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EFFECT OF BROMOLEVAMISOLE AND OTHER IMIDAZO [2,1-b] THIAZOLE DERIVATIVES ON ADENYLATE CYCLASE ACTIVITY

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Abstract—We studied the effect of bromolevamisole (BL) and other imidazo [2,1-b] thiazole derivatives—bromodexamisole (BD) and levamisole (LV)—on adenylate cyclase (AC) activity. BL and BD both inhibited forskolin-activated human thyroid AC, while LV had no effect. This inhibition was non-stereospecific and the IC_{50} values, as measured with 1 mM ATP and 40 μM forskolin, were 0.95 and 0.80 mM for BL and BD, respectively. In contrast, human thyroid alkaline phosphatase (ALP) inhibition was stereospecific, with IC_{50} values of 0.0012 mM for BL and 0.9 mM for BD. LV was a 10-fold weaker inhibitor of ALP than BL. These results show that ALP inhibition is not correlated with forskolin-activated AC inhibition. Furthermore, in the presence of a competitive inhibitor of GTP (0.1 mM guanosine 5′-*O*-(2-thiodiphosphate), BL retained its antagonizing effect on forskolin-activated AC which suggests a direct action on the catalytic subunit. The inhibition was of the mixed type, indicating a complex interaction between BL and AC. Glucagon-activated AC activity in rat liver membranes was also inhibited by BL, although to a slightly lesser degree than thyroid stimulating hormone (TSH)-activated AC from human thyroid for a given BL concentration. In cultured human thyroid cells, BL (0.25 mM) induced a potent decrease in cAMP accumulation after 2 hr of stimulation by TSH. Taken together, these results show that BL inhibits AC and that this inhibition is not organ-specific.

Bromolevamisole (BL†, *S*-(−)-6-(4-bromophenyl) 2,3,5,6-tetrahydroimidazo [2,1-b] thiazole) is a potent stereospecific inhibitor of human bone, liver and kidney alkaline phosphatase (ALP, EC 3.1.3.1) isoenzymes but not placental and intestinal isoenzymes [1]. Its inhibitory potency is 10 times that of levamisole (LV), with an inhibition constant in the micromolar range. BL has also been shown to inhibit diamine oxidase (EC 1.4.3.6) from human placenta [2] at a concentration 100- to 200-fold higher than that required to inhibit ALP [3]. Its high specificity for ALP has made BL a useful compound for measuring ALP in serum [1, 4] and investigating its role in cell metabolism [5, 6]. Recently, we have shown that some ALP inhibitors such as BL, LV, L-homoarginine and cimetidine enhance mitogen-induced lymphocyte proliferation, suggesting that ALP may have a negative effect on the immunomodulating role of these compounds [7]. However, Hadden *et al.* [8] have hypothesized that the effects of LV could result from increased lymphocyte cGMP levels. Furthermore, a decline in lymphocyte cAMP levels has been reported in cancer patients treated with LV, with no significant change in cGMP levels [9]. Other authors have reported that intracellular levels of both cyclic nucleotides increase in polymorphonuclear leukocytes preincubated with LV, relative to untreated cells [10]. The effects of LV on cAMP levels has also been studied in perfused Ascaris suum muscle, in which it prevents serotonin-induced cAMP accumulation [11]. LV has thus been shown to affect the adenylate cyclase (AC) complex or the guanylate cyclase complex in three different cell types, although the target protein is unknown.

Here, we investigated the effect of BL on the activity of human thyroid and rat liver ACs (EC 4.6.1.1), both of which are readily stimulated by agonists. Furthermore, to determine if ALP is involved in the regulation of the catalytic unit of AC, we tested two other imidazo [2,1-b] thiazole derivatives—bromodexamisole (BD, the *R*(+) isomer of bromotetramisole) and LV.

MATERIALS AND METHODS

Chemicals. LV, BL and 3-isobutyl 1-methyl xanthine (IBMX) were purchased from Aldrich Chemie (Steinheim, Germany). Creatine phosphate, creatine kinase, GTP dilithium salt and guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) trilithium salt were purchased from Boehringer (Mannheim GmbH, Germany). Dulbecco's modified Eagle's medium and antibiotic solutions (10,000 U/mL penicillin, 10 mg/mL streptomycin and 25 μg/mL amphotericin B) were purchased from Biochrom (Angoulême, France). Cyclic AMP radioimmunoassay (RIA) kits were purchased from Amersham International (Amersham, U.K.). BD and other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

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† Abbreviations: BL, bromolevamisole; BD, bromodexamisole; LV, levamisole; AC, adenylate cyclase; ALP, alkaline phosphatase; TSH, thyroid-stimulating hormone; IBMX, 3-isobutyl 1-methyl xanthine; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); RIA, radioimmunoassay; TCA, trichloroacetic acid.

Membrane preparation. Cell membranes were prepared by means of an isotonic method, as described previously [12]. Thyroid tissues from patients with non-toxic goitre were obtained at surgery and processed immediately. Rat livers were taken from Wistar males weighing 150–200 g and starved for 16–24 hr before use. The tissue was chopped and homogenized with an Ultra-Turrax homogenizer in 10 volumes of ice-cold buffer (10 mM Tris–HCl, 250 mM sucrose and 1 mM $MgCl_2$, pH 7.4). The homogenate was filtered through four layers of gauze, diluted in five volumes of buffer and centrifuged for 10 min at 400 *g*. The supernatant was then transferred into siliconized plastic tubes and centrifuged at 9000 *g* for 15 min. This step was carried out twice. The final pellet, referred to as the crude membrane preparation, was homogenized with a dounce homogenizer and stored in small aliquots in liquid nitrogen.

The protein concentration was determined using the method of Lowry *et al.* [13], with bovine serum albumin as standard.

AC activity. AC activity was determined by using the method of Birnbaumer *et al.* [14] with modifications. Crude membranes (20–50 μg of protein) were incubated at 32° for 10 min in a final volume of 200 μL, with 20 mM Tris–HCl, pH 7.4, 0.1% bovine serum albumin, 0.5 mM IBMX, 10 mM creatine phosphate, 40 μg creatinine kinase, 2 mM ATP and 4 mM $MgCl_2$. Thyroid stimulating hormone (TSH)-stimulated AC activity was assayed in the presence of 10 μM GTP. The reaction was started by adding the membranes and stopped by adding 200 μL of 12% trichloroacetic acid (TCA) at 4°. The TCA solutions were centrifuged at 1500 *g* for 15 min. After removing TCA with water-saturated diethylether, the supernatants were evaporated in a vacuum oven. The dried extracts were then dissolved in acetate buffer and immediately assayed for cAMP by means of RIA, or stored at −70° until use. According to the manufacturer, the antiserum used in the RIA kit does not cross-react with important compounds such as ATP, ADP, AMP and IBMX. A control solution containing 0.5 mM BL did not interfere with cAMP measurement.

ALP assay. The ALP assay solution contained 100 mM $NaHCO_3$ pH 10.5, 1 mM $MgSO_4$, 20 mM *p*-nitrophenyl phosphate and crude membranes (200 μg protein) in a final volume of 1 mL. *p*-Nitrophenol formation was recorded at 405 nm at 30° using a Kontron spectrophotometer. The specific activity of ALP in crude membranes varied between 6.5 and 9 IU/g protein.

One international unit (IU) corresponds to the amount hydrolysing one μmole of substrate per minute.

Human thyrocyte culture. Normal human thyroid tissues were obtained aseptically from patients undergoing thyroid surgery, usually for uni- or multinodular goitre. Subsequent steps were performed aseptically in a laminar-flow chamber as previously described [15, 16]. The tissue was broken up with 5 mg/mL dispase and 0.1 mg/mL collagenase. The isolated cells and follicles were resuspended in Dulbecco's modified Eagle's medium supplemented with antibiotics, 0.5 g/L albumin, 1% fetal calf serum and 45 mM bicarbonate (medium A) at a density of 5×10^5 cells/mL and cultured in a humidified atmosphere of 5% CO_2 in air in petri dishes (60-mm diameter, Falcon 1007, Becton Dickinson Labware, NJ, U.S.A.).

After about 24 hr, the cells were reseeded at approximately 2×10^4 per well in medium A containing 0.5 mM IBMX, TSH and BL for 2 hr in 96-well microtest plates (Falcon 3072). The incubation was stopped by adding 250 μL of 12% TCA at 4°. The TCA solutions were treated as described above and cAMP was assayed.

Table 1. Inhibition of ALP and forskolin-activated AC in human thyroid membranes by BL, BD and LV

	ALP (IC_{50}, mM)	Forskolin-activated AC (IC_{50}, mM)
BL	0.0012 ± 0.0002	0.95 ± 0.10
BD	0.90 ± 0.15	0.80 ± 0.05
LV	0.011 ± 0.003	NI

Results (means ± SD of triplicate incubations) are expressed as mM of inhibitor which causes 50% inhibition of an enzymatic reaction (IC_{50}). IC_{50} values are obtained by the procedure of Witwicki and Chidambaram [28]. Basal and forskolin (40 μM)-stimulated AC activities, measured with 1 mM ATP, are 3.06 ± 0.24 and 26.82 ± 0.73 pmol cAMP/min/mg protein, respectively.
NI, 15% inhibition at 1 mM.

RESULTS

Comparative inhibition of ALP and AC by imidazo [2,1-b] thiazole derivatives

The comparative effects of BL, BD and LV, three imidazo [2,1-b] thiazole derivatives, on ALP and AC from human thyroid membranes are shown in Table 1. Inhibitions are expressed as IC_{50} values. BL stereospecifically inhibited human thyroid ALP and was 750-fold more potent than BD. The order of potency for ALP inhibition was as follows: BL > LV > BD. These results fall in line with earlier data on "tissue-unspecific" ALP inhibition [17, 18] and suggest that thyroid ALP is the same isoenzyme produced by the gene for liver/bone/kidney ALP.

In contrast, forskolin-activated AC showed no stereospecific inhibition. The results shown in Table 1 indicate that BL is as potent an inhibitor of AC as BD. Furthermore, 1 mM LV inhibited only 15% of forskolin-activated AC activity. The IC_{50} value for the effect of BL on the cyclase, measured with 1 mM ATP, was 700–800 times higher than that for ALP. BD had a similar IC_{50} for both enzymes and LV inhibited ALP at a concentration which had no effect on AC.

Nature of BL inhibition of forskolin-activated AC activity

BL inhibited forskolin-activated AC from human thyroid membranes. The inhibition kinetics were of the mixed type, as indicated by Hanes plots

Table 2. Effect of BL on hormone-activated AC in membranes from human thyroid and rat liver and on cAMP accumulation in human thyroid cells

	AC activity (pmol cAMP/min/mg protein)		cAMP accumulation (pmol cAMP/well)
	Human thyroid	Rat liver	Human thyroid cells
Basal	10.7 ± 0.7	15.4 ± 3.8	0.64 ± 0.18
Hormone	47.5 ± 1.0	46.7 ± 4.6	6.5 ± 1.0
Hormone + BL			
0.25 mM	30.0 ± 1.2	39.2 ± 5.2	1.8 ± 0.4
0.5 mM	18.5 ± 1.2	27.9 ± 4.3	ND

Hormones are: 50 mU/mL TSH for human thyroid membranes, 5 mU/mL for human thyroid cells and 1 μM glucagon for rat liver membranes. Results represent means ± SD of triplicate incubations.
ND, not determined.

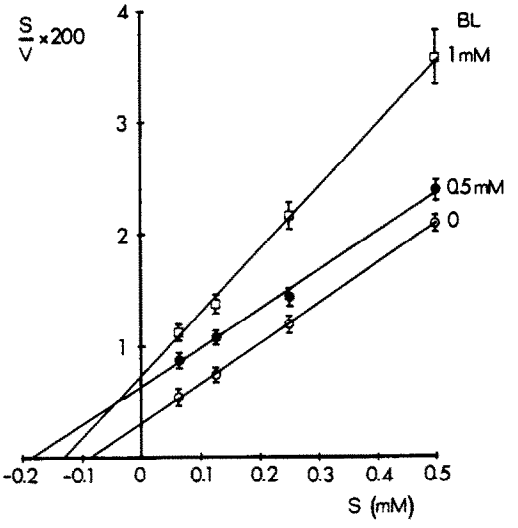

Fig. 1. Hanes plots of inhibition of forskolin-activated AC activity in human thyroid membranes by various concentrations of BL. Basal and 40 μM forskolin-activated enzyme activity (V), at the different ATP concentrations used (S), ranged from 4.16 to 1.98 and 51.45 to 24.34 pmol cAMP/min/mg protein, respectively. Data represent means ± SD of triplicate determinations.

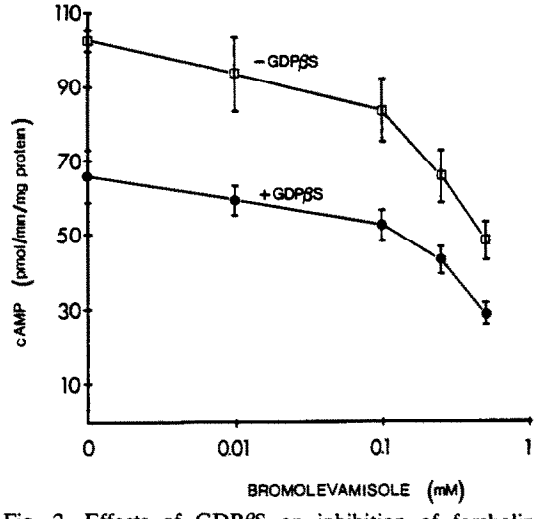

Fig. 2. Effects of GDPβS on inhibition of forskolin-activated AC in human thyroid membranes by BL. Activities were assayed as described in Materials and Methods with 40 μM forskolin and 0.1 mM GDPβS, when present. Basal enzyme activity was 12.2 ± 0.9 pmol cAMP/min/mg protein with or without GDPβS. Data represent means ± SD of triplicate determinations.

(Fig. 1). The K_m value for the enzyme in the absence of BL in Tris buffer (pH 7.4), with ATP as substrate, was 0.08 mM. This value shifted to 0.13 mM and 0.18 mM in the presence of 1 mM and 0.5 mM BL, respectively. The V_{max} in the absence of BL with 40 μM forskolin was 55.6 pmol cAMP/min/mg protein. A similar value was obtained with 0.5 mM BL but the V_{max} was decreased to 34.8 pmol cAMP/min/mg protein with 1 mM BL.

The percentage inhibition of forskolin-activated AC by BL varied with the ATP concentration, being weaker at 0.5 mM ATP than at higher and lower concentrations. It is generally accepted that forskolin has a direct effect on the cyclase catalytic unit [19]. However, GDPβS, a nucleotide analogue that blocks the G protein-coupling function, decreased forskolin-activated AC activity (Fig. 2) in the absence of BL (65.9 and 102.6 pmol cAMP/min/mg protein with and without GDPβS, respectively) which suggests that forskolin acts on the coupling of Gs protein to cyclase. Nevertheless, enzyme inhibition was not modified by BL, with an IC_{50} value of 0.32 mM in the presence and absence of GDPβS, as measured with 2 mM ATP and 40 μM forskolin (Fig. 2).

Effects of BL on the activity of AC from rat liver and cAMP accumulation in cultured human thyroid cells

The inhibition by BL of AC activity in human thyroid membranes was compared with that in rat

liver membranes. Glucagon-activated AC in rat liver membranes was inhibited by BL (Table 2). The percentages of inhibition (after subtraction of the basal values) were 60 and 79 with 0.5 mM BL for rat liver and human thyroid AC, respectively. These inhibitions were of the same order, although a slightly enhanced inhibition of TSH-activated thyroid AC was observed. Table 2 shows that BL also affected cAMP accumulation in human thyroid cells stimulated by TSH. Two hours of incubation with 5 mU/mL TSH elicited a 10-fold increase in cAMP levels, which was reduced by 80% in the presence of 0.25 mM BL. This concentration is within the range of those used for the membranes, suggesting a similar site of action of BL in these systems.

DISCUSSION

The results presented here show that BL inhibits human thyroid AC activated by both forskolin and forskolin plus GDPβS. The fact that forskolin acts on both the cyclase catalytic unit [19] and on Gs protein [20], and GDPβS has a blocking effect on G protein [21], suggests a direct inhibition of AC by BL. This inhibition was of the mixed type, differing from that of ALP or diamine oxidase [3].

BL, a potent "organ-unspecific" ALP inhibitor [17] may serve to investigate the role of ALP in cells. However, although BL inhibited human thyroid ALP with a IC_{50} of 1.2 μM at pH 10.5 with *p*-nitrophenylphosphate as substrate, several of our results show that ALP is not involved in AC regulation. Firstly, forskolin-activated AC inhibition by BL is not stereospecific. BL and BD inhibited the cyclase with similar IC_{50} values, but BL was a 500–1000-fold more potent inhibitor of ALP than BD. Secondly, the IC_{50} values for AC and ALP were different, with BL inhibiting ALP at a concentration which had no effect on AC. Furthermore, LV, another liver/bone/kidney ALP inhibitor, inhibited ALP from human thyroid but not forskolin-activated AC. As shown for melatonin and its analogs [22], the halogenated drugs BL and BD were more potent than LV in inhibiting AC activity. Although there is no easy explanation for this phenomenon, it may be of use in studying structure–inhibition relationships.

The percentage of inhibition of TSH-activated human thyroid AC by BL was not strictly the same in membranes and cells, possibly due to the fact that AC activity was measured in membranes and cAMP accumulation in cells. However, the variation in inhibition with ATP concentration points to another explanation. Effectively, the ATP pool in human thyroid cells is not a controllable value and probably does not correspond to the ATP concentrations tested in membranes. We found that glucagon-activated AC from rat liver membranes is also affected by BL in the range of concentrations used for human thyroid AC. This result, taken together with the lipophilic structure of BL related to the decreased cAMP accumulation in cultured thyroid cells, suggests that AC from several tissues can be inhibited by BL. However, LV is the only imidazo [2,1-b] thiazole derivative used therapeutically [23] and it was found not to affect thyroid AC activity. In the light of our results, the immunomodulating properties of LV (decreasing cAMP levels in lymphocytes of cancer patients) [9], cannot be related to a direct action on the cyclase.

Other membrane enzymes and phosphatases, including 5′nucleotidase, acid phosphatase, ADPase, Na^+/K^+ATPase, Ca^{2+}ATPase, glucose 6-phosphatase and cAMP phosphodiesterase [17, 24–27], are insensitive to inhibition by BL and LV. A possible increase in phosphodiesterase activity induced by BL, as reported for imidazole [27], was not observed with human thyroid membranes either in the presence or absence of IBMX, a cAMP phosphodiesterase inhibitor (data not shown).

In conclusion, our data show that BL inhibits forskolin and TSH-activated human thyroid AC at concentrations above 0.1 mM. The enzyme seems to be affected directly since G proteins and ALP have not been shown to be involved in AC inhibition. Glucagon-activated rat liver AC was also inhibited by BL, showing that this phenomenon is not organ-specific. Further investigations of structure–AC-inhibition relationships could shed light on new properties of imidazo [2,1-b] thiazole derivatives and allow us to synthesize compounds more specific for this enzyme.

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