Vol. 152, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1988 Pages 383-391

RECEPTORS FOR CALCITONIN GENE-RELATED PEPTIDE ON THE RAT LIVER PLASMA MEMBRANES

Akinori Yamaguchi*,Tsutomu Chiba,Yasuhiko Okimura,Toshiyuki Yamatani,Tomoyuki Morishita,Akira Nakamura,Tetsuya Inui,Toshiharu Noda** and Takuo Fujita

Third Division, Department of Medicine, Kobe University School of Medicine, Kobe 650, and **Research Laboratory, Toyo Jozo Co.Ltd, Shizuoka 410-23,Japan

Received March 1, 1988

Summary: Specific binding sites for calcitonin gene-related peptide(CGRP) were identified in the rat liver plasma membrane. The binding of ^{125}I-[Tyr^0]rat CGRP to rat liver plasma membrane was time dependent,saturable and reversible. Scatchard analysis of the data revealed a single class of binding sites with apparent dissociation constant of 260.8 pM and a maximal binding capacity of 26.6 fmol/mg of protein. Rat, chick, and human CGRP and their synthetic analogues inhibited label binding in a dose-dependent manner with relative potencies as follows; chick > rat > human > [Tyr^0]rat CGRP. Salmon, human and [Asu^{1,7}]eel calcitonin also inhibited label binding but only at higher concentrations. These results clearly indicate the presence of specific binding sites for CGRP in rat liver plasma membrane and suggest that CGRP has possible biological actions on the rat liver.

Calcitonin gene-related peptide(CGRP), a 37 amino-acids polypeptide, is produced by alternative tissue specific RNA processing of calcitonin gene(1,2). CGRP has been shown to be more widely distributed than calcitonin and to occur in both the central and peripheral nervous systems by immunocytochemistry(2) as well as by radioimmunoassay(2). Particularly, recent studies have demonstrated that high concentrations of immunoreactive CGRP are present throughout the gut, suggesting an important role for CGRP in gastrointestinal function(3,4). However, the occurrence and distribution of immunoreactive CGRP along the entire length of the gut and pancreas also indicate an important role for CGRP

*To whom correspondence should be addressed.

The abbreviations used are:
EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′ tetraacetic acid
Hepes,4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

0006-291X/88 $1.50

in liver function, in view of the accumulation of CGRP released from the gut in the portal blood. In addition to a variety of biological actions in the gut such as stimulation of amylase secretion from dispersed guinea-pig pancreatic acini(5), inhibition of gastric acid secretion in vivo(6,7) and stimulation of somatostatin release from isolated rat stomach(8), recent studies indeed demonstrated CGRP-induced augmentation of basal glucose levels and enhancement of a rise in blood glucose levels induced by terbutaline(β_2 adrenergic agonist) in the mouse and pig, respectively(9,10). Although the authors explained a part of this glucose rise with the inhibition of insulin secretion as a consequence of a direct action of CGRP on the endocrine pancreas, the possibility of the direct action of CGRP on glucose metabolism in the liver still remains(10). The present study was therefore undertaken to identify and characterize specific binding sites for CGRP in the plasma membrane of rat liver.

Methods

Peptides and radioligands: Synthetic rat CGRP, [Tyr^0]rat CGRP, human and salmon calcitonin, human glucagon, somatostatin and secretin were purchased from Peninsula Lab. Inc.(Belmont, CA). Synthetic neurotensin and substance-P were products of Peptide Institute, Protein Research Foundation(Osaka, Japan). [Asu^{1,7}]eel calcitonin, chicken and human CGRP and analogues and fragments of CGRP were synthesized using an automatic solid phase synthesizer (430-A peptide synthesizer, Applied Biosystem Inc.). [Tyr^0]rat CGRP was labeled with ^{125}I by chloramine-T method to a specific activity of 2000 Ci/mmol and purified by gel chromatography on Sephadex G-25 column(1 × 50 cm) as previously described(5). ^{125}I-human calcitonin(2000 Ci/mmol) was produced by Amersham (Buckinghamshire, England).

Membrane preparation: Rat liver plasma membranes were prepared from adult male Sprague-Dowley rats weighing 300-400 g according to the method described by Pilkis et al.(11). The fraction collecting at 48.2 - 42.5% sucrose interface was removed and washed twice with 10 mM HEPES buffer(PH 7.4) containing 1mg/ml bacitracin. The final pellet was resuspend in 10 mM HEPES buffer (PH 7.4) containing 120 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml bacitracin and 5 mg/ml bovine serum albumin(BSA) (standard buffer) to give a protein concentration of 0.4 mg/ml and was used immediately. Membrane protein levels were estimated by the method of Lowry et al.(12) using BSA as the standard.

^{125}I-[Tyr^0]rat CGRP binding assay: A half milliliter of membrane suspension(200 μg of tissue protein) containing ^{125}I-[Tyr^0]rat CGRP(25pM) was incubated at 24 °C for 60 min with or without various unlabeled peptides unless otherwise indicated. At the end of the incubation, bound and free hormone were separated by centrifugation at 10,000 g for 1 min in a microfuge, and radioactivity remaining bound to the pellets was counted by autogamma counter(Aloka, Japan). To determine nonspecific binding, studies were carried out with unlabeled [Tyr^0]rat CGRP added at 1 μM, and specific binding was obtained by subtracting the non-specific binding from the total binding. For Scatchard analysis, binding studies were carried out by changing label concentrations from 1.5 pM to 770 pM.

Degradation of ^{125}I-[Tyr^0]rat CGRP: The degradation of ^{125}I-[Tyr^0]rat CGRP bound to the membranes and free in the incubation medium after exposure to the membranes were examined by two different methods. First, membranes were incubated in standard buffer with 25 pM label at 24 °C for 60 min. After centrifugation, the supernatant was applied to Sephadex G-25 column. Radioactivity bound to the membrane pellet was also analyzed after 90 min dissociation at 37 °C in 1 ml fresh standard buffer and a dissociated label was applied to Sephadex G-25 gel filtration. The column was eluted with 0.1 N acetic acid containing 0.1 % BSA at a speed of 0.5 ml/min. Second, after the first incubation with membrane, unbound ^{125}I-[Tyr^0]rat CGRP in the supernatant was subjected to second binding studies with fresh liver plasma membrane.

^{125}I-human calcitonin binding: ^{125}I-human calcitonin binding to rat liver plasma membrane was also examined under the same condition used for ^{125}I-[Tyr^0]rat CGRP binding studies.

Results

Specific binding of ^{125}I-[Tyr^0]rat CGRP to rat liver plasma membrane was time dependent, reaching equilibrium within 60 min at 24°C, and a plateau binding was maintained for an additional 60 min(Fig. 1A). This binding was rapidly dissociated either with or without 1 μM unlabeled [Tyr^0]rat CGRP, 50% of the tracer being

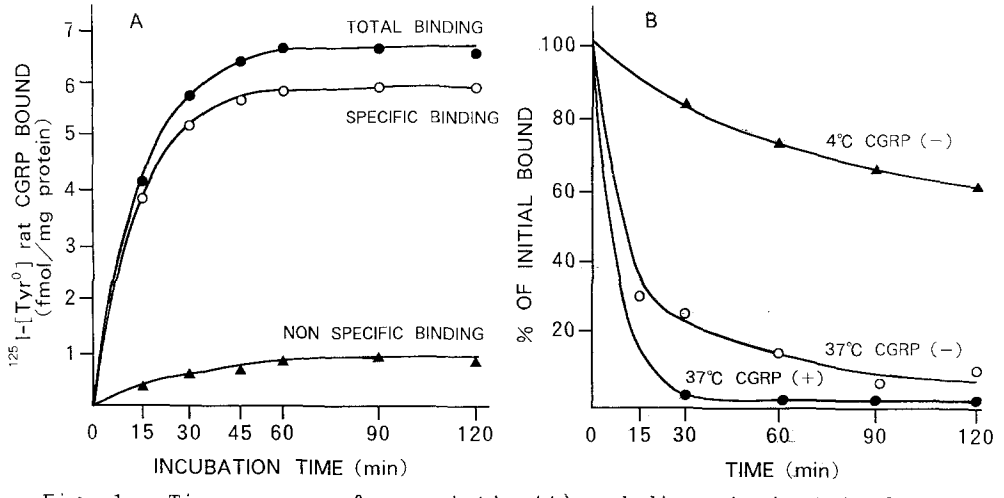


Fig. 1. Time course of association(A) and dissociation(B) of ^{125}I-[Tyr^0]rat CGRP binding to rat liver plasma membrane. Membranes were incubated with 25 pM ^{125}I-[Tyr^0]rat CGRP in the absence(total binding) or presence of 1 μM unlabeled [Tyr^0]rat CGRP(non-specific binding). In the dissociation experiment(B), after 60 min incubation, membrane pellets were reincubated in 1 ml of incubation buffer in the absence of unlabeled CGRP at 37°C(○) and 4°C(▲) or in the presence of 1 μM unlabeled CGRP at 37°C(●), and thereafter, label binding remaining in the pellet was counted at several time points. Each plot shows the mean of duplicate determinations. Results are representative of three separate experiments.

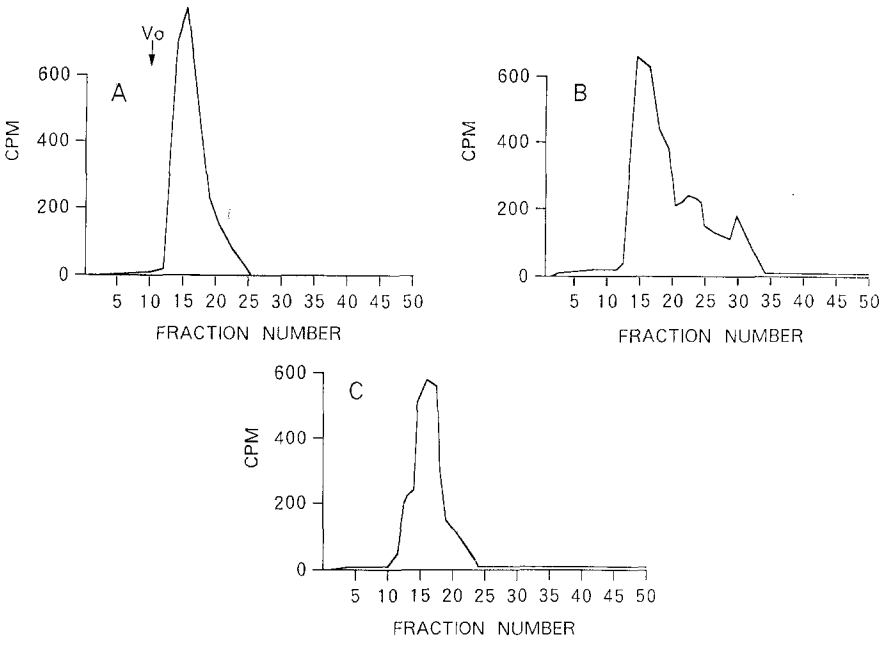
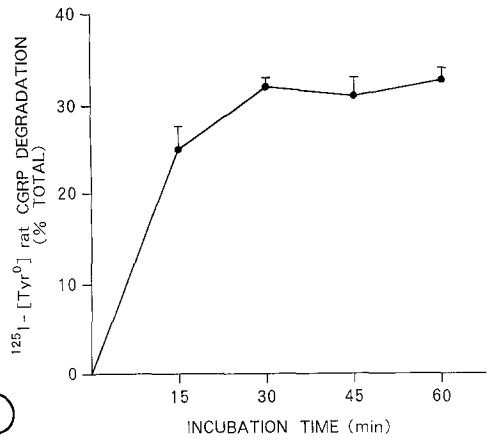


Fig. 2. Analysis of the degradation of ^{125}I-[Tyr^0]rat CGRP as assessed by Sephadex G-25 gel column chromatography. Experimental details are described in Methods.
A:Purified ^{125}I-[Tyr^0]rat CGRP before incubation.
B:Supernatant radioactivity after a 60 min incubation of ^{125}I-[Tyr^0]rat CGRP with membranes at 24°C.
C:Membrane-bound radioactivity after a 90 min dissociation with incubation buffer at 37°C.
V_0 = void volume

released after 10.5 min or 5.3 min at 37°C, respectively(Fig. 1B). Non-specific binding was less than 15% of the total binding.

Degradation of ^{125}I-[Tyr^0]rat CGRP was evaluated by Sephadex G-25 column chromatography. ^{125}I-[Tyr^0]rat CGRP bound to the membrane for 60 min incubation was highly resistant to degradation, since almost all the bound radioactivity was recoverd at the position of intact tracer(Fig. 2). In contrast, the elution pattern of supernatant radioactivity showed three peaks including that of intact tracer(Fig. 2). Two smaller peaks are thought to be degradation products of intact tracer, which represent 35 % of the total radioactivity in the supernatant. In accordance with this data, after 60 min incubation with membrane, the ^{125}I-[Tyr^0] rat CGRP radioactivity in the supernatant had lost 33 % of its binding ability(Fig. 3).

Scatchard analysis of the specific binding data revealed a single class of binding sites with an affinity constant of 260.8 pM and a maximum binding capacity of 26.6 fmol/mg protein(Fig.4).



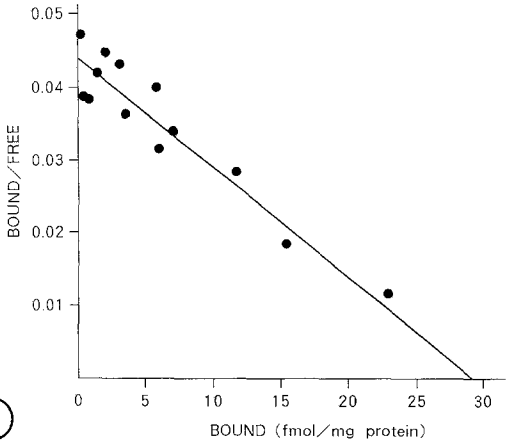


Fig. 3. Time course of ^{125}I-[Tyr^0]rat CGRP degradation by liver plasma membranes. ^{125}I-[Tyr^0]rat CGRP was incubated with liver plasma membrane for various time intervals and degradation of the label was determined by examining the rebinding of supernatant radioactivity to fresh liver plasma membranes. Each value represents the mean ± SE of tripricate determinations.

Fig. 4. Scatchard analysis of specific binding of ^{125}I-[Tyr^0]rat CGRP to liver plasma membrane. Membranes were incubated with varying concentrations of ^{125}I-[Tyr^0]rat CGRP from 1.5 pM to 770 pM for 60 min at 24°C. Results shown are the representative data of the three separate experiments. The line drawn represents the best fit to the data as determined by linear regression analysis.

Chicken, rat and human CGRP and their synthetic analogues and fragments inhibited ^{125}I-[Tyr^0]rat CGRP binding in a dose dependent fashion(Fig. 5 and Table 1). The relative potencies of the peptides tested were chicken CGRP > rat CGRP > human CGRP > [Tyr^0]rat CGRP > (8-37)human CGRP > [Tyr^0](28-37)rat CGRP(Fig. 5 and Table 1). In contrast, 3-cyclo human CGRP did not inhibit label binding up to 10^{-6} M. Furthermore, the binding of ^{125}I-[Tyr^0]rat CGRP was inhibited by salmon calcitonin, [Asu^{1,7}]eel calcitonin and human calcitonin over a concentration range of 10^{-7} M, whereas other brain-gut peptides such as human glucagon, secretin, neurotensin, substance-P and somatostatin had no effect on label binding.

^{125}I-human calcitonin, specifically bound to guinea-pig kidney cell membrane prepared by the same method as that for rat liver plasma membrane, failed to bind to rat liver plasma membrane up to the label concentration of 2 nM, though there was almost no degradation of ^{125}I -human calcitonin during the 60 min incubation period with rat liver plasma membrane as assesed by Sephadex G-25 gel chromatography(data not shown).

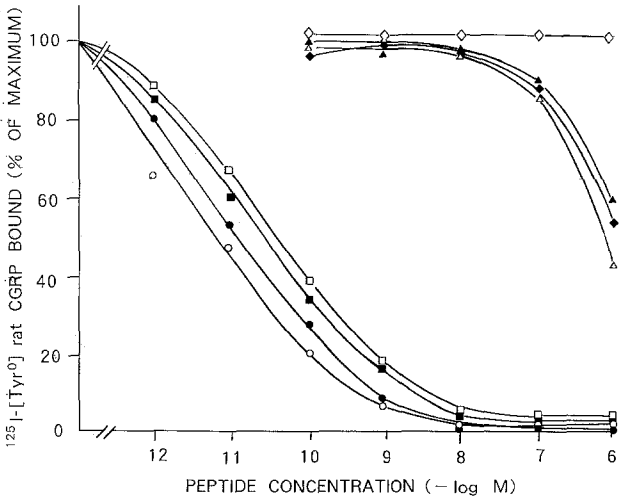


Fig. 5. Competition curves for ^{125}I-[Tyr^0]rat CGRP binding sites by CGRPs, calcitonins, an analogue of CGRP and various brain-gut peptides: rat CGRP(●); chick CGRP(○); human CGRP(■); [tyr^0]rat CGRP(□); [Asu^{1,7}]eel calcitonin(▲); salmon calcitonin(△); human calcitonin(◆); human glucagon, secretin, neurotensin, substance-P, and somatostatin(◇). Results are expressed as the percentage of maximal specific binding in the absence of unlabeled peptides. Each value is the mean of four experiments. Each experiment was done in duplicate.

Discussion

In the present study we found that on rat liver plasma membranes the binding of ^{125}I-[Tyr^0]rat CGRP was time dependent, saturable and reversible. The specificity of ^{125}I-[Tyr^0]rat CGRP binding was demonstrated by its displacement by various CGRPs from different species but not by unrelated peptides. While liver plasma membranes degraded ^{125}I-[Tyr^0]rat CGRP, it is a suitable radioligand because degradation is only a small amount as evaluated by Sephadex G-25 gel chromatography and rebinding to fresh membranes and is restricted to free radioligand in the incubation medium. Thus, as in the case of glucagon degradation in rat liver plasma membranes(13), CGRP bound to its receptors appears to be protected against degradation, and CGRP receptors may not contribute to the degradation of CGRP. From Scatchard analysis, the rat liver plasma membrane CGRP binding sites were found to consist of a single class of high affinity sites. Affinity of these binding sites for CGRP(Kd = 260 pM) is in the same range as those found in rat hypothalamus(14) and rat cerebellum(14) but higher than that observed in guinea-pig pancreatic acini(15). However, the exact Kd value of CGRP binding

Table 1. IC_{50} values and relative affinities of CGRPs, calcitonins and analogues or fragments of CGRP

Peptide	IC_{50}(pM)	Relative Affinity(%)
Rat CGRP	10.5 ± 0.9	
[Tyr^0]rat CGRP	42.3 ± 2.5	24.8
chick CGRP	5.6 ± 1.8	188.7
human CGRP	25.5 ± 3.2	41.2
salmon calcitonin	(0.81 ± 0.06) × 10^6	1.30 × 10^{-3}
[$Asu^{1,7}$] eel calcitonin	(2.0 ± 0.3) × 10^6	0.85 × 10^{-3}
human calcitonin	(1.1 ± 0.4) × 10^6	0.95 × 10^{-3}
(8-37) human CGRP	(98.4 ± 51.1) × 10^3	0.011
[Tyr^0] (28-37) rat CGRP	(1.75 ± 1.12) × 10^6	0.60 × 10^{-3}
3-cyclo human CGRP	NI	

IC_{50} values were defined as the concentration of unlabeled peptide required to inhibit the specific binding of ^{125}I-[Tyr^0]rat CGRP by 50%. The relative affinity was caluculated by comparing the IC_{50} values with that of rat CGRP (taken as 100%). Values are the mean ± SE of three separate experiments. Each experiment was done in duplicate. NI = no interaction

sites on rat liver plasma membranes may be slightly lower than that obtained in this experiment, since the affinity of [Tyr^0]rat CGRP used as radioligand in our study was lower than that of rat CGRP. Taken together, these results suggest that the binding sites observed on liver plasma membranes represent specific receptors for CGRP.

According to the results of the present study, the affinity of CGRP to its receptor on rat liver plasma membranes was different in the species(chick > rat > human). Furthermore, the data revealed that (8-37)human CGRP and [Tyr^0](28-37)rat CGRP, C-terminal fragments lacking ring structure, showed moderate inhibition of label binding whereas 3-cyclo human CGRP which has only ring structure at the N-terminal portion containing a cysteine bond between positions 2 and 7 did not bind to CGRP receptors. Thus, the C-terminal portion of CGRP appears to be essential for binding to its receptors.

In this study, various kinds of calcitonin clearly displaced ^{125}I-[Tyr^0]rat CGRP binding. It is possible to speculate from

this data that a part of ^{125}I-[Tyr^{0}]rat CGRP was bound to calcitonin receptors and this binding was replaced by unlabeled calcitonin as has been demonstrated in LLC-PK$_1$ kidney cells(16). Since we could not find specific binding of ^{125}I-human calcitonin on our rat liver plasma membranes, however, CGRP receptors appear to predominate in rat liver plasma membranes and calcitonin may interact with these CGRP receptors, though the possibility that calcitonin receptors were degraded in our membrane preparation can not be ruled out.

Recent studies have demonstrated that a significant amount of CGRP is present throughout the gut and pancreas(4). Peptides released from gut and pancreas are known to accumulate in the portal blood and have various biological actions in the liver (17-19). Ahrén et al.(10), on the other hand, reported that CGRP augmented hyperglycemic response to terbutaline(β_2-adrenergic agonist) without affecting insulin secretion in pigs. Therefore, whether or not CGRP receptors in rat liver plasma membrane identified in this study are involved in glucose metabolism remains to be elucidated in future studies.

References

1. Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. and Evans, R.M. (1982) Nature 298,240-244.
2. Rosenfeld, M.G., Mermod, J.J., Amara, S.G., Swanson, L.W., Sawchenko, P.E., Rivier, J., Vale, W.W. and Evans, R.M. (1983) Nature 304,129-135.
3. Rodrigo, J., Polak, J.M., Felnandez, L., Ghateri, M.A., Mulderry, P. and Bloom, S.R. (1985) Gastroenterology 88,444-451.
4. Okimura, Y., Chihara, K., Abe, H., Kita, T., Kashio, Y., Sato, M. and Fujita, T. (1987) Regul. Pept. 17,327-337.
5. Zhou, Z.-C., Villanueva, M.L., Noguchi, M., Jones, S.W., Gardner, J.P. and Jensen, R.T. (1986) Am. J. Physiol. 251,G391-G397.
6. Lenz, H.J., Mortrud, M.T., Vale, W.W., Rivier, J.E. and Brown, M.R. (1984) Regul. Pept. 9,271-277.
7. Taché, Y., Pappas, T., Lauffenburger, M., Goto, Y., Walsh, J.H. and Debas, H. (1984) Gastroenterology 87,344-349.
8. Yamatani, T., Kadowaki, S., Chiba, T., Abe, H., Chihara, K., Fukase, M. and Fujita, T. (1986) Endocrinology 118,2144-2145.
9. Petterson, M., Ahrén, B., Böttcher, G. and Sundler, F. (1986) Endocrinology 118,865-869.
10. Ahrén, B., Martensson, H. and Nobin, A. (1987) Diabetologia 30,354-359.
11. Pilkis, S.J., Exton, J.H., Johnson, R.A. and Park, C.R. (1974) Biochem. Biophys. Acta 343,250-267.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193,265-275.
13. Frandsen, E.K. and Bacchus, R.A. (1987) Biochem. Biophys. Acta 929,74-80.
14. Henke, H., Tschopp, F.A. and Fisher, J.A. (1985) Brain Res. 360,165-171.

15. Seiffrt, H., Sawchenko, P.E., Chesnut, J., Rivier, J., Vale, W. and Pandol, S.J. (1985) Am. J. Physiol. 249,G147-G151.
16. Wohlwend, A., Malmstrom, K., Henke, H., Murer, H., Vassalli, J.-D. and Fisher, J.A. (1985) Biochem. Biophys. Res. Commun. 131,537-542.
17. Exton, J.H., Robinson, G.A., Sutherland, E.W. and Park, C.R. (1971) J. Biol. Chem. 246,6166-6177.
18. Wood, C.L. and Blum, J.J. (1982) Am. J. Physiol. 242,E262-E272.
19. Yamatani, K., Sato, N., Takahashi, K., Hara, M. and Sasaki, H. (1983) Biochem. Biophys. Res. Commun. 115,743-748.