counted in a Micromedic 10/600 gamma counter (Horsham, PA). Each sample was assayed in triplicate. The number of specifically bound counts was calculated as the difference between counts in the tubes containing 1 µM unlabeled CRF or no unlabeled CRF. Routinely, approximately 20% of the total ^{125}I-CRF added to each incubation tube was bound and 60-70% of these counts were specifically bound (i.e. displaceable by 1 µM unlabeled CRF). Scatchard analysis was performed by incubating the iodinated CRF with varying amounts of unlabeled CRF (0 to 10^{-6}M) (35). Using 'LIGAND', a computer program system for fitting multiple binding sites (36) the data were analyzed and checked for the number of binding sites by Scatchard plot fit. The preparation of anterior- and neurointermediate-lobe membrane fractions of the pituitary gland and assay conditions for CRF binding were as described earlier (28).

Ethanol Vapor Exposure: Animals were exposed to ethanol vapor for 14 days as described previously (28). Periodic blood samples for alcohol determinations (Sigma diagnostics alcohol procedure no. 332-UV, Sigma Chemical Co., St. Louis, MO) were obtained from the tail artery.

RESULTS

Specific binding of ^{125}I-labeled rCRF to rat erythrocytes and ^{125}I-hCRF to human erythrocytes was rapid and time dependent at 4 °C (Figure 1) with maximal binding occurring at 25 min of incubation which declined thereafter. Incubations at 22°C were not optimal. The binding of rCRF to rat erythrocyte membranes or hCRF to human erythrocyte membranes was found to be dependent on the presence of magnesium chloride. Maximal binding of CRF was observed at 10 mM $MgCl_2$, whereas, at concentrations of 0, 5, 15 or 20 mM MgCl2, CRF binding was reduced by approximately 100, 50, 25 and 35%, respectively, in both rat- and human-derived erythrocytes (data not shown). The specific binding of rCRF to rat erythrocyte and hCRF to human erythrocyte membranes was linear over the range of 50-300 μg membrane protein (data not shown). At protein concentrations greater than 350 μg, non-specific binding increased from approximately 25% to more than 50% of the total binding.

The relative distribution of ^{125}I-labeled rCRF binding sites in the crude microsomal fraction (15,000 - 100,000 x *g* membrane fraction) of rat adrenal, ventral prostate, spleen, liver, kidney and testis, as depicted, was taken from an earlier publication (29) and is presented for comparison to that in rat erythrocyte membranes (Figure 2). In comparison to other peripheral tissue, erythrocytes contain a substantial number of CRF binding sites. Scatchard analysis of hCRF binding to human erythrocyte membranes (Figure 3) or rCRF binding to rat erythrocyte membranes (Figure 4) revealed the existence of a single class of binding site. The apparent affinity constant (K_a) of hCRF binding to human

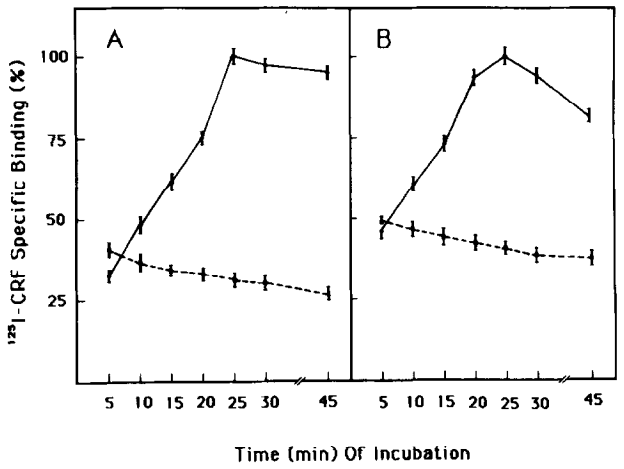
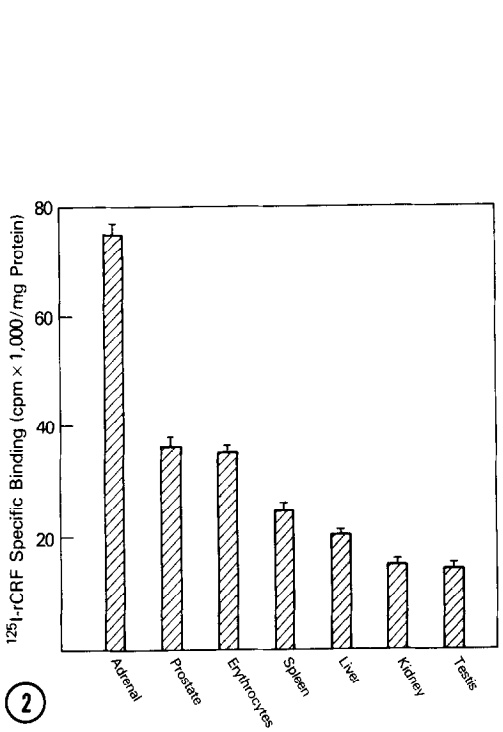


Figure 1: Demonstration of time- and temperature-dependent changes of ^{125}I-hCRF or rCRF binding to erythrocyte membranes obtained from humans (A) or rats (B). Binding was carried out at either 4°C (solid lines) or 22°C (dotted lines) in buffer which consisted of 25 mM Tris-HCl, pH 7.6 containing 10 mM MgCl2 and 0.1% bovine serum albumin. Erythrocytes obtained from 3 adult males (30 to 40 yrs) or 10 adult male rats (250-300 g) were pooled and lysed. Each point represents the mean ± S.E. of triplicate determinations.



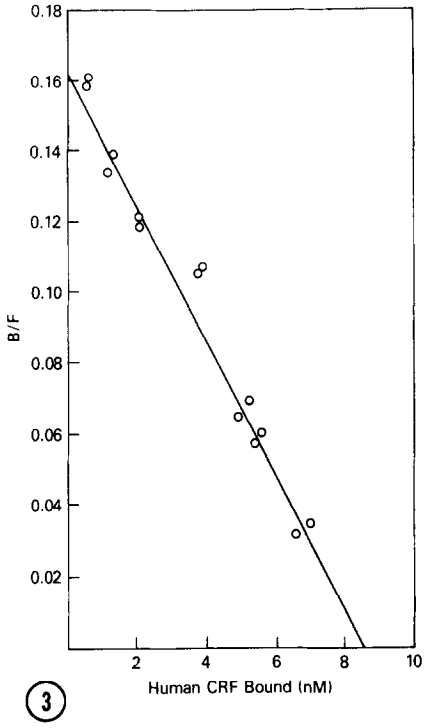


Figure 2: The relative distribution of ^{125}I-rCRF binding to various peripheral tissues and erythrocytes in the rat. CRF binding data for adrenal, prostate, spleen, liver, kidney and testis taken from an earlier study (29). CRF binding was carried out at 4°C for 25 min. A pool of erythrocytes obtained from 3 adult male rats (250-300 g) was used and each bar represents the mean ± S.E. of triplicate determinations.

Figure 3: Scatchard analysis of ^{125}I-human CRF binding to human erythrocyte membranes, suggesting the existence of a single class of binding sites. Binding was carried out at 4°C for 25 min and data are plotted according to the method of Scatchard (35). With the use of 'Ligand', a computer program system for fitting multiple or single binding sites, the data were analyzed and checked for a Scatchard plot fit (36). The amount of membrane protein present was approximately 250 μg. Each point represents the mean of duplicate determinations.

erythrocyte membranes was 0.010 ± 0.001 x 10^9 M^{-1} and the number of hCRF binding sites was 30 ± 5 pmol hCRF/mg protein. The K_a for rCRF binding to rat erythrocyte membranes was 0.006 ± 0.0007 x 10^9 M^{-1} and the number of binding sites was 18 ± 3 pmol rCRF/mg protein (n = 3; mean ± SEM).

Species binding specificity was observed in that ^{125}I-hCRF binding to human erythrocyte membranes in the presence of hCRF (10^{-6}M) inhibited CRF binding by 100%, which was identical to that observed when ^{125}I-rCRF was incubated with 10^{-6}M rCRF in the presence of rat erythrocyte membranes; however, when cross-species binding studies were performed (e.g. ^{125}I-rCRF with oCRF at 10^{-6}M or ^{125}I-hCRF with rCRF at 10^{-6}M) the inhibition of binding was substantial but less than 100% (Table 1). A number of peptides (e.g. AchBE, leucine- and methionine-enkephalin and prolactin) with no

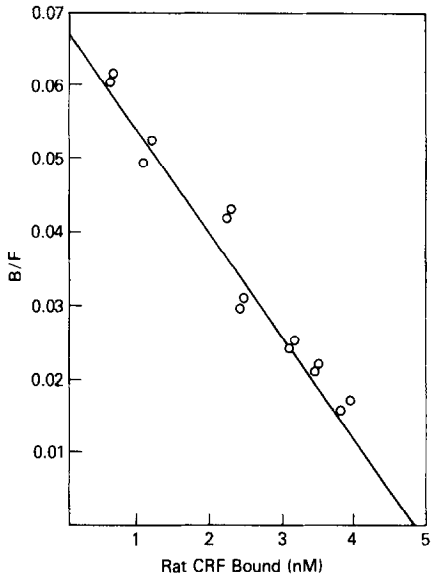


Figure 4: Scatchard analysis of ^{125}I-rat CRF binding to rat erythrocyte membranes, suggesting the existence of a single class of binding sites. Binding was carried out at 4°C for 25 min and data are plotted according to the method of Scatchard (35). With the use of 'Ligand', a computer program system for fitting multiple/single binding sites, the data were analyzed and checked for a Scatchard plot fit (36). The amount of membrane protein present was approximately 230 µg. Each point represents the mean of duplicate determinations.

sequence homologies to CRF at concentrations from 10^{-4}-10^{-6}M resulted in little (4-5%) or no inhibition of specific CRF binding.

Table 1: **The effect of various CRF-like or non-CRF-like peptides on ^{125}I-labeled CRF binding to human and rat erythrocyte membranes**

Compound	Concentration (M)	Inhibition of specific CRF binding (%)	
		Human erythrocyte membranes	Rat erythrocyte membranes
rCRF	10^{-6}	80	100
[Tyr°]rCRF	10^{-6}	80	100
hCRF	10^{-6}	100	85
oCRF	10^{-6}	80	85
AchBE	5×10^{-6}	5	4
Leu-enk	10^{-5}	0	5
Met-enk	10^{-5}	0	5
Prolactin	10^{-4}	0	0

CRF binding was carried out using ^{125}I-hCRF or ^{125}I-rCRF in a assay buffer consisting of 25 mM Tris-HCl, pH 7.6 containing 10 mM MgCl2 and 0.1% BSA for 25 min at 4°C. A pool of erythrocytes obtained from 4 healthy males (ages 30-45 yrs) or 10 adult male rats was used for the competition studies. Each value represents the mean of triplicate determinations from a representative experiment. Peptide abbreviations are as follows: rCRF, rat CRF; [Tyr°]rCRF, N-terminal extension of rat CRF through the addition of a tyrosine residue; hCRF, human CRF; oCRF, ovine CRF, AchBE, acetyl human ß-endorphin; Leu-enk, Leucine-enkephalin; Met-enk, Methionine-enkephalin.

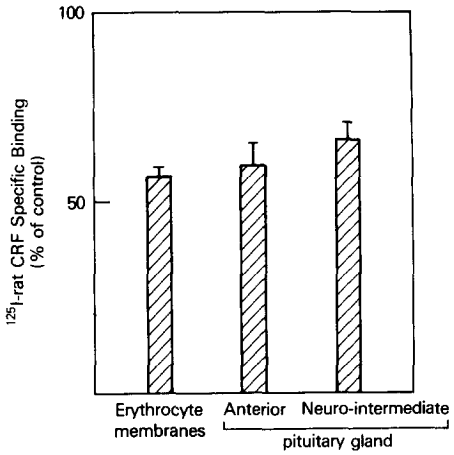


Figure 5: Demonstration that exposure of adult male rats to ethanol vapor for 14 days decreased ^{125}I-rCRF binding to erythrocyte and pituitary membranes. CRF binding was carried out at 4°C for 25 min. Experimental animals (n=18) were exposed to ethanol vapor in an inhalation chamber for 14 days. Control animals were housed in identical chambers in the absence of ethanol vapor. CRF binding data for anterior and neuro-intermediate lobe of the pituitary gland was taken from our earlier study (28). All animals in the experimental group exhibited blood alcohol levels between 120 and 250 mg/100 ml (189 ± 40, mean ± S.D., n=18).

hCRF binding was further studied in erythrocyte-, lymphocyte- or platelet-rich fractions of human blood. The majority of hCRF binding was associated with the erythrocyte-rich fraction, whereas, less than 10% of the total CRF binding was found in the lymphocyte or platelet-rich fractions (Data not shown). Furthermore, the binding of CRF to intact erythrocytes (incubated with labeled CRF with or without the addition of unlabeled CRF in buffer consisting of 0.9% NaCl, 25 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$ and 0.1% BSA) or lysed erythrocyte membranes obtained from rats or human was identical (Data not shown).

Animals exposed to ethanol vapor for 14 days with blood alcohol levels ranging from 120 to 250 mg/100 ml (189 ± 40, mean ± S.D., n = 18) had reduced CRF-binding to erythrocyte, anterior lobe (AL), and neurointermediate lone (NIL) membranes by 45%, 40% and 35%, respectively (Figure 5). Scatchard analysis of rCRF binding revealed that ethanol exposure lowered the number of high-affinity CRF-binding sites without affecting the affinity constant of CRF binding to erythrocyte, AL, or NIL membranes (data not shown).

DISCUSSION

In a recent study, we reported that CRF binding sites are present on membranes obtained from rat adrenal medulla, ventral prostate, spleen, liver, kidney and testes, as well as in bovine chromaffin cells in culture (29). Nanomolar concentrations of rCRF maximally stimulated adenylate cyclase activity in rat adrenal membranes and maximally increased cAMP levels in bovine chromaffin cells. Competition

studies suggested that oCRF, rCRF and [Tyr°]rCRF were similarly potent in inhibiting specific ^{125}I-rCRF binding to adrenal membranes (29). These findings suggested that CRF may have an important regulatory role in various peripheral tissues.

Following our observation that CRF binding sites were present on human and rat erythrocyte membranes, we were interested in knowing if the CRF-erythrocyte binding sites were modulated in a similar direction to those previously observed on pituitary membranes. Establishing such a correlation could provide a window to assess alterations in CRF-pituitary binding *in vivo* and thus an important clinical tool in understanding disorders of the hypothalamic-pituitary-adrenal axis in humans. The results of the present study indicate that virtually all of the CRF binding sites present on cellular blood elements are found in the erythrocyte-rich fraction. Furthermore, the observation that CRF binds equally well to intact erythrocytes or lysed erythrocyte membranes, suggests that this is real membrane binding and not the consequence of CRF uptake by erythrocytes. The only significant differences between CRF binding to erythrocytes in the present study and to adrenal medullary tissue as reported earlier (29) are the types of binding sites present. Only one type of CRF binding site was found on human- or rat-erythrocyte membranes, whereas, two binding-site types were found on rat adrenal tissue. Additional differences between optimal-CRF binding to various peripheral tissues and to erythrocyte membranes were not found.

The regulatory influence of CRF on POMC-derived peptides in the pituitary gland is firmly established. However, the physiological regulatory role(s) that specific CRF binding sites play in peripheral neuroendocrine function is not clear at this time. Activation of peripheral CRF binding sites may result in different tissue-specific physiological responses. It is of particular interest to note that many of the peripheral tissues, which we found in an earlier study (29) to contain CRF-binding sites (e.g. adrenal medulla, kidney and testis), also contain endogenous POMC peptides (21,22,25). In a manner analogous to the known hypothalamic-CRF regulation of POMC peptide release from the pituitary gland, it is possible that occupancy of the peripheral CRF-binding sites may release POMC peptides or other hormones from peripheral organs. Indeed, the results of a recent study, which are consistent with our earlier study (29), demonstrate that CRF can enhance the release of adrenomedullary hormones (30). Furthermore, CRF binding sites on erythrocyte membranes may play a role in transporting CRF from its site(s) of synthesis to peripheral "target" organs and thereby prevent its proteolytic degradation in route.

Chronic exposure of rats to ethanol vapor for 14 days decreased rCRF binding to erythrocyte membranes which is consistent with our earlier finding that chronic ethanol vapor treatment lowered

rCRF binding and activation of adenylate cyclase by CRF in both AL- and NIL-tissue of the pituitary gland. These observations suggest that CRF binding to erythrocyte membranes and pituitary membranes may be modulated in a similar direction. Establishing that changes in CRF binding to erythrocyte membranes parallels CRF binding to pituitary membranes would be an important contribution in the area of clinical neuroendocrinology.

REFERENCES

1. Spiess, J., Rivier, J., Rivier, C. and Vale, W. (1981) Proc. Natl. Acad. Sci. USA. 78, 6512-6521.
2. Vale, W., Spiess, J., Rivier, C. and Rivier, J. (1981) Science. 213, 1394-1397.
3. Turkelson, C.M., Arimura, A., Culler, M.D., Fishback, J.B., Groot, K., Konda, M., Licino, M., Thomas, C.R., Chang, D.,Chang, J.K. and Shimizu,M. (1981) Peptides. 2, 425-429.
4. Al-Noaemi, M.C., Edwardson, J.A. and Hughes, D. (1982) J. Physiol. (Lond) 332, 85-86.
5. Proulx-Ferland, L., Labrie, F., Dumont, D., Cote, J., Coy, D.M. and Sueirab, J. (1982) Science. 217, 62-63.
6. Orth, D.N., Jackson, R.V., DeCherney, G.S., DeBold, C.R., Alexander, A.N., Island, D.P., Rivier, J., Rivier, C., Spiess, J. and Vale, W. (1983) J.Clin.Invest. 71, 587-595.
7. Vale, W., Vaughn, J., Smith, M., Yamamoto, G., Rivier, J. and Rivier, C. (1983) Endocrinology, 113, 1121-1131.
8. Dave, J.R., Eiden, L.E. and Eskay, R.L. (1986) Ann.N.Y.Acad.Sci. (Abstr.) (in press).
9. Sutton, R.E., Koob, G.F., Le Moal, M., Rivier, J. and Vale, W. (1982) Nature. 297, 331-333.
10. Britton, D.R., Koob, G.F., Rivier, J. and Vale, W. (1981) Nature. 297, 331-333.
11. Levine, A.S.,Rogers, B.,Kneip, J.,Grace,M. and Morley, J.E.(1983) Neuropharmacol. 22, 337-339.
12. Ehlers, C.L., Hinriksen, S.J., Wang, S.J., Rivier, J., Vale, W. and Bloom, E.F. (1983) Brain Res. 278, 332-338.
13. Valentino, R.J., Foote, S.L. and Aston-Jones, G. (1983) Brain Res. 270, 363-367.
14. Fischman, A.J. and Moldow, R.L. (1982). Peptides. 3, 149-153.
15. Olschowka, J.A., O'Donohue,T.L.,Mueller,G.P.and Jacobowitz,D.M.(1982)Peptides. 3, 995-1015.
16. Merchenthaler, I., Vigh, S., Petrusz, P. and Schally, A.V. (1982) Am.J.Anat. 165, 385-396.
17. Cote, J., Lefevre, G., Labrie, F. and Barden, N. (1983) Regul. Peptides. 5, 189-195.
18. Hashimoto, K., Murakami, K., Ohno, N., Kageyama, J., Aoki, Y., Takahara, J. and Ota, Z. (1983) Life Sci. 32, 1001-1007.
19. Palkovits, M., Brownstein, M.J. and Vale, W. (1983)Neuroendocrinology. 37, 302-305.
20. Swanson, L.W., Sawchenko, P.E., Rivier, J. and Vale, W.(1983)Neuroendocrinology.37, 302-305.
21. Upton, G.V. and Amatruda, Jr T.T. (1971) N. Engl. J. Med. 285, 419-421.
22. Suda, T., Demura, H., Demura, R., Wakabayashi, I., Nomura, K., Odagiri, E. and Schizume, K. (1977) J. Clin. Endocrinol. Metab. 44, 440-448.
23. Kruseman, A.C.N., Linton, E.A., Lowry, P.J. and Besser, G.M. (1982) Lancet. II, 1245-1246.
24. Petrusz, P., Merchenthaler, I., Maderdrut, J.L., Vigh, S. and Schally, S. (1983) Proc. Natl. Acad. Sci. USA. 80, 1721-1725.
25. Suda, T., Tomori, N., Tozawa, F., Demura, H., Shizume, K., Mouri, T., Miura, Y. and Sasano, N. (1984) J. Clin. Endocrinol. Metab. 58, 919-924.
26. Wynn,P.G.,Aguilera,G.,Morell,J.andCatt,K.J.(1983)Biochem.Biophys.Res.Commun.110,602-608.
27. Desouza, E.B., Perrin, M.H., Rivier, J.,Vale, W. and Kuhar,M.J. (1984) Brain Res. 296, 202-207.
28. Dave, J.R., Eiden, L.E., Karanian, J.W. and Eskay, R.L. (1986) Endocrinology, 118, 280-286.
29. Dave, J.R., Eiden, L.E. and Eskay, R.L. (1985) Endocrinology, 116, 2152-2159.
30. Udelsman, R., Harwood, J.P., Millan, M.A., Chrousos, G.P., Goldstein, D.S., Zimlichman, R., Catt, K.J. and Aguilera, G. (1986) Nature, 319, 147-150.
31. Knazek, R.A., Rizzo, W.B., Schulman, J.D. and Dave, J.R. (1983) J.Clin.Invest. 72, 245-248.
32. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-273.
33. Thorell, J.I. and Johansson, B.G. (1971) Biochim. Biophys. Acta. 251, 363-369.
34. Dave, J.R. and Witorsch, R.J. (1985) Am. J. Physiol. 248, E687-E693.
35. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-673.
36. Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220-226.