BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 80–86 (1996)
ARTICLE NO. 0987

α_{1b}-Adrenoceptor-Mediated Calcium Oscillation is Specific for the S Phase in Cell Cycle and Dependent on the Extracellular Calcium

Takeo Awaji, Shun-ichiro Izumi,[1] Kuniko Horie, Akira Hirasawa, and Gozoh Tsujimoto[2]

Department of Molecular Cell Pharmacology, National Children's Medical Research Center, 3-35-31 Taishido, Setagaya-ku, Tokyo 154, Japan

Received May 23, 1996

Using Chinese hamster ovary cells stably expressing α_{1b}-adrenoceptor (α_{1b}AR) as a model, we examined the effect of the cell cycle on the agonist-promoted intracellular $[Ca^{2+}]_i$ oscillation. In cells synchronized into either the G_1 state or the M phase, no oscillatory behavior was observed under any extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o \leqslant 3$ mM), whereas in cells synchronized into the S phase, norepinephrine caused $[Ca^{2+}]_i$ oscillation in a $[Ca^{2+}]_o$-dependent manner, indicating that α_1AR-mediated $[Ca^{2+}]_i$ oscillation is specific for the S phase in cell cycle and dependent on $[Ca^{2+}]_o$. The S phase-specific occurrence of α_1AR-mediated $[Ca^{2+}]_i$ oscillation is not associated with changes in α_1AR density. As the cells consistently developed $[Ca^{2+}]_i$ oscillation in the S phase, the cells would provide a valuable system to study further the biochemical mechanism for agonist-induced $[Ca^{2+}]_i$ oscillation phenomenon.

Calcium ion (Ca^{2+}) is one of important second messengers regulating a wide range of cellular processes (1). Recent advance in single cell bioimaging technology revealed that a wide variety of nonexcitable cells generate repetitive transient increases in cytosolic calcium ion concentration (''the $[Ca^{2+}]_i$ oscillations'') when receptors that engage the phosphoinositide signaling pathway are stimulated (2). The physiological significance of the $[Ca^{2+}]_i$ oscillations are not fully understood yet, but has been implicated in some cellular functions including exocytosis (3) and fertilization (2). The $[Ca^{2+}]_i$ oscillations vary from one cell to another and also within individual cells responding to different agonists; thus, such enormous variety of oscillatory patterns have hindered progress in studying the biochemical mechanism for the phenomenon, though several hypotheses are now proposed to explain the $[Ca^{2+}]_i$ oscillation phenomenon (4, 5).

α_1-Adrenoceptor (α_1AR) is a prototypic $[Ca^{2+}]_i$-mobilizing hormone receptor. Stimulation of α_1AR caused typical $[Ca^{2+}]_i$ mobilization in several tissues, and induced $[Ca^{2+}]_i$ oscillation in some tissues including hepatocytes (6) and smooth muscle cells (7). We transfected the hamster α_{1b}AR into Chinese hamster ovary (CHO) cells and isolated the cells stably expressing α_{1b}AR (CHOα_{1b} cells) (8). Utilizing the cells, we examined the effects of the cell cycle on the agonist-induced $[Ca^{2+}]_i$ oscillation, since the heterogeneous pattern was observed in $[Ca^{2+}]_i$ response in the cloned cells. We observed that the agonist-induced $[Ca^{2+}]_i$ oscillation occurs when the cells were synchronized into the S phase, but not in the G_1 state or the M phase. Furthermore, the $[Ca^{2+}]_i$ oscillation observed during the S phase was found to be dependent on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). The potentially important roles of the cell cycle and $[Ca^{2+}]_o$ in developing $[Ca^{2+}]_i$ oscillations are discussed.

[1] Present address: Department of OB/GYN, NKK Hospital, Kawasaki, 210 Japan.
[2] To whom correspondence should be addressed. Fax: Japan-3-3419-1252. E-mail: gtsujimoto@nch.go.jp.

0006-291X/96 $18.00

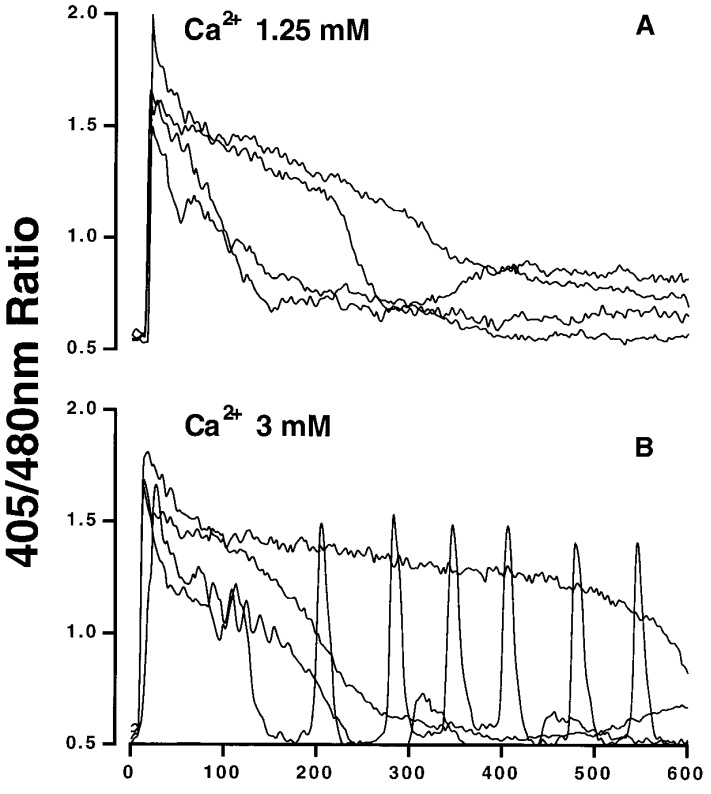

FIG. 1. Adrenergic $[Ca^{2+}]_i$ response in individual CHOα_{1b} cells. (A) NE (10 nM) induced $[Ca^{2+}]_i$ response in all CHOα_{1b} cells under normal extracellular Ca^{2+} concentration (1.25 mM). (B) NE (10 nM) induced $[Ca^{2+}]_i$ response under high extracellular Ca^{2+} concentration (3 mM). The cells used were not synchronized at any cell cycle with the drug described in ''**METHODS.**'' Designated fluorescent ratio represents $[Ca^{2+}]_i$ level of the single cell monitored through a microscopical fluorometer; experimental details are described under ''**METHODS.**'' Data shown are representative of the sextuplicate experiments.

MATERIALS AND METHODS

Cell culture and synchronization of cell cycle. Cell culture and transfection of α_{1b}AR cDNA into CHO cells, and pharmacological properties of the α_{1b}AR stably expressed in CHOα_{1b} cells were described previously (8). Synchronization of cell cycle was performed as follows; cells were pre-incubated with colcemid (40 ng/ml) for 10 h, which treatment synchronizes the cells into the M phase, followed by reincubation for 6 h in fresh medium without colcemid. The treated cells were then incubated for further 10 h in the serum-deprived medium for synchronizing into the G_1 state, or the medium containing 5-fluorouracil (5-FU, 100 nM), methotrexate (MTX, 100 nM), or colcemid (40 ng/ml) for synchronizing into the S phase, the S phase, or the M phase, respectively (9, 10).

Determination of cell cycle. The cells were treated with DNA assay kits (Becton Dickinson & Co., Mountain View, CA) according to the manufacturer manual, and their cell cycle phase was determined by FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA) with Cell FIT program (Becton Dickinson & Co., Mountain View, CA) (11).

Monitoring of $[Ca^{2+}]_i$ *oscillation.* Changes in fluorescent intensity from single CHOα_{1b} cells loaded with a Ca^{2+} fluorescent indicator, Indo-1, were monitored by the single-spot fluorometry (12). Briefly, cultured CHOα_{1b} cells in cover-glass bottom culture dish (MatTek CO., MA) were incubated with 5 μM Indo-1 tetrakisacetoxymethyl ester (Indo-1 AM) dissolved in Tyrode solution (NaCl 135.0 mM, KCl 5.4 mM, NaH_2PO_4 0.33 mM, HEPES 5.0 mM, $MgCl_2$ 0.5 mM, Glucose 5.55 mM, $CaCl_2$ 1.25 mM) containing 0.1% bovine serum albumin (BSA) for 1 h at 37°C. After the cells were washed twice with Tyrode solution, changes in $[Ca^{2+}]_i$ were monitored with sample interval of 3s by OSP-10 fluorescence spectrophotometer (OLYMPUS, Tokyo) with single excitation at 380 nm and emission at 405/480 nm.

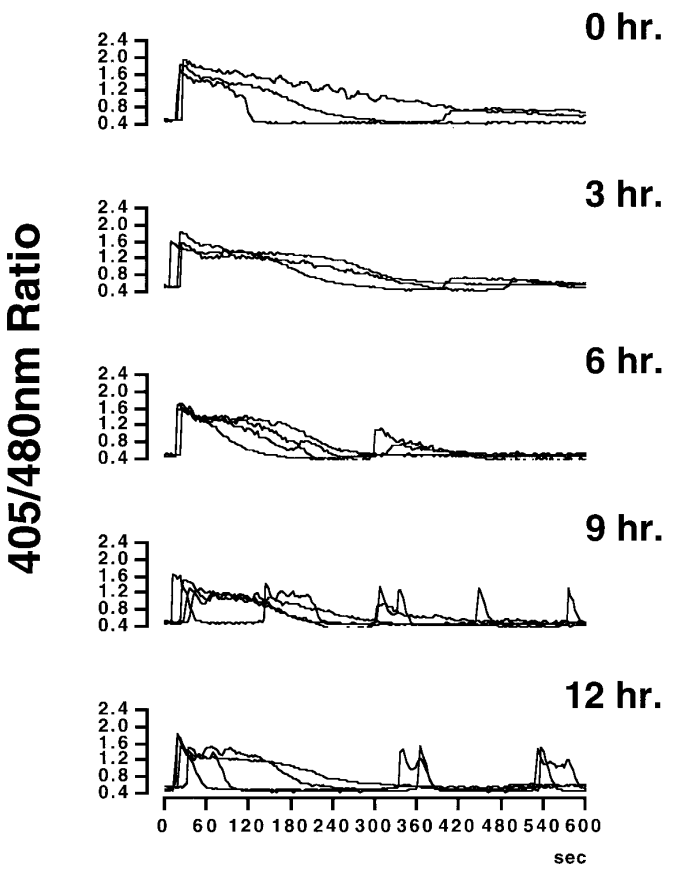

FIG. 2. Time course study on adrenergic $[Ca^{2+}]_i$ response after the cell cycle was synchronized with colcemid. After the cell cycles were synchronized at the M phase with colcemid (40 ng/ml), the cells were transferred to normal medium and monitored by their adrenergic $[Ca^{2+}]_i$ response at every 3 h up to 12 h under 3 mM $[Ca^{2+}]_o$.

Monitoring of receptor density. Radioligand binding studies for α_1AR were performed as described previously (8). Briefly, measurement of specific 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylamino-methyl]tetralone ([^{125}I]HEAT) binding was performed by incubating 0.1 ml of membrane preparation ($\sim$10 μg of protein) with [^{125}I]HEAT in a final volume of 0.25 ml buffer for 60 min at 25 ℃ in the presence or absence of phentolamine (10 μM). Binding assays were always performed in duplicate, and the protein concentration was measured using the bicinchonic acid protein assay kit (PIERCE, Rockford, IL). Data were analyzed by computer with an iterative nonlinear regression program LIGAND.

Materials. Sources of drugs used were as follows: [^{125}I]HEAT (2,200 Ci/mmol; Du Pont -New England Nuclear, Boston, MA); (−)-norepinephrine bitartrate (NE; Sigma, St. Louis, MO); colcemid (Wako, Osaka); Indo-1/AM (Dojindo, Kumamoto); 5-FU (Kyowa Hakko, Tokyo); MTX (Nippon Lederle, Tokyo); All other chemicals were of reagent grade.

Data analysis. Values are expressed as the mean ± SEM. A two-way analysis of variance with 95% confidence limits, followed by a Bonferroni *t*-test on individual sets of data, was performed using analytical software StatView 4.0 (BrainPower Inc., Calabases, CA).

RESULTS

As shown in Fig. 1A, under 1.25 mM of $[Ca^{2+}]_o$ NE (10 nM) immediately caused a rapid increase in $[Ca^{2+}]_i$, consisting of a quick transient peak usually followed by a more sustained component in asynchronous CHOα_{1b} cells; in some cells, however the latter component was not observed (Fig 1A). The $[Ca^{2+}]_i$ responses elicited by NE (10 nM) were

found to be markedly varied, but the responses were monophasic and we could not observe $[Ca^{2+}]_i$ oscillation in any cells examined (n=6). In contrast to the monophasic $[Ca^{2+}]_i$ response observed under 1.25 mM of $[Ca^{2+}]_o$, some CHOα_{1b} cells exhibited $[Ca^{2+}]_i$ oscillation to 10 nM NE when $[Ca^{2+}]_o$ was raised to 3 mM (Fig. 1B). While the NE-induced $[Ca^{2+}]_i$ response varied from one cell to another, there was no statistical difference in either the baseline $[Ca^{2+}]_i$ or the maximum amplitude of responses between either $[Ca^{2+}]_o$; the 405/480 nm ratios for the baseline $[Ca^{2+}]_i$ were 0.51 ± 0.57 and 0.58 ± 0.04, n=6 each, and the 405/480 nm ratios for the maximum amplitude of responses were 1.81 ± 0.14 and 1.75 ± 0.08, n=6 each, under 1.25 mM and 3 mM of $[Ca^{2+}]_o$, respectively. The $[Ca^{2+}]_i$ oscillation was observed only under 3 mM of $[Ca^{2+}]_o$.

As one of possible factors to explain the variety in the development of $[Ca^{2+}]_i$ oscillations under 3 mM of $[Ca^{2+}]_o$ in asynchronous CHOα_{1b} cells, we examined the effect of cell cycle on the occurrence of NE-induced $[Ca^{2+}]_i$ oscillations. We first examined the development of the NE-induced $[Ca^{2+}]_i$ oscillations after the cells were synchronized into the M phase under 3 mM of $[Ca^{2+}]_o$ (Fig. 2). As shown in Fig. 2, NE-induced $[Ca^{2+}]_i$ oscillation was observed only after 6 h of the M phase synchronization, and the incidence of $[Ca^{2+}]_i$ oscillation increased after 9 h, suggesting that NE-induced $[Ca^{2+}]_i$ oscillation occurred not in the M phase but probably in the G_1 state or the S phase. Our FACS analysis confirmed that the treatment of colcemid (40 ng/ml) for 6 h, which was previously found to synchronize the cells into the M phase, resulted in synchronizing over 70-80% of CHOα_{1b} cells into the M phase.

We next examined the effect of cell-cycle synchronization on the development of NE-induced $[Ca^{2+}]_i$ oscillation. We synchronized the cells into the S phase and the G_1 state using either 5-FU (100 nM) and serum deprivation, respectively (9, 10). FACS analysis showed that each treatment resulted in synchronizing over 70-80% of CHOα_{1b} cells into the desired cell-cycle phase; thus, 5-FU treatment and serum deprivation resulted in the $78.8\pm5.4\%$ and $84.1\pm7.5\%$, n=3 each, respectively. The effects of cell-cycle phase and $[Ca^{2+}]_o$ on the incidence of $[Ca^{2+}]_i$ oscillation were summarized in Table 1. As shown in Fig. 3, in CHOα_{1b} cells synchronized into either the G_1 state or the M phase, NE (10 nM) induced a monophasic $[Ca^{2+}]_i$ response under any $[Ca^{2+}]_o$, and the incidence of $[Ca^{2+}]_i$ oscillation was low (less than 20 %; Table 1). In CHOα_{1b} cells synchronized into the S

TABLE 1
The Effects of Cell Cycle Phase and $[Ca^{2+}]_o$ on the Incidence of $[Ca^{2+}]_i$ Oscillation

	$[Ca^{2+}]_o$		
Phase of cell cycle	0[a]	1.25	3
G_1	0/5 (0%)	1/5 (20%)	1/6 (17%)
S	0/5 (0%)	1/6 (17%)	6/6 (100%)
M	0/5 (0%)	1/6 (17%)	1/5 (20)

Note. We analyzed the effect of cell cycle on adrenergic $[Ca^{2+}]_i$ oscillation of CHOα_{1b} cells under the different $[Ca^{2+}]_o$ (free, 1.25 and 3 mM). NE (10 nM) induced $[Ca^{2+}]_i$ elevation in all analyzed cells. Data shown are the percentage of incidence of the $[Ca^{2+}]_i$ oscillations under the indicated condition. Experimental details are described under ''METHODS.''

[a] Nominal

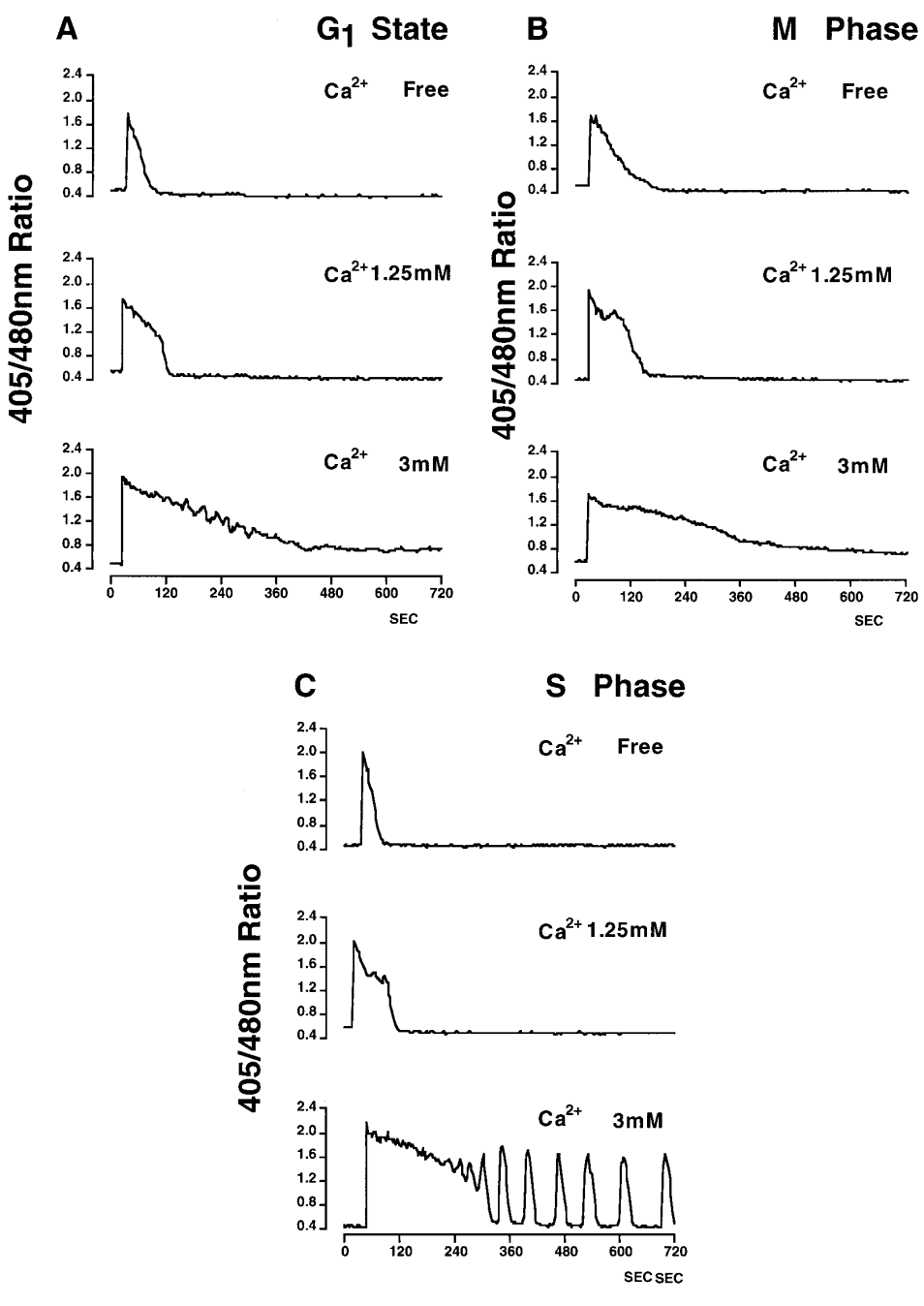

FIG. 3. Adrenergic $[Ca^{2+}]_i$ response at the G_1 state (A), the S phase (B) and the M phase (C) in individual CHOα_{1b} cells. We studied the effect of the cell cycle on adrenergic $[Ca^{2+}]_i$ response of CHOα_{1b} cells under the different $[Ca^{2+}]_o$ (free, 1.25 mM and 3 mM). The cell cycle was synchronized by the drug described in ''**METHODS.**'' Successful synchronization of cell cycle was confirmed by flow cytometry. Designated fluorescent ratio represents $[Ca^{2+}]_i$ level of the single cell monitored through a microscopical fluorometer; experimental details are described under ''**METHODS.**'' Data shown are representative of the quadruplicate runs.

phase, on the other hand, $[Ca^{2+}]_i$ oscillation was observed in higher incidence, and under higher $[Ca^{2+}]_o$ (3 mM) all the CHOα_{1b} cells exhibited $[Ca^{2+}]_i$ oscillation (Fig. 3C; Table 1). Similarly, the higher incidence of oscillatory behavior was also observed in MTX-treated CHOα_{1b} cells, another treatment to synchronize cells into the S phase (data not shown), indicating that the agonist-promoted $[Ca^{2+}]_i$ oscillation is specific for the S phase irrespective of the cell synchronization procedure.

Furthermore, we examined the effect of cell-cycle synchronization on changes in receptor density, by which the effect of cell-cycle synchronization on the development of NE-induced $[Ca^{2+}]_i$ oscillation can be altered. The α_{1b}AR densities monitored by [^{125}I]HEAT binding study are 1,810 ± 280, 1,795 ± 300 and 1,780 ± 250 (fmol/mg protein) in the G_1 state, the S and M phase (n=5 each), respectively. The α_{1b}AR density did not change significantly ($p>0.05$) between each cell cycle phase, providing no evidence to support the idea that change in receptor density alters the incidence of $[Ca^{2+}]_i$ oscillation.

DISCUSSION

In the present study, we found that NE-induced $[Ca^{2+}]_i$ oscillation observed in the CHO cells stably expressing α_{1b}AR is specific for the S phase in the cell cycle and is dependent upon the $[Ca^{2+}]_o$. It was previously reported that IGF-I-activated $[Ca^{2+}]_i$ oscillation occurs not in quiescent the G_o state but when the cells progress into the proliferative cell cycle (13), and our present study extended the study and first demonstrated that the agonist-induced Ca^{2+} oscillation is dependent on the specific stage of cell cycle, the S phase. As Chinese hamster ovary cells stably expressing α_{1b}AR (CHOα_{1b} cells) consistently developed $[Ca^{2+}]_i$ oscillation under the experimental conditions we described, the cells would provide a valuable system to study further the biochemical mechanism for agonist-induced $[Ca^{2+}]_i$ oscillation phenomenon.

Receptors that are coupled to the production of IP_3 are known to cause an increase in $[Ca^{2+}]_i$ as a consequence of both Ca^{2+}-mobilization from intracellular stores and Ca^{2+} influx through the plasma membrane. NE caused a rapid increase in $[Ca^{2+}]_i$ consisting of a quick transient peak and a sustained component in CHOα_{1b} cells, each of which may represent the Ca^{2+}-mobilization from intracellular stores and Ca^{2+} influx through the plasma membrane, respectively (Fig. 3). As shown in Fig. 3 (Ca^{2+} Free), our observation that a quick transient peak, which may reflect the NE-induced Ca^{2+}-mobilization, was not much affected by cell synchronization, suggest that the occurrence of α_1AR-mediated $[Ca^{2+}]_i$ oscillation may not be directly associated to Ca^{2+}-mobilization from intracellular stores. Rather, the present finding that the S phase-specific occurrence of receptor-mediated $[Ca^{2+}]_i$ oscillation is dependent on $[Ca^{2+}]_o$ may indicate that cell cycle affects on the agonist-promoted Ca^{2+} influx mechanism that is closely linked to $[Ca^{2+}]_i$ oscillation. Similarly, the cell cycle-specific failure of agonist to stimulate Ca^{2+} influx but not the Ca^{2+} mobilization during mitosis has been reported in other systems (12). Further studies are required to clarify the molecular mechanism for the effect of cell cycle on the Ca^{2+} influx mechanism that is closely linked to $[Ca^{2+}]_i$ oscillation.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid from Japan Health Foundation (G.T.). We are grateful to Dr. Susanna Cottecchia (Institute de Pharmacologie et Toxocologie, Lausanne, Switzerland) for the generous gift of the cDNA encoding hamster α_{1b}AR.

REFERENCES

1. Berridge, M. J. (1994) *Mol. Cell. Endocrinol.* **98,** 119–124.
2. Berridge, M. J. (1990) *J. Biol. Chem.* **265,** 9583–9586.

3. Tse, A., Tse, F. W., Almers, W., and Hille, B. (1993) *Science* **260,** 82–84.
4. Jacob, R. (1990) *Biochem. biophys. Acta.* **1052,** 427–438.
5. Berridge, M. J. (1993) *Nature* **361,** 315–325.
6. Combettes, L., Tran, D., Tordjmann, T., Laurent, M., Berthon, B., and Claret, M. (1994) *Biochem. J.* **301,** 737–744.
7. Iino, M., Kasai, H., and Yamazawa, T. (1994) *Embo. J.* **13,** 5026–5031.
8. Horie, K., Hirasawa, A., and Tsujimoto, G. (1994) *Eur. J. Pharmacol.* **268,** 399–407.
9. Braunschweiger, P. G., Ting, H. L., and Schiffer, L. M. (1984) *Eur. J. Cancer Clin. Oncol.* **20,** 427–433.
10. Zieve, G. W., Turnbull, D., Mullins, J. M., and McIntosh, J. R. (1980) *Exp. Cell. Res.* **126,** 397–405.
11. Ohtsubo, M., and Roberts, J. M. (1993) *Science* **259,** 1908–1912.
12. Volpi, M., and Berlin, R. D. (1988) *J. Cell. Biol.* **107,** 2533–2539.
13. Kojima, I., Mogami, H., and Ogata, E. (1992) *Am. J. Physiol.* **262,** E307–E311.