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SHORT COMMUNICATIONS

Inhibition of cardiac adenylate cyclase by an irreversible beta-adrenergic blocker

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Many different types of blockers of β-adrenergic mediated effects have been utilized in the biochemical and pharmacological evaluation of the catecholamine-sensitive adenylate cyclase system. Atlas and coworkers [1, 2] showed that a chemically reactive bromoacetylated ethylene diamine derivative of propranolol irreversibly blocks [^{3}H]propranolol binding and (−)epinephrine-stimulated adenylate cyclase [EC 4.6.1.1, ATP pyrophosphate-lyase (cyclizing)] in turkey erythrocytes. However, Lucas *et al.* [3] and Le Fur *et al.* [4, 5] evaluated FM-24, a compound with apparently no reactive groups, and found that it blocks persistently cardiac β-adrenoreceptors and (−)isoproterenol-stimulated cyclase activity. Recently, we have prepared [6] and evaluated a β-adrenergic blocker, bromoacetylated alprenolol menthane (BrAcAlpM), which has high affinity for β-adrenoreceptors and persistently blocks the binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA) to cardiac and pulmonary membranes [7]. This blocker is also functional *in vivo* as indicated by its prolonged ability to block [^{3}H]DHA binding in heart membranes prepared after intraperitoneal injection of the compound. It has been useful in measuring age-related differences in β-adrenoreceptor regeneration in heart and lung membranes of rats [7, 8].

We were interested to discover if BrAcAlpM was indeed blocking biochemically important β-adrenergic binding sites. It is generally agreed that the cellular responses to catecholamine stimulation are initially mediated by adenylate cyclase. Therefore, we examined the ability of BrAcAlpM to block the (−)isoproterenol-stimulated adenylate cyclase activity in homogenates from cardiac tissue and, secondly, we monitored the ability of BrAcAlpM to persistently block [^{3}H]DHA binding and reduce (−)isoproterenol-stimulated cyclase activity in cardiac membranes and homogenates from rats that had been injected previously with BrAcAlpM. The results of this study suggest that this compound is acting as an irreversible blocker of biochemically relevant β-adrenoreceptors.

Materials and Methods

[^{3}H]cAMP (38 Ci/mmole) and [^{3}H]DHA (49 Ci/mmole) were purchased from the New England Nuclear Corp., Boston, MA, U.S.A.; [^{32}P-α]ATP was from ICN, Irvine, CA, U.S.A. (±)Alprenolol–HCl was a gift from Hässle, Mölndal, Sweden. ATP, cAMP, phosphocreatine, and (−)isoproterenol(+)-bitartrate were from the Sigma Chemical Co., St. Louis, MO, U.S.A. BrAcAlpM was prepared as described previously [6]. All other chemicals were of analytical grade and were used without further purification. Male Sprague–Dawley rats were from Taconic Farms, Inc., Germantown, NY, U.S.A.

Cardiac ventricular homogenates were prepared as described by Minneman *et al.* [9]. Approximately 25 vol. of buffer per g wet weight of tissue was used, and these homogenates were used immediately for the cyclase experiments. A crude membrane fraction was then prepared. Equal portions (15 ml) of the homogenate and 80 mM Tris–HCl, pH 7.5, containing 40 mM EDTA and 300 mM sodium perchlorate were mixed and then centrifuged at 4° for 10 min at 48,000 *g*. The pellet was resuspended in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ by use of a Polytron (setting 6, 10 sec) and recentrifuged as above. This washing step was repeated, and the membrane pellet was suspended by Polytron homogenization in 8 ml of 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ to a final protein concentration of about 5 mg/ml.

Binding of [^{3}H]DHA to these membranes was carried out exactly as previously described [10, 11]. A 5 nM concentration of [^{3}H]DHA was utilized in all experiments to measure total binding. Non-specific binding was measured in the presence of 10 μM (±)alprenolol, and specific binding, the difference between total and non-specific, is reported in all cases.

Adenylate cyclase was measured in rat heart homogenates immediately after preparation (short-term incubation) or after a 1-hr preincubation at 0° (long-term incubation) in the absence or presence of BrAcAlpM. Cyclase was measured in duplicate 10-min assays at 30°. Approximately 100 μg of homogenate protein was incubated in a total volume of 100 μl of 50 mM Tris–HCl, pH 7.5, containing the following: 0.1 mM [^{32}P-α]ATP (2.23×10^6 dpm/assay), 1 mM [^{3}H]cAMP ($4–5 \times 10^3$ cpm/assay), 1 mM $MgCl_2$, 0.5 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.75 mM isobutylmethylxanthine (IBMX), 1 mM ascorbate, 0.1 mM dithiothreitol, 10 mM phosphocreatine, and 0.1 mg/ml creatine kinase. [^{32}P]cAMP was isolated by the two column method of Salomon *et al.* [12] using the modification and regeneration procedures of Iyengar *et al.* [13]. In the short-term incubations, homogenates were exposed to blocker only during the 10-min cyclase assay. In the long-term incubations, homogenates were exposed to blocker during the 1-hr preincubation and the 10-min cyclase assay.

Results

In the short-term experiments *in vitro* when BrAcAlpM was exposed to homogenate only during the 10-min cyclase assay, the dose–response curves for (−)isoproterenol-stimulated adenylated cyclase activity were right-shifted with increasing blocker concentrations ($10^{-7}–10^{-5}$ M) (Fig. 1). To obtain a measure of the potency of BrAcAlpM in the cyclase assay, the experimental data in Fig. 1A were analyzed by a Dixon plot (inhibitor concentration vs (cAMP pmoles/mg/min)$^{-1}$ at three different (−)isoproterenol concentrations, 10^{-7}, 10^{-6}, and 10^{-5} M). An apparent K_i value of 1.75×10^{-7} M was calculated which agrees closely with the apparent K_i value of this blocker for inhibiting [^{3}H]DHA binding. The maximum (−)isoproterenol response was almost completely attained in each case except at the highest concentration (10^{-5} M). However, after preincubating homogenates for 1 hr with BrAcAlpM, the maximum (−)isoproterenol stimulation of cyclase was reduced with increasing blocker concentration, while the apparent K_a for isoproterenol was unchanged except at the highest concentration. Basal adenylate cyclase activity in either short-term or long-term experiments was gradually decreased with increasing BrAcAlpM concentrations. This might suggest the presence of small amounts of endogenous catecholamines in these homogenates or a slight non-specific effect of BrAcAlpM. Since extensive washing of membrane preparations can reduce as much as 90% of (−)isoproterenol-stimulated cyclase activity [4, 9], we did not attempt to measure the persistency of the BrAcAlpM

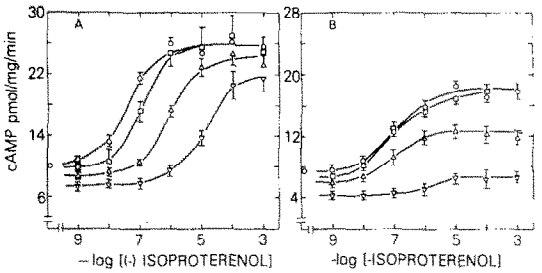

Fig. 1. *In vitro* inhibition of adenylate cyclase by BrAcAlpM. Ventricular homogenates were prepared as described in Materials and Methods and assayed for (−)isoproterenol-stimulated adenylate cyclase activity either immediately (A) or after a 1-hr incubation at 0° (B), in the absence (○——○) or presence of 10^{-7} M (□——□), 10^{-6} M (△——△), and 10^{-5} M (▽——▽) BrAcAlpM. Apparent K_a values were measured as the concentration of (−)isoproterenol giving 50% of maximum stimulation of cyclase above basal levels and were: (A) no preincubation, control, $6.24 \pm 1.7 \times 10^{-8}$ M; 10^{-7} M BrAcAlpM, $1.24 \pm 0.56 \times 10^{-7}$ M; 10^{-6} M BrAcAlpM, $6.09 \pm 0.72 \times 10^{-7}$ M; and 10^{-5} M BrAcAlpM, $7.11 \pm 1.3 \times 10^{-6}$ M; (B) 1-hr preincubation, control, $7.86 \pm 3.2 \times 10^{-8}$ M; 10^{-7} M BrAcAlpM, $7.29 \pm 0.30 \times 10^{-8}$ M; 10^{-6} M BrAcAlpM, $6.07 \pm 1.1 \times 10^{-8}$ M; and 10^{-5} M BrAcAlpM, $1.01 \pm 0.22 \times 10^{-6}$ M. The results are the averages of at least three experiments ± 1 S.E.M.

effect *in vitro*. Rather we monitored both [^{3}H]DHA binding and (−)isoproterenol-stimulated cyclase in heart membranes and homogenates at various times after the intraperitoneal injection (30 mg/kg) of blocker into rats.

Initially, specific [^{3}H]DHA binding decreased to about 45% of control levels at 1 day after injection and returned to control levels with a half-time of approximately 100 hr (Fig. 2). Maximal (−)isoproterenol-stimulated adenylate cyclase activity was also reduced to about 40% of control levels. The recovery of cyclase activity to control levels occurred with a half-life similar to that of binding. The apparent K_a values for (−)isoproterenol in BrAcAlpM-injected cardiac homogenates were not significantly different at any time from control values.

Discussion

Chemically reactive irreversible blockers are important tools *in vitro* and *in vivo* in experimental animals, for studying the function and regulation of the β-adrenergic coupled adenylate cyclase system [14–16]. We have shown previously that BrAcAlpM has high affinity for both amphibian and mammalian β-adrenoreceptors [6, 7]. Our results clearly identify this compound as a potent and persistent blocker of biochemically relevant β-adrenoceptors.

When BrAcAlpM was exposed to cardiac homogenates for short periods of time (10 min of cyclase assay), it appeared to behave as a competitive inhibitor. The dose–response curves for (−)isoproterenol-stimulated adenylate cyclase were right-shifted with increasing blocker concentration, while the maximal response to high concentrations of (−)isoproterenol was reached except at the highest BrAcAlpM concentration (Fig. 1). This response pattern is typical of a reversible blocker. However, when BrAcAlpM was preincubated with homogenates at 0° for 1 hr and then during the 10-min cyclase assay, a different pattern of inhibition emerged. The maximal stimulation of cyclase by (−)isoproterenol was progressively reduced as the concentration of BrAcAlpM was increased, and the apparent K_a for (−)isoproterenol did not change except at the highest BrAcAlpM concentration. This pattern of inhibition is typical of a noncompetitive irreversible inhibitor.

The results of the *in vivo* experiments also suggested that this compound was an irreversible β-blocker. Both the binding of [^{3}H]DHA and (−)isoproterenol-stimulated cyclase activity were persistently reduced (Fig. 2.) In other experiments [20], at similar intraperitoneal doses and subsequent time points (greater than 18 hr), (±)alprenolol had no effect on the specific binding of [^{3}H]DHA. Previously, [7] we showed that BrAcAlpM alters only the number of β-adrenergic binding sites as estimated by Scatchard analysis without any change in apparent affinity of receptors for [^{3}H]DHA so that our measurement of reduced binding at a single [^{3}H]DHA concentration reflects a proportionally reduced number of functional binding sites.

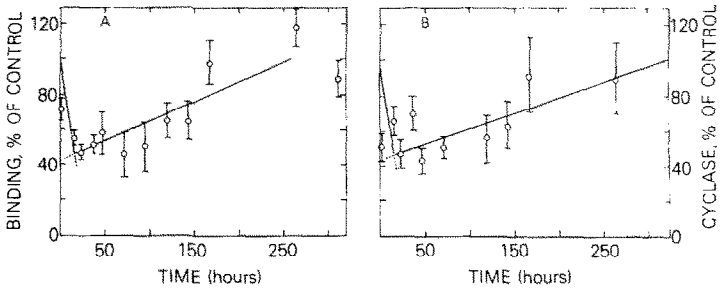

Fig. 2. *In vivo* inhibition of [^{3}H]DHA binding and adenylate cyclase by BrAcAlpM. Ventricular homogenates were prepared and assayed for (−)isoproterenol-stimulated adenylate cyclase (B), and membrane fractions were prepared from portions of the homogenates and assayed for [^{3}H]DHA binding (A), at various times after the intraperitoneal injection (30 mg/kg) of BrAcAlpM into rats. Results are reported as a percentage of control animals injected with vehicle only and killed at various times indicated on the graphs.

Previous studies with irreversible β-adrenergic blockers have indicated that maximal cyclase activity can still be achieved even when a large percentage of receptors is blocked [17, 18]. This is in contrast to our *in vitro* results in Fig. 1B where maximal cyclase activity is reduced. The difference may lie in the tissue preparations that were utilized. Previously, whole cells or intact papillary muscles were used to measure hormone response while our present studies utilize tissue homogenates. In the intact cells and tissues, receptors and receptor–cyclase complexes may be re-arranged to fully active units, or receptors may be able to recycle from previously inaccessible intracellular locations subsequent to treatment with irreversible blocker and thereby illicit a maximal response. In our homogenates, the blocker may have complete access to all receptors or the homogenization process may disrupt cytoskeletal elements or energy gradients necessary for efficient coupling of occupied receptor and cyclase activation. (See Ref. 19 for a discussion of these phenomena.)

The effects of an irreversible β-adrenergic blocker, bromoacetylated alprenolol menthane (BrAcAlpM), were examined on the catecholamine-sensitive adenylate cyclase system. The results suggest that this compound is acting as an irreversible blocker of biochemically relevant β-adrenoceptors. Both *in vitro* and *in vivo* results indicate a significant inhibition of (−)isoproterenol-stimulated adenylate cyclase activity in a manner consistent with an irreversible blockade of receptor function.

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Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumor cells resistant to L-phenylalanine mustard*

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Murine L1210 leukemia cells resistant to the bifunctional alkylating agent L-phenylalanine mustard (L-PAM) have a 2-fold greater intracellular concentration of glutathione (GSH) and glutathione disulfide (GSSG) than do L-PAM sensitive cells [1] and convert L-PAM to its non-cytotoxic derivative 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine [2]. Such L-PAM detoxification is related to the intracellular concentration of GSH and GSSG [2]. These observations prompted investigation into regimens which could be used to reduce the cellular content of GSH and GSSG and to determine whether such reduction was accompanied by altered sensitivity to the drug [1]. It was demonstrated that intracellular GSH and GSSG could be reduced by nutritional deprivation of L-cysteine. Incubation of cells in nutrient medium without L-cystine resulted in a rapid decrease in the cellular content of both glutathione disulfide and glutathione. Intracellular GSSG levels in sensitive and resistant cells decreased and became equivalent approximately 6 h following L-cysteine deprivation while the intracellular concentrations of GSH in the two cells, differing 5-fold in their sensitivity to L-PAM, became equivalent 18 h following deprivation of L-cysteine. L-PAM was equitoxic to resistant cells incubated in medium with a reduced

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