Biochemical and Biophysical Research Communications **254,** 169–173 (1999)
Article ID bbrc.1998.9900, available online at http://www.idealibrary.com on IDEAL®

Cyclosporin A Induces a Biphasic Increase in KCl-Induced Calcium Influx in GH3 Pituitary Cells

Yu-Chi Chou and Jim C. Fong[1]
Institute of Biochemistry, National Yang-Ming University, Taipei, Taiwan, Republic of China

Received November 18, 1998

The role of calcineurin in modulation of calcium channel activity was examined in GH3 pituitary cells by using its selective inhibitor cyclosporin A. While cyclosporin A had little effect on basal activity, it induced a biphasic increase in K^+-induced $^{45}Ca^{2+}$ influx. Cyclosporin A rapidly increased K^+-induced $^{45}Ca^{2+}$ influx to approximately 140% of control in 1 h and the increment maintained at this magnitude for 1-8 h. Thereafter, K^+-induced $^{45}Ca^{2+}$ influx gradually increased further to approximately 220% after 24 h exposure to this compound. In the presence of anisomycin, however, the increase occurred at the latter phase was abolished. In addition, the increased calcium influx in cyclosporin A-pretreated cells had a similar sensitivity to KCl and verapamil as in untreated cells. Measurement of intracellular Ca^{2+} level by Fura-2 analysis indicated that $[Ca^{2+}]i$ increase induced by high K^+ or vasoactive intestinal peptide was similarly augmented in cyclosporin A-pretreated cells. Thus the results of this study suggest that calcineurin may play a tonic control on L-type Ca^{2+} channel, and inhibition of this enzyme may induce a subsequently protein synthesis-dependent higher channel activity.

Voltage-dependent Ca^{2+} channels (VDCC) provide a major pathway for Ca^{2+} entry into cells which, in turn, regulates a wide spectrum of cellular activities including neurotransmitter release, muscle contraction, hormone secretion, gene expression, cell mobility, cell division and cell death (1,2). According to their electrophysiological and pharmacological properties, VDCC has been classified into five groups including L, T, N, P/Q and R (3,4). The L-type Ca^{2+} channels are sensitive to dihydropyridine (3) and their activity may be modulated by phosphorylation and dephosphorylation (4-6). Besides cAMP-dependent protein kinase (PKA), protein kinase C and tyrosine kinase, protein phosphatases may be involved (7-10). Earlier reports have suggested that Ca^{2+}-activated protein phosphatase 2B (calcineurin) may play a negative regulatory role to limit Ca^{2+} current by dephosphorylation of L-type Ca^{2+} channels in nerve cells as well as in GH3 pituitary cells (5,6). A recent study, however, indicated that Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels in GH3 cells did not involve dephosphorylation of the channel proteins by calcineurin (11). The role of calcineurin in modulating Ca^{2+} channel activity, thus, remains to be clarified.

In the present study, we employed cyclosporin A, a selective inhibitor of calcineurin, to further investigate the role of calcineurin in the regulation of $^{45}Ca^{2+}$ influx into GH3 cells. Our results showed that while cyclosporin A had little effect on basal activity, it induced a biphasic increase in K^+-induced $^{45}Ca^{2+}$ influx in a time-dependent manner. Different from its acute effect on Ca^{2+} channel activity, the increase induced by longer incubation with cyclosporin A was abolished in the presence of anisomycin. Thus we have demonstrated for the first time that inhibition of calcineurin may induce a protein synthesis-dependent increase in Ca^{2+} channel activity.

MATERIALS AND METHODS

Materials. GH_3 clonal rat pituitary cell line was purchased from American Type Culture Collection (Rockville, Maryland). $^{45}Ca^{2+}$ (17 mCi/mg) was purchased from New England Nuclear (Boston, MA). Okadaic acid, dibutyryl cAMP, sodium orthovanadate (vanadate), N-2-hydroxyethlpiperaine-N'-2-ethanesulfonic acid (HEPES) and verapamil were obtained from Sigma (St. Louis, MO). Vasoactive intestinal peptide (VIP) was from Bachem (Torrance, CA). Cyclosporin A was from RBI (Natick, MA). Cell culture media and fetal calf serum were from Gibco Laboratories (Grand Island, NY, U.S.A.) and horse serum was from Hyclone (Logan, Utah).

Cell culture. GH_3 cells were grown on 10-mm dishes in Ham's F-10 medium also containing 15% horse serum, 2.5% fetal calf serum, penicillin and streptomycin for 14-16 days. Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C (12).

Measurement of $^{45}Ca^{2+}$ uptake in GH3 cells. Cells were trypsinized and seeded onto 6-well plates (Nunc, Roskilde) with

[1] To whom correspondence and proof should be addressed. Fax: 886-2-28264843. E-mail: ffong@ym.edu.tw.

0006-291X/99 $30.00

approximately 1-2×10^5/ml in serum-containing F-10 medium for three days, replaced with fresh complete medium and maintained in the same medium for two more days. Before experiments, the medium was decanted and the cells were washed three times with F-10 medium. After incubation in the absence or presence of cyclosporin A for the indicated times, 1 μCi/ml $^{45}Ca^{2+}$ ± 50 mM KCl was added and incubation was continued for another 10 min. The medium was then aspirated and the cells were washed three times with Ca^{2+}-free HBBS (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM Hepes, pH 7.4). Cells were solubilized in 2% SDS and measured for radioactivity by liquid scintillation (9).

Measurement of free intracellular calcium. GH_3 cells grown on 100-mm dish (Nunc, Roskilde) were pretreated with or without 1 μM cyclosporin A for 24 h. Cell were then rinsed with 10 ml of modified Krebs-Ringer bicarbonate buffer (KRB)(25 mM HEPES, 120 mM NaCl, 4.75 mM KCl, 1 mM KH_2PO_4, 1.44 mM $MgSO_4$, 5 mM $NaHCO_3$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 2.8 mM glucose, pH 7.4) and removed from dish by pipetting for several times. Cells were harvested by centrifuging at 500 × g for 5 min and incubated in 2 ml KRB also containing 2.5 μM Fura 2-AM for 30 min at 37°C. At the end of incubation, cells were diluted to 25 ml with KRB, centrifuged and the pellet was resuspended in KRB at a density of 2-5x10^6 cells/ml. For the measurement of $[Ca^{2+}]_i$ by spectrofluorometer (SPEX 1681), cells were placed and stirred in a thermostated cuvette at 25°C. After a stable baseline was obtained, 50 mM KCl or 10 nM VIP was added. Maximal fluorescence (Fmax) was obtained by lysing cells with 0.2% Triton X-100 and minimal fluorescence (Fmin) by adding 20 mM EGTA. Intracellular calcium concentrations were calculated from the equation: $[Ca^{2+}]_i = Kd \times (F - F\ min)/(Fmax-F)$ with Kd = 224 nM (13).

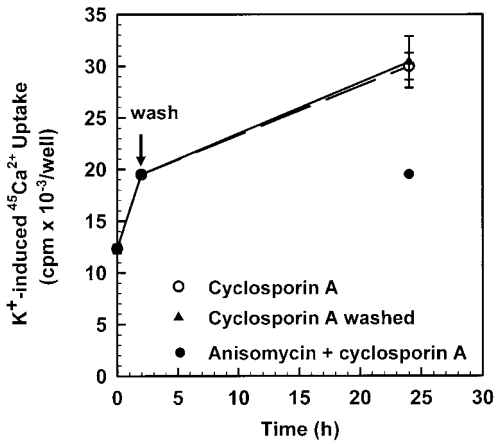

FIG. 2. Effect of anisomycin and washing on the increase of K^+-induced $^{45}Ca^{2+}$ uptake provoked by cyclosporin A. After GH_3 cells were preincubated in the presence of anisomycin for 2 h, cells were washed and incubated in the presence of 1 μM cyclosporin A for another 24 h. 50 mM KCl-induced $^{45}Ca^{2+}$ uptake was then measured as described under Materials and Methods. In another experiment, after 2 h-incubation in the presence of cyclosporin A, cyclosporin A was removed by washing and incubation was continued for another 22 h. K^+-induced $^{45}Ca^{2+}$ uptake was then measured as described above. Values are means ± SE from 3 experiments with duplicated determinations.

RESULTS AND DISCUSSION

To evaluate the role of calcineurin in modulation of Ca^{2+} channel activity, GH3 pituitary cells were treated with a selective calcineurin inhibitor cyclosporin A for various times and measured for basal and 50 mM KCl-induced $^{45}Ca^{2+}$-uptake. While basal $^{45}Ca^{2+}$-uptake was not significantly affected (400-600 cpm/well), pretreatment with cyclosporin A induced a biphasic increase in K^+-induced $^{45}Ca^{2+}$ influx (Fig. 1A). Cyclosporin A rapidly increased K^+-induced $^{45}Ca^{2+}$ influx to approximately 140% of control in 1 h ($P < 0.05$) and the increment sustained for 1-8 h. Longer incubation (>8 h), on the other hand, further increased the calcium channel activity to approximately 220% of control at 24 h ($P < 0.01$). The effect of cyclosporin A was dose-dependent, with maximal effect observed at 1 μM (Fig. 1B). Thus it appears that the effect of cyclosporin A on K^+-induced $^{45}Ca^{2+}$ influx involves two distinct mechanism. Indeed, whereas the short-term (2 h) effect of cyclosporin A was not influenced, the long-term (24 h) effect was reduced to the short-term level in the presence of anisomycin (Fig. 2), suggesting that the long-term effect of cyclosporin A requires continued protein synthesis.

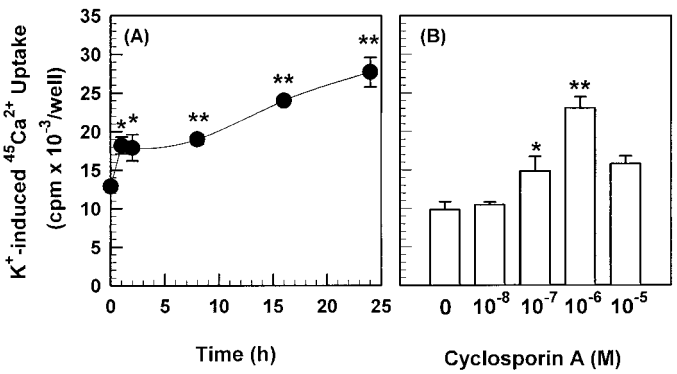

FIG. 1. (A) Time-dependent effect of cyclosporin A-pretreatment on KCl-induced $^{45}Ca^{2+}$ uptake in GH_3 cells. After GH_3 cells were pretreated with 1 μM cyclosporin A for the indicated times, cells were washed and measured for basal and 50 mM KCl-induced $^{45}Ca^{2+}$ uptake as described under Materials and Methods. Basal uptake was measured as 400-600 cpm/well. Values are means ± SE from 3 experiments with duplicated determinations. *$P < 0.05$; **$P < 0.01$ compared to the value measured at time 0. (B) Dose-dependent effects of cyclosporin A-pretreatment on KCl-induced $^{45}Ca^{2+}$ uptake. After GH_3 cells were pretreated with various concentrations of cyclosporin A for 24 h, cells were washed and measured for 50 mM KCl-induced $^{45}Ca^{2+}$ uptake as described under Materials and Methods. Values are means ± SE from 3 experiments with duplicated determinations. *$P < 0.05$; **$P < 0.01$ compared to the value measured in the absence of cyclosporin A.

Since calcineurin might reduce Ca^{2+} influx mediated by L-type Ca^{2+} channel by dephosphorylation of Ca^{2+} channel protein (6) or some other protein modulator, inhibition of this enzyme by cyclosporin A may lead to increased phosphorylation of either Ca^{2+} channel protein or other protein modulator and triggered a signaling cascade, leading to increased Ca^{2+} channel activity. In consistent with this notion, removal of cyclosporin A

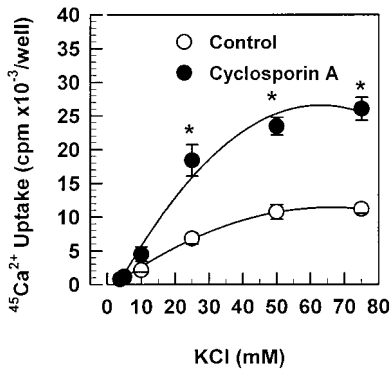

FIG. 3. Dose-dependent effects of KCl on $^{45}Ca^{2+}$ uptake in GH_3 cells pretreated with cyclosporin A. After GH_3 cells were pretreated without (control) or with 1 μM cyclosporin A for 24 h, cells were washed and $^{45}Ca^{2+}$ uptake was measured in response to various concentrations of KCl as described under Materials and Methods. Values are means ± SE from 3 experiments with duplicated determinations. *P < 0.01 compared to the control.

by washing after 2 h incubation did not eliminate or reduce the subsequent long-term effect of cyclosporin A (Fig. 2), implying that the phosphorylation event incurred by 2 h incubation in the presence of cyclosporin A is sufficient to transmit the signal further down the signaling pathway.

To further examine whether or not the increased Ca^{2+} channel activity found in cyclosporin A-pretreated cells was attributed to altered property of L-type Ca^{2+} channel protein, we compared dose-response curves for KCl-induced $^{45}Ca^{2+}$-influx and verapamil inhibition of K^+-induced $^{45}Ca^{2+}$-influx between control and cyclosporin A-pretreated cells. As shown in Fig. 3 and Fig. 4, neither the EC_{50} value for KCl (~20 mM) nor the IC_{50} value for verapamil (~0.3 μM) was significantly changed by cyclosporin A-pretreatment. Therefore, it is plausible that increased amount of L-type Ca^{2+} channel protein in cyclosporin A-pretreated cells may contribute to the increased KCl-induced $^{45}Ca^{2+}$-influx.

Calcineurin has been shown to regulate gene expression by either stimulation or inhibition, depending on the cell types as well as the stimuli involved (14,15). Therefore, inhibiting calcineurin by cyclosporin A may lead to inhibition or stimulation of certain gene expression. For example, cyclosporin A was shown to reduce calcineurin-mediated IL-4 gene expression after ionomycin treatment in murine T-helper cells (14), but enhance transcription rate of NGFI-A gene by A23187 in PC12 cells (15). In the present study, it is then possible that the enhancing effect of cyclosporin A on Ca^{2+} channel activity is mediated by changes in the expression of genes for L-type calcium channel proteins. Nevertheless, we can not rule out the possibility that some other gene may be involved.

The effect of prolonged treatment with cyclosporin A on Ca^{2+} influx is unique. As we surveyed other inhibitors to protein phosphatase 1 & 2A such as okadaic acid (16) or to protein-tyrosine phosphatase such as vanadate (8), neither one induced similar biphasic effect as cyclosporin A. As shown in Fig. 5, pretreatment with okadaic acid or vanadate for 2 h, the basal Ca^{2+} influx was not influenced; however, if the duration of pretreatment was prolonged to 24 h, the basal activity was reduced to 64% of control ($P < 0.01$) by okadaic acid and increased to 121% of control ($P < 0.01$) by vanadate, respectively. On the other hand, whereas pretreatment with okadaic acid for 2 h or 24 h consistently suppressed KCl-induced Ca^{2+} uptake by 65% ($P < 0.01$) or 93% ($P < 0.01$) respectively, the effect of vanadate was biphasic, with an increase in KCl-induced Ca^{2+} uptake (46% over control, $P < 0.01$) for 2 h–pretreatment, but a 41% decrease ($P < 0.01$) for 24 h-pretreatment. The observed short-term effects were consistent with earlier reports that okadaic acid inhibited, whereas vanadate enhanced KCl-induced Ca^{2+} influx (8,17). The effect of long-term (24 h) treatment with these inhibitors, nevertheless, was not reported previously. For comparison, we also examined the effect of dibutyryl cAMP, which decreased KCl-induced Ca^{2+} uptake at high concentration as demonstrated earlier (18).

Do the changes in L-type Ca^{2+} channel activity lead to altered intracellular Ca^{2+} concentration? To answer this question, we measured $[Ca^{2+}]i$ by Fura-2 analysis in cyclosporin A-pretreated cells. As shown in Fig. 6, $[Ca^{2+}]i$ was augmented in response to both 50 mM KCl and 10 nM VIP, which is known to induce Ca^{2+} influx through L-type channel (19), in cyclosporin A-pretreated cells. Thus it seems that the

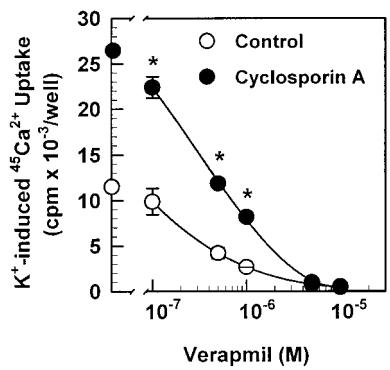

FIG. 4. Dose-dependent inhibition of K^+-induced $^{45}Ca^{2+}$ uptake by verapamil in GH_3 cells pretreated with cyclosporin A. After GH_3 cells were preincubated in the absence (control) or presence of 1 μM cyclosporin A for 24 h, cells were washed and then incubated with varying concentration of verapamil for 30 min and 50 mM KCl-induced $^{45}Ca^{2+}$ uptake was then measured as described under Materials and Methods. Values are means ± SE from 3 experiments with duplicated determinations. *P < 0.01 compared to the control.

phenomenon we observed here is probably of physiological significance. Since GH3 pituitary cells are well established cell model for studying the function of pituitary lactotrophs, the results of the present study suggest that under certain physiological or pathological conditions when the negative regulatory mechanism of calcineurin to limit Ca^{2+} current is suppressed for a long period of time, the cells may become hyper-sensitive to depolarization or neuromodulator such as VIP. It is of interest to examine whether other neuroendocrine or nerve cells are similarly affected by cyclosporin A.

In summary, in the present study we have demonstrated that cyclosporin A may induce a biphasic increase in KCl-induced Ca^{2+} influx in GH3 cells. The increase in KCl-induced Ca^{2+} influx is attributed to enhanced L-type Ca^{2+} channel activity and a protein synthesis-dependent mechanism is responsible for the increase in the latter phase. Since cyclosporin A is a selective inhibitor of calcineurin, the results of this study suggest that calcineurin may play a tonic control on L-type Ca^{2+} channel and sustained inhibition of this enzyme may induce a protein synthesis-dependent higher channel activity.

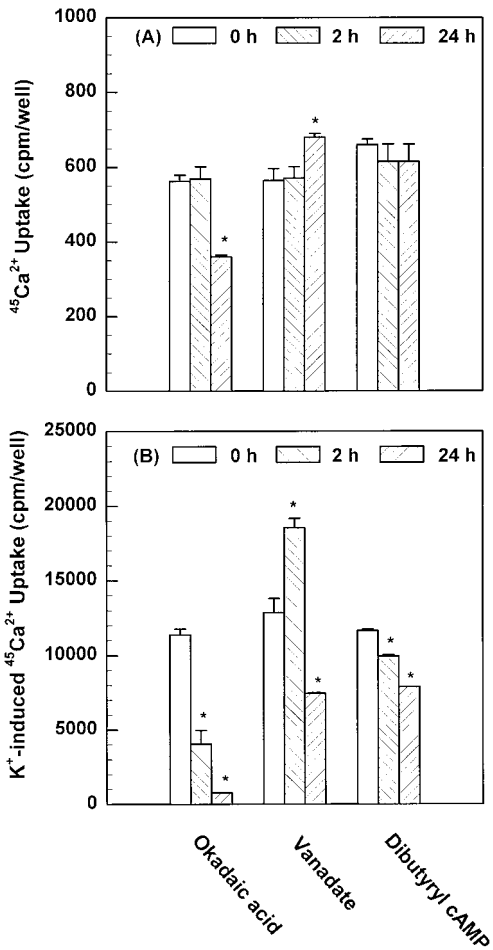

FIG. 5. Effect of okadaic acid, vanadate and dibutyryl cAMP on $^{45}Ca^{2+}$ uptake in GH3 cells. After GH_3 cells were pretreated with 50 nM okadaic acid, 1 mM vanadate or 1 mM dibutyryl cAMP for 0, 2 and 24 h, cells were washed and measured for basal and 50 mM KCl-induced $^{45}Ca^{2+}$ uptake as described under Materials and Methods. Values are means ± SE from 3 experiments with duplicated determinations. *$P < 0.01$ compared to the corresponding 0 h.

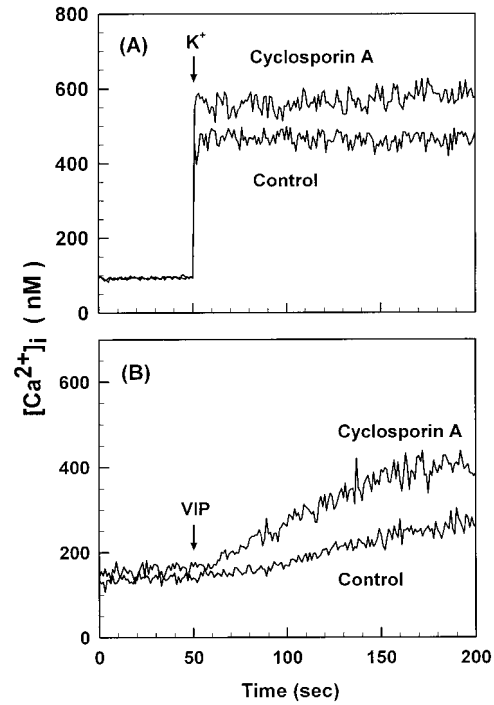

FIG. 6. Effect of cyclosporin A-pretreatment on (A) KCl- and (B) VIP-induced rise in $[Ca^{2+}]_i$ in GH_3 cells.

ACKNOWLEDGMENT

This investigation was supported by Grant NSC85-2331-B010-113-M10 from the National Science Council, Republic of China.

REFERENCES

1. Randall, A. D. (1998) *J. Membrane Biol.* **161,** 207–213.
2. Hosey, M. M., Chien, A. J., and Puri, T. S. (1996) *Trends. Cardiovasc. Med.* **6,** 265–273.
3. Miller, R. J. (1992) *J. Biol. Chem.* **267,** 1403–1406.
4. Walker, D., and Waard, M. D. (1998) *Trends. Neurosci.* **21,** 148–154.
5. Chad, J. E., and Eckert, R. (1986) *J. Physiol.* **378,** 31–51.
6. Kalman, D., O'Lague, P. H., Erxleben, C., and Armstrong, D. L. (1988) *J. Gen. Physiol.* **92,** 531–548.
7. Armstrong, D., and Eckert, R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2518–2522.
8. Cataldi, M., Taglialatela, M., Guerriero, S., Amoroso, S., Lombardi, G., Renzo, G. D., and Annunziato, L. (1996) *J. Biol. Chem.* **271,** 9441–9446.
9. Haymes, A. A., Kwan, Y. W., Arena, J. P., Kass, R. S., and Hinkle, P. M. (1992) *Am. J. Physiol.* **262,** C1211–C1219.

10. Frace, A. M., and Hartzell, H. C. (1993) *J. Physiol.* **472,** 305–326.
11. Victor, R. G., Rusnak, F., Sikkink, R., Marban, E., and O'Rourke, B. (1997) *J. Membrane Biol.* **156,** 53–61.
12. Tashjian, A. H., Yasumura, Y. Jr., Levine, L., Sato, G. H., and Parker, M. L. (1968) *Endocrinology* **82,** 342–352.
13. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1986) *J. Biol. Chem.* **260,** 3440–3450.
14. Rooney, J. W., Hodge, M. R., McCaffrey, P. G., Rao, A., and Glimcher, L. H. (1994) *EMBO J.* **13,** 625–633.
15. Enslen, H., and Soderling, T. R. (1994) *J. Biol. Chem.* **269,** 20872–20877.
16. Cohen, P., Holmes, C. F. B., and Tsukitani, Y. (1990) *Trends. Biochem. Sci.* **15,** 98–102.
17. Ammon, H. P., Heurich, R. O., Kolb, H. A., Lang, F., Schaich, R., Drews, G., and Leiers, T. (1996) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **354,** 95–101.
18. Koh, S. D., and Sanders, K. M. (1996) *Am. J. Physiol.* **271,** C794–803.
19. Jang, Y. C., Kao, L. S., and Wang, F. F. (1998) *Cell. Signal.* **10,** 27–34.