0026-895X/91/060740-05$03.00/0

MOLECULAR PHARMACOLOGY, 39:740-744

Potent G_i-Mediated Inhibition of Adenylyl Cyclase by a Phosphonate Analog of Monooleylphosphatidate

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Received November 26, 1990; Accepted March 14, 1991

SUMMARY

We have previously demonstrated that monooleylphosphatidate (MOPA) and phosphatidate inhibit adenylyl cyclase in cultured fibroblasts. In this study, the specificity of the phospholipid effect was probed by analysis of the effect of phosphonate analogs of these phospholipids on adenylyl cyclase in C6 glioma cells. The MOPA phosphonate analog inhibited adenylyl cyclase, but the comparable phosphonate analog of phosphatidate was ineffective. The IC_{50} for inhibition of adenylyl cyclase by the MOPA phosphonate analog was similar to that of MOPA, the maximal inhibitions were comparable (approximately 45% inhibition of hormone-stimulated adenylyl cyclase), and the effects of both appeared to be mediated by G_i, because treatment with islet-activating protein reduced the inhibition to 5–10%.

There is extensive evidence that lipids are important regulators of numerous cell processes and second messengers (1–3). Neurotransmitters and hormones activate phospholipase C, which increases both the turnover of inositol lipids in a variety of cells and the production of at least two important second messengers, inositol trisphosphate, which has a role in regulating intracellular concentrations of calcium, and diacylglycerol, which stimulates protein kinase C. It also has been well documented that lipids such as platelet-activating factor and prostaglandins regulate second messenger production through direct binding to and activation of membrane-bound receptors.

In addition to these examples, we and others have shown that phosphatidate and MOPA inhibit cAMP accumulation in cultured fibroblasts (4–9), rat-1 cells (9), and hamster adipocytes (10) and, at least in fibroblasts, the inhibition is caused by a decrease in adenylyl cyclase activity (6–8). The effect on adenylyl cyclase resembles hormonal inhibition of this enzyme, because it is GTP dependent and enhanced by low concentrations of Mg^{2+} (6). Furthermore, in fibroblasts and rat-1 cells the inhibitory effects of phospholipids appear to be mediated by the inhibitory coupling protein G_i (6–9, 11), because IAP treatment reduces MOPA and phosphatidate inhibition of adenylyl cyclase to barely discernable levels. These observations, combined with extensive data demonstrating that hormonal stimulation of various cell types leads to an increase in phosphatidate that occurs either through activation of phospholipase C coupled with diacylglycerol kinase or through activation of phospholipase D, which directly increases phosphatidate (12–14), suggest that the inhibition of adenylyl cyclase by phosphatidate and lysophosphatidate may be of physiological significance.

The increasing evidence for phospholipid involvement in the regulation of calcium levels, cAMP metabolism, protein kinase C, and numerous other membrane-associated functions encouraged us to further examine the specificity of the effects of phosphatidate and MOPA, through the use of phosphonate analogs. In addition, the relative stability of the phosphonate analogs, as previously shown by Pagano and Longmuir (15) and Shashidhar *et al.* (16) using phosphonate analogs of phosphatidate and phosphatidylinositol, suggested to us that they could indicate whether dephosphorylation (i.e., conversion of phosphatidate and MOPA to diacyl- and monoacylglycerides, respectively) was required.

We report here that a phosphonate analog of MOPA inhibited both cAMP accumulation and adenylyl cyclase in rat C6 glioma cells, with an IC_{50} similar to that of MOPA. The phosphonate analog of phosphatidate, on the other hand, was ineffective. Because the inhibition required GTP and treatment of cells with IAP attenuated the inhibition, mediation by G_i seems likely. The data also demonstrate the potential usefulness of phosphonate analogs for evaluating phospholipid function.

Materials and Methods

Cell culture. Stock cultures of rat C6 glioma cells maintained in T-75 (Falcon) flasks were grown in a humidified 95% air/5% CO_2 atmos-

This work was supported in part by National Institutes of Health Grant RO1 DK26943.

ABBREVIATIONS: MOPA, monooleylphosphatidate; IAP, islet-activating protein; INE, isoproterenol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

phere, at 37°, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Flow Laboratories) and penicillin-streptomycin (each 100 units/ml). For intact cell studies, the cells were seeded into 35-mm dishes at a concentration of 1.75–2 × 10^5 cells/dish. For broken cell studies, they were seeded into 150-mm dishes at a concentration of 10–12 × 10^6 cells/dish. The cells were used 3–4 days later, when they had reached confluence, with one medium change at approximately 24 hr before the experiment. In the pertussis toxin studies, IAP (100 ng/ml) or vehicle (17) was added to the cells 24 hr before the experiment.

Adenylyl cyclase assay. Adenylyl cyclase was measured by the method of Salomon *et al.* (18). Unless noted otherwise, the assay mixture contained 2 mM $MgCl_2$, 1 mM EDTA, 40 mM HEPES, 8 mM creatine phosphate, 16 units/ml creatine phosphokinase, 0.2 mM ATP, 0.5 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, approximately 2 μCi of [α-^{32}P]ATP, and 60 mM NaCl. The assay was initiated by the addition of 10–30 μg of protein, and a 10-min incubation at 30° was routinely used.

Broken cell studies. Adenylyl cyclase was measured in C6 membranes prepared by the following procedure. The growth medium was removed, and the cells were rinsed three times with a buffer containing 2 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 0.2 mM dithiothreitol (TED). The rinsed dishes were placed briefly in a bath of liquid nitrogen and then removed and stored (5–20 min) at −20° until time of cell harvest. Cells were harvested in the cold (4°) with a rubber policeman. Once collected, the cells were suspended in TED buffer, dispersed with a Pasteur pipette, and homogenized with a Teflon pestle. This cell lysate was layered over a gradient of 43% and 25% sucrose and centrifuged at 100,000 × *g* for 30 min. The membrane layer at the 25%/43% interface was removed, diluted 1:5 with TED buffer, and centrifuged at 45,000 × *g* for 15 min. The pellet was washed once with TED buffer and was then resuspended in TED buffer, 0.3 M sucrose, at a concentration of 2–4 mg/ml. This final pellet was frozen in liquid nitrogen and stored at −80° until time of the assay.

Intact cell studies. The effects of various compounds on cAMP accumulation were determined by a previously described prelabeling technique (19). Briefly, the ATP pool was prelabeled with [2,8-^{3}H] adenine, and then the percentage of conversion of [^{3}H]ATP to [^{3}H] cAMP was calculated by the equation (cAMP)×100/(AXP + cAMP), where AXP is ATP + ADP.

Materials. Phosphatidylinositol was purchased from Sigma. All other lipids were from Serdary Research Laboratories, and they were dispersed in water, before use, by sonication. IAP was a generous gift from Dr. Michio Ui and Dr. Toshiaki Katada (Hokkaido University, Sapporo, Japan). The phosphonate analogs were kindly provided by Dr. Burton Tropp and Dr. Robert Engel (Queens College, Flushing, New York). The structures of the phosphonate analogs are shown in Fig. 1, and the methodology for the synthesis of phosphonate analogs A and B has been published (20).

Results

Specificity of phospholipid inhibition of adenylyl cyclase activity. Phosphatidate and MOPA inhibited INE-stimulated adenylyl cyclase activity in C6 membranes, and, as was previously found with cultured fibroblasts, the inhibition did not appear to be a nonspecific effect of lipids in general. At a concentration of 10 μM, MOPA and phosphatidate attenuated INE-stimulated adenylyl cyclase activity by 58 and 40%, respectively. The same concentration of structurally similar lipids (monooleyphosphatidylcholine, dioleylphosphatidylcholine, 1,2-diolein, phosphatidylinositol, and platelet-activating factor) caused inhibitions no greater than 16% (data not shown).

To further define specificity and determine whether conversion to the mono- or diglyceride of MOPA or phosphatidate, respectively, was a prerequisite for their inhibition of adenylyl

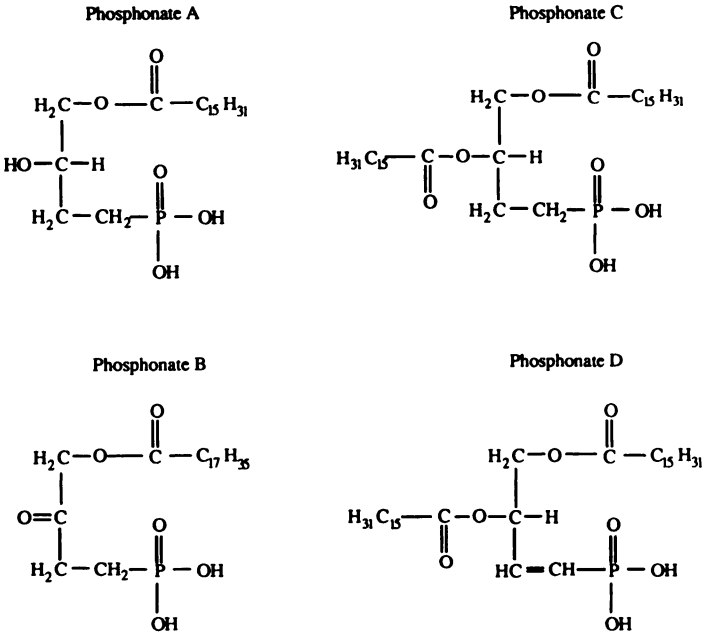

Fig. 1. Structures of phosphonate analogs.

TABLE 1

Effect of MOPA and phosphonates on C6 adenylyl cyclase activity

Adenylyl cyclase was measured in C6 membranes exposed to INE (1 μM) or its vehicle (1 mM thiourea and 0.1 mM ascorbate) and a phospholipid (10 μM) or its vehicle (water). Values shown are the mean ± standard error of three determinations, except where noted.

Additions	Adenylyl cyclase	
	Vehicle	INE (1 μM)
	pmol of cAMP/mg/min	
Vehicle	37 ± 3	213 ± 20
MOPA	19 ± 1 (49)[a]	119 ± 10 (44)
Phosphonate A	21 ± 1 (43)	113 ± 15 (47)[b]
Phosphonate B	39 ± 4	243 ± 24
Phosphonate C	36 ± 3	213 ± 14
Phosphonate D	39 ± 2	225 ± 13

[a] Values shown in parentheses are the percentages of inhibition relative to the control.

[b] Two determinations.

cyclase, the effects of several analogs of these compounds on C6 adenylyl cyclase were examined. The approach taken was to use phosphonates, because previous studies indicated that they were stable to metabolism (15, 16). Only phosphonate A, the structural analog of MOPA, was effective (Table 1). Phosphonate A, like MOPA, inhibited both basal and INE-stimulated adenylyl cyclase, and the percentages of inhibition measured in the presence of maximal concentrations of the two compounds were similar. Replacement of the hydroxyl group on the second carbon of the MOPA analog with a keto group (analog B) abolished the activity of the compound. Neither of the analogs of phosphatidate, phosphonate C or D, had any detectable effect on adenylyl cyclase activity at concentrations of 10 or 30 μM. Analog D differed from analog C in that it had a double bond between the third and fourth carbons.

Characteristics of phospholipid inhibition of adenylyl cyclase activity. The attenuation of INE-stimulated adenylyl cyclase activity in C6 membranes by MOPA, phosphatidate, and phosphonate A was concentration dependent (Fig. 2). MOPA was more potent than phosphatidate and phosphonate A, with the concentrations required for half-maximal inhibition

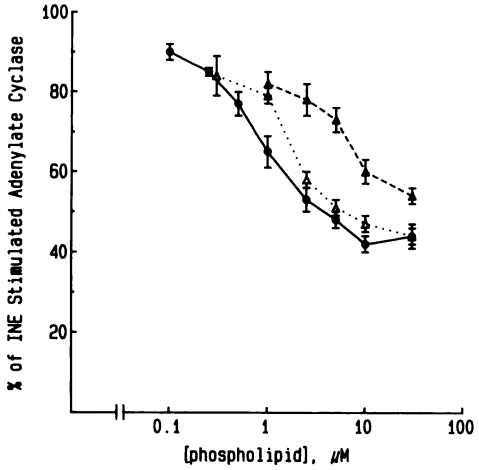

Fig. 2. Effect of various concentrations of MOPA, phosphonate A, or phosphatidate on INE-stimulated adenylyl cyclase in C6 glioma cell membranes. Adenylyl cyclase was measured in membranes from C6 glioma cells exposed to 1 μM INE or 1 μM INE plus various concentrations of MOPA (●), phosphonate A (△), or phosphatidate (▲). Values shown are the percentage of the control INE stimulation and are the mean ± standard error of triplicate determinations.

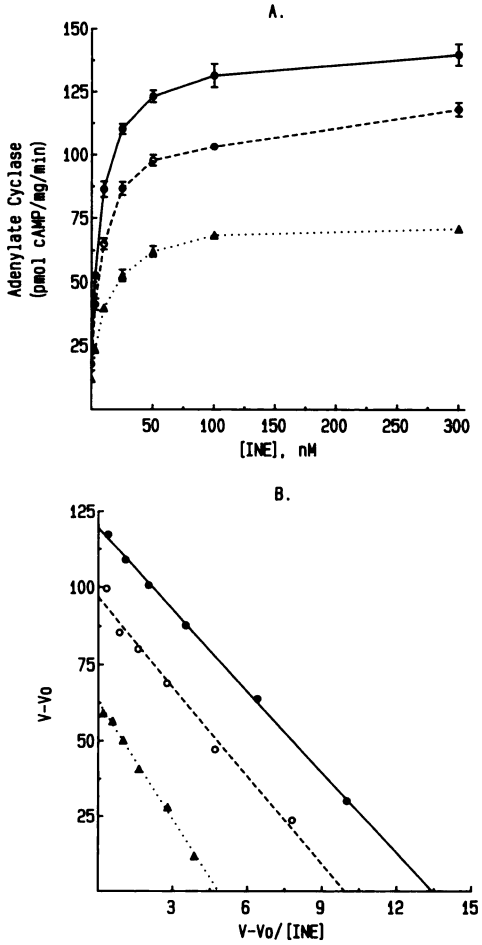

Fig. 3. Effect of INE concentration on MOPA inhibition of INE-stimulated adenylyl cyclase in C6 membranes. A, Adenylyl cyclase was measured in membranes from C6 glioma cells exposed to various concentrations of INE in the absence (●) or presence of 0.3 μM MOPA (○) or 10 μM MOPA (▲). Values shown are the mean ± standard error of triplicate or duplicate determinations. B, Data in A, transformed by the Eadie-Hofstee method.

ranging from 0.2 to 0.7 μM for MOPA, from 1.3 to 3.0 μM for phosphonate A, and from 1.9 to 3.0 μM for phosphatidate. Maximal inhibition was obtained with 10–30 μM concentrations of the phospholipids, with the maximal inhibitions ranging from 30 to 58% for MOPA, from 37 to 54% for phosphonate A, and from 37 to 45% for phosphatidate in different membrane preparations. MOPA, phosphatidate, and phosphonate A also inhibited hormone-stimulated cAMP accumulation in intact C6 cells (data not shown). The maximal inhibitions of cAMP accumulation in intact cells measured in the presence of MOPA, phosphatidate, and phosphonate A were, respectively, 71–87%, 52–65%, and 91%.

MOPA inhibition of adenylyl cyclase was measured in the presence of various concentrations of INE (Fig. 3), and, over the range of INE concentrations examined, relatively constant inhibitions of 18 and 46% were obtained with 0.3 and 10 μM MOPA, respectively. An Eadie-Hofstee transformation of the data showed that the V_{max} decreased from 109 pmol/mg/min to 89 and 59 pmol/mg/min in the presence of 0.3 and 10 μM MOPA, respectively. The K_{act}, which was 8.9 nM when no inhibitor was present, was 9.7 and 13.8 nM in the presence of 0.3 and 10 μM MOPA, respectively. (The V_{max} and K_{act} are the means calculated from the results of two separate experiments.) Thus, although the K_{act} was increased slightly by the high concentration of MOPA, the data were not inconsistent with noncompetitive inhibition, which is a general characteristic of G_i-mediated inhibition of adenylyl cyclase activity (21).

The roles of GTP, Mg^{2+}, and NaCl in MOPA inhibition of adenylyl cyclase were also evaluated, because all have been shown to affect hormonal regulation of cAMP. Inhibition of adenylyl cyclase by hormones is GTP dependent (22), and we demonstrated a similar requirement for MOPA inhibition in C6 cells by measuring MOPA inhibition of forskolin-stimulated adenylyl cyclase in the presence of various concentrations of GTP (data not shown). There was no detectable inhibition when GTP was absent, but with increasing concentrations of GTP there was a progressive increase in the attenuation due to MOPA. The maximal inhibition of 47–49% occurred in the presence of 0.5–1 μM GTP, and 0.5 μM GTP was routinely used for the adenylyl cyclase assay.

The stimulatory and inhibitory regulations of adenylyl cyclase are also affected by Mg^{2+} (23, 24). We previously found that MOPA inhibition of fibroblast adenylyl cyclase activity was more prominent at low concentrations of Mg^{2+} (6). However, this was not the case with MOPA inhibition of C6 adenylyl cyclase. When MOPA inhibition of INE-stimulated adenylyl cyclase was measured in the presence of 0.01–19.0 mM free Mg^{2+}, there was a relatively constant inhibition of about 40%.

Several investigators have studied the role of NaCl in the regulation of adenylyl cyclase, but the results have been varied (24–27). We found no evidence that NaCl was required for MOPA inhibition in C6 cells. MOPA inhibition of INE-stimulated adenylyl cyclase was relatively constant in the presence of 0–120 mM NaCl.

Effect of IAP on phospholipid inhibition of adenylyl cyclase activity. MOPA and phosphonate A inhibitions of INE-stimulated adenylyl cyclase were attenuated in membranes from IAP-treated C6 cells (Table 2). IAP treatment also eliminated the IAP-dependent [^{32}P]NAD labeling of a 40-kDa

TABLE 2

Effect of IAP treatment on MOPA and phosphonate A inhibitions of INE-stimulated adenylyl cyclase in C6 glioma cell membranes

Adenylyl cyclase was measured in membranes from C6 glioma cells treated with vehicle or IAP (100 ng/ml) for 24 hr. The compounds used were INE (1 μM) or its vehicle (1 mM thiourea and 0.1 mM ascorbate), MOPA (10 μM), and phosphonate A (30 μM). Values shown are the mean ± standard error of triplicate determinations.

Additions	Adenylyl cyclase	
	Vehicle	IAP
	pmol of cAMP/mg/min	
Vehicle	40 ± 2	66 ± 3
Vehicle + MOPA	14 ± 1 (65)*	67 ± 4
Vehicle + phosphonate A	16 ± 1 (60)	70 ± 1
INE	148 ± 8	202 ± 2
INE + MOPA	61 ± 1 (59)	186 ± 7 (8)
INE + phosphonate A	65 ± 4 (56)	197 ± 6 (3)

* Values shown in parentheses are the percentages of inhibition relative to the appropriate control.

protein, and it attenuated MOPA inhibition of INE-stimulated cAMP accumulation in intact C6 cells.

Discussion

In this paper, we examined the specificity of phospholipid regulation of cAMP in C6 glioma cells, through the use of analogs of MOPA and phosphatidate. Phosphonate A, the phosphonate analog of MOPA, inhibited both cAMP accumulation in intact cells and adenylyl cyclase activity in purified membranes. It was slightly less potent than MOPA, but the maximal levels of inhibition observed with MOPA and phosphonate A were similar. Phosphonate A and MOPA both appeared to act via G_i, because their effects were attenuated in IAP-treated cells. The lack of effect of the keto analog of MOPA phosphonate suggested that the C-2 hydroxyl was important. The phosphonate analog of phosphatidate, on the other hand, had no detectable effect on adenylyl cyclase.

There are several possible interpretations for the lack of effect of the phosphonate analog of phosphatidate. First, it is certainly possible that phosphatidate is rapidly converted to MOPA by these cells. However, the rapidity of the effects of phosphatidate suggest that this is unlikely. Further, this pathway, presumably involving phospholipase A_2, is likely not a very active pathway in cells. A more likely explanation has recently been provided by the work of Jalink *et al.* (28). This group found that MOPA, but not phosphatidate, caused a potent mobilization of Ca^{2+} in fibroblasts. Of great interest, they found that commercially available preparations of phosphatidate also mobilized Ca^{2+}. Purification of preparations of phosphatidate using standard thin layer chromatographic techniques removed the ability of phosphatidate to mobilize Ca^{2+}, leading them to conclude that the action of phosphatidate was in fact caused by contaminating lysophosphatidate. Our results are most consistent with this interpretation, because the phosphonate analog of phosphatidate was inactive but should be active based on our results with the phosphonate analog of MOPA.

Although it is not possible to determine, from the data presented here, the mechanism by which phosphatidate and MOPA inhibit cAMP synthesis, mechanisms that have been considered by these and other authors involve mediation through a direct action on either G_i or a specific membrane receptor coupled to G_i. Murayama and Ui (8) favor the latter mechanism and have discussed in considerable detail the data supporting this hypothesis. We also favor the membrane receptor-mediated hypothesis, for many of the same reasons. First, MOPA and phosphatidate inhibitions of adenylyl cyclase are cell specific, a characteristic that would not be expected if MOPA and phosphatidate acted directly on G_i. Evidence of the cell specificity is the fact that MOPA and phosphatidate inhibitions of adenylyl cyclase are found in fibroblasts and C6 cells but not in S49 lymphoma cells or platelets, despite the fact that all demonstrate pertussis toxin-sensitive, G_i-mediated inhibition of adenylyl cyclase. Second, MOPA inhibition of adenylyl cyclase activity shows many of the crucial characteristics of hormone/receptor regulation of adenylyl cyclase. In particular, the inhibition does not show a lag, as might be expected if the lipid had to first dissolve in the bilayer to gain access to G_i. Finally, there is precedent for lipids (e.g., platelet-activating factor and prostaglandins) acting through a classic receptor-GTP-binding protein complex to affect adenylyl cyclase, and for the moment there is no reason to expect that MOPA regulation would be otherwise.

In conclusion, we have used phosphonate analogs of MOPA and phosphatidate to further evaluate the specificity of phospholipid regulation of adenylyl cyclase. The experiments reported here demonstrate the potential usefulness of the relatively stable analog of MOPA to further our understanding of phospholipid regulation of cell processes.

Acknowledgments

We would like to thank Dr. R. W. Butcher for his support, advice, and encouragement, Mr. Mark Richardson for expert technical assistance, and Dr. Tom Honeyman, who first suggested that we use the phosphonates.

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