Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 241-248

THE EFFECT OF KN-62, Ca^{2+}/CALMODULIN DEPENDENT PROTEIN KINASE II INHIBITOR ON CELL CYCLE[1]

Hiroyuki Minami, Shigeo Inoue and Hiroyoshi Hidaka[2]

Department of Pharmacology, Nagoya University School of Medicine, Tsurumai 65, Showa-ku, Nagoya 466, Japan

Received January 4, 1994

SUMMARY: The isoquinolinesulfonamide derivative, KN-62 , is a potent and specific inhibitor of Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II)(Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., and Hidaka, H.(1990) J. Biol. Chem. 265, 4315-4320). KN-62 inhibits growth of K562 cells, in a dose-dependent manner. Flow cytometric analysis demonstrates that the treatment of K562 cells with 10 μM KN-62 causes an accumulation of cells in S phase.

Immunoblotting studies showed that specific antibodies against CaM kinase II recognized the 65 kDa of protein in K562 cells . This protein showed protein kinase activity as examined by the activity gel method. The inhibition of this enzyme activity by KN-62 was dose-dependent. The immunoprecipitates with the antibodies from K562 cells phosphorylates the synthetic peptide substrates, syntide-2. These results suggest that CaM kinase II plays an important role in the mechanisms for the cell growth in K562 cells.

The major intracellular Ca^{2+}-receptor calmodulin is present in a wide variety of mammalian tissues. Ca^{2+}/calmodulin (CaM) complex is involved in the regulation of a variety of fundamental intracellular systems such as protein phosphorylation, glycogen and cyclic nucleotide metabolism and microtubule assembly [1-3]. Many pharmacological studies using CaM antagonists have demonstrated the importance of calmodulin in cell function and cell cycle progression [4-7].

Multifunctional CaM dependent protein kinase II (CaM kinase II) was found in most mammalian tissues examined and was purified, characterized from many tissues [8]. The protein

[1] This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

[2] To whom correspondence should be addressed.

The abbreviations used are: CaM, Ca^{2+}/calmodulin; CaM kinase, Ca^{2+}/calmodulin dependent protein kinase; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol bis(2-aminoethylether) tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; KN-62, 1-[N,O-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; MLC, myosin light chain; PAGE, polyacrylamide gel electrophoresis; PBS, Phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; RPMI, Roswell Park Memorial Institute.

0006-291X/94 $5.00

phosphorylation by CaM kinase II may act as a functional switch in the CaM signal transduction. We reported previously the potent and specific inhibitor KN-62 (1-[N,O-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) to clarify the physiological role of CaM kinase II [9].

Previous studies of protein kinases using human erythroleukemia K562 cells have mainly focused on protein kinase C, cyclic AMP dependent protein kinase, myosin light chain kinase or tyrosine kinases [10-15]. No study of CaM kinase II in K562 has been reported. In this study we show that CaM kinase II is found in K562 cells, and that using KN-62 in K562 cells we investigate the role of the phosphorylation by CaM kinase II. A possible involvement of protein phosphorylation by CaM kinase II in cell cycle of K562 cells is discussed.

EXPERIMENTAL PROCEDURES

Cells Culture - K562 cells were cultured in RPMI 1640 (Nissui) supplement with 50 μM 2-mercaptoethanol and 10% fetal calf serum (FCS). All cultures were kept in a humidified atmosphere of 95% air, 5% CO_2 at 37°C [16].

In preparation of K562 cells for cell growth and cell cycle analysis, KN-62 dissolved in 100% dimethyl sulfoxide (DMSO) were added to culture medium. DMSO without KN-62 was added to control cells. The final concentration of DMSO was 0.1% in culture medium. For cell growth analysis, K562 cells were plated in a 3-cm dish at a density 1.4×10^6/dish with 5 ml of culture medium containing various concentration of KN-62. After two days in these condition cell numbers were counted.

Cell Cycle Analysis - For cell cycle analysis, synchrony was achieved by 1 μg/ml aphidicolin block. After removal of aphidicolin, the cells were cultured in the absence or presence of 10 μM KN-62. For flow cytometric analysis, cultured cells were harvested at 1000 r.p.m. for 5 min at room temperature. The cells harvest were washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and collected. The harvests were suspend with small volume of PBS and added 5 ml of PBS-ethanol (3:7) while mixing gently. After 60 min at room temperature, cells were collected and washed twice with PBS. The cells were collected and resuspended with 500 μl of 5 mg/ml RNase A (Sigma) and incubated for 30 min at 37°C. Cells were harvested and stained by addition of an ice cold 2 ml of PBS containing 50 mg/ml propidium iodide (Sigma) and stand on ice in the dark. After 30 min or more, Flow cytometric analysis was carried out using a Coulter EPICS Profile flow cytometer. Ten thousand cells were counted per analysis.

For mitotic (M) and G_1 standard, nocodazole or aphidicolin (Sigma) was added to culture medium at a final concentration of 0.4 or 1.0 μg/ml, respectively. After 24hr, the cells were prepared under the conditions as above.

Preparation of Cell Extracts and Western Blotting - K562 cells were collected and washed twice with PBS. The cells were homogenized in 4 volumes of buffer A containing 40 mM Tris (pH 7.5), 140 mM NaCl, 2 mM EGTA, 50 μg/ml trypsin inhibitor, and 0.1 mM PMSF, and were ultrasonicated 20 sec/ml. The homogenates were centrifuged at 10600xg for 1 hr at 4°C. The supernatant ("cell extract") was collected, 15 μl of aliquots of the supernatant were subjected to SDS-PAGE [17] and were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore) in 48 mM Tris (pH 9.2), 39 mM glycine, 0.375 g/l SDS, and 20% ethanol at 15 V for 30 min. Nonspecific binding of antibody was blocked by incubating the membrane with 1% dry milk in PBS. Then the membrane was probed for 1 hr with 1 μg/ml of anti-CaM kinase II antibody obtained by Tokumitsu et al [9] followed by three times washes with PBS containing 0.05% Tween 20 and treatment with peroxidase-labeled goat anti-rabbit IgG (MBL). Subsequently, the membrane was washed three times with PBS and stained by 3,3'-diaminobenzidine (Sigma) in the presence of hydrogen peroxide.

Immunoprecipitation and Kinase Assay - To avoid nonspecific absorption 10 µl of protein A coupled sepharose 4B (Pharmacia LKB) were added to 500 µl of the cell extract. After 2 hr at 4°C while gently mixing, the suspension was centrifuged 5000 r.p.m for 10 sec. And supernatant was collected, then another 5 µl of protein A coupled sepharose 4B containing 1 µg of anti- CaM kinase II antibody were added. After 12 hr at 4°C, the resin was collected and washed three times with 500 µl of buffer A. The resin were then suspended with 300 µl of buffer A. Using this suspended immunoprecipitates, CaM dependent protein kinase activity was assayed in 35 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 µM calmodulin, 10 µM ATP containing 3.7 kBq [gamma-^{32}P]ATP (Amersham), and 20 µM syntide-2 as a substrate [18]. We incubated assay mixtures at 37°C for 15 min. Forty milliliter of reaction mixture was spotted on phosphocellulose filter, then the filter were washed four times with 0.75% phosphoric acid, and dried at 60°C. Radioactivity on the filter was counted by scintillation counter.

Phosphorylation in Sodium Dodecyl Sulfate-Polyacrylamide Gel - Phosphorylation technique was carried out in the method of Kameshita et al[19]. Briefly 100 µl of suspended immunoprecipitate resolved by SDS-PAGE with 10% acrylamide gel which contained 0.5 mg/ml myosin light chain (MLC) from chicken gizzard. The gels were washed three times with 50 ml of 50 mM Tris (pH 8.0), and 20% 2-mercaptoethanol for 20 min, and finally washed with 100 ml of 50 mM Tris (pH 8.0), and 5 mM 2-mercaptoethanol (buffer B) at room temperature. The proteins were denatured three times in 50 ml of buffer B containing 6 M urea for 20 min. The proteins were renatured with several changes of 100 ml of buffer B containing 0.04% Tween 40 at 4°C. After preincubation in 1.4 ml/lane of 40 mM HEPES (pH 8.0), 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 30 µg/ml calmodulin, and 10% DMSO with or without of various concentration of KN-62 for 30 min at room temperature, gels were incubated in 1.4 ml/lane of same solution containing 50 µM ATP, and 740 kBq [gamma-^{32}P]ATP for 1 hr. After five times washes with 50 ml of 5% trichloroacetic acid and 1% sodium pyrophosphate, the gels were dried and exposed to Fuji imaging plate. Intensities of the radioactive bands of phosphorylated MLC were quantitated using a BioImage Analyser BAS 2000 (Fuji Film, Japan).

RESULTS

Effects of KN-62 on the cell growth and cell cycle of K562 cells

The addition of varying concentration of KN-62 or DMSO as a control to the culture medium produced a dose-dependent inhibition of cell growth in K562 cells (Fig.1). The starting cell number was 1.4×10^6 cells/3cm dishes. Two days after the addition of 10 µM KN-62 to

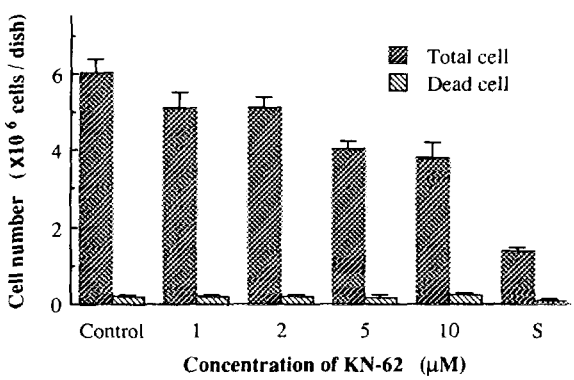

Fig. 1. Effect of KN-62 on K562 cell growth.
K562 cells were cultured in 3 cm dishes with 5 ml of culture medium containing 0 (Control), 1, 2, 5 and 10 µM of KN-62 and 0.1% DMSO. Starting number of cells were 1.4×10^6/dish (S). Two days after, total cells and dead cells (stained by 0.25% trypan blue) were counted. The error bars indicate 2 x standard deviation of triplicate measurements. Data were pooled from three separate experiments.

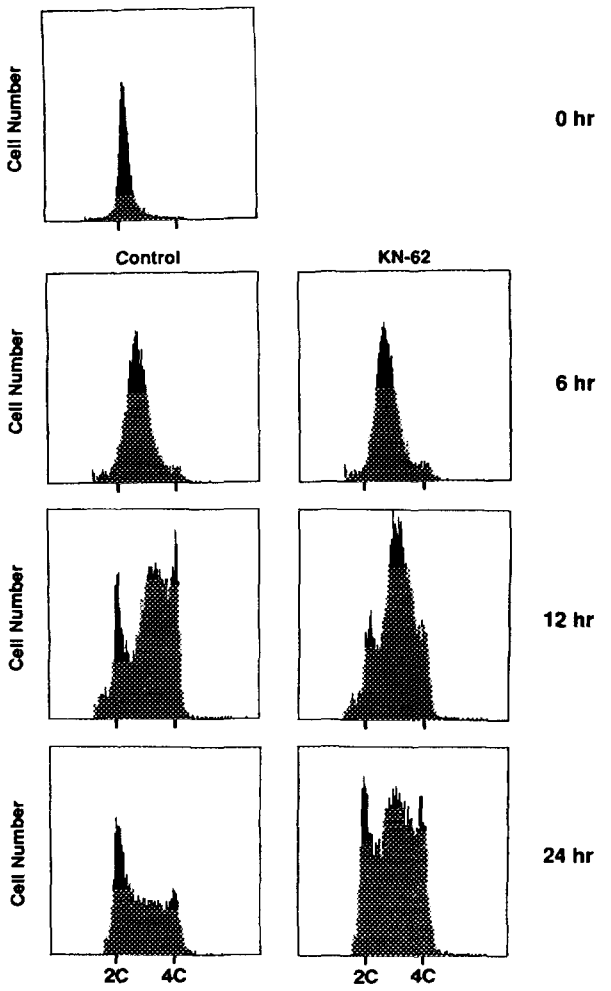

Fig. 2. Effect of KN-62 on K562 cell cycle.
K562 cells, arrested at G1/S boundary by 5 μM aphidicolin for 24 hr (0 hr), were cultured in the absence (control) or presence of 10 μM KN-62. After 6, 12 and 24 hr, the cells were analyzed by a Coulter EPICS Profile flow cytometer. Standard values of 2C (G1) and 4C (G2/M) in profiles were measured as described in Experimental Procedures.

cultures, the cell growth of K562 cells were inhibited to 63% compared to that of control. This inhibition by KN-62 was a dose-dependent. But no increase of dead cells was observed.

We used flow cytometry to assess the effect of KN-62 on the progression of K562 cells through the cell cycle. Synchronized cells with aphidicolin released from G1/S boundary and were cultured in the absence or presence of 10 μM KN-62 from 0 to 24 hr. K562 cells were monitored by flow cytometric analysis of propidium iodide-stained cells (Fig.2). In control cells, at 6hr after release, most of cells entered S phase. Twelve hours after major peak of population profile were seen at end of S phase. After 24 hr the flow cytometric pattern almost returned to

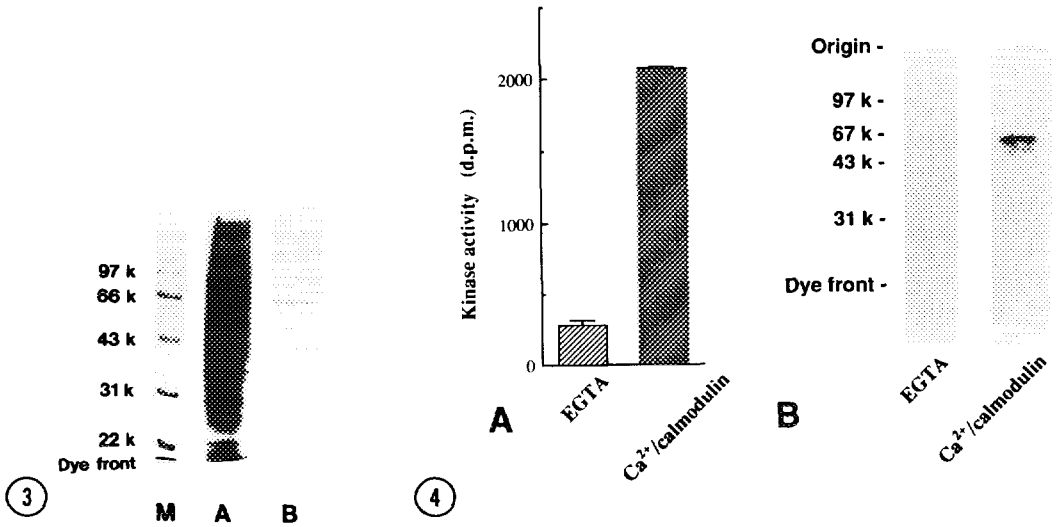

Fig. 3. Detection of 65 kDa protein in K562 cells.
K562 cells were homogenized with 4 volume of Buffer A and were ultrasonicated 20 sec/ml and centrifuged at 10600xg for 60 min at 4°C. 15 µl aliquot of the supernatant were applyed to SDS-PAGE. Lane M: MW marker. Lane A: protein staining by Coomassie blue. Lane B: immunoblotting using anti-CaMK II antibody.

Fig. 4. Ca^{2+}/calmodulin dependency on kinase activity of 65 kDa protein .
65 kDa protein was purified as described in Experimental Procedures. Suspended immunoprecipitates were directly applied to kinase assay using syntide-2 as a substrate (panel A). Immunoprecipitates were boiled with Sample buffer at 100°C for 2 min and was electrophoresed on SDS-polyacrylamide slab gel consisting of 10% acrylamide separation gel containing 0.5 mg/ml myosin light chain. Phospholyration was done under the condition of Experimental Procedures. ^{32}P incorporation was visualized using Fuji BAS 2000 system (panel B). EGTA and Ca^{2+}/calmodulin in each panels were in the absent and the preset of CaM, respectively.

the pattern before synchronization. On the other side, substantial changes occurred in KN-62 treated cells. At the release from aphidicolin the peak of population profile was no different from that of control cells, but even 24 hr after release the major peak still remained in the end of S phase. This result indicates that KN-62 blocked cell cycle progression and caused an accumulation of cells in S phase, as compared with control cells.

Isolation of Ca^{2+}/calmodulin dependent protein kinase II from K562 cells by immunoprecipitation

We examined the presence of the Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II) in K562 cells using the anti-CaM kinase II antibodies [9]. We confirmed that this antibodies possessed reactivity toward both a and b subunits of CaM kinase II by Western blotting using the soluble fraction of rat bovine brain as a standard, and no inhibitory effect on the kinase activity of CaM kinase II (data not shown). As shown in Fig.3, an apparent Mr of 65kDa protein which reacted with the antibodies was detected in K562 cell extracts by immunoblotting. No other protein reacted with the antibodies.

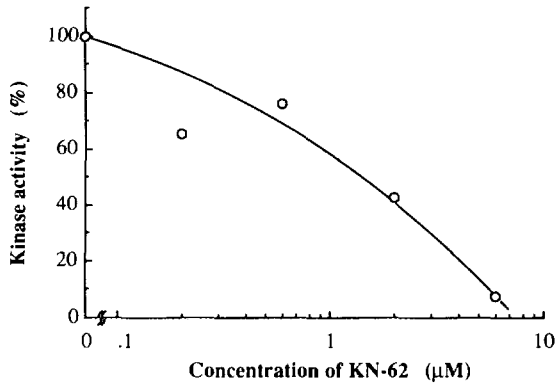

Fig. 5. Effect of KN-62 on kinase activity of 65 kDa protein.
Suspended immunoprecipitates were boiled with Sample buffer at 100°C for 2 min and was electrophoresed on SDS-polyacrylamide slab gel consisting of 10% acrylamide separation gel containing 0.5 mg/ml myosin light chain (MLC). Kinase activity was assayed by activity gel method in the presence of 0, 0.2, 0.6, 2.0 and 6.0 μM of KN-62.

The CaM dependency of the kinase activity were investigated using synthetic peptide, syntide-2, as a substrate. Fig.4A shows that the immunoprecipitated protein had a syntide-2 phosphorylation activity in the presence of Ca^{2+} and calmodulin. No or scanty activity was found in the absence of Ca^{2+} and calmodulin. Autophosphorylation activity was detected in the presence of Ca^{2+} and calmodulin as shown in Fig.4B.

The effect of KN-62 on phosphorylation activity

Then we examined the effect of KN-62 on the activity of this kinase with immunoprecipitates on electrophorated, renatured protein in SDS-PAGE gel using chicken gizzard myosin light chain as a substrate. As shown in Fig.5, KN-62 inhibited this kinase activity in a dose dependent manner. The concentration of KN-62 producing 50 % inhibition was estimated apparently to be 1.8×10^{-6} M. This value is close to the reported dose to the purified CaM kinase II from rat brain [9].

DISCUSSION

In previous report, many Ca^{2+}/calmodulin-dependent protein kinases were identified [20-32]. We reported one of these kinases, Ca^{2+}/calmodulin-dependent protein kinase V (CaM kinase V) [33]. Patterns of distribution for each CaM kinases differ among tissues. It is of interest to note that which kinases K562 cells contain. In the present study we demonstrated that immunoreactive protein kinase with anti-CaM kinase II antibodies was detected in K562 cells (Fig.4). The antibodies which used in this study didn't react with CaM kinase V (data not

shown). And KN-62 , a potent specific CaM kinase II inhibitor, inhibited the kinase activity with myosin light chain as a substrate in a dose-dependent manner (Fig.5). From these results we concluded that the characters of this kinase are very similar to CaM kinase II which was purified from rat brain [32]. But the molecular weight of this kinase differ from any subunits of CaM kinase II from rat brain. This finding suggests that this kinase is another isoenzyme of CaM kinase II.

KN-62 inhibited the cell growth of K562 cells in a dose-dependent manner (Fig.1), and prolonged S phase (Fig.2). We examined the effect of CaM kinase II on DNA polymerase a activity [34], but no significant effect was observed (data not shown). These result suggests that the phosphorylation by CaM kinase II may play an important role in the control of cell growth and progression of cell cycle of K562 cells.

ACKNOWLEDGMENTS

We thank T. Sakurai for instruction on use of the DNA polymerase assay and for kind advice. We also thank H. Mochizuki for his advice and helpful discussions on protein kinases and N. Watanabe for excellent technical assistance.

REFERENCES

(1) Means, A. R.; Dedman, J. R. (1980) *Nature* ***285***, 73-7.
(2) Cheung, W. Y. (1980) *Science* ***207***, 19-27.
(3) Wang, J. H.; Waisman, D. M. (1979) *Curr. Top. Cell Regul.* ***15***, 47-107.
(4) Chafouleas, J. G.; Bolton, W. E.; Hidaka, H.; Boyd, A. 3.; Means, A. R. (1982) *Cell* ***28***, 41-50.
(5) Hidaka, H.; Watanabe, M.; Kobayashi, R. (1991) *Methods Enzymol.* ***201***, 328-39.
(6) Hidaka, H.; Kobayashi, R. (1992) *Annu. Rev. Pharmacol. Toxicol.* ***32***, 377-97.
(7) Hidaka, H.; Ishikawa, T. (1992) *Cell Calcium* ***13***, 465-72.
(8) Colbran, R. J.; Schworer, C. M.; Hashimoto, Y.; Fong, Y. L.; Rich, D. P.; Smith, M. K.; Soderling, T. R. (1989) *Biochem. J.* ***258***, 313-25.
(9) Tokumitsu, H.; Chijiwa, T.; Hagiwara, M.; Mizutani, A.; Terasawa, M.; Hidaka, H. (1990) *J. Biol. Chem.* ***265***, 4315-20.
(10) Barnett, S. C.; Evans, C. H. (1988) *Clin. Exp. Immunol.* ***73***, 505-9.
(11) Elias, L.; Li, A. P.; Longmire, J. (1981) *Cancer Res.* ***41***, 2182-8.
(12) Evans, J. P.; Wickremasinghe, R. G.; Hoffbrand, A. V. (1987) *Leukemia* ***1***, 524-5.
(13) Wakusawa, S.; Nakamura, S.; Tajima, K.; Miyamoto, K.; Hagiwara, M.; Hidaka, H. (1992) *Mol. Pharmacol.* ***41***, 1034-8.
(14) Makishima, M.; Honma, Y.; Hozumi, M.; Sampi, K.; Hattori, M.; Motoyoshi, K. (1991) *FEBS Lett.* ***287***, 175-7.
(15) Allen, H.; Hsuan, J.; Clark, S.; Maziarz, R.; Waterfield, M. D.; Flavell, R. A.; Haley, J. (1990) *Biochem. J.* ***271***, 785-90.
(16) Murate, T.; Kaneda, T.; Hotta, T.; Goto, S. (1988) *Acta-Hematol-Japan.* ***51***, 40-9.

(17) Laemmli, U. K. (1970) *Nature* ***227***, 680-85.
(18) Hashimoto, Y.; Soderling, T. R. (1987) *Arch. Biochem. Biophys.* ***252***, 418-25.
(19) Kameshita, I.; Fujisawa, H. (1989) *Anal. Biochem.* ***183***, 139-43.
(20) Kennedy, M. B.; Bennett, M. K.; Erondu, N. E.; Miller, S. G. (1987) In *Calcium and Cell Function* (W.Y. Cheung, Ed.), Vol. 7, pp 62-107. Academic Press, Orlando, FL.
(21) Beg, Z. H.; Stonik, J. A.; Brewer, H. J. (1987) *J. Biol. Chem.* ***262***, 13228-40.
(22) Bennett, M. K.; Erondu, N. E.; Kennedy, M. B. (1983) *J. Biol. Chem.* ***258***, 12735-44.
(23) Edelman, A. M.; Blumenthal, D. K.; Krebs, E. G. (1987) *Annu. Rev. Biochem.* ***56***, 567-613.
(24) Goldenring, J. R.; Gonzalez, B.; McGuire, J. J.; DeLorenzo, R. J. (1983) *J. Biol. Chem.* ***258***, 12632-40.
(25) Miyano, O.; Kameshita, I.; Fujisawa, H. (1992) *J. Biol. Chem.* ***267***, 1198-203.
(26) Nairn, A. C.; Bhagat, B.; Palfrey, H. C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* ***82***, 7939-43.
(27) Nairn, A. C.; Hemmings, H. J.; Greengard, P. (1985) *Annu. Rev. Biochem.* ***54***, 931-76.
(28) Nairn, A. C.; Greengard, P. (1987) *J. Biol. Chem.* ***262***, 7273-81.
(29) Nairn, A. C.; Palfrey, H. C. (1987) *J. Biol. Chem.* ***262***, 17299-303.
(30) Ohmstede, C. A.; Jensen, K. F.; Sahyoun, N. E. (1989) *J. Biol. Chem.* ***264***, 5866-75.
(31) Schulman, H. (1988) *Adv. Second Messenger Phosphoprotein Res.* ***22***, 39-112.
(32) Yamauchi, T.; Fujisawa, H. (1983) *Eur. J. Biochem.* ***132***, 15-21.
(33) Mochizuki, H.; Ito, T.; Hidaka, H. (1993) *J. Biol. Chem.* ***268***, 9143-47.
(34) Yoshida, S.; Kondo, T.; Ando, T. (1974) *Biochim. Biophys. Acta.* ***353***, 463-474.