Biochemical Pharmacology, Vol. 43, No. 10, pp. 2073–2081, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

PROPERTIES OF BASE-SUBSTITUTED AND CARBOXYL-ESTERIFIED ANALOGUES OF GRISEOLIC ACID, A POTENT cAMP PHOSPHODIESTERASE INHIBITOR

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(*Received* 4 *November* 1991; *accepted* 24 *February* 1992)

Abstract—Griseolic acid (GA) is a potent cyclic AMP (cAMP) phosphodiesterase (PDE) inhibitor that has an adenine base and two carboxyl groups in its molecule (Nakagawa F, Okazaki T, Naito A, Iijima Y and Yamazaki M, *J Antibiot* **38**: 823–829, 1985). GA analogues were synthesized in which the adenine group was substituted with guanine (6-deamino-2-amino-6-hydroxygriseolic acid, G-GA) or hypoxanthine (6-deamino-6-hydroxygriseolic acid, H-GA). Their inhibitory activities to cyclic GMP (cGMP) PDE and cAMP PDE were compared with GA. For cGMP PDE from rod outer segments of bovine retina, the IC_{50} values of GA, G-GA and H-GA were 18, 0.040 and 0.12 μM, respectively, with 0.25 μM cGMP as substrate. For type IV PDE isozyme from mouse 3T3 fibroblast cells, the IC_{50} values of GA, G-GA and H-GA were 0.021, 15 and 11 μM, respectively, with 0.25 μM cAMP as substrate. Thus, GA and G-GA were found to be base-selective inhibitors of type IV PDE of 3T3 cells and type V PDE of bovine retinas, respectively. Esters of carboxylic acids of GA were synthesized in order to increase permeability into cells, and their efficacy was tested by measuring the accumulation of cAMP in 3T3 cells. The dipivaloyloxymethyl ester of GA was found to increase cAMP levels at 0.1 μM, while GA and 3-isobutyl-1-methylxanthine were active only above 100 μM, and the dimethyl ester of GA was inactive. The dipivaloyloxymethyl ester of GA seems to exert its activity after conversion to GA in the cell, since the pivaloyloxymethyl ester was easily hydrolysed by the enzyme action and the dipivaloyloxymethyl ester of GA itself was much less potent an inhibitor of PDE. The dipivaloyloxymethyl ester of GA inhibited thrombin-induced aggregation of platelets and stimulated lipolysis of adipocytes at low concentrations.

Griseolic acid (GA§) (Fig. 1), a competitive inhibitor of cAMP phosphodiesterase (PDE), is a fermentation product of *Streptomyces griseoaurantiacus* [1]. GA has a similar structure to cAMP, with an adenine base and a ribose-like moiety, but it has two carboxyl groups instead of a phosphate group [1]. GA inhibited the cAMP PDE reaction at low concentrations; the IC_{50} was 0.16 μM with the supernatant fraction of rat brain homogenate as an enzyme and 0.14 μM cAMP as substrate [1]. GA stimulated lipolysis in isolated rat adipocytes [2], insulin release from pancreatic islets [3], and neurite formation from neuro 2a cells [4]. When GA (10 mg/kg) was subcutaneously administered to rats, the cAMP level was increased several-fold in liver and plasma [2]. An increase in blood glucose and a decrease in liver glycogen were observed in mice following intravenous injection of GA [2]. This compound has no other biological activities, such as interference with adenosine receptors and activation or inhibition activity to cAMP-dependent protein kinase [2].

Although GA is an active inhibitor of cAMP PDE in a cell-free system, considerably higher concentrations of GA were required for the accumulation of cAMP in isolated tissue and cells [2]. Furthermore, GA showed no inhibitory effects on platelet aggregation and on the relaxation of guinea pig ileum [2]. A plausible explanation of these findings is that GA does not readily cross the cell membrane of these tissues due to its polar structure with two carboxyl groups.

The present study was undertaken to investigate the biochemical properties of GA using its synthetic analogues. At first, GA derivatives whose adenine group was substituted with guanine (6-deamino-2-amino-6-hydroxygriseolic acid, G-GA) or hypoxanthine (6-deamino-6-hydroxygriseolic acid, H-GA) were synthesized, and their inhibitory effects on cGMP PDE of bovine retina and on PDE isozymes in mouse 3T3 fibroblast cells were investigated. Next, carboxyl esters of GA (Fig. 1) were synthesized to increase the permeability into cells, and their effects on cAMP accumulation in 3T3 fibroblast cells were tested. The dipivaloyloxymethyl (diPOM) ester of GA was found to increase the cAMP level at much lower concentrations than GA. The effects of the diPOM ester on rabbit platelets and rat adipocytes were also investigated.

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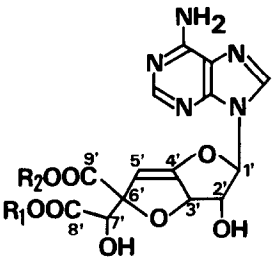
§ Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid; GA, griseolic acid; G-GA, 6-deamino-2-amino-6-hydroxygriseolic acid; H-GA, 6-deamino-6-hydroxygriseolic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; PDE, phosphodiesterase; POM, pivaloyloxymethyl; ROS, rod outer segment.



R_1	R_2	
H	H	Griseolic acid
POM	POM	DiPOM ester
CH_3	CH_3	Dimethyl ester
POM	H	MonoPOM ester
CH_3	H	Monomethyl ester

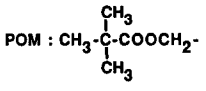


Fig. 1. Structures of griseolic acid and its derivatives.

MATERIALS AND METHODS

Preparation and assay of cGMP PDE from rod outer segments of bovine retina

Fresh bovine eyes, kept on ice after excision, were obtained from a local slaughterhouse. Bovine rod outer segment (ROS) PDE was prepared according to the method of Papermaster and William [5] as modified by Baehr *et al.* [6]. ROS PDE was activated by trypsin [6] and assayed with [^{3}H]cGMP (910 GBq (24.6 Ci)/mmol, Amersham) as substrate, according to the method of Pichard and Cheung [7] with the following modifications: the reaction buffer consisted of 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin and 100 mM Tris–HCl (pH 7.5); and incubation was performed at 30° for 4 min. 5′-Nucleotidase reaction was stopped by adding a resin slurry of Amberlite IRP-58 (Rohm and Haas).

Separation and assay of cyclic nucleotide PDE activities of 3T3 cells

Balb 3T3 cells were grown in plastic culture dishes under a humid atmosphere containing 5% CO_2. The culture medium used was Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. After cells were grown to confluency, medium was replaced with phosphate-buffered saline (PBS) and cells were harvested with a cell scraper. Cells were then washed twice with PBS by centrifugation at 100 *g* for 2 min. The cells were stored at −100°.

Cyclic nucleotide PDE activities were separated as described by Sakai *et al.* [8] with some modifications using an FPLC system (Pharmacia). 3T3 cells collected (1×10^{10}) were suspended in 30 mL of buffer that consisted of 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM Tris–HCl (pH 7.4); they were then homogenized with a Dounce homogenizer. The homogenate was centrifuged at 100,000 *g* for 60 min, and the supernatant fraction (400 mg protein) was applied to a DEAE-5PW anion exchange column (21.5 × 150 mm, TOSOH Corporation), which was pre-equilibrated with 0.07 M sodium acetate (pH 6.5) containing 5 mM 2-mercaptoethanol and 0.1 mM EGTA. The column was washed with 100 mL of 0.07 M sodium acetate containing 5 mM 2-mercaptoethanol and 0.1 mM EGTA. No detectable PDE activity was found in this initial wash. PDE activities were then eluted with 320 mL of a linear gradient of 0.07–1.2 M sodium acetate (pH 6.5) containing 5 mM 2-mercaptoethanol and 0.1 mM EGTA. These processes were performed with a flow rate of 4 mL/min. The eluate was collected in 4-mL fractions, and 2 mL of ethyleneglycol was added for storage. Fractions were stored at −20°.

PDE activities were assayed with [^{14}C]cAMP (10.2 GBq (276 mCi)/mmol, Amersham) or [^{3}H]-cGMP as substrate in buffer that consisted of 5 mM $MgCl_2$, 0.05 mM $CaCl_2$, 5 mM 2-mercaptoethanol, 0.1 mg/mL bovine serum albumin, and 40 mM Tris–HCl (pH 7.6), in the manner described above. In the assay of Ca^{2+}/calmodulin-dependent PDE, calmodulin was further supplemented in the buffer at a concentration of 10 U/mL. Incubation was performed at 30° for 10 min.

Determination of the cAMP level in 3T3 cell suspension

3T3 cells were grown as described above. After the cells were grown to complete confluency, the cells were harvested by treatment with trypsin, washed with a buffer that consisted of 140 mM NaCl, 3 mM $MgCl_2$ and 10 mM Hepes (pH 7.4), and suspended in the same buffer. The cAMP level in the cell suspension was determined by essentially the same method as described by Murayama and Ui [9]. The cell suspension (2×10^5 cells/tube) was incubated at 30° for the time indicated with GA, GA ester, IBMX or vehicle, in 200 μL of the buffer further supplemented with 1 U/mL adenosine deaminase, which was added to eliminate the effect of endogenous adenosine, and 2 mM $CaCl_2$. The reaction was terminated by the addition of 20 μL of 1 M HCl, and the tubes were centrifuged at 3000 rpm for 5 min. The cellular cAMP transferred to the supernatant fraction upon acidification was determined by radioimmunoassay as described by Honma *et al.* [10]. GA showed no interference in cAMP determination, even at a concentration of 1 mM.

Determination of enzymatic hydrolysis of the GA esters

3T3 cells were grown, harvested by a cell scraper, and washed by PBS as described above. The cells (2×10^8) were suspended in 3.5 mL of buffer that consisted of 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM Tris–HCl (pH 7.4), followed by homogenization with a Dounce homogenizer. The homogenate was centrifuged at 100,000 *g* for 60 min and the supernatant fraction was desalted by ultrafiltration. The supernatant

fraction thus obtained was transferred to an ultracent-30 cartridge (TOSOH Corporation) and centrifuged at 3000 *g* for 30 min at 4°. Tris–HCl buffer (20 mM, pH 7.4), the same volume as the filtrate, was added to the residue, followed by centrifugation under the same conditions. These steps were repeated six times. The residue obtained (10.4 mg protein/mL) was diluted with the same volume of 20 mM Tris–HCl buffer (pH 7.4) and used as a source of esterase. The reaction was started by adding 50-μL aliquots of esterase fraction into 50 μL of 0.2 mM monoPOM or monomethyl ester of GA dissolved in 20 mM Tris–HCl buffer (pH 7.4). The reaction proceeded at 30° and was terminated by adding 50 μL of 6% trichloroacetic acid in methanol. The tubes were immersed in an ice bath for 10 min and centrifuged at 3000 rpm for 10 min. GA produced and GA ester that remained in the supernatant fraction were separated by HPLC. The HPLC system was equipped with an ODS reverse phase column (A-312 S-5 120A, Yamamura Chemical Laboratories Co. Ltd, Kyoto, Japan). The mobile phase consisted of 70% of 10 mM ammonium acetate containing 2.5 mM PIC A reagent and 30% of acetonitrile. All separations were performed with a flow rate of 1.0 mL/min, and GA and its analogues were detected by UV absorption at 260 nm. Under these conditions, GA esters were not hydrolysed nonenzymatically.

Measurements of the cAMP level in washed platelets and platelet aggregation

Rabbit washed platelets were prepared by the method of Hamberg *et al.* [11]. The washed platelet suspension (2×10^7 cells/tube) was incubated at 37° with GA, GA diPOM ester, 3-isobutyl-1-methylxanthine (IBMX) or vehicle, in 200 μL of a buffer that consisted of 137 mM NaCl, 3.3 mM K_2HPO_4, 0.7 mM $MgCl_2$, 5 mM glucose, 0.5 U/mL apyrase [12] and 10 mM Hepes (pH 7.4). The reaction was terminated by adding 20 μL of 1 M HCl, and the tubes were centrifuged at 3000 rpm for 5 min. The cAMP level in the supernatant fraction was determined as described above. Platelet aggregation was determined with a Born aggregometer. The washed platelet suspension was preincubated at 37° for 3 min with GA, GA diPOM ester, IBMX or vehicle, and thrombin was added into the tubes to a final concentration of 0.5 U/mL. Aggregation was recorded as the change in light transmittance during continuous stirring.

Measurements of the cAMP level and glycerol production in rat isolated adipocytes

Adipocytes were isolated from the epididymal fat pads of 180–200 *g* Sprague–Dawley rats. The fat pads were digested with collagenase according to the method of Rodbell [13] as modified by Cushman [14]. All incubations were carried out in Krebs–Ringer buffer, pH 7.4, supplemented with 25 mM Hepes, 10 mM $NaHCO_3$, 4% (w/v) bovine serum albumin and 2 mM glucose. The adipocyte suspension (4×10^5 cells/tube) was incubated at 37° with GA, GA diPOM ester, IBMX or vehicle, in 400 μL of the buffer containing 1 U/mL adenosine deaminase. The reaction was terminated by immersing the tubes in a boiling water bath for 1 min. The tubes were centrifuged at 4° for 5 min at 3000 rpm, and the infranate fraction was transferred to Costar spin-X centrifugre filter units. Then the filter units were centrifuged at 10,000 rpm for 5 min. A 100-μL aliquot of the filtrate was assayed for cAMP level, and another 100-μL aliquot was assayed for glycerol content, which served as the measure of lipolysis. The cAMP level was determined as described above, and the glycerol content was determined by UV method using F-kit (Boehringer Mannheim).

Materials

Griseolic acid was isolated from a cultured broth of *Streptomyces griseoaurantiacus* [1].

GA derivtives whose adenine base was substituted with guanine [15, 16] or hypoxanthine [17] and dimethyl ester of GA [18] were synthesized as described previously.

8′-*Monomethyl ester.* To a solution of 800 mg of dimethyl griseolate in 32 mL of dimethylformamide were added 8 mL of saturated aqueous sodium bicarbonate and 8 mL of water, and the solution was stirred at room temperature for 130 hr. The pH of the reaction solution was adjusted to 2.3 with 1 M hydrochloric acid. The reaction mixture was purified by a prepacked RP-8 column eluted with water containing 5% acetonitrile after washing the column with water. The fractions of the main peak were collected and concentrated to about 10 mL under reduced pressure, and the solution was kept in a refrigerator. The resulting crystalline powder was collected by filtration, to give 522 mg of 8′-methyl griseolate. NMR δ ppm (DMSO-d_6): 8.33,1H,s (2 or 8-H); 8.19,1H,s (2 or 8-H); 6.49,1H,s (1′-H); 6.04,1H,dd, (J = 2.4, 4.9 Hz), (3′-H); 5.09,1H,d (J = 2.0 Hz), (5′-H); 4.59,1H,s (7′-H); 4.55,1H,d (J = 4.9 Hz)(2′-H); 3.64, 3H,s(CH_3).

8′,9′-*Dipivaloyloxymethyl ester.* To a suspension of 1 g of griseolic acid in 100 mL of acetonitrile were added 1.21 g of 1,8-diazabicyclo[5,4,0]-7-undecene and 2.56 g of iodomethylpivalate under a stream of nitrogen. The solution was stirred for 1 hr at room temperature. The solvent was removed under reduced pressure. The residue was dissolved into a mixture of 60 mL of ethyl acetate and 30 mL of 0.5 M aqueous hydrochloric acid. The organic layer was washed with an additional 30 mL of 0.5 M aqueous hydrochloric acid and 30 mL of 5% sodium bicarbonate solution, followed by drying with magnesium sulfate. The solvent was evaporated under reduced pressure. The residue was purified through a silica gel column and eluted with a 2:1 mixture of ethyl acetate and acetonitrile, to give 970 mg of 8′,9′-dipivaloyloxymethyl griseolate. NMR δ ppm (DMSO-d_6): 8.30,1H,s (2 or 8-H); 8.17,1H,s (2 or 8-H); 6.54,1H,s (1′-H); 6.08,1H,dd, (J = 2.4, 4.9 Hz), (3′-H); 5.07,1H,d (J = 2.0 Hz), (5′-H); 4.63,2H,m, (2′ and 7′-H); 7.39,2H,br.s, (NH_2); 6.20,1H,d (J = 4.4 Hz)(2′-OH); 5.68-5.77,5H,m (CH_2,7′-OH); 1.17,3H,d (CH_3); 1.09,3H,s (CH_3).

8′-*Pivaloyloxymethyl ester.* Griseolic acid (5 g) was dissolved in 15 mL of dimethyl sulfoxide, and 2.17 mL of 1,8-diazabicyclo[5,4,0]-7-undecene was added under a stream of nitrogen. Acetonitrile (15 mL) and 3.83 g of iodomethylpivalate were added to the resulting mixture under ice-cooling, and the

Table 1. K_m values and inhibition constates of inhibitors for PDE isozymes from bovine ROS and 3T3 cells

Substrate	K_m (μM)	K_i (IC_{50}) (μM) GA	H-GA	G-GA	IBMX
Bovine ROS PDE					
cGMP	160	25 (18)	0.13 (0.12)	0.013 (0.040)	9.0 (9.0)
3T3 cell PDE					
Peak No.					
1 cGMP	4.7	0.50 (1.1)	0.10 (0.20)	0.081 (0.12)	3.2 (5.2)
1 cAMP	17	0.48 (0.40)	0.080 (0.38)	0.075 (0.32)	3.5 (4.6)
2 cAMP	7.3	0.043 (0.062)	29 (43)	33 (38)	18 (24)
3 cAMP	3.3	0.021 (0.021)	22 (11)	15 (15)	17 (19)

PDE activities of ROSs were assayed at 30° for 4 min after they were activated by trypsin, and PDE activities from 3T3 cells were assayed at 30° for 10 min, as described in Materials and Methods. In the assay of peak 1 PDE isozyme of 3T3 cells, the buffer was further supplemented with 10 U/mL calmodulin. K_m values were determined by Lineweaver–Burk plots. K_i values were determined by Dixon plots. IC_{50} values were determined with 0.25 μM cAMP or cGMP as substrate. Each value was estimated from the mean of duplicate experiments.

mixture was allowed to react at room temperature for 5 hr. Thereafter, the acetonitrile was removed by evaporation under reduced pressure, and the residue was dissolved in 300 mL of ethyl acetate and 300 mL of water. A cold 10% (w/v) aqueous solution of sodium bicarbonate was then added to the resulting solution, to cause the aqueous layer to separate. The ethyl acetate layer was then further extracted with 100 mL of water, and the aqueous extract and the previously separated aqueous layer were combined. The combined aqueous solution was adjusted to a pH value of 3.1 by adding 1 M aqueous hydrochloric acid, and then the solution was purified by chromatography through a prepacked column RP-8 (Merck & Co. Ltd), followed by elution with water containing successively 5%, 10% and 20% by volume of acetonitrile. Fractions containing the desired compound were collected and lyophilized, to give 688 mg (yield 10.6%) of the 8′-pivaloyloxymethyl ester. NMR δ ppm (DMSO-d_6): 8.33,1H,s (2 or 8-H); 8.18,1H,s (2 or 8-H); 6.49,1H,s (1′-H); 6.03,1H,dd (J = 2.4, 4.9 Hz), (3′-H); 5.08,1H,d (J = 2.4 Hz), (5′-H); 4.58,2H,m (2′ and 7′-H); 7.40,2H,bs (NH_2); 6.26,1H,d (J = 4.9 Hz)(2′-OH); 5.73,2H,s (CH_2); 1.16,9H,s [$C(CH_3)_3$].

Radioimmunoassay kit for cAMP was purchased from Yamasa Shoyu, Co. Ltd (Chiba, Japan). RO 20-1724 was a gift from Nippon Roche K.K. (Kamakura, Japan). Milrinone was prepared by the Sankyo Co. Ltd. Calmodulin (bovine brain), snake venom (*Crotalus atrox*), trypsin, soybean trypsin inhibitor, collagenase (type II), 2-mercaptoethanol, phenylmethylsulfonyl fluoride, cGMP, and IBMX were purchased from Sigma. Adenosine deaminase was from Boehringer Mannheim. Thrombin was from Mochida Pharmaceutical Co. Ltd (Tokyo, Japan). PIC A reagent was from Waters. All other chemicals were reagent grade.

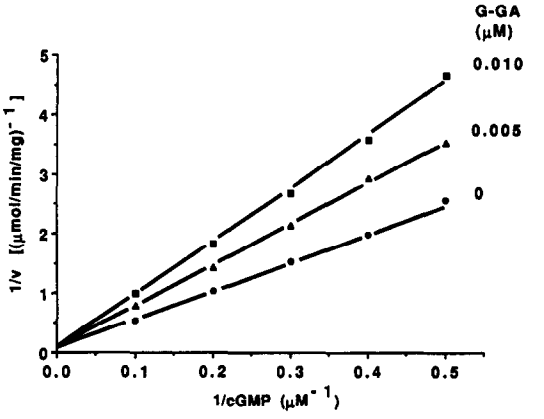


Fig. 2. The double reciprocal plots of the cGMP concentration versus the reaction velocity of bovine retinal PDE. The PDE was activated by trypsin and assayed as described in Materials and Methods. Each point is the mean of duplicate experiments.

RESULTS

Effects of GA and its adenine base-substituted derivatives on cGMP PDE from bovine retina

GA and its derivatives, in which the adenine group was substituted with a guanine group or a hypoxanthine group, were tested for their inhibitory effects on cGMP PDE from ROSs of bovine retina (Table 1). G-GA was a far more potent inhibitor than GA and H-GA for the cGMP PDE, and the IC_{50} of G-GA was less than one four-hundredth that of GA. G-GA exhibited competitive inhibition with regard to cGMP on this cGMP PDE (Fig. 2). The inhibition of retinal PDE by GA, H-GA or IBMX was competitive with regard to cGMP (data not

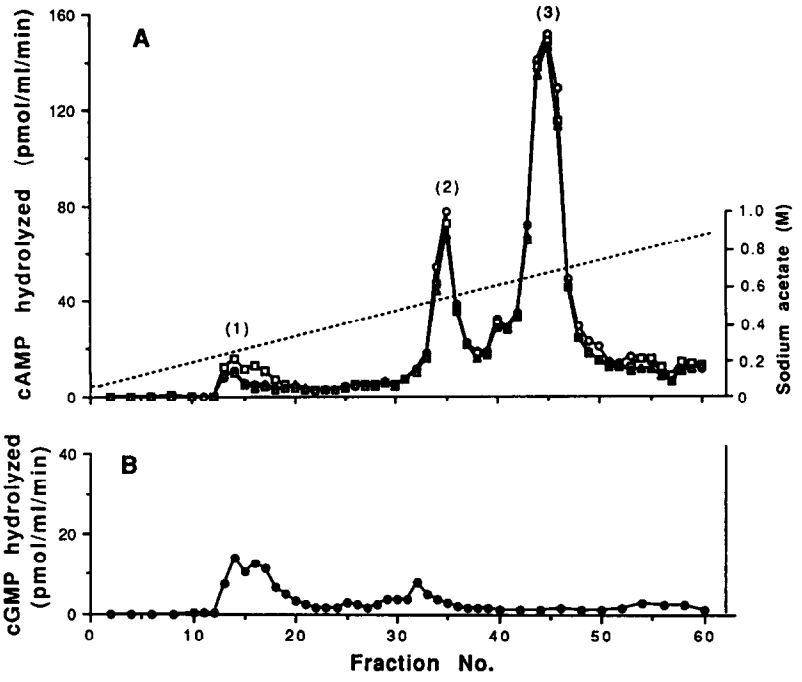


Fig. 3. Separation profiles of cyclic nucleotide PDE activities from 3T3 cells. (A) Fractions were assayed with 0.25 μM cAMP (○), 0.25 μM cAMP and 10 U/mL calmodulin (□), or 0.25 μM cAMP and 1 μM cGMP (△). (B) Fractions were assayed with 0.25 μM cGMP (●). Each point is a mean of duplicate experiments.

shown). The K_i values were estimated from Dixon plots [19] (Table 1).

Effects of GA and its adenine base-substituted derivatives on PDEs from 3T3 cells

Cytosolic fraction from 3T3 cell homogenate was applied to a DEAE-5PW column and eluted with a linear sodium acetate gradient. As shown in Fig. 3, cAMP PDE activities were separated into three major peaks. The profile of these activities was not altered by the presence of 1 μM cGMP. Cyclic GMP PDE activity was detected only in the first peak fractions and both cAMP PDE and cGMP PDE activities in this peak were enhanced by calmodulin: cGMP PDE activity was activated 4-fold, whereas cAMP PDE activity was activated only 2-fold, by 10 U/mL of calmodulin. The hydrolysis of cAMP and cGMP by peak 1 PDE isozyme and that of cAMP by peak 2 and 3 PDE isozymes followed linear Michaelis–Menten kinetics, and K_m values were estimated from Lineweaver–Burk plots. The peak 1 PDE had a higher affinity for cGMP than cAMP, the K_m values for cGMP and cAMP in the presence of 10 U/mL of calmodulin being 4.7 and 17 μM, respectively (Table 1). The PDE isozymes of peaks 2 and 3 are cAMP specific, and their K_m values for cAMP were 7.3 and 3.3 μM, respectively (Table 1).

GA and its derivatives whose adenine group was substituted with a guanine group or a hypoxanthine group were tested for their inhibitory activity on the PDE isozymes from 3T3 cells. GA, G-GA, H-GA and IBMX exhibited competitive inhibition with regard to cAMP or cGMP on all PDE isozymes tested (data not shown). The K_i values were estimated from Dixon plots [19] and are summarized in Table 1. For peak 1 PDE isozyme, the K_i value of GA was about 0.5 μM, and those of H-GA and G-GA were much lower, being 0.10 and 0.075 μM, respectively. In contrast, for peak 2 and 3 PDE isozymes, the K_i values of GA were 0.043 and 0.021 μM, respectively, and those of H-GA and G-GA were much higher; between 11 and 43 μM.

Under the same conditions, inhibitory effects of RO 20-1724 and milrinone on the PDE isozymes from 3T3 cells were investigated. The IC_{50} values of these inhibitors for peak 1, 2 and 3 PDE isozyme are summarized in Table 2.

Synthesis and properties of membrane-permeable GA derivatives

Esters of the carboxyl groups of GA were synthesized, to increase membrane permeability, and their effects on cAMP accumulation in 3T3 cells were investigated. GA diPOM ester was found to increase the cAMP accumulation at 1 μM, while GA and IBMX were active only at higher concentrations, over 100 μM (Fig. 4). In the time course studies, GA diPOM ester at 10 μM increased markedly the cAMP accumulation, but GA, GA dimethyl ester and IBMX failed to increase the cAMP level under the same conditions (Fig. 5). Forskolin at 1 μM increased the cAMP accumulation more rapidly than GA diPOM ester when it was applied with 0.5 mM

Table 2. IC_{50} values of inhibitors for PDE isozymes from 3T3 cells

Peak No.	IC_{50} (μM) RO 20-1724	Milrinone
1	>100	100
2	10	44
3	5.6	26

PDE activities were assayed at 30° for 10 min as described in Materials and Methods. In the assay of peak 1 PDE isozyme, the buffer was further supplemented with 10 U/mL calmodulin. IC_{50} values were determined with 0.25 μM cAMP as substrate from the mean of duplicate experiments.

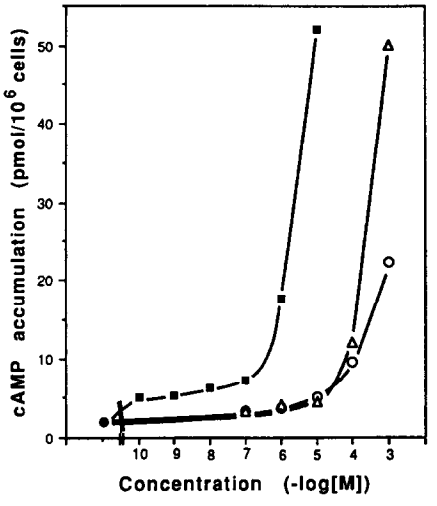


Fig. 4. Concentration–response curve for cAMP accumulation in 3T3 cells. The 3T3 cells were incubated for 10 min with increasing concentrations of GA (○), GA diPOM ester (■) or IBMX (△), in the presence of 1 U/mL adenosine deaminase as described in Materials and Methods. Each point is the mean of duplicate experiments.

IBMX, while it was less potent than GA diPOM ester without IBMX (Fig. 5).

Inhibitory activities of GA diPOM ester and dimethyl ester on three PDE isozymes from 3T3 cells were investigated, and their inhibitory activities were compared with those of GA. The inhibitory activities of these esters of GA were much lower than GA: the IC_{50} values of diPOM ester and dimethyl ester of GA for peak 1–3 PDE isozymes were greater than 10 μM with 0.25 μM cAMP as substrate.

Since the GA esters showed much lower activities of PDE inhibition than GA, enzymatic hydrolysis of GA esters by esterase of 3T3 cells was investigated using water-soluble monoesters. When GA mono-POM ester at 100 μM was incubated with the supernatant fraction of homogenate of 3T3 cells, 75% of the ester was converted to GA within 30

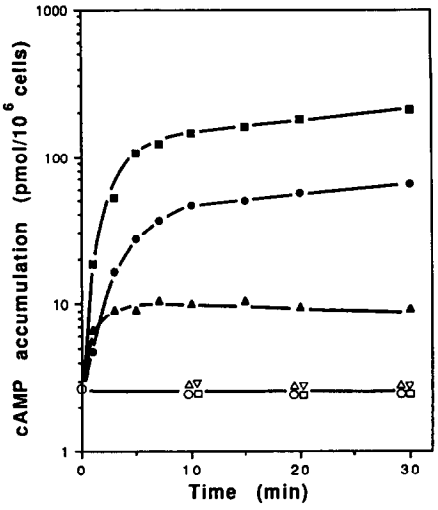


Fig. 5. Time course of cAMP accumulation in 3T3 cells. The 3T3 cells were incubated for the times indicated with 10 μM GA (△), 10 μM GA diPOM ester (●), 10 μM GA dimethyl ester (▽), 10 μM IBMX (□), 1 μM forskolin (▲), 1 μM forskolin and 0.5 mM IBMX (■) or vehicle (○), in the presence of 1 U/mL adenosine deaminase as described in Materials and Methods. Each point is the mean of duplicate experiments.

min. Under the same conditions, GA monomethyl ester was not hydrolysed at all. Therefore, the diPOM ester of GA seems to exert its activity after conversion to GA by esterase in the cells.

Effect of GA diPOM ester on platelets and adipocytes

Investigation was made on the effects of GA diPOM ester on rabbit washed platelets. As shown in Fig. 6, GA diPOM ester at 10 μM caused an accumulation of cAMP and completely inhibited thrombin-induced aggregation of platelets. GA and IBMX at the same concentration neither caused an accumulation of cAMP nor inhibited the aggregation. Even at 500 μM GA showed no effects on the platelets.

The effects of GA diPOM ester on cAMP accumulation and lipolysis in rat isolated adipocytes were also investigated. GA diPOM ester at 10 μM caused an accumulation of cAMP (Fig. 7A) and stimulated glycerol production, i.e. lipolysis (Fig. 7B). GA and IBMX at the same concentration, 10 μM, neither caused an accumulation of cAMP nor stimulated glycerol production. In contrast with 3T3 cells, cAMP accumulation reached a maximum after 15 min incubation in adipocytes, but glycerol production was still increasing at 30 min.

DISCUSSION

In the present study, GA analogues whose adenine group was substituted with a guanine or hypoxanthine group were tested for their inhibitory activity on PDE isozymes from bovine retina and 3T3 fibroblast cells. As a result, GA more potently inhibited cAMP-specific PDE than G-GA and H-GA. In

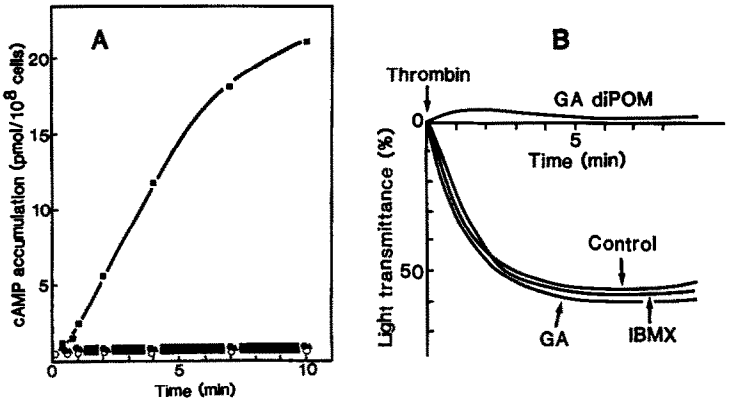


Fig. 6. Time course of cAMP accumulation in rabbit washed platelets (panel A) and the inhibition of aggregation (panel B). In panel A, the platelets were incubated for the time indicated with 10 μM GA (●), 10 μM GA diPOM ester (■), 10 μM IBMX (▲) or vehicle (○), as described in Materials and Methods. Each point is the mean of duplicate experiments. In panel B, platelets were preincubated at 37° for 3 min with 10 μM GA, 10 μM GA diPOM ester, 10 μM IBMX or vehicle. Then thrombin (0.5 U/mL) was added and the change in light transmittance was recorded as described in Materials and Methods.

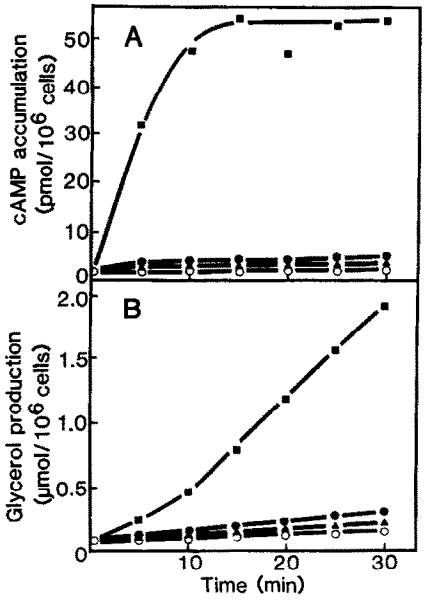


Fig. 7. Time course of cAMP accumulation (panel A) and glycerol production (panel B) in rat isolated adipocytes. Rat isolated adipocytes were incubated at 37° for the times indicated with 10 μM GA (●), 10 μM GA diPOM ester (■), 10 μM IBMX (▲) or vehicle (○), in the presence of 1 U/mL adenosine deaminase as described in Materials and Methods. Each point is the mean of duplicate experiments.

contrast, G-GA and H-GA inhibited cGMP-specific PDE more potently than GA. In particular, the cGMP PDE activity in ROS of the retina was inhibited by G-GA at the order of 0.01 μM. Therefore, it is apparent that the adenine or guanine group of these compounds is involved in the selective inhibition of these cyclic nucleotide PDEs. A number of selective inhibitors of various PDE isozyme families have been reported, and all of these isozyme selective inhibitors exhibit at least 20-fold selectivity as inhibitors of a particular isozyme family [20]. In contrast, GA and G-GA seem to be unique base-selective inhibitors of type IV PDE of 3T3 cells and type V PDE of bovine retinas, respectively. GA and G-GA will be useful tools for the investigation of cyclic nucleotides and PDE isozymes in various biological systems.

PDE isozymes have been isolated from many tissues and cells and classified into types I to V by their properties [20–22]. In the present study, three isozyme peaks of PDE activity were isolated from cytosolic fraction of 3T3 cells. Peak 1 isozyme was Ca^{2+}/calmodulin-dependent and had higher affinity for cGMP than for cAMP; its K_m for cGMP was below one-third that for cAMP. This peak seems to be of the type I family (Ca^{2+}/calmodulin-dependent family) [20–22]. Peak 2 and 3 isozymes had high affinity for cAMP, their K_m values being 7.3 and 3.3 μM, respectively, and their activities were not altered by cGMP. RO 20-1724, which is a specific inhibitor of cAMP-specific PDE (type IV) [20], inhibited these isozymes with micromolar potencies. Furthermore, milrinone, which specifically inhibits cGMP-inhibited PDE (type III) at submicromolar concentrations [20], had only slight inhibitory effects. Therefore, peak 2 and 3 isozymes of PDE seem to belong to isozymes of the cAMP-specific (type IV) family. In the cytosolic fraction of 3T3 cell homogenates, type II cGMP-activated PDE activity, type III cGMP-inhibited PDE activity and type V cGMP-specific PDE activity were not detected.

Esters of the carboxylic acids of GA were synthesized and tested for their activity in 3T3 fibroblast cells. As a result, the diPOM ester of GA

was found to increase the cAMP level at as low a concentration as 0.1 μM. These results may be explained by the difference in membrane permeability between GA and its diPOM ester: the diPOM ester of GA can readily cross the cell membrane, but GA cannot cross it easily. The diPOM ester of GA is though to exert its activity after conversion to GA by esterase in cells for the following reasons: (1) the diPOM ester of GA was much less potent than GA in inhibition of PDE, but the POM ester of GA was easily hydrolysed to GA by esterase in the supernatant fraction of 3T3 cell homogenate; and (2) dimethyl ester of GA was also less potent in inhibition of PDE than GA and the methyl ester was not hydrolysed by esterase. In accordance with these findings, the dimethyl ester of GA cannot increase the cAMP level in 3T3 cells. The acyloxymethyl esters are used to enhance intestinal absorption of β-lactam antibiotics [23] or to load calcium indicator Quin II into cells [24]. The accepted mechanism of the POM ester hydrolysis is enzymatic cleavage of the acyl ester linkage, followed by rapid spontaneous release of formaldehyde [25]. The reason that the methyl ester of GA was not hydrolysed by esterase may be explained by steric hindrance of the GA ester toward the attack of the enzyme.

In rabbit washed platelets, the diPOM ester of GA showed an accumulation of cAMP and inhibition of aggregation. In rat adipocytes, the diPOM ester of GA enhanced accumulation of cAMP and stimulated lipolysis and the inhibition of type III PDE in platelets [26] or adipocytes [27] contributes respectively to the inhibition of aggregation or to the stimulation of lipolysis. In the preliminary study, the IC_{50} of GA for type III PDE from canine ventricular myocardium was 0.028 μM with 0.25 μM cAMP as substrate.* Thus, the effects of GA on platelets or adipocytes may include the inhibition of type III PDE. Furthermore, Mishima *et al.* [28] reported that the monoPOM ester of GA lowered intraocular pressure of rabbits with topical administration. Since forskolin has been reported to lower the intraocular presure of rabbits [29], the intraocular pressure lowering effect of GA ester seems to be associated with an increase of the cAMP level. GA analogues may be useful as anti-glaucoma agents or as pharmacological tools.

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