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Modulation of Cyclic AMP Levels in a Clonal Neural Cell Line by Inhibitors of Tyrosine Phosphorylation

*Teresa M. Stringfield and Bruce H. Morimoto**
DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY, WEST LAFAYETTE, IN 47907-1393, U.S.A.

ABSTRACT. The convergence of tyrosine kinase and cyclic AMP (cAMP) signal transduction pathways was investigated in the HT4.7 neural cell line with inhibitors of tyrosine kinases and tyrosine phosphatases. The protein tyrosine kinase inhibitor genistein inhibited isoproterenol-stimulated cAMP production by 40–60% in whole cells, with no effect on basal cAMP levels. In both whole cells and membranes, genistein also inhibited cAMP produced in response to direct stimulation of adenylyl cyclase with forskolin. However, in the absence of phosphodiesterase inhibitors, genistein presentation resulted in an increase in cAMP levels. Genistein inhibited phosphodiesterase activity by 80–85%, indicating that tyrosine phosphorylation stimulates both cAMP synthesis and degradation. The decrease in cAMP levels by genistein was not merely competitive inhibition of adenylyl cyclase with respect to ATP, since the K_m of adenylyl cyclase for ATP remained essentially the same in either the presence or the absence of genistein. Another tyrosine kinase inhibitor, herbimycin A, which inhibits by a different mechanism than genistein, also decreased forskolin-stimulated cAMP in whole cells. As would be expected for the involvement of tyrosine phosphorylation in the control of cAMP production, inhibition of tyrosine phosphatases by vandate increased forskolin-stimulated cAMP production. These results suggest that cAMP production can be regulated by tyrosine phosphorylation, and the simultaneous activation of both cAMP synthesis and degradation may serve to alter the duration of cAMP elevation. BIOCHEM PHARMACOL 53;9:1271–1278, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. adenylyl cyclase; phosphodiesterase; tyrosine phosphorylation; regulation of cAMP production

Production of the second messenger cAMP† can be regulated by numerous intra- and extracellular signals including hormones, neurotransmitters, heterotrimeric G-proteins, intracellular calcium, and serine-threonine protein kinases [1, 2]. Recently, evidence is mounting for the regulation of cAMP levels by protein tyrosine kinases.

Receptor tyrosine kinases have been shown to modulate cAMP production. For example, insulin decreases the ability of the β-adrenergic receptor to respond to agonists, and leads to *in vivo* tyrosine phosphorylation of the adrenergic receptor [3]. In reconstitution assays, the insulin receptor itself phosphorylates the β-adrenergic receptor [4]. There is also evidence that the insulin receptor can phosphorylate some G-protein α subunits [5].

EGF can also regulate cAMP production in a number of cell types. In cardiac myocytes, EGF elicits cAMP production [6, 7], and compounds that selectively inhibit the EGF receptor kinase attenuate cAMP production in response to EGF, while having no effect on β-adrenergic receptor-stimulated cAMP production. Furthermore, antisera generated against a decapeptide of the carboxy-terminus of $G_{\alpha s}$ abolished EGF-stimulated cAMP production, implicating the stimulatory G-protein in EGF-induced cAMP production [8]. Additionally, in A431 cells, which overexpress the EGF receptor, EGF presentation confers a 5-fold potentiation of cAMP production in response to agents that normally elevate cAMP [9].

In addition to receptor tyrosine kinases, overexpression of the cytoplasmic tyrosine kinase $pp60^{c\text{-}src}$ leads to an elevated cAMP response to β-adrenergic receptor stimulation [10]. This effect was not observed following overexpression of a kinase-deficient $pp60^{c\text{-}src}$ [10], and mutation of the PKC phosphorylation site on $pp60^{c\text{-}src}$ as well as alterations in the SH2 domain also abolished the enhancement of cAMP production [11]. This mechanism for cAMP regulation appears to involve heterotrimeric G-proteins, since $pp60^{c\text{-}src}$ can phosphorylate both the long and short variants of $G_{\alpha s}$ as well as $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$, and $G_{\alpha t}$ [12, 13]. PKC may be upstream of $pp60^{c\text{-}src}$ in its ability to elevate cAMP levels, since pretreatment with the PKC-activating phorbol esters enhances phosphorylation of G_α subunits by $pp60^{c\text{-}src}$ [12]. This potential cascade of events in which PKC increases cAMP production via *src* could

* Corresponding author. Tel. (765) 494-0382; FAX (765) 494-0239; E-mail: morimoto@chem.purdue.edu

† *Abbreviations:* cAMP, cyclic AMP; EGF, epidermal growth factor; $G_{\alpha s}$, stimulatory guanine nucleotide binding protein; PKC, protein kinase C; ScAMP-TME, 2′-O-succinyl adenosine 3′,5′ cyclic monophosphate tyrosyl methyl ester; MES, 2-(*N*-morpholino)-ethanesulfonic acid; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; DTT, dithiothreitol; IBMX, isobutylmethylxanthine; RIA, radioimmunoassay; and IPT, isoproterenol.

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explain the conditional cAMP activation by phorbol esters that we observed in the HT4 neural cell line [14]. This prompted us to consider the potential role for tyrosine phosphorylation in conditional cAMP regulation.

MATERIALS AND METHODS

Materials

Na[^{125}I] (17 Ci/mg) was purchased from DuPont/New England Nuclear (Boston, MA). Iodination of ScAMP-TME with Na[^{125}I] and chloramine T was as previously described [15]. Genistein and daidzein were purchased from the ICN Chemical Co. (Costa Mesa, CA). Herbimycin A was obtained from Gibco/BRL (Gaithersburg, MD). Anti-cyclic AMP antibody was purchased from Calbiochem (La Jolla, CA). DMEM containing high glucose (4.5 g/L) was obtained from Irvine Scientific (Santa Ana, CA). Forskolin, IBMX, ScAMP, ScAMP-TME, insoluble protein A, and FBS were obtained from the Sigma Chemical Co. (St. Louis, MO). Isoproterenol and Ro 20-1724 were purchased from Research Biochemicals International (Natick, MA).

Cell Growth

HT4 cells were obtained from Ronald McKay [16], and subcloned by limiting dilution. A single clone, HT4.7, was selected for all subsequent studies.

HT4.7 cells were grown to confluence in DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin for 3–5 days at 33°C in a 5% CO_2/95% air atmosphere.

Generation of cAMP Antibodies

ScAMP was cross-linked to BSA essentially as described by Brooker *et al.* [15]. Briefly, 9.8 mg BSA and 5.2 mg ScAMP were dissolved in a total volume of 2.7 mL of 0.1 M MES, pH 5.5. The BSA/ScAMP mixture was incubated at room temperature for 16 hr in the presence of 5.7 mg of 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide, after which time the ScAMP-conjugated BSA was separated by gel filtration chromatography. Two female rabbits were immunized according to established protocols by the Purdue University Cancer Center. Antibody specificity was tested with AMP, ADP, ATP, cGMP, GMP, GDP, GTP, and cIMP. The anti-cAMP antibody was 10,000 times more specific for cAMP when compared with all other nucleotides except cIMP, in which the antibody had only a 100-fold greater affinity for cAMP.

cAMP RIA

Cyclic AMP was determined by competition binding with [^{125}I]ScAMP-TME [15]. Radioactive tracer solution containing approximately 200,000 cpm/mL of [^{125}I]ScAMP-TME was prepared in 50 mM sodium acetate, pH 4.75, containing 0.1% (w/v) NaN_3. The RIA was performed in a 96-well filtration plate (Multiscreen HV, Millipore, Bedford, MA), containing 50 μL of neutralized sample, 50 μL of tracer, and 50 μL of 1:4000 diluted anti-cAMP antibody. The assay was incubated for 16–20 hr at 4°, and terminated with 0.2 mL of 0.1% (w/v) insoluble protein A. The amount of bound radioactivity was separated by vacuum filtration, and determined by gamma counting. The assay is able to detect 1–100 pmol cAMP, and 3–200 fmol if the samples are acetylated.

cAMP Measurements in Whole Cells

Medium was removed and cells were washed with LK buffer (125 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM K_2HPO_4, 10 mM glucose, and 20 mM HEPES, pH 7.4). Various stimuli were added in LK plus 50 μM IBMX and 20 μM Ro 20-1724 unless otherwise indicated in the figure legend, and incubated at room temperature for 6 min. For herbimycin A pretreatment, the cells were grown to confluence, and then herbimycin A was added to the growth medium (DMEM + 10% FBS) for 18–25 hr. The cells were then treated as described above. The stimulus was removed and cells were lysed with 0.4 M $HClO_4$. Samples were neutralized with 1/6 vol. 2.4 M $KHCO_3$, and cAMP was determined by RIA. All experiments were performed a minimum of three times.

Cell Extract and Membrane Preparation

Growth medium was removed from confluent cells, and the cells were washed with LK buffer. Cells were removed into lysis buffer (20 mM HEPES, pH 8, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) and transferred to ice for 5 min. The cells were Dounce homogenized (30 strokes), and the cell lysate was centrifuged at 1600 *g* for 10 min at 4°C. The supernatant was then adjusted to a protein concentration of 1 to 1.5 mg/mL and used to measure phosphodiesterase activity. Alternatively, membranes were collected by an additional centrifugation of the supernatant at 424,000 *g* for 30 min at 4°C. The pellets were resuspended in lysis buffer at a concentration of 0.5 to 1 mg/mL.

Membrane cAMP Assay

Membranes were added to 20 mM HEPES, pH 8, containing the appropriate stimulus and a final concentration of 5 mM $MgCl_2$, 0.5 mM ATP, 100 μM IBMX, and 100 μM Ro 20-1724 unless otherwise indicated in the figure legend. Incubations were stopped by the addition of $HClO_4$ to give a final concentration of 0.4 M. Samples were neutralized with $KHCO_3$, and 200 μL was acetylated with triethylamine and acetic anhydride at a ratio of 2:1 for 10–20 min at room temperature. The acetylated samples were diluted 1:10 to 1:15 in 50 mM sodium acetate, pH 4.75, containing 0.1% (w/v) NaN_3, and cAMP was determined by RIA.

Phosphodiesterase Assay

Extracts of HT4.7 cells (0.6 mg/mL protein, final concentration) were incubated in a final concentration of 20 mM HEPES, pH 7.4, containing 90 mM KCl, 5 mM $MgCl_2$, 0.75 mM $CaCl_2$, and 100 nM cAMP in the presence or absence of 100 μM genistein or daidzein. The reactions were terminated at various times by acidifying 300 μL with $HClO_4$ to give a final concentration of 0.4 M $HClO_4$. The acid extract was deproteinized by centrifugation and neutralized with 1/6 vol. of $KHCO_3$. cAMP levels were measured by RIA on acetylated and diluted (1:50) samples.

Computer Simulation

The effect of tyrosine phosphorylation on the time-dependent change in cAMP concentration was modeled using Stella II (High Performance Systems, Inc., Hanover, NH). The concentration of cAMP at any given time was determined by numerical integration of Equation (1).

$$\frac{d[cAMP]}{dt} = rate\ of\ synthesis - rate\ of\ degradation \quad (1)$$

In the model, a stimulus that increased the rate of cAMP synthesis by 100 units was provided for 1 time unit every 10 time units. The rate of cAMP degradation was determined by the first-order Michaelis–Menten equation:

$$\frac{(V_{max} \cdot [cAMP]_t)}{(K_m + [cAMP]_t)}.$$

Tyrosine phosphorylation was introduced into the model so that is would increase the velocity of both adenylyl cyclase and phosphodiesterase.

RESULTS

Effect of Genistein on IPT-and Forskolin-Stimulated cAMP Production

To assess the involvement of tyrosine phosphorylation in the regulation of cAMP production, the tyrosine kinase inhibitor genistein was used. This isoflavone inhibits tyrosine kinases with IC_{50} values ranging from 20 to 30 μM, while possessing little activity towards serine/threonine kinases [17]. Increasing concentrations of genistein inhibited β-adrenergic receptor-mediated cAMP production (Fig. 1, open circles). The β-adrenergic receptor agonist IPT at 300 nM increased cAMP levels from 3.0 ± 0.1 to 79 ± 7 pmol/mg/min, and 10 μM forskolin also resulted in an increase in cAMP levels to 197 ± 6 pmol/mg/min. For either IPT or forskolin, the presence of genistein inhibited cAMP levels in a concentration-dependent manner, with an IC_{50} of approximately 20 μM (Fig. 1). Genistein had no appreciable effect on basal cAMP levels (data not shown); however, the basal amount of cAMP produced is at the lower end of the RIA detection limit and a decrease would be difficult to observe.

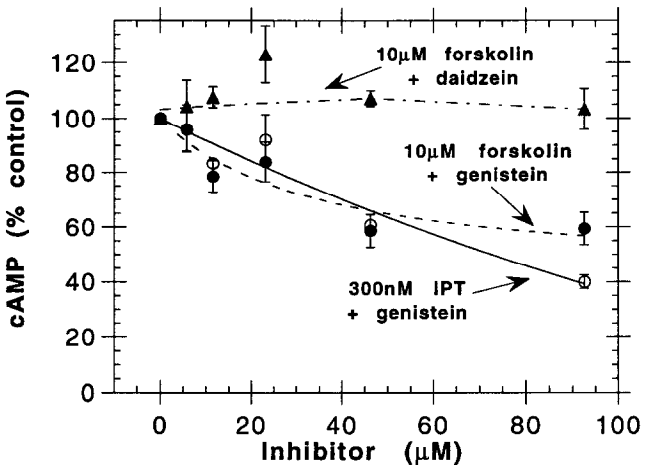

FIG. 1. Genistein-mediated inhibition of cAMP production. HT4.7 cells were incubated for 6 min with various concentrations of genistein (circles) or daidzein (triangles) in the presence of 50 μM IBMX and 20 μM Ro 20-1724, and 300 nM IPT (open symbols) or 10 μM forskolin (closed symbols). The data presented are the means ± SEM for N = 4. Basal cAMP (3.2 ± 0.1 pmol/mg/min for the IPT experiment and 61.3 ± 15.6 pmol/mg/min for the forskolin experiment) was subtracted from each value, and 100% of control is defined as the cAMP produced by 300 nM IPT (79.2 ± 10.2 pmol/mg/min) or 10 μM forskolin (196.9 ± 6.1 pmol/mg/min) in the absence of tyrosine kinase inhibitors.

As a control for specificity of tyrosine kinase inhibition, daidzein, an inactive analog of genistein, was tested (Fig. 1, triangles). Increasing concentrations of daidzein had no effect on cAMP production. Since it has been shown that genistein can lead to enhanced cAMP production in the absence of phosphodiesterase inhibitors [18, 19], it is important to note that all incubations were performed in the presence of the phosphodiesterase inhibitors IBMX and Ro 20-1724, suggesting that in HT4.7 cells the observed effect of genistein was mediated by an alteration in cAMP production rather than cAMP degradation.

Effect of Phosphodiesterase Inhibitors and Genistein on cAMP Levels

To assess the role for tyrosine phosphorylation in cAMP degradation, the effect of genistein on forskolin-stimulated cAMP levels was determined in the absence of the phosphodiesterase inhibitors (Fig. 2A). Increasing concentrations of genistein increased cAMP levels 160% in the absence of phosphodiesterase inhibitors, whereas a decrease to 60% was observed in the presence of IBMX and Ro 20-1724 (Fig. 1). The 160% increase in cAMP levels was the net result of a 60% decrease in cAMP production and the effect of tyrosine phosphorylation can be inferred to increase phosphodiesterase activity by 220%.

To directly assess the effect of tyrosine phosphorylation on basal phosphodiesterase activity, HT4.7 cell extracts

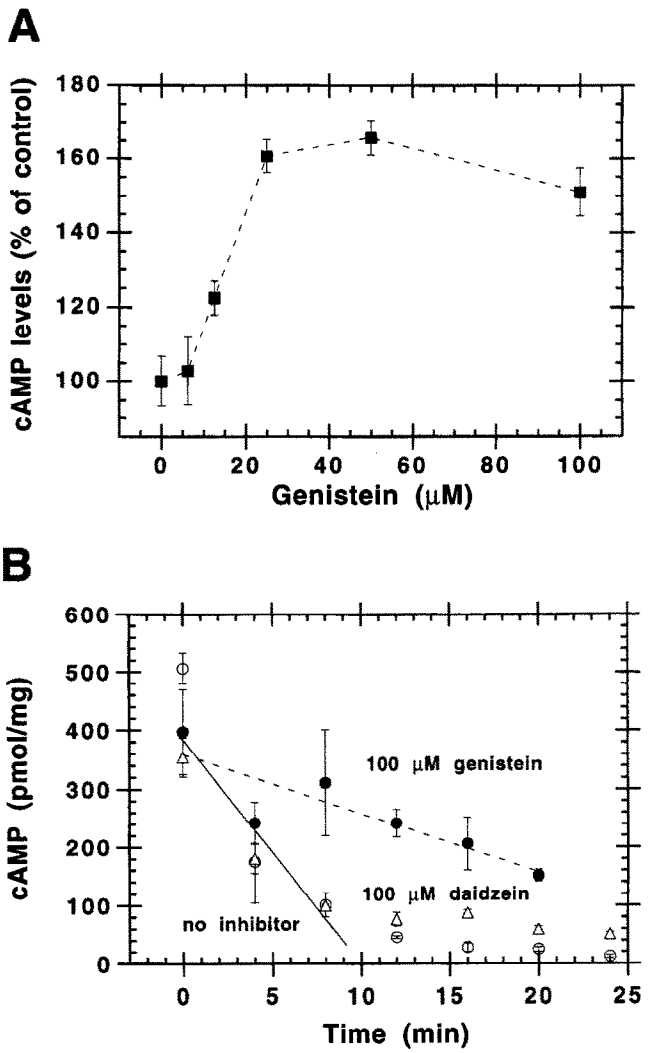

FIG. 2. Effect of genistein on cAMP levels and phosphodiesterase activity. (A) HT4.7 cells were treated as in Fig. 1 with 10 μM forskolin in the absence of 50 μM IBMX and 20 μM Ro 20-1724. The data shown are the means ± SEM for N = 4. Basal cAMP (23.8 ± 1.7 pmol/mg/min) was subtracted from each value, and 100% of control is defined as the amount of cAMP produced in the presence of 10 μM forskolin only (56.5 ± 6.6 pmol/mg/min). (B) HT4.7 cell extract (0.6 mg/mL protein) was incubated with 100 nM cAMP in the presence (open circles) or absence (closed circles) of 100 μM genistein or 100 μM daidzein (open triangles). Phosphodiesterase activity was determined by the slope of the initial rate of cAMP disappearance.

were incubated with 100 nM cAMP in the presence or absence of 100 μM genistein (Fig. 2B). The amount of cAMP remaining was determined by RIA as a function of time. In the absence of genistein or in the presence of the inactive analog daidzein, phosphodiesterase activity was 50 pmol/min/mg. The inclusion of 100 μM genistein resulted in an 80% decrease in phosphodiesterase activity to 10 pmol/min/mg. This not only confirms the reported ability of tyrosine phosphorylation to control phosphodiesterase activity [20], but also suggests that tyrosine phosphorylation in HT4.7 cells can modulate both cAMP production and degradation.

Effect of Genistein on the Kinetics of cAMP Production

Genistein is a non-selective, ATP-competitive tyrosine kinase inhibitor [17]. Therefore, these results must be interpreted carefully since ATP is also a substrate for adenylyl cyclase. Genistein may decrease cAMP production by acting as a competitive inhibitor of adenylyl cyclase. To address this possibility, the kinetics of cAMP production were determined. Membranes from HT4.7 cells were incubated with 10 μM forskolin and various concentrations of ATP for 15 min. cAMP production under these conditions was linear at all time points tested. No appreciable change in the K_m for ATP in the presence (K_m = 65 ± 16 μM) or absence (K_m = 47 ± 9 μM) of 100 μM genistein was observed (Fig. 3), whereas the V_{max} decreased from 6.9 ± 0.3 to 4.7 ± 0.4 pmol/mg/min in the presence of genistein.

Effect of Herbimycin A on Forskolin-Stimulated cAMP Production

As an additional control to ensure that the inhibition of cAMP production by genistein was mediated by tyrosine kinase inhibition, another tyrosine kinase inhibitor, herbimycin A, was employed. The mechanism for herbimycin A inhibition is different from that of genistein. Herbimycin A decreases the levels of tyrosine kinases as well as inhibits their activity [21–25]. HT4.7 cells were pretreated for 25 hr with various concentrations of herbimycin A. Cells were then presented with 10 μM forskolin for 6 min and cAMP was determined by RIA (Fig. 4). Concentrations of herbimycin A greater than 0.5 μM resulted in a 60% decrease in cAMP production. The IC_{50} of the herbimycin A-mediated inhibition of cAMP production was approximately 200 nM. Consistent with herbimycin A's mechanism of decreasing

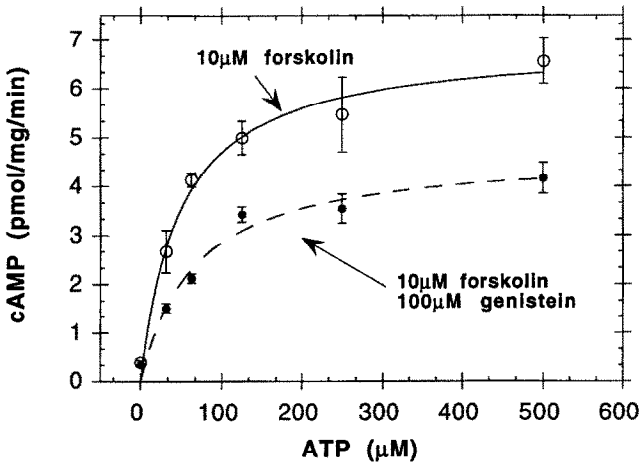

FIG. 3. Effect of genistein on the kinetics of cAMP production. Membranes were prepared from HT4.7 cells, as described in Materials and Methods, and incubated for 15 min with 10 μM forskolin in the presence of various concentrations of ATP in the presence (closed symbols) or absence (open symbols) of 100 μM genistein. The K_m and V_{max} were derived from a non-linear regression of the data.

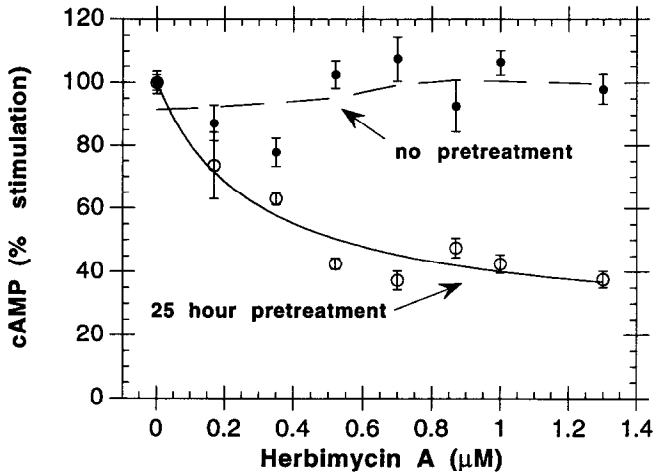

FIG. 4. Herbimycin A-mediated inhibition of cAMP production. Various concentrations of herbimycin A were added to the medium of confluent HT4.7 cells and preincubated for either 25 hr (open circles) or 0 hr (closed circles). The medium was removed and the cells were washed with LK prior to the addition of 10 μM forskolin. Basal cAMP (27.2 ± 0.3 pmol/mg/min for the 25-hr pretreatment and 13.4 ± 0.7 pmol/mg/min for the 0-hr pretreatment) was subtracted from each value, and 100% of control is defined as the amount of cAMP produced in the presence of 10 μM forskolin only (236.2 ± 5.1 pmol/mg/min for the 25-hr pretreatment and 185.8 ± 6.0 pmol/mg/min for the 0-hr pretreatment).

tyrosine kinase protein levels, no effect on cAMP production was observed in the absence of pretreatment (Fig. 4).

Effect of Genistein and Orthovanadate on Forskolin-Stimulated cAMP Production

The role of tyrosine phosphorylation in the control of cAMP production was further tested by inhibiting tyrosine phosphatases. Since tyrosine kinase inhibitors decreased cAMP production, one would expect tyrosine phosphatase inhibitors to enhance cAMP production. HT4.7 membranes were treated with the non-selective tyrosine phosphatase inhibitor sodium orthovanadate (NaOVa). This resulted in an increase in forskolin-stimulated cAMP production (Fig. 5), with an EC_{50} of approximately 5 μM. The fact that the inhibition of tyrosine kinases and the inhibition of tyrosine phosphatases led to opposite effects on cAMP production lends support to the hypothesis that genistein does not directly inhibit adenylyl cyclase.

Computer Simulation of the Effect of Tyrosine Phosphorylation on cAMP Levels

The data presented in Figs. 1 and 2 suggest that tyrosine phosphorylation can increase both cAMP synthesis and degradation. In the cell, tyrosine phosphorylation increased cAMP production by approximately 60% and cAMP degradation by 220%. This is assuming that the effect of tyrosine phosphorylation on cAMP degradation (Fig. 2A) would be the sum of the effect of genistein on cAMP levels in the absence of phosphodiesterase inhibitors (160%) plus the contribution of tyrosine phosphorylation on cAMP production (60%). In an attempt to explain the significance of increasing both the rate of synthesis and the rate of degradation, a computer simulation monitoring the temporal effect of tyrosine phosphorylation on the concentration of cAMP was performed.

In the computer model, the concentration of cAMP was described as a function of the rate of synthesis and degradation. Synthesis of cAMP was assumed to be affected by a change in the velocity of adenylyl cyclase. The rate of cAMP degradation was described by a first order Michaelis–Menten process dependent on the concentration of cAMP.

A 100-unit stimulus was given for 1 time unit every 10 time units. As expected, cAMP levels were elevated transiently, with an initial rapid increase due to activation of adenylyl cyclase (cAMP synthesis), and then decay back to basal levels (Fig. 6A, from 0 to 20 time units). To observe the effect of tyrosine phosphorylation on cAMP concentration, an increase in the velocity of adenylyl cyclase (60%) and phosphodiesterase (220%) was included from 20 to 40 time units. Tyrosine phosphorylation resulted in an increase in the basal cAMP levels. Moreover, the duration of cAMP elevation was shortened significantly. Non-linear analysis of the rate of cAMP decay indicated that the $T_{1/2}$ decreased 3-fold, from 0.77 time units in the absence of tyrosine phosphorylation to 0.26 time units in the presence of tyrosine phosphorylation (Fig. 6B). This suggests that the latency of cAMP elevation could be shortened significantly when a tyrosine phosphorylation signal is provided simultaneously with a cAMP generating signal.

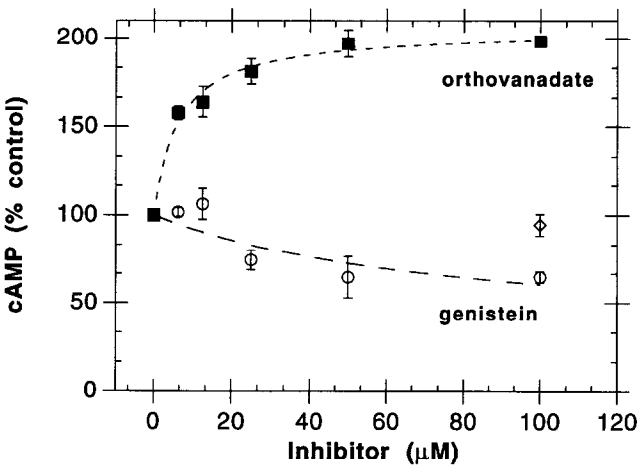

FIG. 5. Effect of genistein and sodium orthovanadate on cAMP production. Membranes were prepared from HT4.7 cells, as described in Materials and Methods, and incubated with 10 μM forskolin in the presence of various concentrations of genistein (open circles), sodium orthovanadate (closed squares), or 100 μM daidzein (open diamond) for 15 min prior to cAMP determination. Basal cAMP (0.45 ± 0.02 pmol/mg/min) was subtracted from each value, and 100% of control is defined as the amount of cAMP produced in the presence of 10 μM forskolin only (3.1 ± 0.08 pmol/mg/min).

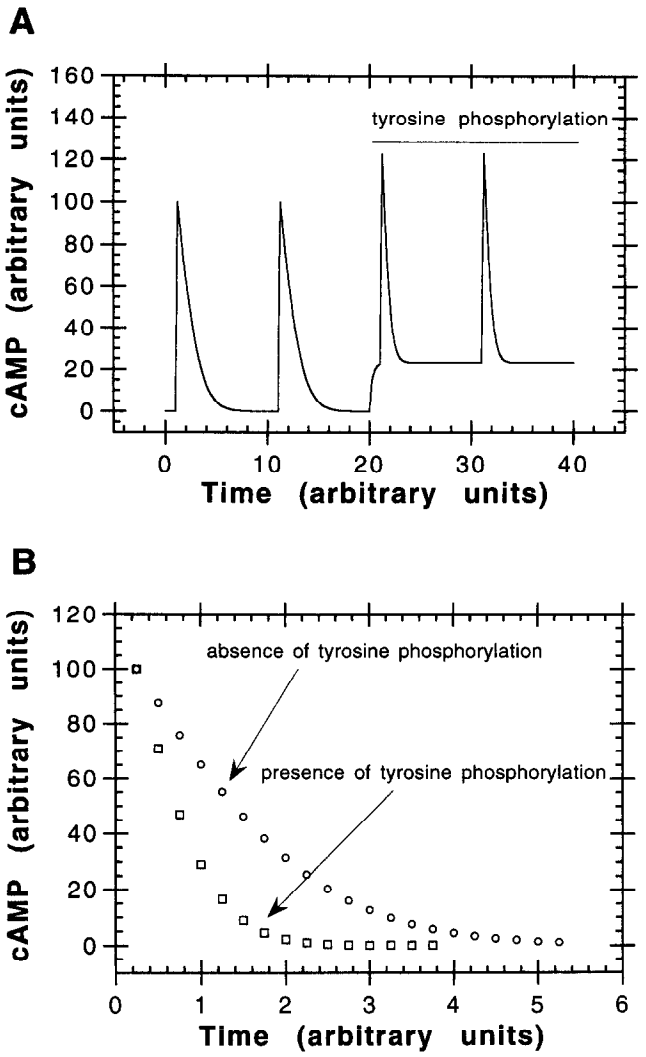

FIG. 6. Computer simulation of the effect of tyrosine phosphorylation on cAMP levels. (A) Theoretical change in cAMP levels as a function of time. A 100-unit stimulus was presented every 10 time units. At t = 20 time units, tyrosine phosphorylation was included in the stimulation. (B) Effect of tyrosine phosphorylation on the decay of cAMP. Basal cAMP levels were subtracted from the data presented in (A), and the half-life of cAMP in the presence or absence or tyrosine phosphorylation was determined by non-linear regression.

DISCUSSION

The results of this study indicate that cAMP levels in the HT4.7 cell line can be regulated by tyrosine phosphorylation. Tyrosine kinase inhibitors decreased cAMP production in response to forskolin and IPT (Fig. 1). The IC_{50} for cAMP inhibition was similar to that reported for tyrosine kinase inhibition [17, 21]. In the absence of phosphodiesterase inhibitors, genistein increased cAMP levels (Fig. 2). This is in agreement with the work of Ueki *et al.* [20] which demonstrates the control of phosphodiesterase activity by tyrosine phosphorylation.

The convergence of tyrosine phosphorylation and cAMP levels is of interest, and may vary with cell type depending on the expression of various components of the signaling pathways involved. For example, EGF stimulates cAMP production in cardiac myocytes and this stimulation has been shown to involve $G_{\alpha s}$ [7]. Recently, purified EGF receptor was found to tyrosine phosphorylate purified $G_{\alpha s}$ [26]. However, in non-myocytes, which also express $G_{\alpha s}$ and the EGF receptor, cAMP production is not responsive to EGF presentation [8]. This difference has been attributed to the adenylyl cyclase type V isoform, which is absent in the non-myocytes and is present in the cardiac myocytes. In fact, when adenylyl cyclase types I, II, V, and VI are transfected individually into HEK 293 cells, only those cells expressing the type V isoform exhibit EGF-mediated cAMP production [27]. This evidence suggests a mode of differential regulation of various adenylyl cyclase isoforms by tyrosine kinases.

The identity of the protein tyrosine kinase regulating cAMP levels in HT4.7 cells remains to be determined. It is interesting to note that in our experiments, tyrosine kinases were not specifically activated. Tyrosine kinase inhibitors have been shown to affect cAMP levels in the absence of a tyrosine kinase activating stimulus. For example, in rat pinealocytes, tyrosine kinase inhibitors alone can potentiate cAMP production induced by various cAMP elevating agents [18].

For HT4.7 cells, one interpretation could be that a constitutively active tyrosine kinase is involved in the regulation of cAMP levels. If so, perhaps control of cAMP levels by tyrosine phosphorylation is exerted by the regulation of a tyrosine phosphatase. It is also possible that since HT4.7 is a transformed cell line containing a temperature-sensitive SV40 large T antigen [16], the process of transformation may, in itself, activate endogenous tyrosine kinases that are involved in the regulation of cAMP levels.

An alternative explanation may involve a serum-activated kinase, since HT4.7 cells are grown in the presence of serum. Serum could stimulate a growth factor receptor tyrosine kinase, which could directly phosphorylate some component of the cAMP signal transduction pathway. Stimulation of a growth factor receptor could also activate a variety of signalling cascades that would ultimately lead to alterations in cAMP levels. For example, inositol phospholipid phospholipase C-γ can be activated by a variety of growth factor receptors resulting in the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) and the activation of some isoforms of PKC [28, 29]. Growth factor receptors have also been shown to increase the activity of phosphatidylinositol 3-kinase activity, which may be involved in the activation of certain other isoforms of PKC [30, 31]. This mechanism would be consistent with our previous observations that PKC activation results in conditional activation of cAMP production [14].

The ability of tyrosine phosphorylation to modulate cAMP levels represents convergence or cross talk between these signal transduction pathways. Moreover, the ability of

tyrosine phosphorylation to regulate both cAMP production and degradation in the same cell may be a mechanism by which the magnitude and duration of cAMP elevation can be controlled. The data presented for the HT4.7 cell line indicate that tyrosine phosphorylation enhances both cAMP production and cAMP degradation. This establishes a potential metabolic regulatory cascade in which tyrosine phosphorylation regulates to different extents both synthesis and degradation of the second messenger. This could alter significantly the duration of the cAMP response to a given stimulus as seen in the computer simulation (Fig. 6).

Tyrosine phosphorylation may be a general mechanism for the control of cAMP levels in response to a given stimulus. Varying the extent of tyrosine phosphorylation by modulating kinase and phosphatase activities could precisely regulate both the magnitude and duration of cAMP elevation. To illustrate this point, the effect of tyrosine phosphorylation on cAMP synthesis and degradation was independently varied from 0 to 100% in our computer model. This resulted in a 2-fold change in the magnitude of cAMP levels, and the duration varied with a $T_{1/2}$ from 0.37 to 3.0 (data not shown).

From the work presented in this study, along with that of others, it appears that tyrosine phosphorylation can regulate cAMP levels. Although the physiological consequence of tyrosine phosphorylation in the control of cAMP levels remains to be determined, the computer stimulations based on our experimental data indicate that tyrosine phosphorylation can alter the temporal characteristics of cAMP elevation. Tyrosine phosphorylation may therefore allow the cell to control the dynamics of a cAMP response to a particular stimuli, namely the length of time cAMP remains elevated in the cell. If cAMP is produced at a particular location in the cell, then the concentration of cAMP at a distal point would be dependent on the diffusion of cAMP. Diffusion, in turn, depends not only on the concentration of cAMP produced but also on the duration in which cAMP remains elevated. This may serve to discriminate as to which downstream effectors of cAMP are activated or not.

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