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Protein Kinase C Potentiation of the Tyrosine Kinase Inhibitor-Stimulated Cyclic GMP Production in Rat Pinealocytes

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ABSTRACT. Inhibition of tyrosine kinase activities elevates cyclic GMP (cGMP) levels in rat pinealocytes. Since protein kinase C (PKC) and intracellular Ca^{2+} both interact with the agonist-stimulated cGMP accumulation, in this study their interactions with the tyrosine kinase inhibitor-mediated cGMP response were investigated. Two tyrosine kinase inhibitors, genistein and tyrphostin B42, increased basal cGMP accumulation concentration dose-dependently. This increase in cGMP accumulation was potentiated by 4β-phorbol 12-myristate 13-acetate (PMA), an activator of PKC, and blocked by calphostin C, a specific PKC inhibitor. The tyrosine kinase inhibitors had no effect on the *in vitro* or PMA-mediated translocation of PKC activity. However, when the phosphodiesterase was inhibited by isobutylmethylxanthine (IBMX), neither the tyrosine kinase inhibitors alone nor in combination with PMA had an effect on cGMP accumulation, suggesting that phosphodiesterase is a probable site of action of the inhibitors. In comparison, elevation of intracellular Ca^{2+} by BayK 8644, ionomycin, or KCl inhibited the genistein- or tyrphostin B42-mediated increase in cGMP accumulation. This inhibition persisted in the presence of IBMX and was partly reversed by a Ca^{2+}/calmodulin inhibitor. These results suggest that PKC modulates the rate of cGMP degradation through signalling pathways involving tyrosine phosphorylation. However, the inhibitory effect of the Ca^{2+}-elevating agents on the tyrosine kinase inhibitor-stimulated cGMP accumulation appears to be independent of phosphodiesterase inhibition. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:95–102, 1997.

KEY WORDS. genistein; phosphodiesterase; pineal; protein kinase C; tyrosine kinase; tyrphostin B42

The regulation of cAMP§ and cGMP production in rat pinealocytes involves interaction of several G-protein-coupled signal transduction pathways [1–3]. Stimulation of the β-adrenoceptors or activation of G_s-protein by cholera toxin produces a 5-fold increase in cAMP and cGMP accumulation. Selective activation of α_1-adrenoceptors, while having no effect on its own, potentiates these stimulated cAMP and cGMP responses, resulting in 100-fold increases [1–3]. At least two intracellular mechanisms are involved in this potentiation: the α_1-adrenergic-mediated activation of PKC and elevation of $[Ca^{2+}]_i$ [1, 3]. However, in the case of the cGMP response, several other signal transduction pathways have also been shown to play a significant modulating role. For example, it has been shown that the norepinephrine-stimulated cGMP response is more sensitive than the cAMP response to inhibition of phospholipase A_2 [4] and Ca^{2+}/calmodulin-dependent kinase [5], as well as changes in intracellular pH [6, 7].

Another known cross-talk mechanism is the interaction between the growth factor-mediated tyrosine kinase pathway and G-protein-coupled signal transduction mechanisms [8, 9]. For example, it has been shown that tyrosine kinase can interact with the receptor-coupled phospholipase C signal transduction mechanism [10] and the cAMP signalling pathways [11]. Recently, we have shown that treatment with tyrosine kinase inhibitors causes an increase in cGMP accumulation [12], suggesting that tyrosine kinase may be directly involved in the regulation of cGMP accumulation in rat pinealocytes. Unlike the G-protein-coupled signal transduction pathways that modulate the pineal cGMP response by changing its rate of synthesis, tyrosine kinase appears to regulate the cGMP level by altering the phosphodiesterase activity [12].

In view of the possible cross-talk between tyrosine kinase and G-protein-coupled signalling pathways, in this study we investigated the roles of PKC and $[Ca^{2+}]_i$ on tyrosine kinase inhibitor-mediated cGMP accumulation. The two tyrosine kinase inhibitors chosen for the present study were genistein [13, 14] and tyrphostin B42 [15]. Genistein, an

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§ *Abbreviations:* cAMP, cyclic AMP; cGMP, cyclic GMP; 4α-PDD, 4α-phorbol 12,13-didecanoate; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, intracellular Ca^{2+}; IBMX, isobutylmethylxanthine; ISO, isoproterenol; and DMEM, Dulbecco's modified Eagle's medium.

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established tyrosine kinase inhibitor, was shown to stimulate pineal cGMP accumulation in our previous study [12], while tyrphostin B42 was the most potent inhibitor that stimulated pineal cGMP accumulation (unpublished observation).

MATERIALS AND METHODS

Materials

IBMX, ISO, calphopstin C, phorbol esters, culture medium, and fetal bovine serum were obtained from the Sigma Chemical Co. (St. Louis, MO). BayK 8644, calphostin C, EGTA-AM, genistein, daidzein, and tyrphostin analogues were obtained from Calbiochem (La Jolla, CA). The PKC substrate peptide Ac-MBP (4–14) was obtained from Gibco/BRL (Gaithersburg, MD). [^{125}I]cGMP, [^{125}I]cAMP, and [γ-^{32}P]ATP were obtained from ICN Immunobiologicals (Lisle, IL). All other chemicals were of the purest grades available and were obtained commercially. Antibodies for the radioimmunoassays of cGMP and cAMP were gifts from Dr. A. Baukal (National Institute of Child Health and Human Development, NIH, Bethesda, MD).

Preparation and Treatment of Rat Pinealocytes

Pinealocytes were prepared from male Sprague–Dawley rats (150 g, University of Alberta Animal Unit) by trypsinization as previously described [16, 17]. The cells were suspended in DMEM containing 10% fetal bovine serum and maintained (37°) for 24 hr in a gas mixture of 95% air and 5% CO_2 before experimental treatment.

Aliquots of cells (2×10^4 cells/0.4 mL) were treated with drugs that had been prepared in concentrated solutions in water or dimethyl sulfoxide. The final concentration of the latter never exceeded 0.5%. At this concentration, dimethyl sulfoxide had no effect on basal or agonist-stimulated cGMP or cAMP responses. The duration of the drug treatment period was 30 min. At the end of the treatment period, cells were collected by centrifugation (2 min, 10,000 *g*), the supernatant was aspirated, and the tube was placed on solid CO_2. The frozen cell pellets were lysed by the addition of 5 mM acetic acid (100 μL) and boiling (5 min). The lysates were stored frozen at −20° until analysis.

For the determination of the PKC translocation, aliquots of cells (2×10^5 cells/0.5 mL) were treated with drugs for 6 min, and the cells were collected by centrifugation. To separate the membrane and cytosolic fractions, the pinealocytes were permeabilized by resuspension and incubation (7 min, 4°) in 100 μL of 50 μM digitonin in buffer A (20 mM Tris–HCl, containing 0.5 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulphonyl fluoride, 25 μg/mL of leupeptin and aprotinin, pH 7.5) as described [18]. The samples were then centrifuged (1 min, 12,000 g) to complete the separation. The membrane fraction was solubilized in 0.1% Triton-X (in buffer A) before assay. The presence of EGTA and EDTA ensured that only the chelator-stable form of membrane-associated PKC was analyzed.

Cyclic Nucleotide Assays

The lysates were centrifuged (12,000 *g*, 10 min), and samples of the supernatant were used to determine cellular cGMP and cAMP contents, using a radioimmunoassay procedure in which samples were acetylated prior to analysis [5, 19]. Because there was a small batch-to-batch variation of the cyclic nucleotide responses between cell preparations, all comparisons were performed within the same batch of cells.

PKC Assay

PKC activity was measured in duplicate [18]. The reaction mixture contained 20 mM Tris-HCl, 1.0 mM $CaCl_2$, 20 mM $MgCl_2$, 50 μM Ac-MBP (4–14) as substrate [20], 0.5 mg/mL leupeptin, 0.1 mM ATP ($1–5 \times 10^6$ cpm of [γ-^{32}P]ATP). Phosphatidylserine (280 μg/mL) and PMA (10 μM) were added to some tubes to demonstrate phospholipid-dependent protein kinase activity. The reaction was initiated by adding 1–2 μg of protein, and the incubation (6 min, 37°) was stopped by immediate spotting of the reaction mixture onto phosphocellulose discs. The discs were then washed twice with 1% phosphoric acid and three times with distilled water. The radioactivity retained by the filter disc was determined by scintillation counting. PKC activity was calculated from the difference in ^{32}P incorporated into the PKC substrate peptide in the presence and absence of added phospholipids.

Statistical Analysis

Data are presented as the mean ± SEM of the amount of cyclic nucleotide or PKC activity in four aliquots of cells. Each experiment was repeated at least three times with different cell preparations. Statistical comparisons were analyzed by the unpaired *t*-test and analysis of variance with the Newman–Keuls test where appropriate, with statistical significance set at $P < 0.05$.

RESULTS

Effect of Tyrosine Kinase Inhibitors on Basal cAMP and cGMP Levels in Rat Pinealocytes

Treatment with genistein (10–300 μM) or tyrphostin B42 (10–300 μM) alone increased cGMP accumulation concentration dependently in rat pinealocytes (Fig. 1A) without causing a concomitant increase in cAMP accumulation (Fig. 1B). At 300 μM, genistein and tyrphostin B42 increased cGMP accumulation 5- and 20-fold, respectively (Fig. 1A). In comparison, similar concentrations of daidzein and tyrphostin A1, the corresponding inactive analogues of the tyrosine kinase inhibitors, had no effect on cGMP accumulation. Higher concentrations (1 mM) of both tyrosine kinase inhibitors and the inactive analogues resulted in significant cell death (as determined by trypan blue exclusion staining) after 15 min and were not used in subsequent studies (data not shown).

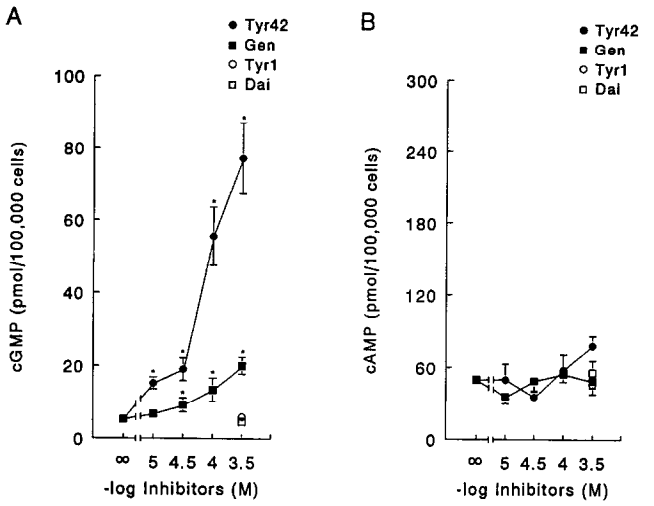

FIG. 1. Effects of tyrosine kinase inhibitors on cGMP (A) and cAMP (B) accumulation in rat pinealocytes. Pinealocytes (2×10^4 cells/0.4 mL) were incubated in DMEM and stimulated with various concentrations of genistein (Gen) and tyrphostin B42 (Tyr42) for 30 min. Daidzein (Dai, 300 μM) and tyrphostin A1 (Tyr1, 300 μM) were included as negative controls. Each value is the mean ± SEM of determinations in four samples of cells. For further details, see Materials and Methods. Key: (*) significantly different from control ($P < 0.05$).

Effect of PKC Activator and Inhibitor on the Tyrosine Kinase Inhibitor-Stimulated cGMP Accumulation

To study the interaction between PKC and tyrosine kinase on the cGMP accumulation, we investigated the effect of PMA, a potent PKC activator, on the tyrphostin B42- and genistein-stimulated cGMP responses. PMA, while having no effect on its own, potentiated the tyrphostin B42- and genistein-stimulated cGMP responses in a concentration dependent manner (Fig. 2). In comparison, 4α-PDD, an inactive analogue of PMA, did not potentiate the effect of the tyrosine kinase inhibitors (Table 1). To further establish the role of PKC, calphostin C, a specific PKC inhibitor [21], was used. Calphostin C, which had no effect on its own, abolished the enhancing effect of PMA on the tyrphostin B42- and genistein-stimulated cGMP responses (Table 1). Interestingly, calphostin C, which had no effect on the ISO (1 μM)-stimulated cGMP response, suppressed the tyrosine kinase inhibitor-stimulated cGMP response in the absence of PMA. These results indicate that PKC selectively potentiates the tyrosine kinase inhibitor- but not the β-adrenoceptor-stimulated cGMP response, and PKC may be directly involved in the action of tyrosine kinase inhibitors on the cGMP response.

Previously, we reported that the effect of tyrosine kinase inhibitors on cGMP accumulation was due to inhibition of cGMP metabolism [12]. In the following study, we investigated whether the potentiating effect of PMA was also mediated through phosphodiesterase. In the presence of maximal phosphodiesterase inhibition with 1 mM IBMX, neither tyrphostin B42 nor genistein had an effect on cGMP accumulation (Table 2). Under this condition, PMA also had no effect on the tyrosine kinase inhibitor-stimulated cGMP responses (Table 2).

Effect of Changes in $[Ca^{2+}]_i$ on the Tyrosine Kinase Inhibitor-Stimulated cGMP Accumulation

To determine the role of $[Ca^{2+}]_i$ in the effect of tyrosine kinase inhibitors on cGMP accumulation, $[Ca^{2+}]_i$ was elevated by BayK 8644, a depolarizing concentration of K^+, or ionomycin. Addition of BayK 8644 (10 μM) alone had no effect on the basal cGMP level. In contrast to the effect of PMA, BayK 8644 inhibited the tyrphostin B42- and genistein-stimulated cGMP responses concentration dependently (Fig. 3). Similar inhibitory effects were also observed when $[Ca^{2+}]_i$ was elevated by a depolarizing concentration of K^+ (30 mM) or ionomycin (10 μM) (Table 3). In comparison, reducing $[Ca^{2+}]_i$ with the cell-permeable Ca^{2+}

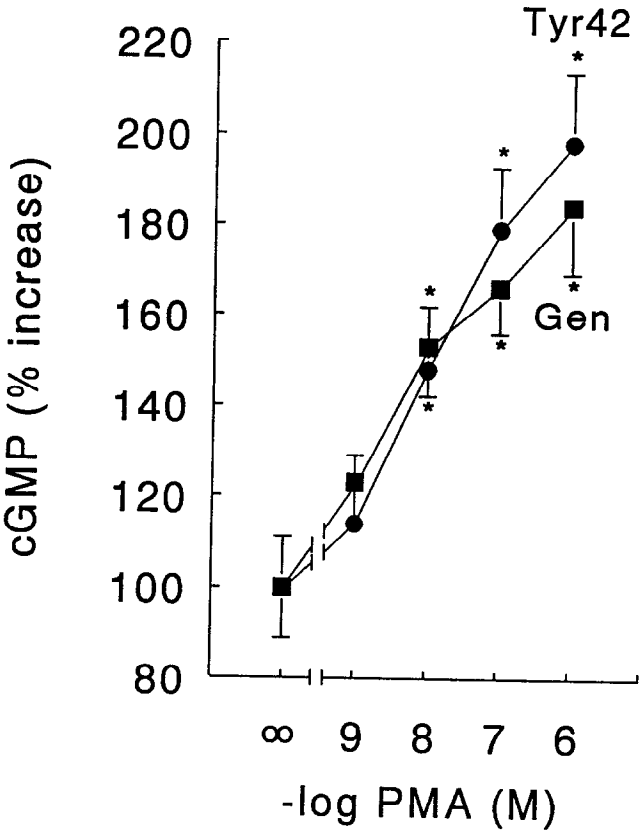

FIG. 2. Effect of PMA on tyrosine kinase inhibitor-stimulated cGMP accumulation in rat pinealocytes. Pinealocytes (2×10^4 cells/0.4 mL) were incubated in DMEM and stimulated with tyrphostin B42 (Tyr42, 300 μM) or genistein (Gen, 300 μM) for 30 min in the presence or absence of various concentrations of PMA. The cGMP values for cells stimulated with Tyr42 or Gen and for the untreated control were 82.0 ± 7.6, 16.3 ± 3.0, and 4.8 ± 1.5 pmol/10^5 cells, respectively. Each value is the mean ± SEM of determinations in four samples of cells. For further details, see Materials and Methods. Key: (*) significantly different from the corresponding treatment without PMA ($P < 0.05$).

TABLE 1. Effect of PKC activator and inhibitor on cGMP accumulation stimulated by tryosine kinase inhibitors in rat pinealocytes

Treatment	cGMP (pmol/10^5 cells) - Calphostin C	+ Calphostin C (1 μM)
Control	5.8 ± 0.7	6.8 ± 0.9
PMA (100 nM)	6.9 ± 1.1	ND*
4α-PDD (100 nM)	7.2 ± 1.1	ND
ISO (1 μM)	22.4 ± 1.2†	19.9 ± 1.1†
Tryphostin B42 (300 μM)	78.7 ± 8.6†	17.1 ± 2.7‡
+ PMA (100 nM)	145.5 ± 33.8†§	29.3 ± 7.6‡§
+ 4α-PDD (100 nM)	87.6 ± 17.0†	ND
Genistein (300 μM)	17.9 ± 0.3†	6.9 ± 1.1‡
+ PMA (100 nM)	35.7 ± 4.9§	13.3 ± 2.2‡§
+ 4α-PDD (100 nM)	19.3 ± 0.1†	ND

Pinealocytes (2×10^4 cells/0.4 mL) were incubated DMEM and treated with tyrosine kinase inhibitors for 30 min in the presence or absence of PMA, 4α-PDD, or calphostin C. The effect of calphostin C on the ISO-stimulated cGMP response was included for comparison. Each value is the mean ± SEM of cGMP in four samples of cells. For further details, See Materials and Methods.

* ND, not determined.

† Significantly different from control ($P < 0.05$).

‡ Significantly different from the corresponding treatment without calphostin C ($P < 0.05$).

§ Significantly different from the corresponding treatment with tyrosine kinase inhibitor alone ($P < 0.05$).

chelator, EGTA-AM, significantly enhanced the tyrphostin B42- and genistein-stimulated cGMP responses (Table 3).

To determine the site of interaction between $[Ca^{2+}]_i$ elevating agents and the tyrosine kinase inhibitors, IBMX was used. Unlike PMA, which had no potentiating effect on the cGMP response in the presence of IBMX, the inhibitory effect of BayK 8644 or ionomycin on tyrphostin B42- and genistein-stimulated cGMP accumulation persisted in the presence of phosphodiesterase inhibition

TABLE 2. Effect of PMA on the cGMP accumulation in rat pinealocytes stimulated by tryosine kinase inhibitors in the presence of maximal phosphodiesterase inhibition

Treatment	cGMP (pmol/10^5 cells) - IBMX	+ IBMX
Control	5.2 ± 1.4	124.5 ± 5.6
PMA (100 nM)	8.6 ± 2.0	119.0 ± 6.8
Tyrphostin B42 (300 μM)	76.3 ± 5.8*	125.0 ± 5.9
+ PMA (100 nM)	113.6 ± 5.9†	123.2 ± 9.4
Genistein (300 μM)	24.4 ± 3.5*	106.5 ± 11.9
+ PMA (100 nM)	40.3 ± 3.1†	109.7 ± 8.6

Pinealocytes 2×10^4 cells/0.4 mL) were incubated in DMEM and treated with tyrosine kinase inhibitors and PMA for 30 min in the presence or absence of IBMX (1 mM). Each value is the mean ± SEM of cGMP in four samples of cells. For further details, see Materials and Methods.

* Significantly different from control ($P < 0.05$).

† Significantly different from the corrsponding treatments without PMA ($P < 0.05$).

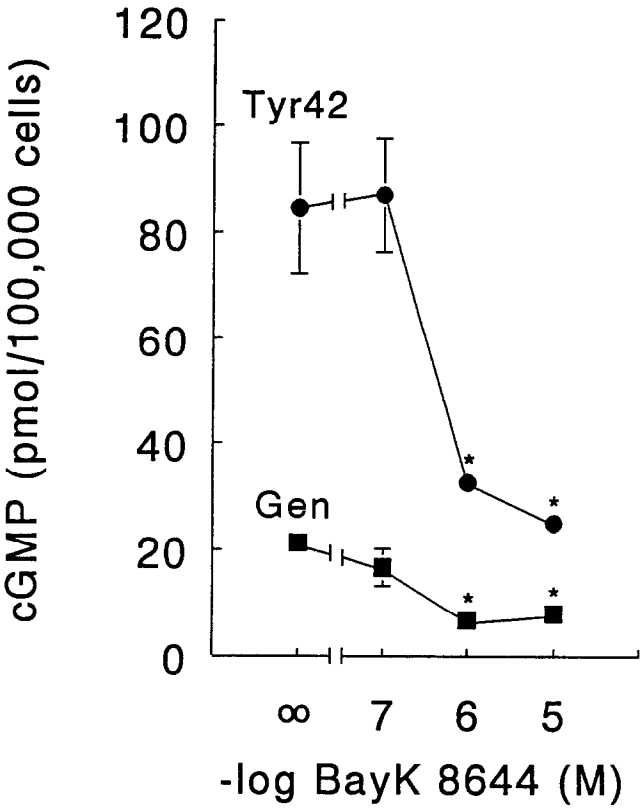

FIG. 3. Effect of BayK 8644 on tyrosine kinase inhibitor-stimulated cGMP accumulation in rat pinealocytes. Pinealocytes (2×10^4 cells/0.4 mL) were incubated in DMEM and stimulated with tyrphostin B42 (Tyr42, 300 μM) or genistein (Gen, 300 μM) for 30 min in the presence or absence of various concentrations of BayK 8644. The cGMP values for cells stimulated with tyrphostin B42 or genistein and for the untreated control were 84.5 ± 12.3, 21.3 ± 1.7, and 5.0 ± 1.5 pmol/10^5 cells, respectively. Each value is the mean ± SEM of determinations in four samples of cells. For further details, see Materials and Methods. Key: (*) significantly different from corresponding treatment without BayK 8644 ($P < 0.05$).

(Table 4). However, BayK 8644 or ionomycin alone also reduced the IBMX-mediated increase in cGMP accumulation (Table 4). These data, therefore, suggest that the inhibitory effect of elevating $[Ca^{2+}]_i$ on the cGMP level is mediated through a step that is directly involved in the synthesis of cGMP and may not be directly related to tyrosine kinase.

To investigate the intracellular mechanism involved in the effect of $[Ca^{2+}]_i$-elevating agents, the effect of W7, a calmodulin inhibitor [22], on the tyrosine kinase inhibitor-stimulated cGMP accumulation was determined. W7 (1 μM), which had no effect on basal cGMP accumulation, partially reversed the inhibitory effect of BayK 8644 on tyrphostin B42- and genistein-stimulated cGMP responses (Table 5).

Effect of Tyrosine Kinase Inhibitors on the Basal and PMA-Stimulated Distribution of PKC Activities

To further investigate the interaction between the tyrosine kinase inhibitors and PKC, the effect of tyrosine kinase

Table 3. Effect of Ca^{2+}-elevating agents on cGMP accumulation stimulated by tyrosine kinase inhibitors in rat pinealocytes

Treatment	cGMP (pmol/10^5 cells)
Control	5.8 ± 1.0
KCl (30 mM)	5.1 ± 1.0
Ionomycin (10 μM)	5.3 ± 1.0
EGTA-AM (10 μM)	5.8 ± 1.2
Tyrphostin B42 (300 μM)	80.3 ± 4.5*
+ KCl (30 mM)	30.7 ± 6.3†
+ Ionomycin (10 μM)	20.4 ± 0.5†
+ EGTA-AM (10 μM)	113.3 ± 18.7†
Genistein (300 mM)	20.3 ± 4.4*
+ KCl (30mM)	7.7 ± 0.6†
+ Ionomycin (10 μM)	5.3 ± 0.2†
+ EGTA-AM (10 μM)	37.8 ± 6.7†

Pinealocytes (2 × 10^4 cells/0.4 mL) were incubated in DMEM and treated with the tyrosine kinase inhibitors and KCl (30 mM) or ionomycin (10 μM) or EGTA-AM (10 μM) for 30 min. Each value is the mean ± SEM of cGMP in four samples of cells. For further details, see Materials and Methods.

* Significantly different from control ($P < 0.05$).

† Significantly different from the corresponding treatment with tyrosine kinase inhibitor alone ($P < 0.05$).

inhibitors on the PMA-mediated translocation of PKC in intact pinealocytes was investigated. As shown in Fig. 4, PMA (0.1 μM) caused a 4-fold increase in PKC activities associated with the particulate fraction while reducing the cytosolic fraction by 60% [18, 23]. Treatment with tyrphostin B42 (300 μM) had no significant effect on the basal or PMA-stimulated distribution of PKC activities (Fig. 4). Addition of tyrphostin B42 (300 μM) directly into the PKC assay also had no effect on the PKC activities (Fig. 5).

Table 4. Effect of BayK 8644 on cGMP accumulation in rat pinealocytes stimulated by tyrosine kinase inhibitors in the prescence of maximal phosphidiesterase inhibition

	cGMP (pmol/10^5 cells)	
Treatment	-IBMX	+IBMX
Control	5.3 ± 1.4	134.3 ± 10.3
BayK 8644 (10 μM)	4.5 ± 1.2	76.4 ± 7.2*
Ionomycin (10 μM)	5.5 ± 1.2	52.4 ± 6.2*
Tyrphostin B42 (300 μM)	89.4 ± 13.0†	123.7 ± 17.8
+ BayK 8644 (10 μM)	26.4 ± 0.6*	69.9 ± 5.7*
+ Ionomycin (10 μM)	12.3 ± 0.7*	38.0 ± 2.8*
Genistein (300 μM)	22.1 ± 3.5†	129.5 ± 8.5
+ BayK 8644 (10 μM)	6.1 ± 0.8*	59.8 ± 1.7*
+ Ionomycin (10μM)	5.3 ± 0.4*	38.0 ± 2.8*

Pinealocytes (2 × 10^4 cells/0.4 mL) were incubated in DMEM and treated with the tyrosine kinase inhibitors and BayK 8644 or ionomycin (10 μM) for 30 min in the prescence or abscence of IBMX (1 mM). Each value is the mean ± SEM of cGMP in four samples of cells. For further details, see Materials and Methods.

* Significantly different from the corresponding treatment without Ca^{2+}-elevating agents ($P < 0.05$).

† Significantly different from control ($P < 0.05$).

Table 5. Effect of a Ca^{2+}/calmodulin inhibitor on cGMP accumulation stimulated by tyrosine kinase inhibitors and BayK 8644 in rat pinealocytes

	cGMP (pmol/10^5 cells)	
Treatment	-W7	+W7
Control	5.8 ± 1.0	7.8 ± 1.5
BayK 8644 (10 μM)	4.4 ± 0.9	5.2 ± 0.6
Tyrphostin B42 (300 μM)	80.3 ± 4.5*	90.4 ± 10.7*
+ BayK 8644 (10 μM)	34.2 ± 4.6†	44.0 ± 0.5†‡
Genistein (300 μM)	20.3 ± 4.4*	22.4 ± 1.1*
+ BayK 8644 (10 μM)	6.6 ± 0.9†	15.0 ± 2.7†‡

Pinealocytes (2 × 10^4 cells/0.4 mL) were incubated in DMEM and treated with the tyrosine kinase inhibitors and BayK 8644 or ionomycin (10 μM) for 30 min in the prescence or abscence of W7 (1 μM). Each value is the mean ± SEM of cGMP in four samples of cells. For further details, see Materials and Methods.

* Significantly different from control ($P < 0.05$).

† Significantly different from the corresponding treatment without BayK 8644 ($P < 0.05$).

‡ Significantly different from the corresponding treatment without W7 ($P < 0.05$).

DISCUSSION

We have shown recently that tyrosine kinase inhibitors elevate pineal cGMP levels by inhibiting the metabolism of cGMP [12]. In the present study, we have further demonstrated that the effect of tyrosine kinase inhibitors on cGMP accumulation was selective since this occurred in the absence of any changes in cAMP accumulation. Similar results were observed with tyrphostin B42 and genistein, two specific tyrosine kinase inhibitors with different sites of action, while tyrphostin A1 and daidzein, their corresponding inactive analogues, had no effect on cGMP accumulation. These results indicate that the effects of tyrphostin B42 and genistein on cGMP accumulation are likely related to inhibition of tyrosine kinase activity.

The interactions of PKC and $[Ca^{2+}]_i$ with the tyrosine kinase inhibitor-mediated cGMP elevation are of interest. Although an activator of PRL poteniates the tyrosine kinase inhibitor-mediated increase in cGMP accumulation, $[Ca^{2+}]_i$-elevating agents have the opposite effect. In comparison, both PKC activation and elevated $[Ca^{2+}]_i$ are known to potentiate the β-adrenergic- or vasoactive intestinal peptide-stimulated cGMP accumulation in rat pinealocytes [17, 24, 25]. These results suggest that there are distinct difference between the interactions of PKC and $[Ca^{2+}]_i$ with the tyrosine kinase inhibitor- versus the receptor-mediated cGMP accumulation.

Investigation into the interaction between PKC and tyrosine kinase inhibitors on the cGMP response showed that activation of PKC by PMA, but not the inactive 4α-PDD, significantly potentiated the tyrosine kinase inhibitor-stimulated cGMP accumulation. However, the stimulatory effects on cGMP accumulation by either tyrosine kinase inhibitors alone or in combination with PMA were not observed in the presence of maximal phosphodiesterase inhibition. These observations suggest that both PMA and

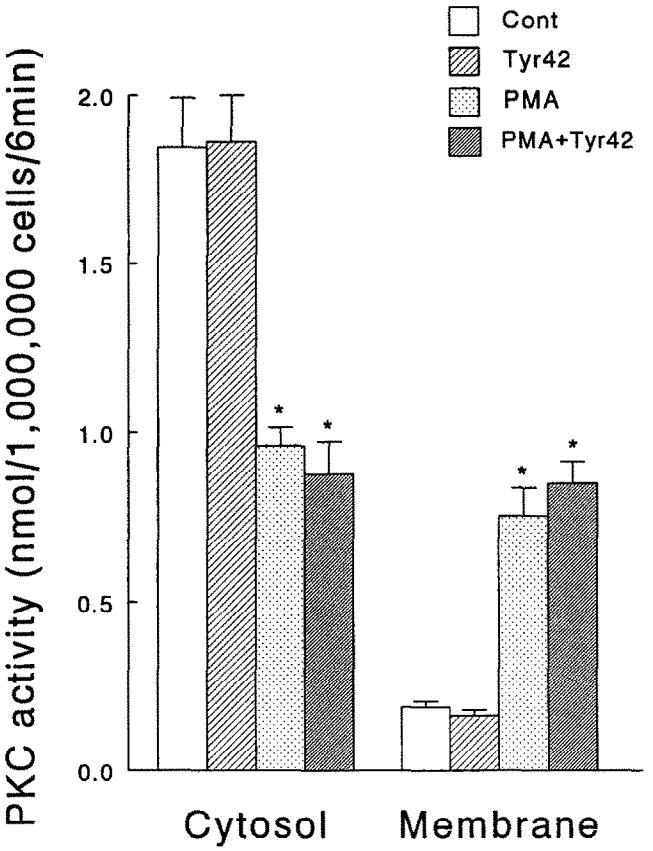

FIG. 4. Effect of tyrphostin B42 on the PMA-mediated translocation of PKC activity in rat pinealocytes. PKC activity of the membrane and cytosolic fractions of pinealocytes was determined in control and PMA (100 nM)-treated cells in the absence or presence of tyrphostin B42 (Tyr42, 300 μM). Each value is the mean ± SEM of determinations done in duplicate on three samples of cell preparations. For further details see Materials and Methods. Key: (*) significantly different from control ($P < 0.05$).

the tyrosine kinase inhibitors are working through phosphodiesterase in modulating cGMP accumulation. Although PKC has a direct effect on phosphodiesterase activity in several tissues [26–29], this is an unlikely mechanism in rat pinealocytes since activation of PKC alone has no effect on basal cGMP accumulation. Considering that PMA has no effect on its own, these results further suggest that activation of PKC is modulating the effect of the tyrosine phosphorylation on the phosphodiesterase rather than having a direct effect on the cGMP.

In addition to the effect of activated PKC activity, our results also suggest that basal PKC activity may play a significant role in modulating the regulation of phosphodiesterase by protein tyrosine phosphorylation. This is based on the observation that treatment with calphostin C reduces the tyrosine kinase inhibitor-stimulated cGMP accumulation. Since this occurs in the absence of PMA treatment, it suggests that the interaction between tyrosine kinase and phosphodiesterase is subjected to the tonic regulation by basal PKC activity. Of interest, calphostin C has no effect on the ISO-stimulated cGMP response, further indicating that the effect of calphostin C is specific for the tyrosine kinase inhibitor-mediated response and is not shared by other agonists.

Another possible explanation for the interaction between PKC and tyrosine kinase is that PKC activation may occur as a consequence of tyrosine kinase inhibition. Activation of phospholipase C-γ through tyrosine phosphorylation may lead to generation of diacylglycerol which can activate PKC [10]. However, this is not likely in rat pinealocytes since tyrosine kinase inhibitors, apart from having no effect on the *in vitro* PKC activity, also have no effect on the basal or PMA-mediated PKC distribution. Therefore, the more likely explanation is that PKC could reduce tyrosine phosphorylatiion in rat pinealocytes which, in turn, modulate phosphodiesterase activity. This is supported by previous studies which showed that PKC could phosphorylate the epidermal growth factor and insulin receptors, resulting in a reduction of tyrosine kinase activities [30, 31]. Furthermore, a cone-type cGMP phosphodiesterase is expressed in the rat pineal gland [32], and the inhibitory

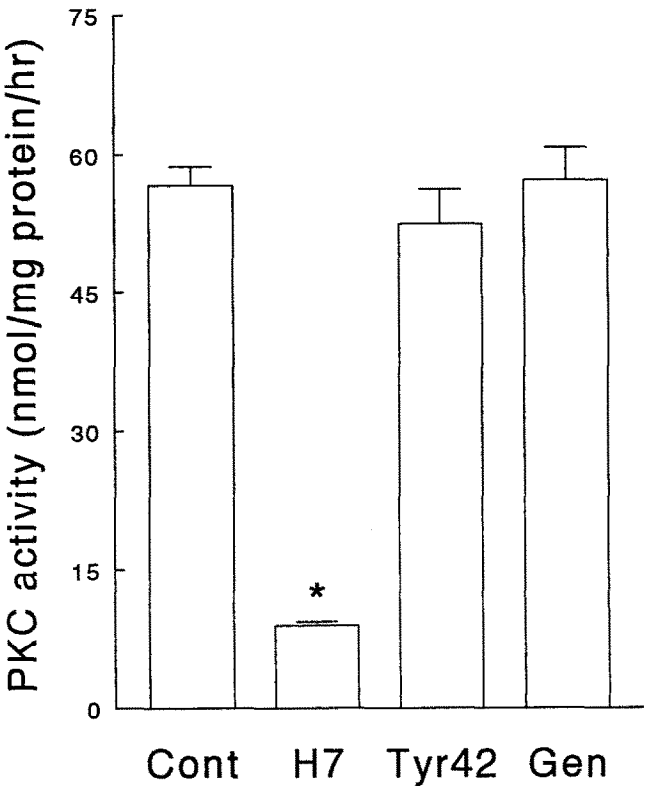

FIG. 5. Effect of tyrphostin B42 and genistein on *in vitro* PKC activity. PKC activity of the cytosolic fractions of pinealocytes was determined in the absence or presence of tyrphostin B42 (Tyr42, 300 μM) or genistein (Gen, 300 μM). Ac-MBP(4-14) was used as substrate. H7 (100 μM) was included in this experiment as a positive control. Each value is the mean ± SEM of determinations done in duplicate on three samples of cell preparations. For further details see Materials and Methods. Key: (*) significant inhibition of the *in vitro* PKC activity ($P < 0.05$).

subunit of this phosphodiesterase can be phosphorylated by PKC [29]. This may explain the modulating effects of tyrosine kinase inhibitors and PKC activators observed in this study. However, it should be mentioned that in other tissues such as glomerular mesangial cells and hepatoma cells, activation of PKC may also lead to tyrosine kinase activation [33, 34], indicating that the nature of the interaction between PKC and tyrosine phosphorylation is tissue specific.

In addition to directly affecting the tyrosine kinase activities, activation of PKC may also affect the phosphotyrosine phosphatase activities as shown in other studies [35]. This may result in an alteration of the state of tyrosine phosphorylation. Whether PKC potentiates the effect of the tyrosine kinase inhibitors by reducing tyrosine kinase activities or enhancing the phosphotyrosine phosphatase activities in rat pinealocytes remains to be determined.

In contrast to PKC activation, elevation of $[Ca^{2+}]_i$ significantly decreases the tyrosine kinase inhibitor-mediated increase in cGMP accumulation while EGTA-AM, which reduces $[Ca^{2+}]_i$, increases the effects of these inhibitors. The inhibitory effect of $[Ca^{2+}]_i$-elevating agents appears to be partly mediated through the Ca^{2+}/calmodulin-dependent kinase since the BayK 8644- or KCl-mediated inhibition of the tyrphostin B42- and genistein-stimulated cGMP accumulation was partly blocked by W7, a selective Ca^{2+}/calmodulin inhibitor. More interestingly, the interaction between $[Ca^{2+}]_i$ and tyrosine kinase in regulating cGMP accumulation is different from that between PKC and tyrosine kinase. While the potentiating effect of PMA is abolished by phosphodiesterase inhibition, the inhibitory effect of elevated $[Ca^{2+}]_i$ persists in the presence of phosphodiesterase inhibition. This result indicates that changes in $[Ca^{2+}]_i$ are affecting the synthesis of cGMP rather than its degradation. This effect of $[Ca^{2+}]_i$ is also consistent with the observation that high $[Ca^{2+}]_i$ can inhibit guanylyl cyclase through guanylyl cyclase-activating protein [36, 37].

The roles of PKC and $[Ca^{2+}]_i$ on the cyclic nucleotide accumulation in rat pinealocytes have been investigated previously [1, 3]. In the earlier studies, the focus was on the modulation of cyclic nucleotide accumulation stimulated by the G-protein-coupled signal transduction pathway. These included activation of membrane receptors (β-adrenergic or vasoactive intestinal peptide receptors), G-protein (by cholera toxin), or direct activation of the cyclases (by forskolin). In all cases, the agonist-stimulated cGMP accumulation was potentiated by elevation of $[Ca^{2+}]_i$ alone or by PKC activation in the presence of $[Ca^{2+}]_i$ elevation [17]. The mechanism of the potentiation was due to enhanced synthesis of the cyclic nucleotide. However, our present findings indicate that, although PKC activation alone has no effect on the cGMP level in the rat pinealocytes, it may utilize multiple signalling pathways in modulating cGMP accumulation indirectly. In addition to the above-mentioned potentiation of the stimulated cGMP response by increasing synthesis, PKC also modulates the rate of cGMP degradation through signalling pathways involving tyrosine phosphorylation.

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References

1. Chik CL and Ho AK, Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. *Prog Biophys Mol Biol* **53:** 197–203, 1989.
2. Klein DC, Photoneural regulation of the mammalian pineal gland. In: *Photoperiodism, Melatonin and the Pineal* (Eds. Evered D and Clark S), Vol. 119, pp. 38–56. Ciba Foundation Symposium, Pitman, London, 1985.
3. Klein DC, Chik CL, Weller J and Ho AK, Adrenergic regulation of pineal cAMP and cGMP: Evidence for a gating mechanism. In: *Progress in Catecholamine Research* (Eds. Dahlstrom A, Belmaker RH and Sandler M), Vol. 1, pp. 415–422. Alan R. Liss, New York, 1988.
4. Vanecek J, Sugden D, Weller J and Klein DC, Atypical synergistic α_1- and β-adrenergic regulation of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate in rat pinealocytes. *Endocrinology* **116:** 2167–2173, 1985.
5. Ho AK, Young I and Chik CL, Evidence for a role of calmodulin in regulation of pinealocyte cyclic nucleotides. *Biochem Pharmacol* **41:** 896–903, 1991.
6. Ho AK, Chik CL, Weller JL, Cragoe EJ Jr and Klein DC, Evidence of α_1-adrenergic → protein kinase C → Na^+/H^+ antiporter-dependent increase in pinealocyte intracellular pH. Role in the adrenergic stimulation of cGMP accumulation. *J Biol Chem* **264:** 12983–12988, 1989.
7. Ho AK, Girard M, Young I and Chik CL, Intracellular pH on adrenergic-stimulated cAMP and cGMP production in rat pinealocytes. *Am J Physiol* **261:** C642–C649, 1991.
8. Hadcock JR, Port JD, Gelman MS and Malbon CC, Cross-talk between tyrosine kinase and G-protein-linked receptors. *J Biol Chem* **267:** 26017–26022, 1992.
9. Nair BG, Parikh B, Milligan G and Patel TB, $G_{s\alpha}$ mediates epidermal growth factor-elicted stimulation of rat cardiac adenylate cyclase. *J Biol Chem* **265:** 21317–21322, 1990.
10. Meisenhelder J, Suh P-S, Rhee SG and Hunter T, Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases *in vivo* and *in vitro*. *Cell* **57:** 1109–1122, 1989.
11. Ball RL, Tanner KD and Carpenter G, Epidermal growth factor potentiates cyclic AMP accumulation in A-431 cells. *J Biol Chem* **265:** 12836–12845, 1990.
12. Ogiwara T, Murdoch G, Chik CL and Ho AK, Tyrosine kinase inhibitors enhance cGMP production in rat pinealocytes. *Biochem Biophys Res Commun* **207:** 994–1002, 1995.
13. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M and Fukami Y, Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* **262:** 5592–5595, 1987.
14. Gaudette DC and Holub BJ, Effect of genistein, a tyrosine kinase inhibitors, on U46619-induced phosphoinositide phosphorylation in human platelets. *Biochem Biophys Res Commun* **170:** 238–242, 1990.
15. Levitzki A and Gilon C, Tyrphostins as molecular tools and anti-proliferative drugs. *Trends Pharmacol Sci* **12:** 171–174, 1991.

16. Buda M and Klein DC, A suspension culture of pinealocytes: Regulation of *N*-acetyltransferase activity. *Endocrinology* **103:** 1483–1493, 1978.
17. Ho AK, Chik CL and Klein DC, Protein kinase C is involved in the adrenergic stimulation of pineal cGMP accumulation. *J Biol Chem* **262:** 10059–10064, 1987.
18. Ho AK, Thomas TP, Chik CL, Anderson WB and Klein DC, Protein kinase C: Subcellular redistribution by agents which increase Ca^{2+} influx. *J Biol Chem* **263:** 9292–9297, 1988.
19. Harper JF and Brooker GJ, Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′-0 acetylation by acetic anhydride in aqueous solution. *J Cyclic Nucleotide Res* **1:** 207–218, 1975.
20. Yasuda I, Kishimoto A, Tanaka S, Tominaga M, Sakurai A and Nishizuka Y, A synthetic peptide substrate for selective assay of protein kinase C. *Biochem Biophys Res Commun* **166:** 1220–1227, 1990.
21. Kobayashi E, Nakano H, Morimoto H and Tamaoki T, Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159:** 548–553, 1989.
22. Hidaka H, Sasaki Y, Tanaka T, Endo T, Ohno S, Fujii Y and Nagata T, *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc Natl Acad Sci USA* **78:** 4354–4357, 1981.
23. Ho AK and Chik CL, Acute elevation of intracellular pH translocates protein kinase C activities in rat pinealocytes. *Biochem Biophys Res Commun* **200:** 569–576, 1994.
24. Sugden D, Vanecek J, Klein DC, Thomas TP and Anderson WB, Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. *Nature* **314:** 359–360, 1985.
25. Chik CL, Ho AK and Klein DC, α_1-Adrenergic potentiation of vasoactive intestinal peptide stimulation of rat pinealocyte adenosine 3′,5′-monophosphate and guanosine 3′,5′,-monophosphate: Evidence for a role of calcium and protein kinase C. *Endocrinology* **122:** 702–708, 1988.
26. Irvine F, Pyne NJ and Houslay MD, The phorbol ester TPA inhibits cyclic AMP phosphodiesterase activity in intact hepatocytes. *FEBS Lett* **208:** 455–459, 1986.
27. Lee HC, Cai JJ and Yu H, Effect of protein kinase C on cyclic 3′,5′-adenosine monophosphate-dependent phosphodiesterase in hypertrophic cardiomyopathic hamster hearts. *J Pharmacol Exp Ther* **270:** 1171–1176, 1994.
28. Udovichenko IP, Cunnick J, Gonzales K and Takemoto DJ, Phosphorylation of bovine rod photoreceptor cyclic GMP phosphodiesterase. *Biochem J* **295:** 49–55, 1993.
29. Udovichenko IP, Cunnick J, Gonzalez K and Takemoto DJ, Functional effect of phosphorylation of the photoreceptor phosphodiesterase inhibitory subunit by protein kinase C. *J Biol Chem* **269:** 9850–9856, 1994.
30. Cochet C, Gill GN, Meisenhelder J, Cooper JA and Hunter T, C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* **259:** 2553–2558, 1984.
31. Takayama S, White MF and Kahn CR, Phorbol ester-induced serine phosphorylation of the insulin receptor decreases its tyrosine kinase activity. *J Biol Chem* **263:** 3440–3447, 1988.
32. Carcamo B, Hurwitz MY, Craft CM and Hurwitz RL, The mammalian pineal expresses the cone but not the rod cyclic GMP phosphodiesterase. *J Neurochem* **65:** 1085–1092, 1995.
33. Force T, Kyriakis JM, Avruch J and Bonventre JV, Endothelin, vasopressin, and angiogtensin II enhance tyrosine phosphorylation by protein kinase C-dependent and -independent pathways in glomerular mesangial cells. *J Biol Chem* **266:** 6650–6656, 1991.
34. Ogawa W, Hosomi Y and Roth RA, Activation of protein kinase C stimulates the tyrosine phosphorylation and guanosine triphosphatase-activating protein association of P60 in rat hepatoma cells. *Endocrinology* **136:** 476–481, 1995.
35. Garton AJ and Tonks NK, PTP-PEST: A protein tyrosine phosphatase regulated by serine phosphorylation. *EMBO J* **13:** 3763–3771, 1994.
36. Koch KW and Stryer L, Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. *Nature* **334:** 64–66, 1988.
37. Gorczyca WA, Gray-Keller MP, Detwiler PB and Palczewski K, Purification and physiological evaluation of a guanylate cyclase activating protein from retinal rods. *Proc Natl Acad Sci USA* **91:** 4014–4018, 1994.