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THE A_1 ADENOSINE RECEPTOR ANTAGONIST 1,3, DIPROPYL-8-CYCLOPENTYLXANTHINE (DPCPX) DISPLAYS ADENOSINE AGONIST PROPERTIES IN THE FRTL5 THYROID CELL LINE

Albert G. Frauman and Alan C. Moses

Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory of Beth Israel Hospital, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Mass.

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We have evaluated whether the type I adenosine receptor mediates adenosine's ability to inhibit thyrotropin-stimulated cyclic AMP generation and DNA synthesis in FRTL5 cells. The xanthine derivative 1,3-dipropyl-8-cyclopentylxanthine, a selective antagonist for the type 1 adenosine receptor, binds to FRTL5 with high affinity and specificity. 1,3-Dipropyl-8-cyclopentylxanthine does not alter basal cyclic AMP levels but does reverse adenosine's ability to inhibit thyrotropin-stimulated cyclic AMP generation. 1,3-Dipropyl-8-cyclopentylxanthine also potently inhibits thyrotropin-stimulated and dibutyryl cyclic AMP-stimulated [^{3}H]-thymidine incorporation into DNA in FRTL5 cells. Thus, in FRTL5 cells, 1,3-dipropyl-8-cyclopentyl-xanthine displays both adenosine antagonist and adenosine agonist properties, the latter occuring at a site distal to cyclic AMP generation.

Adenosine is a ubiquitous nucleoside which, in addition to serving as a substrate for ATP, acts through cell surface receptors to modulate a variety of cellular functions. Adenosine is capable of both inhibiting cellular cAMP generation through A_1 adenosine receptors and stimulating cAMP generation through A_2 adenosine receptors. The distribution of A_1 and A_2 adenosine receptors varies among different tissues. A diverse number of tissues respond to adenosine, including adipocytes (1-3), brain (4), vascular smooth muscle (5), Leydig tumor cells (6), pheochromocytoma cells (7) and platelets (6). The range of adenosine biological responses differs for different

ABBREVIATIONS: DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; A_1, type 1 adenosine receptor; A_2, type 2 adenosine receptor; IGF-I, insulin-like growth factor I; BSA, bovine serum albumin; KRB, Kreb's Ringer's buffer; NECA, N-ethylcarboxamidoadenosine; 2-CA, 2-chloroadenosine; IBMX, isobutylmethylxanthine; Bt_2cAMP, dibutyryl cyclic adenosine monophosphate.

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target tissues: for example, vasodilation (8) and negative chronotropic effects on the sino-atrial node (9) or inhibition of glycerol formation in adipocytes (2). Some effects of adenosine may be modified by specific nucleoside transporters that promote intracellular adenosine uptake (11). Despite recent advances, large gaps remain in our understanding of the diverse effects and mechanisms of action of adenosine.

Nonmetabolizable adenosine analogs have helped to define the dual nature of adenosine's action. Xanthine derivatives antagonize adenosine binding to its receptors and adenosine's biological effects (11). The xanthine DPCPX is a highly potent A_1 receptor ligand and antagonist that has been utilized to antagonize the cardiac effects of adenosine (12) and to selectively block A_1 adenosine receptors in order to visualize non A_1 binding sites in rat brain (13).

We previously have demonstrated that in the FRTL5 rat thyroid cell line adenosine inhibits TSH-stimulated DNA synthesis via cAMP-dependent pathways (14). Furthermore, adenosine potentiates IGF-I stimulated DNA synthesis via cAMP-independent pathways (14). In the present study, we have utilized DPCPX to investigate the role of one class of adenosine (A_1) receptors in FRTL5 cells. In addition to its expected antagonist activity, DPCPX possesses adenosine-like activity at a site distal to cAMP generation.

METHODS

Cell culture and [^{3}H]-thymidine incorporation assay: FRTL5 cells were grown to subconfluent density as previously described (15). Test reagents in serum-free medium containing 0.1% BSA were added to each well of cells. DPCPX (Research Biochemicals Inc., Natick, MA.) was studied in the presence of 25 mU/ml adenosine deaminase (Calbiochem, LaJolla, CA) to inactivate endogenous adenosine. After 48 hours, test media were removed and ^{3}H-thymidine (New England Nuclear, Burlington, MA.) incorporation into DNA was determined as published previously (15). Each experiment was performed in triplicate on at least 2 occasions. Statistical analyses were performed by paired t-tests on a Hewlett Packard 9845 B (Core Laboratory, Clinical Research Center, Beth Israel Hospital). Purified bovine TSH (bTSH) was obtained from the Pituitary Hormone Distribution Program of the National Institutes of Health (Bethesda, MD). Recombinant human IGF-I was a gift from Creative Biomolecules (Hopkinton, MA.)

Binding experiments: Cells were grown to confluence in the presence of 5% calf serum and 10^{-9} M TSH and then were switched to media lacking TSH for at least 5 days. [^{3}H]-DPCPX (0.05-2.2 pmoles) was added in saturation binding experiments. In competitive binding experiments, [^{3}H]-DPCPX (0.22

pmoles) (Amersham, Arlington Heights, IL.), unlabeled DPCPX, or other adenosine analogs were added and incubated overnight at 4°C. All reagents were added in modified KRB containing 280 mM sucrose instead of sodium chloride, and 0.1% BSA (15). Cells then were washed with buffer, solubilized with 1N NaOH, and counted for cell-associated radioactivity.

Unlabeled NECA (Boehringer Mannheim Biochemicals, Indianapolis, IN), adenosine (Boehringer Mannheim Biochemicals, Indianapolis IN), and 2-chloroadenosine (Sigma Chemical Co., St. Louis, MO.) were utilized in competition binding studies.

Cellular cAMP Determination: Cells were grown to confluence as noted above for the binding experiments. Test reagents were added for 30 minutes in the presence of 1mM IBMX in KRB buffer containing 0.1% BSA. Buffer was aspirated and frozen at -20°C and cells were extracted in absolute ethanol overnight (-20° C). Cell extract was lyophilized, reconstituted in buffer and combined with the extracellular material and assayed for cAMP content using a commercial radioimmunossay kit (Immuno Nuclear Corp., Stillwater,MN.).

RESULTS

Binding of DPCPX to FRTL5 monolayers: [^{3}H]-DPCPX bound to monolayers of FRTL5 in a saturable fashion with a Kd of 0.5 nM (Fig 1). Nonspecific binding was 1% at the K_d. In equilibrium competitive binding experiments performed in the presence of 0.22 pmoles [^{3}H]-DPCPX, unlabeled DPCPX was the most potent ligand tested for its ability to compete for [^{3}H]-DPCPX binding (Fig.2). The ED_{50} for DPCPX was 3nM compared to 10 μM for the

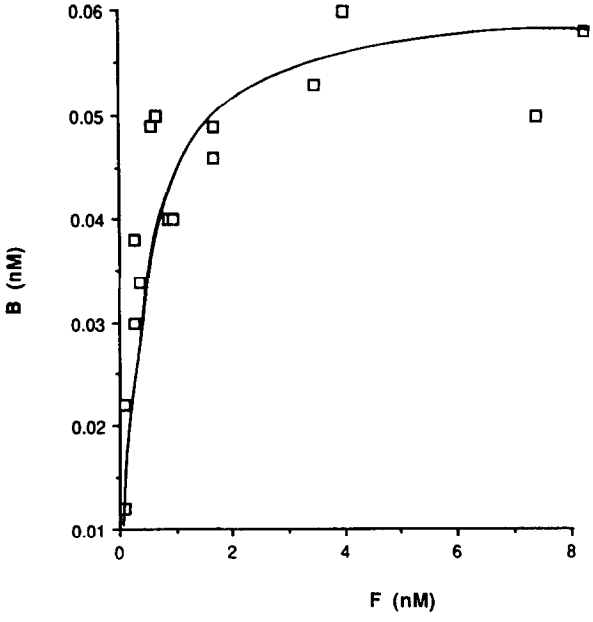

Fig. 1. Saturation binding of [^{3}H]-DPCPX to monolayers of FRTL5 cells. Confluent FRTL5 cells were incubated with [^{3}H]-DPCPX ranging from 0.05-22 pmoles/well.

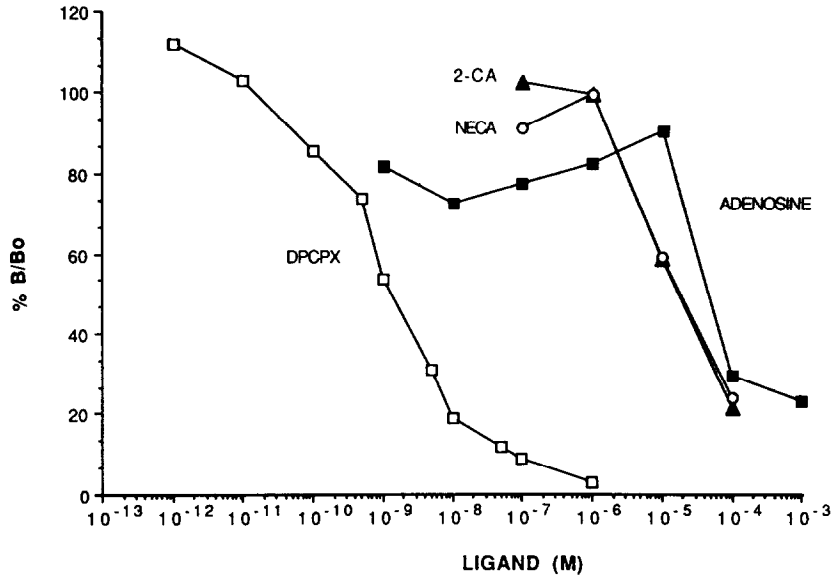

Fig. 2. Equilibrium competitive binding of [^{3}H]DPCPX to FRTL5 by unlabeled adenosine analogs.

A_1 agonist 2-chloroadenosine, 10μM for the A_2 agonist NECA, and 40μM for adenosine itself. Scatchard analysis of competitive binding data and saturation binding studies revealed a single binding site with a Kd of 3 nM.

Effects of DPCPX on TSH-stimulated cAMP accumulation and [^{3}H]-thymidine incorporation: In FRTL5 cells, TSH is a potent stimulus for cAMP accumulation and for ^{3}H-thymidine incorporation into DNA (15). 10^{-9}M bTSH markedly increases total cAMP content to 60 times control levels (fig. 3) following a 30 minute incubation in the presence of IBMX. Adenosine (1 mM) markedly inhibits this effect of TSH (Fig. 3).

Consistent with its activity as a potent A_1 receptor antagonist, DPCPX produces a dose-dependent blockade of TSH-stimulated cAMP generation (Fig. 3). DPCPX by itself does not inhibit TSH-stimulated cAMP generation and does not alter basal cAMP levels (data not shown).

Contrary to our expectations, DPCPX potently inhibits TSH-stimulated [^{3}H]-thymidine incorporation in FRTL5 (Fig. 4A). Significant inhibition ($p<0.01$) occurs at 10^{-5}M and DPCPX is more potent than adenosine in this activity (Fig. 4A). Since DPCPX, unlike adenosine, does not alter TSH-

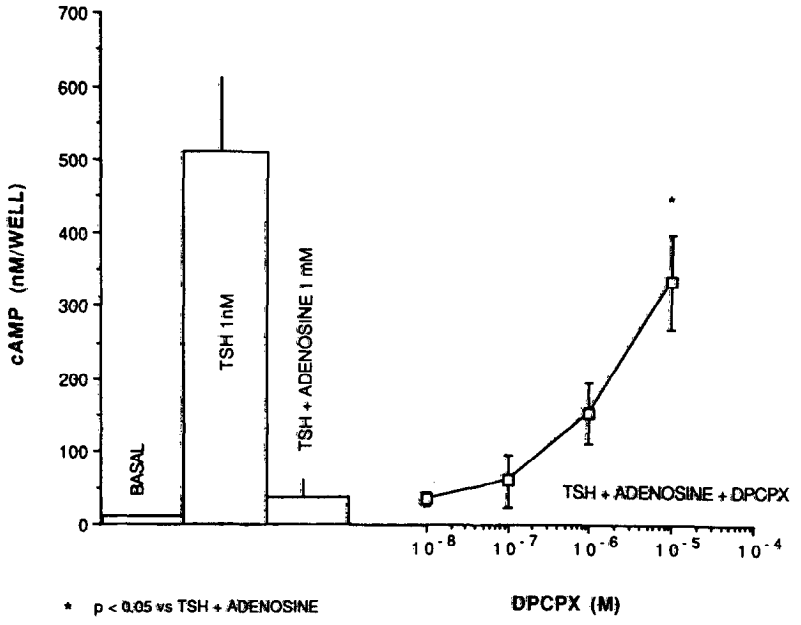

Fig. 3. Cellular cAMP responses to TSH in the presence and absence of adenosine and DPCPX.

stimulated cAMP accumulation, we investigated whether DPCPX could inhibit DNA synthesis stimulated by pathways distal to cAMP generation. Bt_2cAMP stimulates DNA synthesis in FRTL5 cells while bypassing adenylyl cyclase activation (16). DPCPX inhibits Bt_2cAMP-stimulated [^{3}H]-thymidine

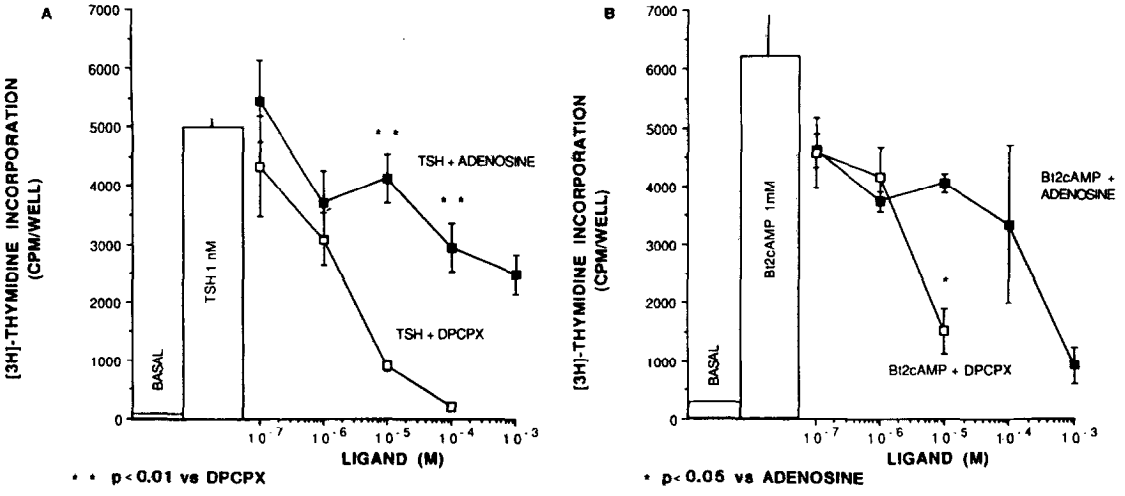

Fig. 4. Panel A. [^{3}H]-Thymidine incorporation into DNA in FRTL5 cells in response to TSH in the absence and presence of adenosine and DPCPX.
Panel B. [^{3}H]-Thymidine incorporation into DNA in FRTL5 cells in response to Bt_2cAMP in the absence and presence of adenosine and DPCPX.

incorporation. DPCPX is more potent and produces a greater degree of inhibition than does adenosine, which also partially inhibits Bt_2cAMP stimulated DNA synthesis (Fig. 4B).

DPCPX does not alter basal [^{3}H]thymidine incorporation and neither inhibits nor enhances IGF-I stimulated DNA synthesis (data not shown).

DISCUSSION

Adenosine is a ubiquitous nucleoside which can interact with at least two distinct cell surface receptors to modify cAMP production and hormone action in many cell types (1-7). Few data exist on the effects of adenosine on cell growth or the pathways subserving these effects.

Methylxanthines are antagonists of adenosine action at both A_1 and A_2 receptors (11). Although all xanthine derivatives act as phosphodiesterase inhibitors, the Ki for phosphodiesterase inhibition in brain membrane and platelet preparations is typically several times greater than the Kd for competitive binding for adenosine (11). Thus the site of action of xanthine derivatives as adenosine antagonists appears to reside primarily at adenosine receptors.

The xanthine derivative 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) is a potent A_1 ligand antagonist as assessed by the competition for the binding of other A_1 ligands to brain membrane preparations (13) and by the inhibition of the negative chronotropic effects of adenosine in the isolated rat heart (12). However, little is known about the effects of DPCPX on cell growth or intracellular cAMP levels.

The present work demonstrates that DPCPX binds to FRTL5 cells with high affinity and specificity consistent with its binding to an A_1 adenosine receptor. As expected, DPCPX acts as an adenosine antagonist since it reverses adenosine's ability to inhibit TSH-stimulated cAMP generation in FRTL5. Surprisingly, DPCPX, like adenosine itself, also potently inhibits TSH-stimulated DNA synthesis. This inhibition of DNA synthesis is not likely to be due to nonspecific toxic effects since DPCPX neither alters basal

^{3}H-thymidine incorporation nor inhibits IGF-I stimulated DNA synthesis. Thus, in FRTL5 cells, DPCPX displays both adenosine antagonist and adenosine-like agonist activity.

The mechanism(s) of DPCPX's adenosine-like agonist properties are not clear. They are not entirely explained by DPCPX interacting with an adenosine receptor. First there is no correlation between the K_i of DPCPX's biological effects (2 μM; Fig. 4A) and K_d of the binding of DPCPX (3nM; Fig. 2) to the type 1 receptor. This finding would suggest a site(s) of action of DPCPX distinct from its interactions with the A_1 receptor. Secondly, DPCPX appears to act as a locus distal to cAMP generation. DPCPX did not inhibit TSH-induced cAMP generation and did not alter basal cAMP levels. Importantly, DPCPX did inhibit DNA synthesis stimulated by Bt_2cAMP, a cAMP analog that is transported intracellularly. Adenosine itself may, in part, be acting via this pathway as it also inhibits Bt_2cAMP-stimulated DNA synthesis. Other instances of cAMP-independent actions of adenosine have been described previously. For example, adenosine potentiates insulin-stimulated glucose transport in adipocytes (17) and inhibition of superoxide production by neutrophils (18) independent of changes in cAMP generation. These effects of adenosine may result from activation of G proteins independently of adenylate cyclase activation, since pertussis toxin (which irreversibly ADP-ribosylates the $G_i\alpha$ subunit) may attenuate these effects (19). Other potential pathways mediating these effects of adenosine have not been clearly defined although calcium mobilization may play a role (20). There are no data available to suggest a role for DPCPX in altering adenosine transport.

Data obtained with DPCPX suggest that the A_1 receptor mediates adenosine's effects on TSH-stimulated cAMP generation and DNA synthesis, although a site(s) of action distal to cAMP generation also appears to be operative. The mechanisms of these pathways require additional investigation and may lead to further understanding of the role of adenosine in modifying the signaling pathways of other hormones in FRTL5 and other cell systems.

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