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INHIBITION OF ADENYLATE CYCLASE BY *p*-BROMOPHENACYL BROMIDE

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Abstract—*p*-Bromophenacyl bromide (BPB) is an alkylating agent which has been used in biochemical studies as an inhibitor of phospholipase A_2 activity. We report here that BPB irreversibly inhibited adenylate cyclase activity stimulated by hormones, forskolin, GppNHp, NaF, and cholera toxin. The action of BPB in S49 lymphoma cell membranes (wild type and cyc$^-$) indicates that it can inhibit adenylate cyclase function in the absence of G_s. In the presence of G_s, however, inhibition of adenylate cyclase by BPB was enhanced, suggesting that BPB may covalently modify the catalytic protein on a site involved in activated catalytic functioning or critical to its interaction with G_s and/or additionally on the α_s protein.

p-Bromophenacyl bromide (BPB‡) is a potent alkylating agent that will attack nucleophilic groups on proteins including a critical histidine residue on phospholipase A_2 [1, 2]. This compound is an important tool for studies describing how eicosanoids can mediate changes in cellular functions [for review, see Refs. 3–5]. BPB has been used extensively as an inhibitor of phospholipase A_2 activity in studies aimed at blocking arachidonic acid release in intact cells [6–8]. During the course of investigating the relationship between hormone-regulated phospholipid metabolism and cyclic AMP accumulation, we found that BPB inhibits G_s-regulated adenylate cyclase activity directly. The work presented here describes how this alkylating agent produces its effects on the adenylate cyclase system.

MATERIALS AND METHODS

Materials. All tissue culture media, sera, and antibiotics were purchased from Hazelton Research Products. Cholera toxin was purchased from List Biological Laboratories Inc. BPB was purchased from the Sigma Chemical Co. and stored dessicated in the dark. [^{32}P]ATP was purchased from ICN Laboratories Inc. and [^{3}H]cyclic AMP was from New England Nuclear.

Cell maintenance and membrane preparations. N18TG2 cells (passage number 25–40) were grown on 175 cm^2 culture flasks in Dulbecco's modified Eagle's/Ham's F12 (1:1) medium containing 10% heat-inactivated calf serum, 50 I.U./mL penicillin and 50 μg/mL streptomycin. S49 lymphoma cells [wild type (wt) and cyc$^-$] were grown to a density of 2×10^6 cells/mL in spinner bottles in the same medium as above except that 10% heat-inactivated calf serum was replaced by 10% heat-inactivated horse serum. Confluent N18TG2 cells were harvested by gentle pipetting with phosphate-buffered saline containing 0.625 mM EDTA (PBS-EDTA). Partially purified plasma membranes were prepared by differential and sucrose density gradient sedimentation according to Howlett [9], and stored in aliquots in 20 mM sodium Hepes, pH 8.0, 2 mM $MgCl_2$ and 1 mM EDTA (HME) buffer at −80° until used. A soluble adenylate cyclase devoid of G-protein regulation was prepared from the cytosol of testes from Sprague–Dawley rats according to the procedure of Braun and Dodds [10]. Protein was determined by the method of Bradford [11].

p-Bromophenacyl bromide pretreatment. Plasma membranes prepared from N18TG2 or S49 lymphoma cells were incubated with 100 μM BPB or vehicle (0.1% ethanol in HME buffer) for 15 min at 4°. Cold HME buffer (3 vol.) was added and the membranes were sedimented at 100,000 *g* for 10 min. The membranes were resuspended in HME buffer and immediately assayed for adenylate cyclase activity.

Determination of adenylate cyclase activity. Plasma membranes from N18TG2 or S49 lymphoma cells were incubated at 30° for 20 min in a 100 μL volume containing the following: 50 mM sodium Hepes, pH 8.0, 0.1 mM Ro 20-1724, 1 mM EDTA, 10 mM $MgCl_2$, 1.5 mM potassium phosphoenolpyruvate, 0.01% fatty acid-deficient bovine serum albumin, 0.01 mg/mL pyruvate kinase, 0.5 mM ATP, 0.1 mM cyclic AMP, 0.5 μCi of [α-^{32}P]ATP, and 10 nCi of [^{3}H]cyclic AMP. Cyclic [^{32}P]AMP was isolated according to Salomon *et al.* [12]. All determinations were made in triplicate and the data shown are means ± SEM.

Cellular cyclic AMP determination. Confluent N18TG2 cells were dissociated from the flask with

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‡ Abbreviations: BPB, *p*-bromophenacyl bromide; GppNHp, guanylyl β,γ-imido diphosphate; and NEM, *N*-ethylmaleimide.

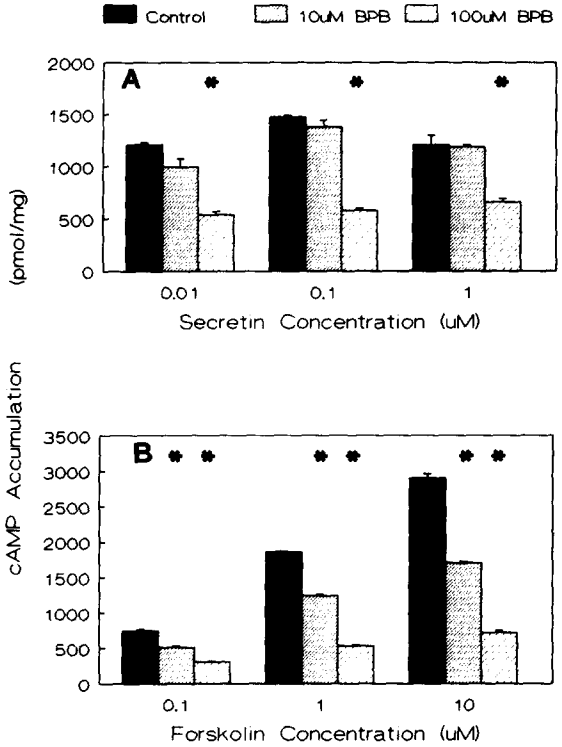

Fig. 1. Inhibition of secretin-stimulated (A) and forskolin-activated (B) cyclic AMP accumulation in N18TG2 cells by BPB. Cells were incubated with the indicated concentration of secretin or forskolin alone or in combination with 10 or 100 μM BPB. The data are means ± SEM for N = 3 replicates; the asterisk (*) indicates a significant difference from control at $P < 0.01$ according to ANOVA followed by Tukey's multiple comparison test. These experiments are representative of four similar experiments.

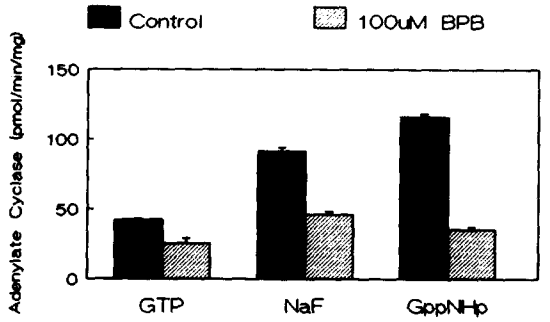

Fig. 2. Inhibition of G protein-regulated adenylate cyclase activity in N18TG2 membranes by BPB. Membranes were incubated with the indicated regulatory agents (1 μM GTP; 0.1 mM GDP plus 10 mM NaF; 1 μM GppNHp) either alone or in the presence of 100 μM BPB. The data are means ± SEM for N = 3 replicates from a single experiment representative of four.

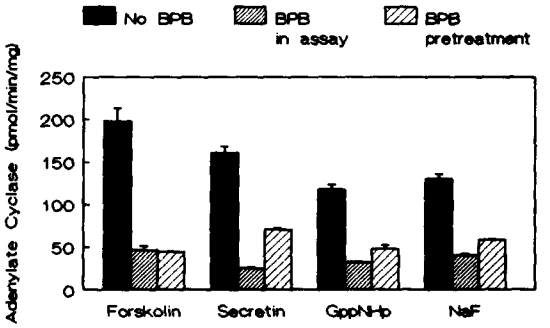

Fig. 3. Irreversibility of the inhibition of adenylate cyclase by BPB. N18TG2 membranes were exposed to 100 μM BPB either by a 15-min preincubation with subsequent washing, or by the presence of BPB in the assay. Agents present in the assay were: 1 μM forskolin plus 1 μM GTP; 400 nM secretin plus 1 μM GTP; 1 μM GppNHp; or 10 mM NaF plus 100 μM GDP. The data are means ± SEM for N = 3 replicates from a single experiment which was repeated with identical results.

PBS-EDTA, rinsed once and resuspended at 2×10^6 cells/mL in Gey's Balanced Salt Solution (GBSS: 129 mM $NaCl_2$, 5.07 mM KCL, 2.48 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM glucose) plus 15 mM sodium Hepes, pH 7.4, and 0.01% fatty acid deficient bovine serum albumin. Following a 30-min incubation at 37° with a cyclic nucleotide phosphodiesterase inhibitor (100 μM Ro 20-1724), cells were added to test tubes containing the indicated compounds or vehicle in a 500 μL final volume and allowed to incubate for an additional 4 min at 37°. The incubation was terminated by addition of 50 μL of 500 mM sodium acetate, pH 4.5, and boiling for 4 min. The particulate matter was sedimented for protein determinations [11] and the cyclic AMP content of the supernatant was assayed according to the Brostrom and Kon [13] modification of the Gilman [14] protein kinase binding method. Intact cell experiments were performed in triplicate, and the protein and cyclic AMP determinations were performed in duplicate.

RESULTS

Figure 1A shows that 100 μM BPB decreased secretin-stimulated cyclic AMP accumulation by 45–60% in intact N18TG2 cells. BPB also inhibited forskolin-activated cyclic AMP accumulation in N18TG2 cells in the absence of a hormone–receptor interaction (Fig. 1B). At 10 and 100 μM BPB, the forskolin response was inhibited 30–40 and 60–75%, respectively. It is not possible to increase BPB concentrations greater than 100 μM without raising the solvent concentration in the assay to unacceptable levels (0.1% EtOH), and thus the experiments shown depict the results using 100 μM BPB.

BPB also inhibited adenylate cyclase activity in N18TG2 cell membranes (Fig. 2). This suggests that BPB is probably acting directly at the level of adenylate cyclase and its associated receptors and G proteins rather than indirectly through a cellular event that would subsequently affect the adenylate cyclase system. It would be predicted that if BPB alkylates a critical component of the adenylate cyclase system, then the response would be irreversible. Figure 3 shows that when N18TG2

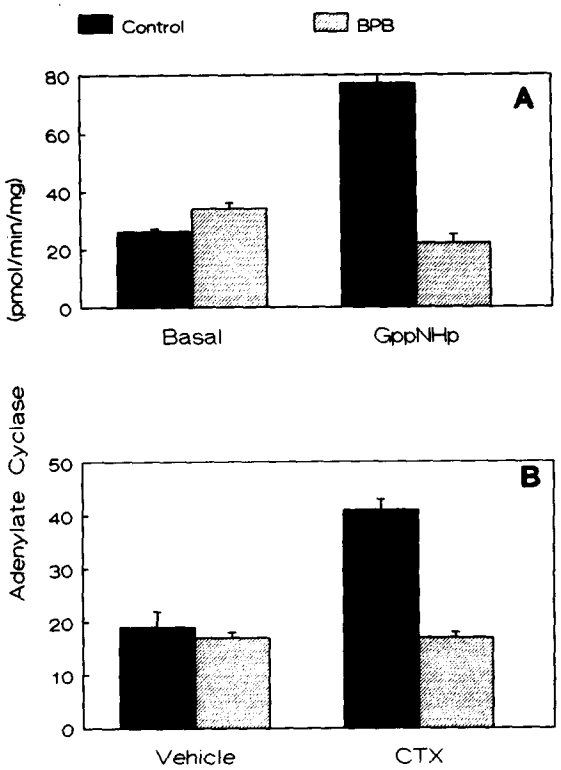

Fig. 4. Inhibition by BPB of adenylate cyclase that has been preactivated with GppNHp (A) or cholera toxin (B). (A) N18TG2 membranes were incubated with 75 μM GppNHp or vehicle for 5 min at 30°. After the incubation, cold HME (3 vol.) was added, the membranes were sedimented at 100,000 *g*, and resuspended membranes were assayed immediately for adenylate cyclase activity in the presence or absence of BPB. (B) Confluent N18TG2 cells were treated with cholera toxin (1 μg/mL) or vehicle (H_2O) for 2 hr, and then dissociated from the flask with PBS-EDTA. After being allowed to swell in HME buffer for 5 min, the cells were homogenized and the lysate was sedimented at 2000 *g* for 10 min. The supernatant was centrifuged at 38,000 *g* for 30 min, and the resulting pellet was resuspended in HME buffer and assayed immediately for adenylate cyclase activity in the presence or absence of BPB. The data are means ± SEM for N = 3 replicates from single representative experiments.

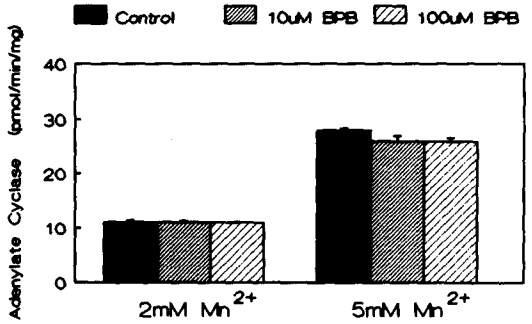

Fig. 5. Failure of BPB to inhibit cytosolic adenylate cyclase from sperm. Adenylate cyclase obtained from the cytosolic fraction of rat testes was assayed in the presence of Mn^{2+} alone or in combination with BPB in the assay. The data are means ± SEM for N = 3 replicates from a single experiment representative of three.

membranes were preincubated with BPB and then thoroughly washed, the inhibition of adenylate cyclase activity was still evident. Figure 2 shows that 100 μM BPB blocked the responses to NaF and the non-hydrolyzable GTP analog, GppNHp, both acting at the level of G_s. BPB partially attenuated the basal activity.

Because NaF and GppNHp activate G_s, the target for BPB's action may be at G_s, the α_s protein interaction with the catalytic protein, or the catalytic protein itself. Figure 4 shows that BPB could fully abolish the activity following pretreatment with cholera toxin or GppNHp. GppNHp displaces bound GDP and causes subunit dissociation, increasing the availability of free active α_s. Cholera toxin increases availability of free α_s by modifying the α_s protein such that GTP cannot be hydrolyzed. Because these treatments should maximally increase the pool of active α_s, these results would indicate that the effects of BPB occurred distal to the activation and dissociation of G_s.

If BPB alters the coupling between α_s and the catalytic protein, its site of action could be on either protein. To examine this issue, we utilized two systems in which catalytic proteins were not regulated by G_s: the cytosolic adenylate cyclase from rat sperm [10], and the S49 lymphoma cyc$^-$ mutant which fails to express G_s [15]. Figure 5 shows that neither 10 nor 100 μM BPB inhibited the Mn^{2+}-activated adenylate cyclase from sperm cytosol. In contrast, BPB was able to inhibit the S49 cyc$^-$ adenylate cyclase activity. The membranes were pretreated with BPB and washed prior to assay for forskolin-stimulated activity. At 10 or 30 μM forskolin, BPB inhibited adenylate cyclase activity by 30 or 38%, respectively (Fig. 6A). Thus, BPB appears to directly affect catalytic activity in the membrane-associated adenylate cyclase, but not in the cytosolic enzyme.

Figure 6B depicts the action of forskolin and BPB on the S49 lymphoma wt membranes that had been pretreated with BPB. In the G_s-containing membranes, forskolin was more efficacious, indicating that forskolin maximally activates adenylate cyclase when G_s is expressed. The presence of G_s allowed BPB to have a greater effect on adenylate cyclase activity: at all forskolin concentrations, BPB pretreatment inhibited the activity by approximately 90%. The increased efficacy of BPB in wt compared with its action in the cyc$^-$ membranes, was not dependent on the extent of stimulation by forskolin. In the cyc$^-$ membranes, 10 μM forskolin stimulated adenylate cyclase activity to the same extent as 1 μM forskolin did in the wt membranes, i.e. 54 pmol/min/mg. However, in the cyc$^-$ membranes, 100 μM BPB inhibited this activity by 30% compared with the 93% inhibition observed in the wt membranes.

To determine whether BPB affects G protein coupling to the catalytic protein, we determined the effects of BPB on the ability of GppNHp to regulate forskolin-activated adenylate cyclase. Because the cyc$^-$ membranes do not contain G_s, addition of GppNHp can be expected to indicate the extent of

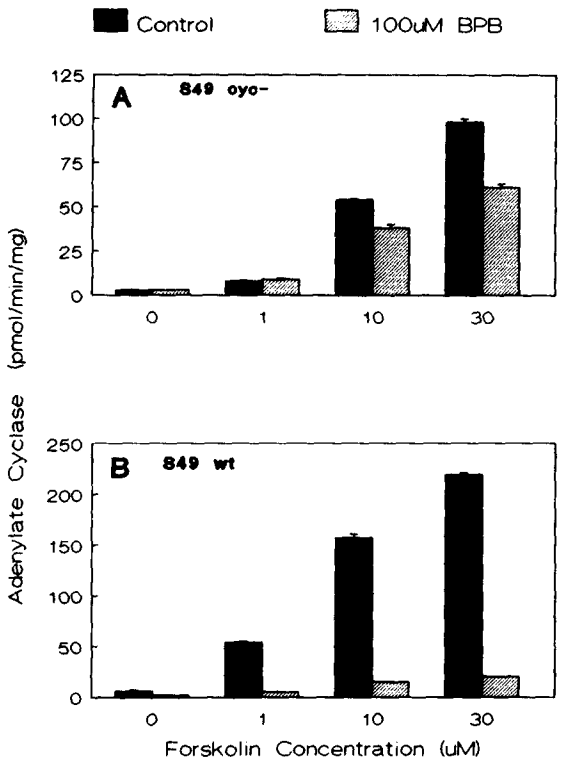

Fig. 6. Inhibition by BPB of forskolin-activated adenylate cyclase in membranes from S49 lymphoma cyc⁻ (A) and wt (B) cells. Membranes were pretreated with BPB or vehicle prior to adenylate cyclase assay as described in the text. The data are means ± SEM for N = 3 replicates from a single experiment representative of four.

G_i coupling to the adenylate cyclase. Table 1 shows that in the absence of BPB pretreatment, 1 μM GppNHp inhibited 30 μM forskolin activity by 34% in cyc⁻ membrane. After 100 μM BPB pretreatment, GppNHp was able to inhibit forskolin activity by 21%. In the S49 wt membranes, GppNHp (1 μM) activated adenylate cyclase, and thus, gives an indication of the coupling between G_s and the catalytic protein. Table 1 shows that GppNHp augmented forskolin activation by 2.4-fold. Following BPB treatment, GppNHp also increased forskolin stimulation (1.7-fold). This series of experiments would suggest that BPB curtails adenylate cyclase activity by inhibiting the catalytic activity of the enzyme, particularly in its activated state. However, regulation of this activity at the level of G protein coupling was only marginally affected by BPB treatment.

DISCUSSION

The present work describes the effects of BPB on adenylate cyclase function. BPB irreversibly blocked activity regulated by hormone receptors, GppNHp, NaF, forskolin, and cholera toxin. Its wide ranging effect and the fact that it is a potent alkylating agent indicate that BPB is disrupting catalytic and/or G protein activity through a covalent modification. There are a number of steps involved in G protein activation of adenylate cyclase: (1) GDP is released; (2) GTP is bound and the G_s enters an activated state; (3) the G protein subunits dissociate; (4) α_s-GTP couples to the adenylate cyclase such that the catalytic protein accelerates the reaction synthesizing cyclic AMP; (5) GTP is hydrolyzed, returning the α_s to the basal state; and (6) the G_s subunits reassociate (see description and analysis in the review by Birnbaumer [16]). BPB did not prevent GDP release because the fluoride response which relies on bound GDP for its action [17] was inhibited by BPB. Because GppNHp activation was attenuated, BPB probably did not accelerate the turn-off mechanism of GTP hydrolysis. BPB did not prevent binding and/or subunit dissociation because the inhibition by BPB was observed after the G protein was fully activated and α_s had been released by pretreatment with GppNHp or cholera toxin. From the experiments that demonstrate that the effects of activated α_s could be blocked, we conclude that BPB is probably acting to inhibit catalytic protein that has been activated by interaction with active α_s.

Many previous studies have shown that the adenylate cyclase system is susceptible to alkylating agents, especially sulfhydryl reagents such as *N*-ethylmaleimide (NEM). Components within the

Table 1. BPB effects on G protein regulation of adenylate cyclase activity

	Adenylate cyclase activity (pmol cAMP/min/mg protein)	
BPB (μM)	Forskolin	Forskolin plus GppNHp
S49 cyc⁻ memranes		
0	98 ± 2	65 ± 2
100	61 ± 2	48 ± 2
S49 wt membranes		
0	219 ± 2	522 ± 24
100	20 ± 0.3	33 ± 1

Membranes were pretreated with BPB or vehicle as described in the text and assayed for adenylate cyclase activity in the presence of forskolin (30 μM) alone or in combination with GppNHp (1 μM). The data are means ± SEM of N = 3 replicates within a single representative experiment.

adenylate cyclase system show a differential sensitivity to NEM [18]. For instance, membranes treated with 1 mM NEM show less than 10% of the original adenylate cyclase activity although G_s interaction with the β-adrenergic receptor remains normal. Treatment with 10 mM NEM abolishes both processes. Similarly, Korner and colleagues [19] showed differential sensitivity to NEM and additionally suggested that there are at least two critical sulfhydryl groups on G_s. They suggested three different targets: (1) 0.5 mM NEM at 4° caused complete blockade of catalytic activity although G_s coupling to the catalytic protein remained intact; (2) 0.5 mM NEM at 30° resulted in a 75% loss of the ability of G_s to couple to the catalytic protein; and (3) 0.5 mM NEM at 30° in the presence of a β-adrenergic agonist opened up a second site on G_s resulting in alteration of receptor–G protein interaction. Evidence demonstrating that G_i is also susceptible to NEM comes from the work of Jakobs and colleagues [20], which indicated that NEM pretreatment of cyc^- membranes eliminates G_i inhibition of forskolin-activated adenylate cyclase. Winslow and colleagues [21] purified the β subunit from G_o and found that three molecules of NEM could be incorporated per mole of protein. When they identified the labeled cysteine residues, they found that one was shared by all α subunits and two were shared by α_i.

Our work has shown that like NEM, BPB may differentially inhibit adenylate cyclase components. Table 1 indicates that 100 μM BPB pretreatment of wt membranes caused >90% inhibition of catalytic activity, although G_s and G_i coupling was decreased by only about 30%. These results are consistent with those showing NEM to be a more potent inhibitor of the catalytic activity compared with its effect on the G proteins.

In contrast to the relative amino acid specificity of NEM, BPB can also alkylate the nucleophilic arginine, lysine and histidine residues of proteins. A histidine residue which is part of the active site is the target for BPB's alkylation of phospholipases A_2 [1, 2]. The primary amino acid sequence has been deduced for mammalian adenylate cyclase and a tertiary structure model has been proposed [22]. Several critical histidine residues and a lysine appear to be present in a pore-like intramembranous region, but these may not be critical for the catalytic conversion of ATP to cyclic AMP [22]. It might be speculated that certain of the conserved nucleophilic residues contained within the large central cytoplasmic loop and C-terminal domains [22] may be the target(s) for alkylation of the adenylate cyclase.

It is interesting to note that treatment of S49 cyc^- membranes with BPB inhibited catalytic activity by only 38% in contrast to the >90% inhibition in the S49 wt membranes (Fig. 6 and Table 1). The difference may be attributed to the presence of G_s. It would appear that the catalytic protein in its activated form or the catalytic protein–α_s complex may be more vulnerable to the modification produced by BPB. This interpretation is also supported by the evidence that the cytosolic adenylate cyclase from sperm, which fails to be activated by forskolin [23] or to interact with G_s [24] is not a target for the action of BPB. Perhaps a critical site(s) necessary for the increased rate of cyclic AMP synthesis resulting from the interaction with activated α_s or forskolin is alkylated by BPB on the membrane-associated enzyme but is lacking on the cytosolic isozyme.

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