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Inhibition of stimulated Jurkat cell adenosine 3′,5′-cyclic monophosphate synthesis by the immunomodulatory compound HR325

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Abstract

HR325 (2-cyano-3-cyclopropyl-3-hydroxy-*N*-[3′-methyl-4′(trifluoromethyl)-phenyl]-propenamide) is an immunomodulatory compound through pyrimidine biosynthesis inhibition with antiproliferative properties which was derived from the isoxazol compound A77 1726 [2-cyano-3-cyclopropyl-3-hydroxy-enoic acid (4-trifluoromethylphenyl)-amide]. During studies of the effects on early signal transduction events of this type of compound, it was found that HR325 dose-dependently inhibited adenosine 3′,5′-cyclic monophosphate (cAMP) synthesis by Jurkat cells stimulated with prostaglandin E_2, (PGE_2), cholera toxin (CTX), or forskolin (FKN). The potency of inhibition by HR325 of FKN-stimulated cells (IC_{50} 30.4 μM) was approximately 3-fold higher than that of the other agonists (11.6 and 11.7 μM) and was independent of time of preincubation for both PGE_2 and FKN. Interestingly, A77 1726, an analogue of HR325, displayed a markedly different profile of stimulus-dependent potencies. The inhibition of cAMP synthesis by HR325 when stimulated by both PGE_2 and FKN was unaffected by glucose supplementation, in contrast to HR325-inhibited ATP levels, which were restored under such conditions. Further studies revealed that HR325 reduced intracellular ATP levels by uncoupling oxidative phosphorylation, albeit with a 1000-fold lower potency than the antihelmintic drug niclosamide. In addition, glucose supplementation experiments showed that, in contrast to HR325, the niclosamide-mediated reduction of ATP levels was wholly responsible for its inhibition of PGE_2- and FKN-stimulated cAMP synthesis.

Keywords: Jurkat; cAMP synthesis; Immunomodulation; Oxidative phosphorylation; Uncoupling; Cellular respiration

1. Introduction

HR 325 (2-cyano-3-cyclopropyl-3-hydroxy-*N*-[3′-methyl-4′(trifluoromethyl)-phenyl]-propenamide) is a novel immunomodulatory compound that has recently been shown to bind, with high affinity, to the mitochondrial protein DHO-DH [1], the enzyme responsible for conversion of dihydroorotate (DHO) to orotate during *de novo* pyrimidine synthesis. Binding of HR325 results in inhibition of enzyme activity non-competitively [1] with respect to both substrate (dihydroorotate) and coenzyme (ubiquinone). HR 325 is an analogue of A77 1726 (2-cyano-3-cyclopropyl-3-hydroxy-enoic acid (4-trifluoromethylphenyl)-amide), the active metabolite of leflunomide, which has also been shown to bind and inhibit DHO-DH [2]. Leflunomide is a novel immunomodulatory compound that has been shown to be effective in animal models of arthritis, transplant rejection, and autoimmune disease[6] [3]. Inhibition of DHO-DH is responsi-

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Abbreviations: PGE_2, prostaglandin E_2; FKN, forskolin; CTX, cholera toxin; cAMP, adenosine 3′,5′-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine; NCA, niclosamide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; DHO-DH, dihydroorotate dehydrogenase; IL-2, interleukin-2; and HBSS, Hanks' balanced salt solution.

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ble for some of the *in vivo* and *in vitro* activities of these compounds [1,2].

Studies of the potential mechanisms of action of HR325 have included investigations on second messenger systems of proliferating cells, and data on one such system, cAMP generation in a human T-cell line, are presented. The role of cAMP in the control of immune responses is poorly defined and has been suggested to be both immunosuppressive and immunosupportive [4,5]. Many studies indicate a general down-regulation of T-lymphocyte function by cAMP via inhibition of IL-2 production [6–8], IL-2 receptor expression [9], phosphoinositide turnover [10,11], and T-cell cytotoxic activity [12,13]. In contrast, Koh *et al*. [5] demonstrated dose-dependent enhancement of phorbol myristic acid/ionomycin-stimulated lymphoproliferation by dibutyryl cAMP. Furthermore, elevated cAMP levels have been shown to augment T-cell proliferation indirectly by stimulating monocytes to secrete factors that mediate the up-regulation of IL-2 synthesis in T-lymphocytes [14]. Increases in intracellular cAMP levels have been reported following stimulation of T-cells with specific antigen [15] or monoclonal antibodies against CD2 or CD3 [16,17]. The requirement for both a rise and fall in intracellular cAMP levels during lymphocyte activation has also been suggested [18].

The role of cAMP in the control of B-cell function has been less well studied. It has been shown to inhibit IL-2-driven B-cell proliferation [19], whilst enhancing B-cell activation in combination with interleukin-1 [20]. It has also been implicated in prostaglandin E-mediated immunoglobulin class switching [21].

Here, we show that HR325 inhibits cAMP synthesis by the human T-cell line, Jurkat, induced by treatment with receptor-dependent, i.e. receptor-specific PGE_2 stimulation and receptor-coupled G-protein stimulation, and receptor-independent stimuli, i.e. FKN stimulation. Furthermore, HR325 is capable of reducing intracellular ATP concentrations by uncoupling mitochondrial respiration, and whilst this effect is inhibitory towards cAMP synthesis (as shown by treatment with other respiratory uncouplers and inhibitors), restoration of cellular ATP levels by glucose supplementation does not abrogate the inhibition of cAMP synthesis. These results are consistent with inhibition of cAMP synthesis by both substrate-dependent and substrate-independent mechanisms.

2. Materials and methods

2.1. Reagents

Jurkat J6 cells were obtained from the European Collection of Animal Cell Cultures (Centre for Applied Microbiology and Research, Porton Down, Salisbury). RPMI-1640, foetal bovine serum, antibiotic/antimycotic solution, and glutamine were supplied by GIBCO (GIBCO, Life Technologies Ltd). CTX was obtained from Interchim (List Biological) and Sigma Chemical Co. Test compounds HR325 and A77 1726 were synthesised in the Chemistry Department, Roussel Laboratories Ltd. All other reagents were supplied by Sigma.

2.2. Preparation of cells

Cells were cultured at 37° in a humidified atmosphere containing 5% CO_2 at a concentration of 1–5 $\times$ 10^6 cells/mL in RPMI-1640 supplemented with 10% foetal bovine serum, 2 mM glutamine, and antibiotic/antimycotic. Prior to use in the cAMP assays, cells were pelleted (240 *g* for 5 min), washed 3 times in 20 mM HEPES balanced salt solution (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, pH 7.4; HBSS) and resuspended to 1 $\times$ 10^6 cells/mL in the same buffer.

2.3. Stimulation of cAMP production

Aliquots (1 mL) of Jurkat cells were equilibrated to 37° for 10 min and test compounds added. Following incubation for 50 min at 37° in the presence of test compounds, 3 IBMX was added to a final concentration of 1 mM and the cells incubated for a further 10 min. The cells were then stimulated with 100 nM PGE_2 or 50 μM FKN for 10 min. CTX-induced cAMP production was initiated by stimulating cells with 1 ng/mL of CTX for 30 min in the absence of IBMX, followed by a 10-min capture incubation in the presence of 1 mM IBMX. Incubations with all stimuli were stopped by the addition of 1 mL of ice-cold absolute ethanol and 50 μL of 80 mM EDTA (2 mM final), the latter to inhibit phosphodiesterase activity. Cell debris was removed by centrifugation at 2° (13,000 *g*, 10 min) and the supernatants decanted and dried in a vacuum evaporator. Dried samples were reconstituted in 500 μL double-distilled water and stored at −80° until assay.

Investigation of the effect of glucose supplementation on cAMP inhibition was carried out in an identical manner, using PGE_2 or FKN stimulation, except that the cells were washed in HBSS without glucose, resuspended at 2 $\times$ 10^6 cells/mL, and 0.5 mL aliquots added to 0.5 mL HBSS containing glucose at double concentration and equilibrated to 37°.

2.4. Stimulation of cAMP production in isolated Jurkat cell membranes

Jurkat cells were washed three times in HBSS and resuspended in disruption buffer (20 mM HEPES, pH 7.4, 4 mM EDTA, aprotinin, leupeptin, and pepstatin, all at 2 μg/mL) and stored on ice for 30 min. They were then homogenised in a Potter–Elvehjem homogeniser until at least 90% cell disruption was attained (as determined by trypan blue exclusion). A membrane pellet was prepared by centrifugation at 25,000 *g* at 4° for 20 min and resuspended

in disruption buffer at 1×10^6 cell equivalents/mL and stored at −80° until use. Aliquots (50 μL) of the membrane preparation were added to tubes containing 950 μL buffer (20 mM HEPES, pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM IBMX) at 30°. Test reagents were added in 1-μL aliquots (from 1000 × stock solutions in DMSO) and the membranes incubated for 1 hr prior to stimulation with 50 μM FKN. After 30 min, the reaction was stopped by the addition of 1 mL of ice-cold absolute ethanol and 50 μL of 80 mM EDTA (2 mM final). Cell debris was removed by centrifugation at 2° (13,000 *g*, 10 min) and the supernatants decanted and dried in a vacuum evaporator. Dried samples were reconstituted in 500 μL double-distilled water and stored at −80° until use.

2.5. cAMP determinations

Levels of cAMP in the samples were assayed using a commercial [^{3}H]cAMP competition binding assay (Amersham International). Each supernatant was assayed in duplicate.

2.6. Measurement of oxygen uptake by rat liver mitochondria

Two rat livers were excised (male, 200–250 g, Wistar) and immediately homogenised in 2 volumes of buffer (10 mM TRIS pH 7.4, 250 mM sucrose, 1 mM EGTA) at 4°. A postnuclear pellet was collected by centrifugation at 600 *g* for 7 min and the supernatant centrifuged at 10,000 *g* for 10 min. The enriched mitochondrial pellet was washed once and finally resuspended in the buffer and stored on ice until use. Measurement of the rate of oxygen uptake was performed using a Clark-type oxygen electrode and recorded on an XY chart recorder. Prewarmed (37°) and oxygenated buffer containing 120 mM KCl and 7 mM phosphate (pH 7.4) was placed in the jacketed chamber of the electrode and 50 μL of the mitochondrial suspension and 30 μL of a 0.5 M stock solution of sodium glutamate (8.9 mM final) added. The electrode was lowered into the chamber and the oxygen uptake monitored in the absence of phosphate acceptor (state 4 respiration). Two microlitres of a 200-mM stock solution of ADP was added (0.4 μmol) and the mitochondrial oxygen uptake measured (state 3) until the rate returned to state 4 (at depletion of ADP), at which time 1.6 μL of a 1000 × stock solution of the test reagent was added. The rate of oxygen uptake was monitored until linear before addition of a further 2 μL of ADP. Rates of oxygen uptake were measured for state 4, state 3, in the presence of test reagent, and in the presence of both test reagent and ADP.

2.7. Measurement of intracellular ATP levels in Jurkat cells

Jurkat cells were washed 3 times in a buffer containing 20 mM HEPES pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM Na_2HPO_4. They were resuspended in the same buffer (prewarmed to 37° and containing 100 mM glucose in the relevant experiment) and 0.5 mL aliquots placed in tubes containing 0.5 μL of the relevant test reagent (100 × solutions in DMSO). After incubation at 37° for 30 min with regular agitation, the tubes were immersed in a boiling water bath for 5 min. Cell debris was removed by centrifugation (25,000 *g*, 10 min) and the supernatants stored on ice until assay.

Attempts to measure ATP concentrations using the luciferase/luciferin assay were unsuccessful due to inhibition of the reaction by HR325, a property common to mitochondrial uncouplers [22], and hence cell supernatants were assayed using an NADPH-linked spectrophotometric assay utilising hexokinase and glucose-6-phosphate dehydrogenase (G6PDH; [23]). Assay buffer was prepared at room temperature (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 5 mM EDTA, 10 mM glucose) and 0.5 mL aliquots placed in cuvettes. After addition of 20 μL of a 10 mg/mL solution of NADP and 450 μL of supernatant, the absorbance at 340 nm was recorded. Two microlitres each of hexokinase and G6PDH were added and after mixing, the cuvette was incubated for 10 min at room temperature, allowing the reaction to reach completion. The 340-nm absorbance was read again and the change in absorbance noted. ATP concentrations were calculated using the extinction coefficient of NADPH (6.22×10^3 M^{-1}). Absorbance changes were consistently reduced by 40–50% in all experiments using known respiratory uncouplers and inhibitors at concentrations up to 100 times saturating. Since these compounds are known to completely deplete intracellular ATP in the absence of glucose [24], negative control absorbance changes were calculated by treatment of cells with a saturating concentration of uncoupler (FCCP or niclosamide) in each experiment and defined as 100% inhibition of ATP concentration.

3. Results

3.1. Inhibition of cAMP synthesis

Preliminary experiments were carried out to determine optimum conditions for cAMP stimulation with PGE_2, CTX, and FKN in Jurkat cells (data not shown). All agonists displayed time- and dose-dependent stimulation of cAMP synthesis, although optimum doses and times were specific to each agonist. From these experiments conditions were chosen, as described in the Methods section, such that levels of cAMP synthesis were similar for each of the agonists. PGE_2 (100 nM) stimulation for 10 min led to synthesis of 40.1 ± 9.9 pmol cAMP/10^6 cells (N = 8), CTX (1 ng/mL) for 40 min, 31.7 ± 12.0 pmol cAMP/10^6 cells (N = 3), and FKN (50 μM) for 10 min, 56.1 ± 11.7 pmol cAMP/10^6 cells (N = 7). Basal levels of cAMP in unstimulated cells

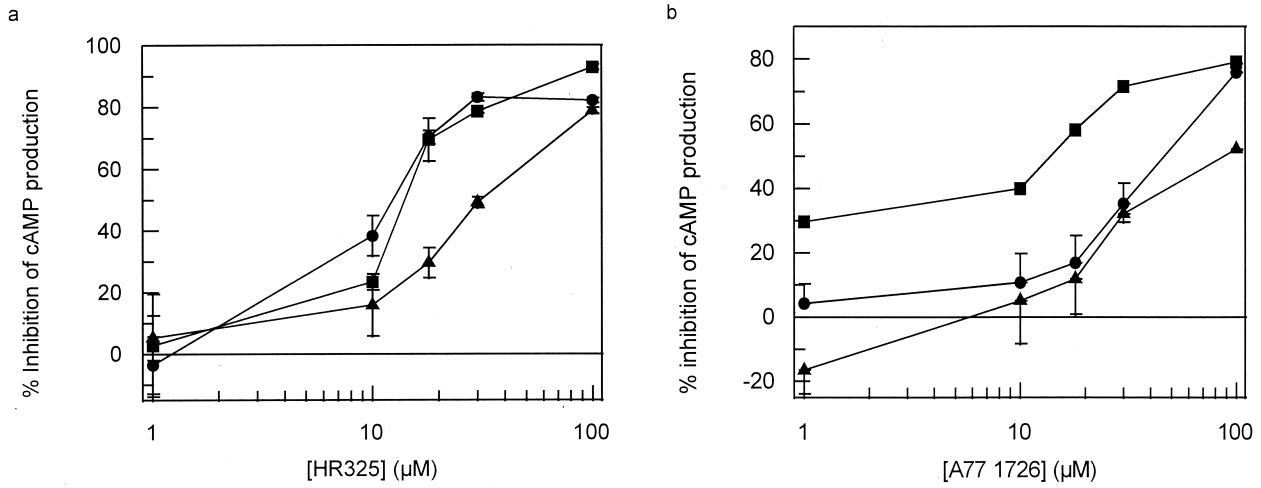

Fig. 1. Inhibition of cAMP production by HR325 and A77 1726. Cells were stimulated with PGE_2, (circles), CTX (squares), or FKN (triangles) in the presence of increasing concentrations of (a) HR325 or (b) A77 1726 as described in the Methods section. Percentage inhibition values were calculated for each concentration of compound with respect to control incubations containing vehicle and mean percentage inhibition values prepared from replicate experiments. Typical control levels of cAMP were (pmol cAMP/10^6 cells ± SD): PGE_2, 40.1 ± 9.9 (N = 8); FKN, 56.1 ± 11.7 (N = 7); CTX, 31.7 ± 12.0 (N = 3).

were 1.7 ± 0.8 pmol/10^6 cells (N = 13). All values shown are means ± SD.

HR325 dose-dependently inhibited cAMP synthesis by Jurkat cells (Fig. 1) stimulated by PGE_2, CTX, and FKN. IC_{50} values were calculated by 4-parameter logistic fits of replicate experiments and yielded mean values of 11.6 ± 3.3 μM, 11.7 ± 1.7 μM, and 30.4 ± 4.0 μM against each of the agonists, respectively (means ± SD, N = 6, 3, 6). The IC_{50} values for PGE_2 and CTX stimulation were both significantly different from that for FKN stimulation ($P < 0.001$). A77 1726 inhibited cAMP synthesis (Fig. 1) with a rank order of potencies different from that of HR325. Potencies were 5- to 10-fold lower than HR325 when stimulated by PGE_2 (52.4 ± 17.6 μM; means ± SD, N = 4) and CTX (92.4 ± 0.2 μM; mean ± range, N = 2). Its potency against FKN-stimulated cAMP synthesis (24.7 μM; N = 1) was, however, similar to that of HR325. Profiles of inhibition of cAMP by HR325 were independent of the length of preincubation when cells were stimulated with PGE_2 or FKN (Fig. 2), as were the derived IC_{50} values (Table 1).

The effect of HR325 on cell-free adenylyl cyclase activity was studied using isolated Jurkat membranes stimulated with FKN. Experiments were conducted in the absence of GTP and in the presence of high purity ATP to circumvent G-protein involvement. cAMP was produced by the isolated membranes stimulated with 50 μM FKN in the presence of 1 mM ATP (14.68 ± 1.36 pmol cAMP/5 × 10^6 cell equivalents, mean ± SD, N = 3). HR325 demonstrated no inhibition of FKN-stimulated cAMP synthesis at any concentration up to 100 μM (data not shown).

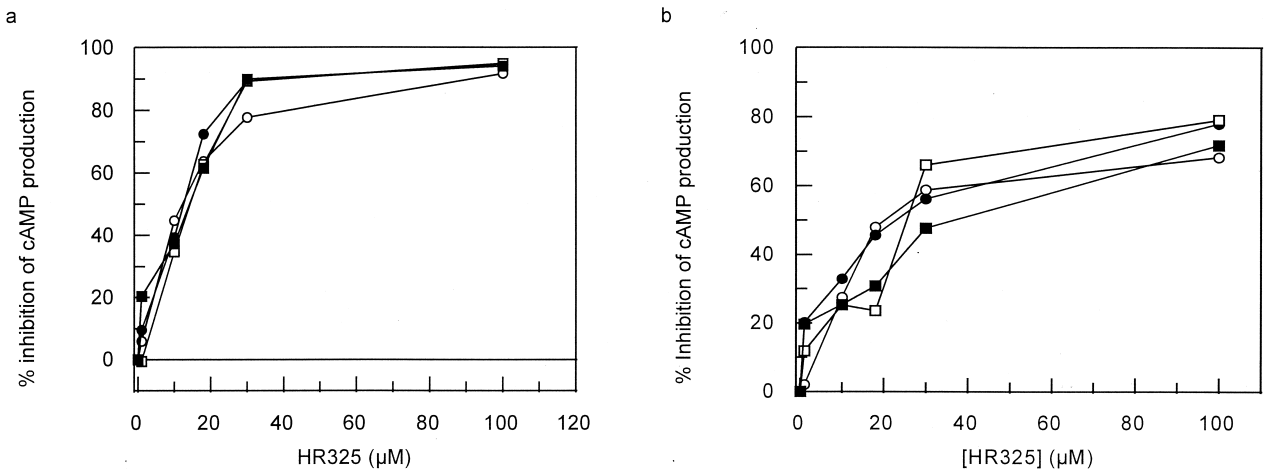

Fig. 2. Effect of HR325 preincubation time on inhibition of PGE_2- and FKN-stimulated cAMP synthesis. Jurkat cells were incubated with HR325 for 0 (open circles), 10 (closed circles), 20 (open squares), and 60 (closed squares) min and then stimulated for 10 min with (a) 100 nM PGE_2 or (b) 50 μM FKN. Mean cAMP levels from duplicate determinations were calculated and percentage inhibition values determined with respect to agonist control cAMP levels at each time point.

Table 1
Effect of time of preincubation with HR325 on inhibition of PGE_2- and FKN-stimulated cAMP production

Time of preincubation (min)	IC_{50} (μM)	
	PGE_2	FKN
0	11.8	19.7
10	12.1	22.6
20	13.4	25.2
60	13.9	32.6

Cells were incubated as described in Fig. 2. Percentage inhibition values were calculated with respect to vehicle-treated cells and IC_{50} values calculated by iterative fitting of the dose–response curves to a four-parameter logistic equation.

3.2. Oxidative phosphorylation and ATP levels

The effect of HR325 on rat liver mitochondrial respiration was investigated by monitoring oxygen uptake in the presence of glutamate as substrate (Fig. 3), in the absence (state 4 respiration) or presence (state 3 respiration) of ADP. Uncoupling activity was assessed by the effect of compounds on the state 4 respiration and inhibitory activity by their effect on state 3 respiration. Rates of oxygen uptake were expressed relative to the untreated state 3 respiratory rate. At concentrations up to 50 μM, HR325 stimulated oxygen uptake in the absence of ADP with an EC_{50} (the concentration of HR325 stimulating oxygen uptake to 50% of that driven by ADP alone) of approximately 25 μM. Niclosamide also stimulated the rate of oxygen uptake in the absence of ADP with an EC_{50} of 22 nM, and at 1 μM stimulated the rate to 110% of that in the presence of ADP alone.

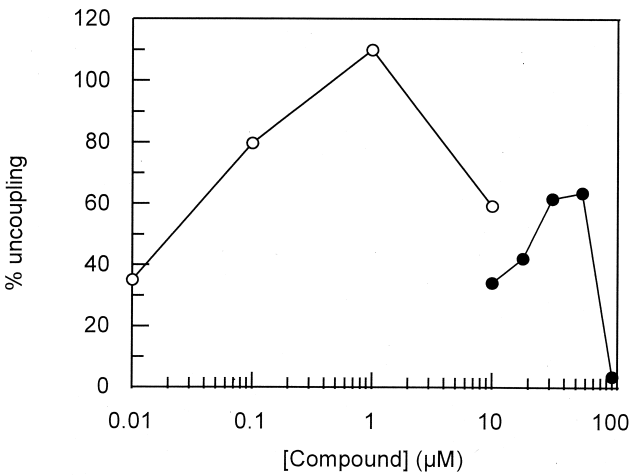

Fig. 3. Effect of HR325 and niclosamide on rat liver oxygen consumption. The rate of oxygen consumption by rat liver mitochondria was measured in the presence of HR325 (closed symbols) and niclosamide (open symbols) in the absence of ADP. Percentage uncoupling was calculated with respect to the state 3 rate of mitochondrial respiration (in the presence of ADP alone).

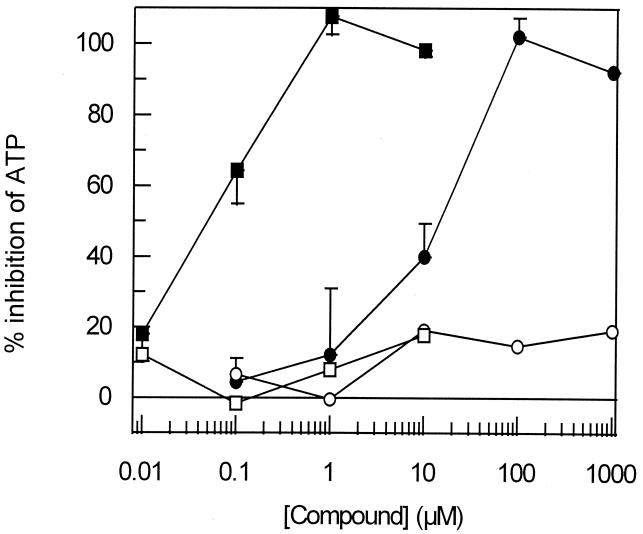

Fig. 4. Effect of HR325 and niclosamide on intracellular ATP levels in Jurkat cells. ATP concentrations in cell-free supernatants were measured as described in the Methods section following incubation of Jurkat cells with HR325 (circles) or niclosamide (squares) and percentage inhibition values calculated with respect to vehicle-treated cells. The mean results of two replicate experiments are shown (error bars show range of data). The effect of glucose supplementation was assessed by inclusion of 100 mM glucose in the cell incubations (open symbols).

Intracellular ATP concentrations of Jurkat cells were assessed using a spectrophotometric assay coupled to oxidation of NADPH (Fig. 4). Niclosamide reduced intracellular ATP levels to negative control values at concentrations of 1 μM and above, and displayed an IC_{50} of 60 nM. HR325 demonstrated maximal inhibition at concentrations of 100 μM and above with an IC_{50} of 20 μM. In the presence of 100 mM glucose, ATP concentrations were not affected by any of the concentrations of the compounds.

The effect of reduction of the ATP supply on the synthesis on cAMP by Jurkat cells was investigated using a number of compounds known to disrupt oxidative phosphorylation. Both respiratory inhibitors (potassium cyanide, rotenone) and uncouplers (2,4-dinitrophenol, niclosamide, FCCP) demonstrated dose-dependent inhibition of PGE_2-stimulated cAMP synthesis (Fig. 5). At the highest doses tested inhibition was almost complete (80–91%). In the presence of increasing concentrations of glucose, inhibition of cAMP synthesis by all of the above compounds was dose-dependently abrogated (Fig. 6). In contrast, inhibition of PGE_2-stimulated cAMP synthesis by both intermediate (data not shown) and maximally effective doses of HR325 and A77 1726 was unaffected by glucose supplementation at concentrations up to 250 mM (Fig. 6). In a similar manner, inhibition of FKN-stimulated cAMP synthesis by niclosamide was completely overcome by glucose supplementation, whereas HR325 and A77 1726 inhibition displayed no such reversal (Fig. 7).

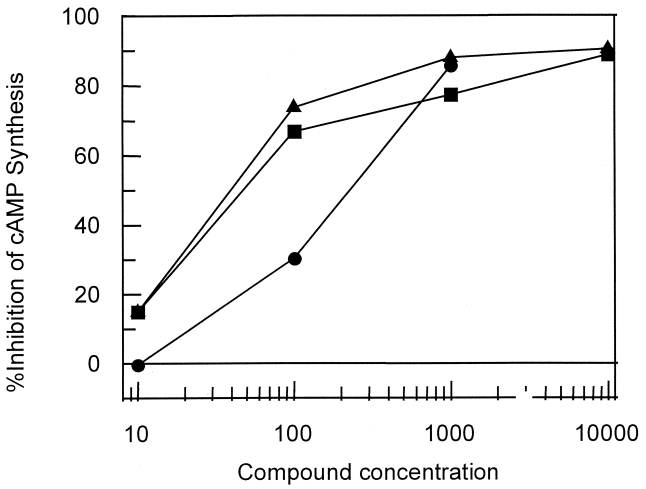

Fig. 5. Effect of mitochondrial poisons on cAMP synthesis. Jurkat cells were stimulated with 100 nM PGE_2 as described in the Methods section in the presence of increasing concentrations of KCN (circles; mM), FCCP (squares; nM), or NCA (triangles; nM). Percentage inhibition values were calculated for each concentration of compound with respect to control incubations containing vehicle only.

4. Discussion

As the agonists used to stimulate cAMP synthesis in this study displayed stimulus-specific dose and time dependence, direct comparison with the levels of cAMP synthesised in other published reports, in which different concentrations and times are used, is difficult. However, the range of concentrations detected (32–56 pmol/10^6 cells) is similar to previous results in both Jurkat cells (34–90 pmol/10^6 cells; [6]) and in human peripheral blood mononuclear cells (10–30 pmol/10^6 cells; [9]). HR325 inhibited cAMP synthesis by the Jurkat cells stimulated by both receptor-dependent (PGE_2 stimulation) and -independent (FKN stimulation) mechanisms, although a consistently lower potency was observed with the latter. Whilst this difference is statistically significant, its biological significance is unknown at present. Interestingly, whilst the analogue A77 1726 also inhibited cAMP synthesis, IC_{50} values for receptor-dependent stimulation were 5- to 10-fold higher than those for HR325. In contrast, the potency of A77 1726 against FKN-stimulated cAMP synthesis was very similar to that of HR325. These differing profiles of potencies are consistent with the possibility that HR325 and A77 1726 display different mechanisms of action against cAMP synthesis, as reported by Jöckel *et al.* [24].

Certain features of the structure of HR325, namely its weakly acidic hydroxyl, its hydrophobic nature, and its potential for charge dissipation and anion stabilisation through formation of a six-membered ring by H bonding between the oxyanion and the NH, are characteristic of uncouplers of oxidative phosphorylation [24,25]. In addition, trialkyl tin compounds, which interfere with oxidative phosphorylation, have been shown to inhibit cAMP synthesis through substrate depletion [26]. In view of this, the effects of HR325 on oxidative phosphorylation and intracellular ATP were examined to determine whether HR325 may affect cAMP through ATP depletion. Consistent with this possibility, the potency of inhibition of cAMP synthesis

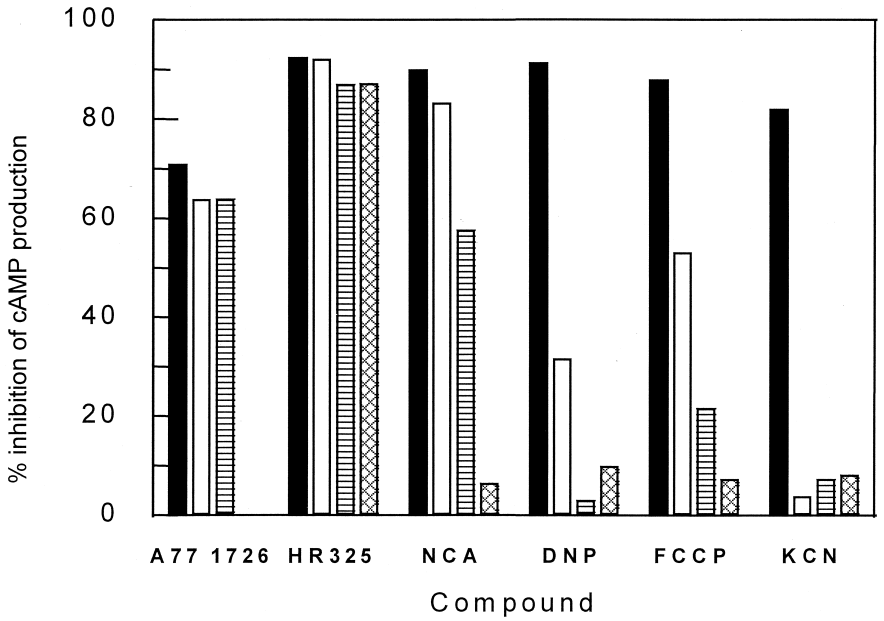

Fig. 6. Effect of glucose supplementation on inhibition of PGE_2-stimulated cAMP synthesis by several inhibitors of oxidative phosphorylation. cAMP synthesis was measured in Jurkat cells stimulated with 100 nM PGE_2 in the presence of A77 1726 (100 μM), HR325 (100 μM), NCA (10 μM), DNP (500 μM), FCCP (10 μM), or KCN (1 mM) as described in the Methods section. Percentage inhibition values were calculated with respect to vehicle-treated PGE_2-stimulated controls at 0 (filled), 5 mM (unfilled), 50 mM (horizontal), or 250 mM (hatched) glucose (no data for A77 1726 at 250 mM glucose). Values represent the mean inhibition of two replicate experiments except for DNP, which was a single experiment.

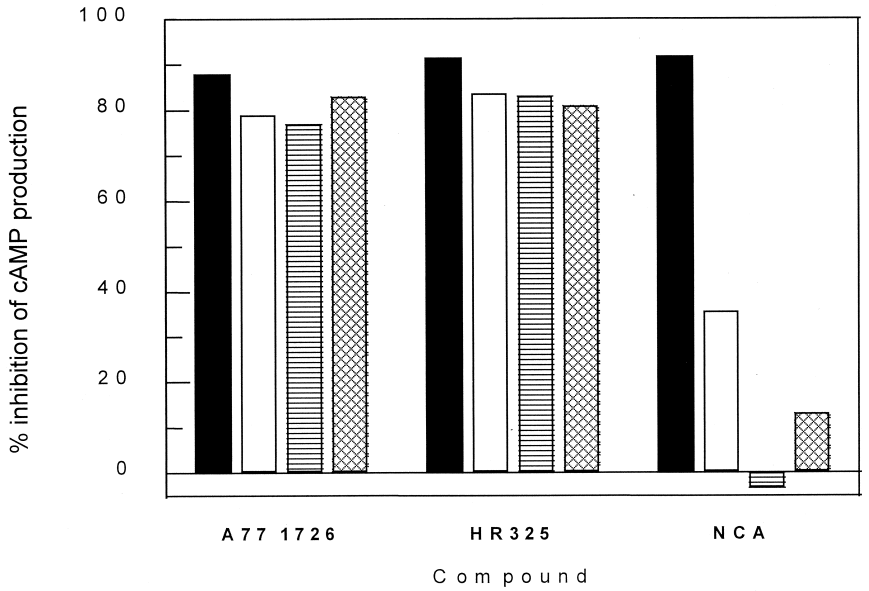

Fig. 7. Effect of glucose supplementation on inhibition of FKN-stimulated cAMP synthesis. cAMP synthesis was measured in Jurkat cells stimulated with 50 μM FKN in the presence of A77 1726 (300 μM), HR325 (100 μM), or NCA (10 μM) as described in the Methods section. Percentage inhibition values were calculated with respect to vehicle-treated FKN-stimulated controls at 0 (filled), 5 mM (unfilled), 50 mM (horizontal), or 250 mM (hatched) glucose. Values represent the mean inhibition of two replicate experiments.

by HR325 (10–30 μM) is very similar to its potency in both uncoupling (25 μM) and cellular ATP depletion (20 μM). Similarly, the antihelmintic compound NCA [27] displayed consistent potencies for inhibition of cAMP synthesis ($<$40 nM), uncoupling (22 nM), and ATP depletion (60 nM), while FCCP displayed potencies for inhibition of cAMP synthesis (45 nM) and depletion of ATP (50 nM). Although potency comparisons alone are not sufficient to establish a relationship between these effects, these results are in line with previous results [24].

Rapidly growing tumour cells display increased aerobic glycolysis and are insensitive to the effects of uncouplers or inhibitors of electron transport in the presence of glucose [28]. That depletion of ATP levels, via uncoupling of oxidative phosphorylation, was solely responsible for the inhibition of cAMP synthesis by NCA was confirmed by the fact that glucose restored both cellular ATP and cAMP synthesis in NCA-treated cells. This effect was confirmed with other uncouplers and respiratory inhibitors. However, whilst ATP depletion by HR325 would also lead to decreased cAMP synthesis, a further ATP-independent mechanism is inferred, since restoration of ATP levels was unable to overcome the inhibition of cAMP synthesis. Furthermore, since ATP depletion of human lymphocytes by mitochondrial poisons is time-dependent over a period of at least 30 min [29], simple substrate depletion should also show some time-dependent potency. This, however, is not the case for HR325, further supporting the existence of a substrate-independent mechanism of inhibition of cAMP synthesis which is inoperative in isolated membranes. Thus, HR325 and compounds of this class could inhibit cAMP production by acting at the receptor level, either through uncoupling of oxidative phosphorylation or the stopping of electron transport, or by an as yet unknown mechanism at the level of the adenylyl cyclase [24].

The primary antiproliferative effect of HR325 and A77 1726 is the result of pyrimidine depletion following inhibition of DHO-DH [2,30–33]. However, overcoming the effects of DHO-DH inhibition by uridine supplementation is only able to partially abrogate the antiproliferative activity of 50 μM A77 1726 [31]. Similarly, it has been shown that uridine only partially prevents the antiproliferative effect of HR325 against phytohaemagglutinin/IL-2-stimulated human peripheral blood mononuclear cells, elevating the IC_{50} from 30 μM to approximately 110 μM [30]. Since proliferation was measured in the presence of 10% foetal bovine serum, we investigated the effect of serum inclusion on the inhibition of cAMP synthesis by HR325. The IC_{50} was raised, presumably due to serum protein binding [34], from 11 to 92 μM (data not shown), similar to the antiproliferative potency of HR325 in the presence of uridine. It is thus possible that inhibition of cAMP synthesis is responsible for the lower potency antiproliferative activity of HR325 and the uridine-insensitive effects of A77 1726 previously noted [30,31]. Similarly, A77 1726 has been shown to be a very low potency inhibitor of tyrosine kinase activity in whole cells, with IC_{50} values of 5–170 μM for various substrates, including ζ chain of the CD3 T-cell receptor complex, phospholipase C-γ1, and epithelial growth factor receptor [35,36]. Finally, it has been shown that both leflunomide and HR325 inhibit prostaglandin endoperoxidase H synthase-1 and -2, although with lower potency than classical non-inflammatory steroids [37]. The possibility that such

effects could be related to the activities noted herein requires investigation.

We have shown that HR325 inhibits cAMP synthesis in the Jurkat T-cell line stimulated with receptor-dependent and -independent stimuli. Furthermore, two potential mechanisms of inhibition have been implicated: a reduction of the ATP supply in the absence of glycolytic substrate related to this class of compounds and a putative specific inhibition of the adenylyl cyclase system by an as yet unknown mechanism. Both of these mechanisms, however, are low potency compared with inhibition of DHODH.

Acknowledgments

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