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PHLORETIN AND RELATED COMPOUNDS INHIBIT AGONIST STIMULATED cAMP ACCUMULATION IN CULTURED CELLS OF CNS ORIGIN

RAINER ORTMANN,* DORIS NUTTO and JÜRG WALDMEYER†

Pharmakologisches Institut der Universität Freiburg, Hermann-Herder Str. 5, D-78 Freiburg i.B., West-Germany and †Institut für Physik der Universtät Basel, Klingelbergstr. 82, CH-4056 Basel, Switzerland

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Abstract—The dihydrochalcone phloretin, related flavonoids and diethylstilbestrol inhibit agonist induced cAMP accumulation with different potencies in human astrocytoma cells 1321N1 and murine neuroblastoma cells N4TG3. The inhibition is not agonist specific, abolished by glycosidation of the inhibitor, irreversible by washing, calcium independent and increases with time during a preincubation period. Measurement of dipole moments and estimation of lipid solubility of the inhibitors indicate that lipophilicity is important for their inhibitory potency. The structure–activity relationship of different inhibitors resembles their inhibitory potency in the hexose transport system of red blood cells.

The dihydrochalcone phloretin and its glucoside phlorizin are reversible inhibitors of various transport processes across membranes [1–3]. Both compounds inhibit with different potencies sugar, urea, glycerol and ion transport systems. Phosphorylated polymers of phloretin were originally synthesized as enzyme inhibitors [4] and were later shown to possess anti-prostaglandin activity in various smooth muscle preparations [5–9]. During our studies of the effects of phosphorylated derivatives of phloretin on the adenylate cyclase system of cultured cells *in vitro* [10] we found that phloretin itself had an inhibitory effect on agonist stimulated cAMP accumulation in human astrocytoma cells 1321N1 and the murine neuroblastoma cell line N4TG3. In order to elucidate the difference in potency and agonist specificity of the inhibitory actions of phloretin and its phosphorylated derivatives, we studied the inhibitory effect of phloretin and related molecules in these 2 cell lines. Our data suggest that these substances inhibit cAMP accumulation not by specific interaction with agonist receptor sites but by interacting with membrane lipids.

MATERIALS AND METHODS

Materials. All the flavonoids were purchased from Roth, Karlsruhe, West-Germany. Phlorizin, phloroglucinol, diethylstilbestrol, D,L-isoproterenol HCl were from Sigma, München. PGE_1 was a gift from Dr. J. Pike, Upjohn Comp., MI.

Cell culture conditions. The isolation and growth of the human astrocytoma cell line 1321N1 [11–13] and the murine neuroblastoma cell line N4TG3 [14, 15] have been described. 1321N1 Cells were from R. B. Clark, Worcester, MA, N4TG3 cells from B. Hamprecht, München. 35 mm plastic dishes ("Lux") were seeded with 1×10^5 (N4TG3) or 2×10^5 1321N1 cells in 2 ml Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum and grown for 3 days.

Experimental incubation conditions. For experiments the growth medium was aspirated, the dishes washed twice with serum-free medium and challenged for 5 minutes with agonists in 1 ml serum-free growth medium containing 1 mM isobutyl-methylxanthine (IBMX) or 0.1 mM papaverine. Incubations were stopped by aspirating the medium and adding 1 ml 5% trichloroacetic acid (TCA). All inhibitors were dissolved in ethylenglycolmonoethylether, diethylstilbestrol in ethanol, prostaglandin E_1 (PGE_1) in 10% ethanol, D,L-isoproterenol and adenosine in 0.001 N HCl. Agonists and antagonists were diluted 100-fold into the incubation medium. cAMP was determined by radioimmunoassay as described elsewhere [16]. At the highest concentrations used none of the antagonists interfered with the determination of cAMP. Solvents were routinely added to control dishes, but had no significant effect on cAMP levels of either cell line.

Determination of dipole moments. Static dielectric constants were determined at room temperature by direct reflection time domain spectroscopy in dioxane (0.1 M solution) [17–21]. This method yielded the dielectric spectra in the frequency range between 100 MHz ard 1 GHz. The real part of the permittivity in this frequency band was extrapolated to the static value. Refractive indices were measured in an Abbé refractometer. The dipole moments were derived using the equation of Halverstadt and Kumler [20, 21]. In this procedure the atomic polarization was supposed to be negligible compared to the electronic polarization. The uncertainty of the dipole moments is mainly characterized by the standard deviation of the averaged dielectric constants due to small unwanted reflections from the discontinuities in the coaxial line system.

Estimation of the paraffin/water partition coefficients. Relative paraffin/water partition coefficients

* Send offprint requests to R. Ortmann. Present address: Research Department, Pharmaceutical Division, Ciba-Geigy, CH-4002 Basel, Switzerland.

were determined by thin layer chromatography on paraffin coated Kieselgur plates, using different water/methanol mixtures as solvent systems [22]. The solvent system with the lowest concentration of methanol which generated R_f-values greater than 0.5 was determined for each substance and used as an indication of its lipid solubility.

RESULTS

Inhibition of PGE_1 effects on cAMP accumulation

cAMP accumulation of 1321N1 cells is stimulated by prostaglandin E_1 (1140 pmoles cAMP/mg protein/5 min at 100 μM), catecholamines (2200 pmoles cAMP/mg protein/5 min at 100 μM isoproterenol) and adenosine (330 pmoles cAMP/mg protein/5 min at 330 μM) by 3 independent receptors [13, 23, 24]. cAMP levels of N4TG3 cells are increased by prostaglandins (1200 pmoles cAMP/mg protein/5 min with 100 μM PGE_1) and adenosine (600 pmoles cAMP/mg protein/5 min at 300 μM) [15]. Basal levels of cAMP were 20–24 pmoles/mg protein in both cell lines. All incubations were done at half-maximal concentrations of the agonists. Figure 1 depicts dose–response curves of the dihydrochalcone phloretin, the flavonols quercetin and dihydroquercetin, the flavone apigenin and the flavanone naringenin in the presence of PGE_1 in both cell lines. There are significant differences in the inhibitory potency of the tested compounds in the 2 cell lines: While phloretin, quercetin and apigenin are equipotent in 1321N1 cells, phloretin is more potent that quercetin or apigenin in N4TG3 cells. This difference is most obvious for apigenin which is a good inhibitor in the 1321N1 line ($IC_{50} = 110 \pm 30$ μM) but does almost not inhibit cAMP accumulation in N4TG3 cells at the highest concentration tested (300 μM). In addition naringenin is a better inhibitor of PGE_1 effects than apigenin in the neuroblastoma cells, while the reverse is true in 1321N1 cells.

Agonist specificity of the inhibitors

Table 1 summarizes the potencies of different inhibitors to inhibit cAMP accumulation stimulated by PGE_1, isoproterenol and adenosine. While the stimulation of cAMP accumulation by PGE_1 and adenosine was affected by the same inhibitor concentration in both cell lines, isoproterenol effects in 1321N1 cells were inhibited only by 2–4 times higher concentrations of the inhibitors. However the sequence of inhibitory potency was the same for different agonists in both cell lines. Phloretin at 300 μM did not significantly decrease basal cAMP levels in 1321N1 and N4TG3 cells.

Effect of glycosidation on inhibitory potency

Table 2 shows the effect of phlorizin (phloretin-2′-β-glucoside), rutin (quercetin-3-rutinoside) and apiin (apigenin-7-apiosylglucoside) on PGE_1-stimulated cAMP accumulation in both cell lines. All 3 glycosides are virtually devoid of inhibitory activity. The same results were obtained when these compounds were tested in the presence of isoproterenol or adenosine.

Effects of other structurally related compounds

The results summarized in Table 3 indicate that diethylstilbesterol was a more potent inhibitor of PGE_1 stimulated cAMP accumulation than phloretin in both cell lines. Both phloroglucinol and phloroacetophenone did not significantly inhibit at 1 mM concentration; phloroacetophenone was more potent than phloroglucinol at 10 mM.

Characterization of phloretin inhibition

1. *Onset and reversal of inhibitory action of phloretin.* The inhibitory effect of 100 μM phloretin on PGE_1 stimulated cAMP accumulation increased during a 30 min preincubation period without PGE_1 (data not shown) and was almost irreversible by washing the cells after this preincubation period. When 1321N1 cells

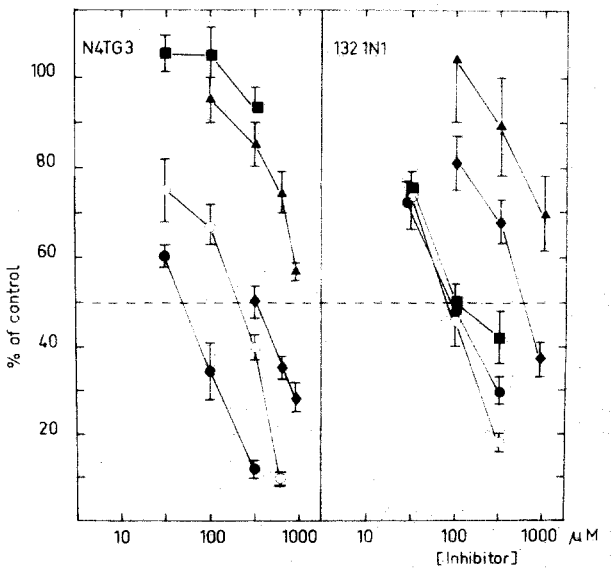

Fig. 1. N4TG3 cells (left) were incubated with 0.03 μM PGE_1 1321N1 cells with 1 μM pGE_1 and the indicated concentrations of phloretin (●——●), quercetin (○——○), dihydroquercetin (▲——▲), apigenin (■——■) and naringenin (◆——◆). Data represent the mean of 3 dishes assayed in duplicates ± S.D.

Table 1. Effect of various inhibitors on cAMP accumulation stimulated by PGE_1, isoproterenol and adenosine in N4TG3 and 1321N1 cells

	IC_{50} (μM)				
	1321N1	N4TG3	1321N1	1321N1	N4TG3
	PGE_1		Isoproterenol	Adenosine	
	1 μM	0.03 μM	0.1 μM	50 μM	20 μM
Phloretin	160 ± 90 (4)	120 ± 70 (6)	400 ± 100 (3)	130 ± 60 (3)	380 ± 150 (4)
Apigenin*	110 ± 30 (4)	> 300 (3)	300 ± 10 (3)	60 (2)	> 300 (2)
Naringenin	460 ± 110 (5)	530 ± 170 (4)	900 ± 100 (3)	450 (2)	600 ± 100 (3)
Quercetin	80 ± 20 (5)	300 ± 80 (4)	300 ± 100 (3)	60 (2)	500 ± 200 (3)
Dihydroquercetin	> 900 (2)	> 900 (2)	> 900 (3)	> 900 (3)	> 900 (2)

Cells were incubated as described in experimental procedure. IC_{50} values were graphically determined from dose–effect curves of the inhibitors in the presence of the indicated agonist concentrations. Results represent means ± S.D. when $n \geqslant 3$, with n (number of experiments) in parentheses.

* 300 μM represents the highest concentration soluble in the incubation medium.

Table 2. Glycosidation abolishes inhibitory potency of phloretin, quercetin and apigenin

		% of control	
Inhibitor	(μM)	1321N1 PGE_1 (1 μM)	N4TG3 PGE_1 (0.03 μM)
Phlorizin (phloretin-2′-β-glucoside)	300	100	98
	900	81,87	90
Rutin (quercetin-3-rutinoside)	300	91	102
	900	84	91
Apiin (apigenin-7-apiosylglucosid)	300*	93	88 ± 8

Cells were incubated under standard conditions in the presence of the indicated concentrations of PGE_1 and the inhibitors. Results are the average of 2 dishes, or means ± S.D. of 3 dishes.

* Highest concentration soluble in the incubation medium.

Table 3. Effect of phloroglucinol, phloroacetophenone and diethylstilbestrol on PGE_1-stimulated cAMP accumulation

		% of control	
Inhibitor	(mM)	1321N1 (PGE_1 1 μM)	N4TG3 (PGE_1 0.03 μM)
Phloroglucinol	1	124	87 ± 12, 82, 82, 101
	10	73 ± 5, 82	51 ± 10, 70, 64, 83
Phloroacetophenone	1	99	78 ± 8, 97, 73
	10	48	22 ± 4, 38, 37, 45
Diethylstilbestrol	0.01	87 ± 5	61 ± 10
	0.03	45 ± 10, 40 ± 4	14 ± 1

Incubation of cells under standard conditions. Phloroglucinol and phloroacetophenone were directly dissolved in the incubation medium. Results are the average of 2 dishes or means ± S.D. of 3 dishes. Results of different experiments are given.

Table 4. Phloretin inhibition of PGE_1 effects is dependent on the pH of the incubation medium

Phloretin (μM)	% of control ($\bar{x} \pm$ S.D.) pH 6.0	pH 7.3	pH 8.3
100	54 ± 4 (95)	70 ± 6 (50)	75 ± 5 (11)
190		57 ± 5 (95)	

1321N1 cells were incubated for 5 min in Hepes-buffered (20 mM) incubation medium adjusted to the indicated pH. Number in parentheses indicate concentration (μM) of undissociated phloretin. $n = 3$.

were preincubated for 30 min with 100 μM phloretin, then washed 3 times with 2 ml serum-free incubation medium and challenged with 1 μM PGE_1 cAMP accumulation was only 21 ± 4% of that of control dishes which received no phloretin during the preincubation period. When 100 μM phloretin was present only during the incubation with PGE_1 cAMP accumulation was 25 ± 4% of control dishes. These results are in marked contrast to the reported reversibility of phloretin effects on ion transport [3] and to the results reported for phosphorylated derivatives of phloretin [10].

2. *Effects of calcium and of the pH of the incubation medium.* Un-ionized phloretin was shown to be bound to the red blood cell membrane (2, 25). Since phloretin is a weak acid (pK = 7.3), its inhibitory effect should be greater at low pH if uncharged phloretin is responsible for its inhibitory activity in the adenylate cyclase system. Table 4 indicates that the inhibition of PGE_1-stimulated cAMP accumulation by phloretin is indeed higher at pH 6.0 (95% uncharged) than at pH 7.3 (50% uncharged) or at pH 8.3 (11% uncharged). The degree of inhibition therefore seems to be dependent on the concentration of uncharged phloretin. Omission of calcium from the incubation medium did not alter the inhibitory effect of 100 μM phloretin. The same results were reported for the inhibitory action of the phosphorylated derivative of phloretin: di-4-phloretinphosphate [10].

Dipole moments and estimation of lipid solubility

The dipole moments (measured in dioxane) and the lipophilicity of different compounds (estimated by thin layer chromatography on paraffin coated Kieselgur plates) are summarized in Table 5. While the dipole moments of the flavonoids do not vary significantly, there are great differences in their lipid solubility: Inhibitors with a C_2—C_3 double bond (quercetin, apigenin) are much more lipophilic than their dihydro-derivatives (dihydroquercetin, naringenin). Together with dihydroquercetin, the glycosides rutin and phlorizin were the least lipid soluble substances. Addition of HCl to the solvent system did not change the R_f values found with solvents of pH 7.

DISCUSSION

Comparison of the effects of phloretin and di-4-phloretinphosphate

Although there are several reports on the prostaglandin inhibiting activity of phosphorylated derivatives of phloretin [5–9], few reports dealt with the inhibition of prostaglandin effects by phloretin itself. Eakins found [8] that in an isolated jird colon preparation phloretin and phlorizin were 20–40 times less active than polyphloretinphosphate (PPP) and not prostaglandin-specific. We reported that phloretin was 9 and 30 times less potent than di-4-phloretinphosphate (DPP) to inhibit PGE_1-stimulated cAMP accumulation in 1321N1 and N4TG3 cells, respectively [10]. The data of this report confirm that all tested inhibitors exert no prostaglandin specific inhibition, since adenosine and isoproterenol effects are inhibited in the same concentration range of the inhibitors (Table 1). In addition to these differences between DPP and phloretin we show here that in contrast to the results reported for DPP [10] the inhibitory effect of phloretin is essentially irreversible by washing after a 30-min preincubation period. Since we did not study uptake or binding of phloretin to the cells under study we do not know to what extent these processes contribute to the kinetic pattern observed. A very fast, nonsaturable and reversible binding of phloretin to membranes of red blood cells has been demonstrated [25].

Table 5. Dipole moments and estimated paraffin/water partition coefficients of the inhibitors tested.

Inhibitor	Estimate of paraffin/water partition coefficient: % methanol of the solvent which produces R_f greater than 0.5	Dipole moment μ (D) ± S.D.
Rutin	0	n.d.
Phlorizin	0	n.d.
Dihydroquercetin	0	5.6 ± 0.2
Naringenin	20	4.9 ± 0.3
Phloretin	30	5.4 ± 0.3
Apiin	60	n.d.
Apigenin	60	n.d.
Quercetin	60	5.2 ± 0.3
Diethylstilbestrol	60	3.8 ± 0.4
Phloroglucinol	n.d.	3.8 ± 0.5

Dipole moments and paraffin/water partition coefficients were determined as described in experimental procedure. Methanol concentrations were increased by steps of 10%. n.d. = not determined.

Comparison of phloretin effects on the cAMP system and the hexose transport system of red blood cells

The hexose transport system of red blood cells has been studied in detail by LeFevre [1, 2]. The following properties of phloretin inhibition of the hexose transport system of red blood cells and of adenylate cyclase activity in 1321N1 and N4TG3 cells are very similar: (i) glycosidation of phloretin (quercetin, apigenin) abolishes all inhibitory activity; (ii) diethylstilbestrol is more potent and the flavanone naringenin less potent than phloretin; (iii) the inhibition is pH-dependent and omission of calcium or addition of calcium chelating agents has no effect; and (iv) phloroglucinol or phloroacetophenone are very weak inhibitors. In addition we show here that the inhibitory effect on the adenylate cyclase system is not restricted to the phloretin molecule since quercetin and apigenin are as potent inhibitors as phloretin in 1321N1 cells. The significant differences in the inhibitory potencies of the tested compounds (Fig. 1) between the two cell lines studied show that the inhibitors do not interact with the same binding site in both cell types.

Are dipole moments of the inhibitors important?

Andersen *et al.* [3] assumed that due to its large dipole moment phloretin could alter the orientation or conformation of molecules mediating the transport of molecules across biological membranes or lipid bilayers. They found a correlation between the dipole moments of several compounds structurally related to phloretin and their ability to influence ion transport across lipid bilayers and the human red blood cell membrane. Our result suggest that the observed inhibition of cAMP accumulation by phloretin is caused by a different mechanism: Dipole moments of aglycones with different inhibitory potency did not vary significantly and the dipole moments of the most potent inhibitor diethylstilbestrol and the almost inactive phloroglucinol were essentially the same (Table 5). Furthermore, diethylstilbestrol was shown to have no inhibitory activity on the transport of ions across lipid bilayers and the red blood cell membrane [3], phlorogulcinol (10 mM) being inactive and phloroacetophenone having a strong inhibitory activity at a 10 times higher concentration than the maximal phloretin concentration (250 μM). We found that diethylstilbestrol is even more potent than phloretin and the inhibitory activity of phloroglucinol and phloroacetophenone differed only at the highest concentration tested (10 mM).

The interaction with membrane lipids

Jenning *et al.* [25] described a high affinity ($K_{Diss} = 1.5\ \mu M$) and a low affinity binding site ($K_{Diss} = 54\ \mu M$) for the binding of phloretin to ghosts of red blood cells. Assuming a similar situation for the cell lines used for this study we could ascribe the inhibitory effect of phloretin and of the other inhibitors to an interaction with the low affinity binding site, representing the membrane lipids in Jennings model [25]. Membrane lipids have been shown to be of essential importance for the hormone stimulation of the adenylate cyclase system in a variety of systems [26]. The estimated paraffin/water partition coefficients of various inhibitors support this hypothesis: A certain degree of lipid solubility of a given inhibitor seems to be necessary for inhibitory activity, since all compounds with R_f values greater than 0.5 in pure water as solvent system are very poor inhibitors (Table 5). However, lipid solubility alone is not sufficient for inhibitory activity, since apiin is as lipid soluble as the potent inhibitors quercetin or diethylstilbesterol but inactive as inhibitor of PGE_1-stimulated cAMP accumulation (compare Tables 2 and 5). Therefore lipophilicity seems to be an important requirement for inhibitory activity of phloretin and related molecules, but not the only parameter which governs their structure–inhibitory activity relationship. This is supported by the finding that the sequence of potencies of the tested inhibitors is different in the 2 cell lines studied.

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