BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **221,** 25–30 (1996)
ARTICLE NOS. 0538

Adrenocorticotropin Induces Calcium Oscillations in Adrenal Fasciculata Cells: Single Cell Imaging[1]

Tetsuya Kimoto, Yoshihiro Ohta, and Suguru Kawato[2]

Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, University of Tokyo at Komaba, Meguro, Tokyo 153, Japan

Received February 22, 1996

With fluorescence microscopic imaging, we have demonstrated that the Ca^{2+} signaling occurred in individual Calcium Green-1 loaded bovine adrenal fasciculata cells upon stimulation with adrenocorticotropin (ACTH) at physiological concentration of 0.1–100 pM. We observed three patterns of Ca^{2+} signaling which were Ca^{2+} oscillations (33%), step-like increase in Ca^{2+} concentration (10%), and Ca^{2+} oscillations superimposed on step-like increase in Ca^{2+} (57%). The oscillation in intracellular Ca^{2+} concentration occurred with a frequency around 0.04 Hz. When Ca^{2+} signaling upon ACTH stimulation was inhibited by the treatment with EGTA, the corticoid production was considerably suppressed. The results suggest that the Ca^{2+} signaling is a probable candidate of the second messenger for ACTH-induced steroid hormone synthesis in zona fasciculata cells.

Adrenocorticotropic hormone (ACTH) is the essential hormonal stimulus of corticoid production in adrenal fasciculata cells. Upon ACTH stimulation, the signal transduction may occur sequentially through hormone receptor in plasma membrane → movement of second messengers in cytoplasm → steroid hydroxylation by cytochrome P450s in mitochondria and endoplasmic reticulum (ER), resulting in corticoid production.

The mechanisms of ACTH action remained subject to controversy especially about the movement of second messengers. cAMP is a candidate of intracellular messenger of ACTH action on steroidogenesis in adrenocortical cells (1,2). For ACTH stimulation, calcium ions (Ca^{2+}) may also play an important role in steroidogenesis since their removal or the addition of calcium channel blockers reduced or abolished the corticoid response. In bovine adrenal glomerulosa cells, Kojima and Ogata (3) showed that a high dose of 1 nM ACTH increased transiently the cytoplasmic free calcium concentration $[Ca^2]_i$ in cell population measurements. Yanagibashi et al. (4) showed in bovine fasciculata cells that low doses of ACTH, lower than 10 pM, did not increase cAMP but stimulated steroidogenesis. Until the present study for fasciculata cells, there was no direct evidence which indicated that ACTH induced Ca^{2+} signaling.

MATERIALS AND METHODS

Chemicals. Calcium Green-1/AM was purchased from Molecular Probes (OR, USA); thapsigargin, CPA and nifedipine from Wako Pure Chemicals (Japan); ACTH 1–24 peptide (Cortrosyn) from Dai-ichi Seiyaku (Japan).

Preparation of fasciculata cell cultures. Adrenocortical zona fasciculata cells were aseptically isolated by collagenase-DNase digestion from beef adrenal glands. The cortical tissue was minced and digested with collagenase (0.1%) and DNase (0.005%) dissolved in Krebs-Ringer bicarbonate glucose buffer, containing 125mM NaCl, 6mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.2mM $CaCl_2$, 0.01mM EGTA, 25.3mM $NaHCO_3$, 0.2% glucose, 0.3% BSA, 0.005% gentamicin, pH 7.4. The digestion was performed with 1 hour incubation at 37-°C under 95% O_2-5% CO_2 mixture as a gas phase. The isolated cells were cultured in Ham's F-10 medium supplemented with 5% fetal calf serum, 10% newborn calf serum, 2.5% horse serum, 100U/ml penicillin G, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. Cells were plated onto glass-bottom

[1] This work is supported by grants from the Ministry of Education, Science and Culture in Japan (to S.K. and Y.O.) and JSPS research fellowships for young scientists (to T.K.).

[2] To whom correspondence should be addressed. FAX: 81-3-5454-6517, E-mail: kawato@phys3.c.u-tokyo.ac.jp.

Abbreviations: AM, acetoxymethyl ester; CPA, cyclopiazonic acid; ER, endoplasmic reticulum.

0006-291X/96 $18.00

dishes previously coated with collagen. The cells were cultured for 3–6 days at a cell density of 0.7×10^4 cells/cm^2 or 2×10^5 cells/cm^2.

Cell loading with Calcium Green-1. Cells were loaded for 10 min at 37°C with 3 μM Calcium Green-1/AM (from 300 μM stock solution in dimethyl sulfoxide) in the presence of 0.01% Triton X-100 and 0.02% Pluronic F-127 in 1ml of Hepes buffer medium pH 7.4, containing 120 mM NaCl, 4.0 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 5.0 mM $MgSO_4$, 20 mM Hepes supplemented with 0.1% glucose and 0.1% BSA.

Fluorescence imaging and analysis. We used a video-enhanced fluorescence microscope which consisted of an inverted microscope (Nikon TMD-300, Japan) equipped with a xenon lamp for excitation and a SIT camera (Hamamatsu Photonics C-1145, Japan). The glass-bottom dish was mounted on the microscope equipped with the temperature chamber which maintained the air atmosphere at 37°C with high humidity, using a warm air-supplying system. For Calcium Green-1, we selected the excitation wavelength of 450–490 nm and fluorescence above 520 nm with excitation filter, dichroic mirror, and emission filter. Data acquision on a video tape was performed with VIP-8800 and PIP-4000 system (ADS, Japan). For image analysis with ARGUS-50 system (Hamamatsu Photonics, Japan), the video output was degitized and the images were stored in frame memory. For Figure 1, Panel A, successive 16 video frames were averaged to improve the signal-to-noise ratio of each image. For generation of time-course traces of fluorescence intensity as shown in Figures 1 and 2, average was performed over an area of 5×5 pixels and 15 successive video frames were averaged, resulting in a 0.5 sec time resolution.

Assay of corticoid production. For measurements of corticoid production, the cultured cells were incubated in multiwell dishes with various reagents such as ACTH, EGTA, nifedipine for 1hr at 37°C in KRBGA. Corticoids were extracted from the incubation medium with dichloromethane and measured fluorometrically by the method of Silber et al. (5) using cortisol as a standard. Excitation at 470 nm and emission at 520 nm were employed.

RESULTS

ACTH-Induced Ca^{2+} Signaling

We have observed that the application of ACTH (0.1 pM -100 pM) induces oscillations or sustained elevation in intracellular calcium concentration $[Ca^{2+}]_i$ in bovine adrenal fasciculata cells, as shown in Figures 1 and 2 using single cell imaging of Calcium Green-1 loaded cells. For about 33% of Ca^{2+} signaling cells, ACTH induced Ca^{2+} oscillations which consist of repetitive sharp Ca^{2+} spikes with frequency of around 0.04 Hz. The half width was around 10 sec for each Ca^{2+} spike. The plateau level of $[Ca^{2+}]_i$ between Ca^{2+} spikes was almost the same as the basal level. The phase of Ca^{2+} oscillations was different between individual cells. Not only stable Ca^{2+} oscillations, without changes in the peak height of each repetitive Ca^{2+} spike but also damped Ca^{2+} oscillations were observed. For about 10% of cells, we observed a step-like increase in Ca^{2+} concentration which consisted of a 20 sec rise phase and a sustained plateau phase lasting for more than 10 min. For about 57% of cells, we observed Ca^{2+} oscillations superimposed on a step-like increase in Ca^{2+}. The relative proportion of cells which showed three different signals was 33% (24 cells, oscillation), 10% (7cells, step-like increase) and 57% (41 cells, oscillation plus step-like increase) upon 1 pM ACTH stimulation for 72 cells from 12 independent experiments. These numbers of relative proportion of cells were not altered significantly by changing the ACTH concentration from 0.1 pM to 100 pM. Typical Ca^{2+} oscillations lasted for several minutes. In some cells, Ca^{2+} oscillations continued for 120 min. There was a latency period of 10–60 sec prior to the onset of Ca^{2+} signaling. The increase in ACTH concentration from 0.1 pM to 100 pM did not considerably affect the characteristics of Ca^{2+} signaling about the frequency of Ca^{2+} oscillations, the shape of each Ca^{2+} spike and the relative proportion of cells which showed three different patterns of Ca^{2+} signaling. The total population of Ca^{2+} signaling cells was significantly increased from 38% (31 cells out of 81 cells, 0.1 pM) to 85% (59 cells out of 74 cells, 100 pM) by the increase in ACTH concentration from 0.1 pM to 100 pM.

The Ca^{2+} oscillations induced by 1 pM ACTH were almost completely suppressed by the addition of EGTA, thapsigargin and CPA (see Fig. 2). Upon addition of thapsigargin and CPA to inhibit the activity of calcium pump in ER, the Ca^{2+} transient appeared due to the blocking of Ca^{2+} uptake into ER, and thereafter Ca^{2+} oscillations did not appear again. The addition of EGTA and these inhibitors prior to the ACTH stimulation also suppressed Ca^{2+} signaling considerably.

We have achieved nearly a complete loading of Calcium Green-1/AM for more than 90% of

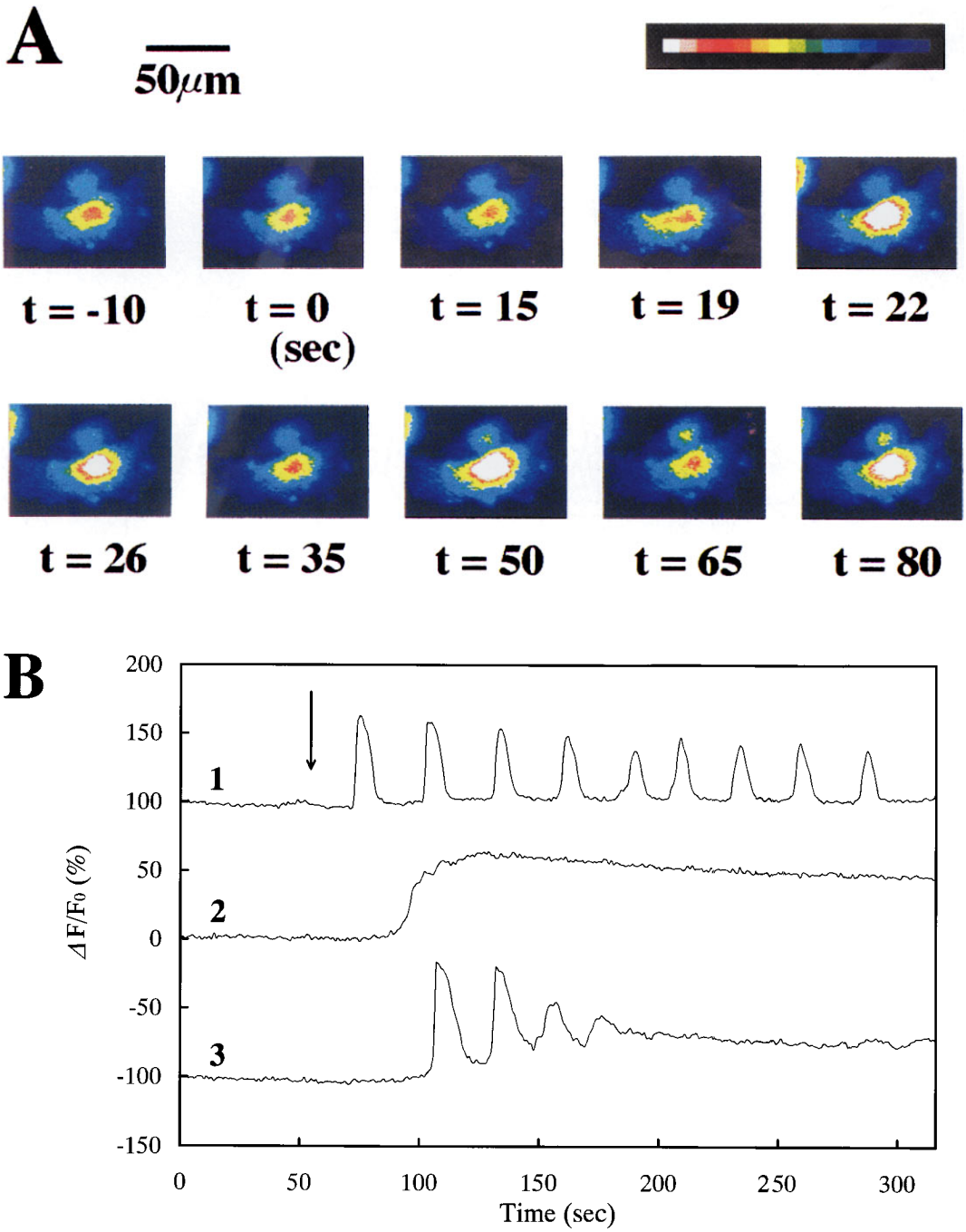

FIG. 1. Time-dependent images and time course of Ca^{2+} signaling induced upon 1 pM ACTH stimulation. Panel A: Pseudocoloured images demonstrate that the adrenal fasciculata cells show oscillations of intracellular $[Ca^{2+}]i$ upon 1 pM ACTH stimulation at t = 0 sec. The fluorescence intensity of Calcium Green-1 is indicated with a colour bar from blue (low intensity) to red/white (high intensity). Time t is indicated in seconds. The spacial scale is indicated with a horizontal black bar of 50 μm. Panel B: The time course of typical Ca^{2+} signaling induced by 1 pM ACTH. Curve 1, Ca^{2+} oscillations; curve 2, step-like increase in Ca^{2+}; curve 3, Ca^{2+} oscillations superimposed on step-like increase in Ca^{2+}. The vertical scale ($\Delta F/F_0$) is the ratio of the fluorescence intensity change ($F-F_0$) to the basal fluorescence F_0 for Calcium Green-1. The arrow indicates the addition of ACTH. Curves 1 and 3 were vertically displaced for clarity in order to avoid overlapping each other.

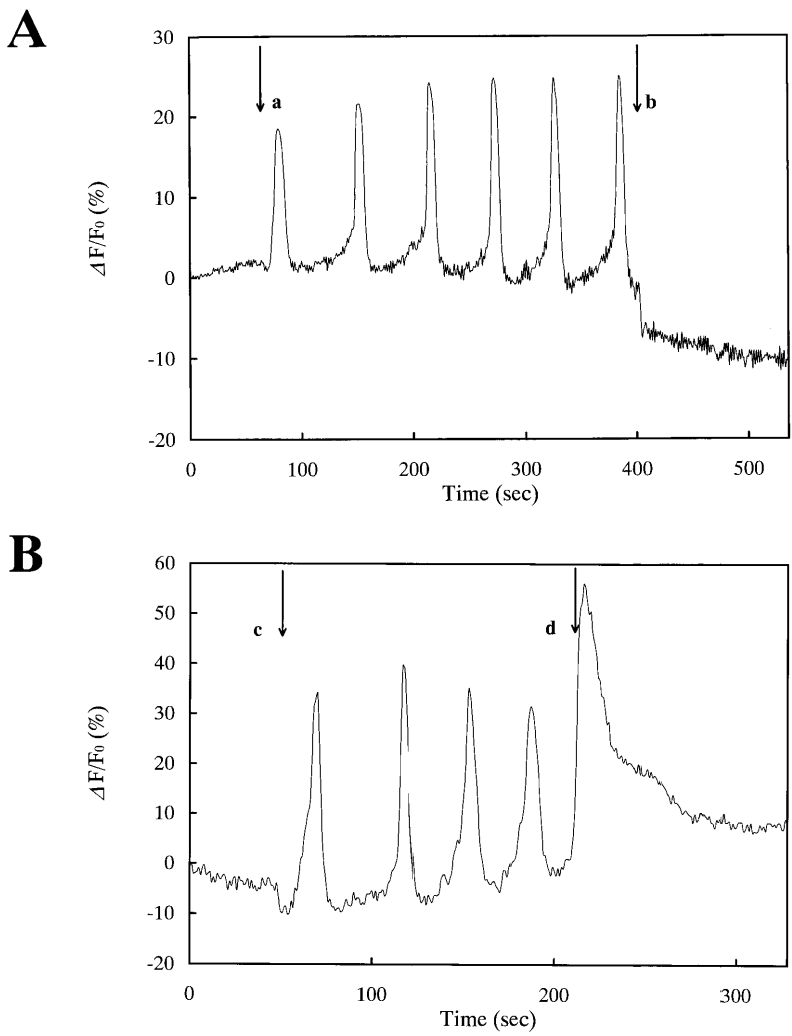

FIG. 2. Time course of Ca^{2+} oscillations induced with ACTH and suppressed with EGTA and thapsigargin. The vertical scale ($\Delta F/F_0$) is the ratio of the fluorescence intensity change ($F-F_0$) to the basal fluorescence F_0 for Calcium Green-1. The arrows indicate the addition of ACTH, EGTA and thapsigargin. Panel A: Ca^{2+} oscillations are induced by 1 pM ACTH (arrow a) and then suppressed by the addition of 2 mM EGTA (arrow b) in the outer medium. Panel B: Ca^{2+} oscillations are induced by 1 pM ACTH (arrow c) and then abolished by the addition of 20 μM thapsigargin (arrow d) which induces a cytosolic Ca^{2+} transient.

fasciculata cells in the presence of 0.01% of Triton X-100 in Hepes buffer. Without this trace amount of Triton X-100 even in the presence of Pluronic F-127, only a small populations of adrenocortical cells were successfully loaded with Calcium Green-1/AM. It should be noted that the presence of 0.01% Triton X-100 did not significantly disturb the corticoid production activity of adrenocortical cells (see Fig. 3) nor reduce the viability (kept around 95%) of the cells examined with trypan blue staining. The difficulty of sufficient loading of the acetoxymethyl ester type of calcium dyes was a particular problem for adrenocortical fasciculata cells of which plasma membrane was resistant for incorporation of these dyes. Triton X-100 treatment for sufficient loading of Calcium Green-1/AM, Fura-2 /AM, and Fluo-3/AM, however, was not necessary for other cells examined such as rat glial primary culture cells, C6BU-1 glioma cells, rat primary culture hepatocytes, cloned immune T-cells (6,7).

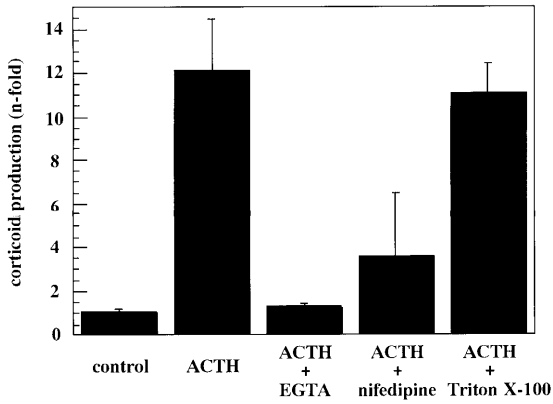

FIG. 3. Influence of ACTH, inhibitors on corticoid production activity in adrenocortical fasciculata cells. For illustrated experiments from left to right, we applied 0 pM ACTH, 100 pM ACTH, 100 pM ACTH plus 2 mM EGTA, 100 pM ACTH plus 10 μM nifedipine, 100 pM ACTH plus 0.01% Triton X-100 and 0.02% Pluronic F127, respectively. Vertical axis is chosen as normalizing all values to the corticoid production (551 ± 113 pmol/10^6 cells/hr) of non-stimulated control cells. Standard error is shown as a bar on the figure. Corticoid production was normalized by living cell numbers which were counted with trypan blue staining by a hemocytometer after the incubation. All the activity values are averaged ones obtained from several independent experiments.

Corticoid Production Assay

In an attempt to correlate the Ca^{2+} signaling with corticoid production, a series of experiments were performed as shown in Fig. 3. Control non-stimulated cells produced 551 ± 113 pmol of corticoid/10^6cells/hr. One hundred pM ACTH significantly enhanced the corticoid production by 12 fold of the control level. Since we observed that ACTH-induced Ca^{2+} oscillations were almost completely suppressed by chelating Ca^{2+} in the outer medium with EGTA, possible inhibitory effects of EGTA on corticoid production were investigated. The presence of 2 mM EGTA, reducing the Ca^{2+} concentration to about 1.2 μM in the outer medium, considerably decreased corticoid production to 11 % of that of fasciculata cells stimulated with 100 pM ACTH. The importance of the Ca^{2+} influx was demonstrated because the addition of calcium channel blocker nifedipine decreased corticoid production to 30%.

DISCUSSION

Until this study, ACTH was not observed to induce Ca^{2+} signaling in zona fasciculata cells. This may be due to an insufficient loading of calcium dyes such as Fura-2/AM and Calcium Green-1/AM into cells without Triton X-100 treatment, resulting in a very small fluorescence change. The Ca^{2+} signaling occurred in 83% of cells with 1 pM ACTH. For generation of Ca^{2+} oscillations, Ca^{2+} pumps in ER would be involved, since Ca^{2+} oscillations were abolished with inhibitors of Ca^{2+} pumps such as thapsigargin and CPA. The influx of extracellular Ca^{2+} would also be necessary for Ca^{2+} oscillations, because Ca^{2+} oscillations were disappeared when extracellular Ca^{2+} was chelated with EGTA. There are investigations which support that steroidogenic activity requires the presence of extracellular calcium for ACTH stimulation of fasciculata cells (8, 9, 10). The requirement of Ca^{2+} signaling was demonstrated with our assay for corticoid production. The suppression of Ca^{2+} signaling by EGTA considerably reduced corticoid production. These results suggest that the Ca^{2+} signaling is a probable candidate of the second messenger for ACTH action on steroidogenesis of adrenocortical fasciculata cells.

In contrast to fasciculata cells, glomerulosa cells have been extensively investigated with respect to Ca^{2+} signaling by hormonal stimulation with ACTH, since they showed intense fluorescence Ca^{2+} signals (11, 12). A single Ca^{2+} spike induced by ACTH was demonstrated with aequorin-

loaded cell population measurements of glomerulosa cells (3). On the other hand, for Fura-2 loaded cultured cells, ACTH induced a gradual increase in $[Ca^{2+}]_i$ over 10–30 min with single cell imaging (11). These conflictions are still unresolved in glomerulosa cells. So far no Ca^{2+} oscillation signals were observed for glomerulosa cells.

When ACTH was applied at high concentrations, there were reports implying that cAMP may be an intracellular messenger of ACTH action on steroidogenesis in adrenal cells. Nanomolar concentration of ACTH induced cAMP production over 10–30 min (11). It could therefore be claimed that the observed Ca^{2+} signals might not be essential for steroidogenesis. However, Yanagibashi et al. (4) showed in bovine fasciculata cells that as low as 10 pM doses of ACTH did not increase cAMP but stimulated steroidogenesis, implying that cAMP does not play a role of second messenger upon 1 pM ACTH stimulation used in the present study.

REFERENCES

1. Grahame-Smith, D. G., Butcher, R. W., Ney, R. L., and Sutherland, E. W. (1967) *J. Biol. Chem.* **242,** 5535–5541.
2. Hayashi, K., Sala, G., Catt, K., and Dufau, M. L. (1979) *J. Biol. Chem.* **254,** 6678–6683.
3. Kojima, I., and Ogata, E. (1986) *J. Biol. Chem.* **261,** 9832–9838.
4. Yanagibashi, K., Papadopoulos, V., Masaki, E., Iwaki, T., Kawamura, M., and Hall, P. F. (1989) *Endocrinology* **124,** 2383–2391.
5. Silber, R. H., Busch, R. D., and Oslapas, R. (1958) *Clin. Chem.* **4,** 278–285.
6. Nakato, K., Furuno, T., Inagaki, K., Teshima, R., Terao, T., and Nakanishi, M. (1992) *Eur. J. Biochem.* **209,** 745–749.
7. Kawanishi, T., Blank, L. M., Harootunian, A. T., Smith, M. T., and Tsien, R. Y. (1989) *J. Biol. Chem.* **264,** 12859–12866.
8. Kojima, I., Kojima, K., and Rasmussen, H. (1985) *J. Biol. Chem.* **260,** 9177–9184.
9. Fakunding, J. L., Chow, R., and Catt, K. (1979) *Endocrinology* **105,** 327–333.
10. Yanagibashi, K., Kawamura, M., and Hall, P. F. (1990) *Endocrinology* **127,** 311–318.
11. Tremblay, E., Payet, M. D., and Gallo-Payet, N. (1991) *Cell Calcium* **12,** 655–673.
12. Iida, S., Widmaier, E. P., and Hall, P. F. (1986) *Biochem. J.* **236,** 53–59.