A Novel Partial Agonist of the A1-Adenosine Receptor and Evidence of Receptor Homogeneity in Adipocytes

Marjan Fatholahi, Yiwen Xiang, Yuzhi Wu, Yuan Li, Lin Wu, Arvinder K. Dhalla, Luiz Belardinelli, and John C. Shryock

CV Therapeutics, Inc., Palo Alto, California (M.F., Y.W., Y.L., A.K.D., L.B., J.C.S.); and Division of Cardiovascular Medicine, University of Florida, Gainesville, Florida (Y.X., J.C.S.)

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ABSTRACT

This study characterizes the receptor binding and functional effects of CVT-3619, 2-[6-[[1(R,2R)-2-hydroxycyclopentyl]amino]purin-9-yl](4S,5S,2R,3R)-5-[[2-fluorophenylthio)methyl]-oxolane-3,4-diol], a novel N6-5′-substituted adenosine analog and A1-adenosine receptor (A1AdoR) agonist, on rat epididymal and inguinal adipocytes and on the isolated heart and compares these effects with those caused by the full agonist N6-cyclopentyladenosine (CPA). In addition, the hypothesis that adipocyte A1AdoR are a heterogeneous population with regard to their affinities for ligands was tested. CVT-3619 was 10–100-fold selective for A1AdoR versus other AdoR and bound to adipocyte membranes with high (Kd = 14 nM) and low (Kd = 5.4 μM) affinities. CVT-3619 reduced cyclic AMP content and release of nonesterified fatty acids from epididymal adipocytes with IC50 values of 6 and 44 nM, respectively. CVT-3619 was a partial agonist relative to CPA to reduce lipolysis in epididymal and inguinal adipocytes. CVT-3619 did not change atrial rate in rat heart and caused a small (6-ms) prolongation of the stimulus-to-His bundle interval without causing atrioventricular block in guinea pig heart (effects mediated by A1AdoR), whereas CPA caused atrioventricular block and near cessation of atrial electrical activity. CVT-3619 increased coronary conductance (effect mediated by A2aAdoR) only at concentrations ≥10 μM. Rat epididymal adipocyte A1AdoR had similar affinities for the antagonist 8-cyclopentyl-1,3-dipropylxanthine in the presence of three dissimilar A1AdoR agonists (2-chloro-N6-cyclopentyladenosine, N6-sulfophenyladenosine, and N-5′-ethylcarboxamidoadenosine) as determined by Schild analysis. It was concluded that rat epididymal adipocyte A1AdoR are a homogeneous receptor population with regard to affinities for ligands and that CVT-3619 is a partial agonist with selectivity for A1AdoR and inhibition of lipolysis.
maximal functional response because receptor density is high and receptor reserve is great (Liang et al., 2002). Partial A₁AdoR agonists have been shown to reduce lipolysis at concentrations that do not cause effects on heart rate (van Schaick et al., 1998; van der Graaf et al., 1999; Wu et al., 2001).

Functional selectivity of an A₁AdoR agonist for inhibition of lipolysis could also be achieved if adipocyte A₁AdoR could be distinguished pharmacologically from A₂AdoR in other tissues. However, available evidence indicates that the amino acid sequences of A₁AdoR in adipose and brain tissues are not different (Stiles, 1986; Nakata, 1993). Nonetheless, the finding that adipocyte A₁AdoR are tonically active (Liang et al., 2002) suggests the possibility that there are subclasses of these receptors—possibly in the same cell—with inherently different activities and/or coupling to different signaling systems, either in the absence or presence of an agonist. The presence of subclasses of A₁AdoR in detergent-permeabilized chick myocytes has been demonstrated (Ma et al., 1994). If subclasses of A₁AdoR can be shown to be present in adipocytes, then each subclass is potentially an individual drug target of a selective agonist.

Our goals in this study were: 1) to characterize the effects of a novel A₁AdoR agonist, CVT-3619 [2-(6-[(1R,2R)-2-hydroxy(cyclopentyl)amino]purin-9-yl)(4S,5S,2R,3R)-5-[(2-fluorophenylthio)methyl]oxolane-3,4-diol], and demonstrate that the compound is a partial agonist relative to the refer-

### Materials and Methods

**Chemical and Biological Reagents.** CVT-3619 (Lot number 735-87) was synthesized by the Department of Medicinal and Bio-

4-enoic acid, 5'-N-acetyl-5'-deoxyadenosine (NEA), 5'-N-ethylcarboxamidoadenosine (NECA), on intact adipocytes.

**Isolation of Adipocytes.** Adipocytes were isolated by collagenase digestion of epididymal (abdominal) and/or inguinal (subcu-

**Assay of Cyclic AMP Content of Rat Adipocytes.** To deter-

**NEFA Release from Rat Adipocytes.** To determine the effects of A₁AdoR agonists on adipocyte cyclic AMP content, aliquots (100 μl, containing 50,000–90,000 cells, unless otherwise noted) of freshly isolated adipocytes were incubated for 6 min at 36°C with appropriate drugs: 3 μM forskolin, 1 U/ml ADA, and 1% defatted BSA in KRH buffer. Cyclic AMP phosphodiesterase inhibitors (10 μM rolipram and 1 μM cilostamide) were used when indicated to increase the accumulation of cyclic AMP. The effects of forskolin to increase CAMP content of rat adipocytes was near-maximal at 3 and maximal at a concentration of 30 μM.

The assay of cyclic AMP in experiments to investigate the heter-

**Rat Adipocyte Membrane Preparation.** Isolated cells were washed once with fresh KRH buffer (without nicotinic acid) and then collected and added to a chilled (4°C) Tris-EDTA buffer containing 250 mM sucrose, 10 mM Tris, 1 mM EDTA, and protease inhibitor cocktail, pH 7.4. The cell suspension was homogenized using a
Wheaton Potter-Elvehjelm tissue grinder. The homogenate was centrifuged at 450g for 10 min at 4°C. The supernatant fatty cake was discarded, and the infranate was removed to fresh tubes. Membranes were collected by centrifugation of the infranate at 16,000g for 15 min at 4°C. The membrane pellet was resuspended, and the centrifugation process was repeated. The final pellet was resuspended in a small volume of 250 mM sucrose with 10 mM Tris, pH 7.4, and frozen in liquid nitrogen. The protein content of membrane preparations was determined using a Bio-Rad D, protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard.

**Binding of [3H]CPX, CVT-3619, and CPA to Rat Adipocyte Membranes.** Adipocyte membranes were prepared as described above and used to determine the affinities of A1AdoR antagonist [3H]CPX to adipocyte membranes, increasing concentrations of [3H]CPX (0.075–9.6 nM) were incubated with adipocyte membranes (25 μg) for 90 min at 25°C in a 150-μl volume of HEPES-EDTA buffer, pH 7.4, containing 10 mM HEPES, 1 mM EDTA, 5 mM MgCl2, 0.1 mM benzamidine chloride, 1 U/ml ADA, 0.02% sodium azide, and protease inhibitor cocktail. At the end of the incubation, bound and free radioligands were separated by rapid filtration of the membrane suspension using a cell harvester. Collected membranes (with bound radioactivity) were washed three times with ice-cold buffer containing 10 mM Tris-HCl and 1 mM MgCl2, pH 7.4, and membrane-bound radioactivity was quantified with a microplate scintillation counter (TopCount). Specific binding of [3H]CPX was calculated as the difference between total binding and nonspecific binding. Radioactivity bound to membranes in the presence of 3 μM unlabeled CPX was assumed to be nonspecific binding. Nonspecific binding was determined at each concentration of radioligand. Competitive radioligand binding assays were used to determine the affinities of adipocyte A1AdoR for CVT-3619 and CPA. Aliquots of adipocyte membranes (25 μg) were incubated for 90 min at 25°C in 150 μl of HEPES-EDTA buffer + ADA (1 U/ml), pH 7.4, increasing concentrations of agonist, and 2 nM [3H]CPX. All assays were done in triplicate.

**Expression of A1AdoR in Rat Epididymal and Inguinal Adipose Tissues.** RNA was isolated from freshly prepared adipocytes using an Absolutely RNA Reverse Transcription-PCR Miniprep kit (Stratagene, La Jolla, CA). cDNA was transcribed by reverse transcription using a TaqMan kit (Applied Biosystems) and a GeneAmp PCR System 9700 (Applied Biosystems). To quantify A1AdoR expression levels, 2 μl of the cDNA was amplified (in triplicate) by PCR using a SYBR Green kit (PE Biosystems, Warrington, UK) with bovine serum albumin as internal control. Primer expression (Applied Biosystems) software. Forward and reverse primers (5′-TCCACCGAAGCTTCCATG-3′ and 3′-CCCTCTCGAGGATAT-5′, respectively) for the A1AdoR were designed.

**Determination of A1AdoR Population Heterogeneity in Epididymal Adipocytes.** Isolated rat epididymal adipocytes (20–50,000 cells/ml) were incubated in KRH buffer (6 min, 36°C) with 0.1 μM isoproterenol (to stimulate activity of adenyl cyclase), 1 mM ascorbic acid, 10 μM erythron-9-(2-hydroxy-3-nonyl)-adenine (EHNH, an inhibitor of ADA), and 1 nM to 10 nM CPX in the absence or presence of 0.1, 1, and 10 μM adenosine. Incubations were terminated by the addition of HCl, and lysates were harvested for cAMP content. In another set of experiments, isolated epididymal adipocytes (20–50,000 cells/ml) were incubated (6 min, 36°C) with 0.1 μM isoproterenol, 10 μM EHNH, and CPA (0.01, 0.1, or 1 μM) in the presence of either CCPA (0.01 nM-10 μM), NECA (0.01 nM-30 μM), or CPA (0.3 nM-100 μM). The effect of isoproterenol to increase CAMP content of adipocytes was near-maximal at 0.1 and maximal at 1 μM. Concentration-response relationships for agonists to decrease adipocyte cyclic AMP content and values of Kd for CPA to antagonize agonist-mediated responses (from Schild plots) were determined using GraphPad Prism version 3.0 (GraphPad Software, Inc., San Diego, CA).

**Effects of CVT-3619 and CPA on Rat and Guinea Pig Isolated Perfused Hearts.** Rat and guinea pig hearts were isolated and perfused by the method of Langendorff at a constant flow of 10 ml/min with modified Krebs-Henseleit solution at a temperature of 36.0 ± 0.5°C as described previously (Wu et al., 2001).

The effects of CVT-3619 and CPA to decrease spontaneous atrial rate of rat hearts were measured as described previously (Frolid and Belardinelli, 1990) to enable the determination of agonist functional selectivity for adipose tissue versus heart. Average atrial rate (de-polarizations/minute) was determined during a steady-state response to each concentration of drug. To determine the selectivities of CVT-3619 and CPA to elicit functional responses mediated by A1AdoR versus A2AAdoR in the same heart, the guinea pig was used as the animal model because it is more responsive to AdoR agonists than is the rat (Frolid and Belardinelli, 1990). To record the effects of drugs on the S-H interval, parts of the left and right atrial tissues, including the region of the sinoatrial node, were removed to decrease the spontaneous heart rate and to expose the atrial septum for electrode placement. Hearts were electrically paced at a rate of 3.2 Hz, and the His bundle electrogram was recorded and displayed continuously in real time (Tektronix Inc., Beaverton, OR) at a sweep speed of 10 ms/cm. The duration of the time from the first pacing artifact to the maximal deflection of the His bundle signal was used as the S-H interval. To measure the effects of drug on the coronary perfusion pressure, a pressure transducer was connected to a port on the aortic perfusion cannula, and pressure signals were analyzed using a Power Lab acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia) and a computer. Drugs were infused via the aortic cannula, and steady-state responses were recorded for analysis. Coronary conductance (in milliliter minute−1 mm Hg−1) was calculated as the ratio of coronary flow (10 ml/min) to coronary perfusion pressure (in mm Hg).

**Data Analysis.** All data are reported either as mean ± S.E.M. or as mean with 95% confidence limits given in parentheses (e.g., for values of EC50 and IC50). Values of IC50 and maximal effects of CVT-3619 and CPA in functional assays were determined by nonlinear regression analysis of drug concentration-response relationships using Prism. Values of Kd and maximal binding (Bmax) of [3H]CPX in saturation binding assays and values of Kd, Ki, and KI in competitive radioligand binding assays were determined by nonlinear regression analysis using Prism.

Statistical analysis of data from experiments with two treatment groups (i.e., maximal effect of CPA and CVT-3619 on S-H interval) was performed using the unpaired Student’s t test. Repeated measures one-way analysis of variance followed by Student-Newman-Keuls test was used to compare values of measurements obtained from experiments with more than two treatment or time groups.
The affinities of CPA for high- and low-affinity agonist 

The percentages of receptors with high affinity for CVT-3619

expression of 

assess the selectivity of the compound for the A1AdoR. The

concentration-dependent and biphasic manner (Fig. 1). The

values of $K_a$ from inguinal adipocytes with $B_{max}$ and $K_a$ values of 355 ± 32 (287–423) fmol/mg and 1.7 (1.0–2.4) nM ($n = 2$), respectively. 

The differences between values of $B_{max}$ and $K_a$ for 

binding to epididymal and inguinal preparations were not significant ($p > 0.05$). The levels of expression of A1AdoR in epididymal and inguinal fat tissues were 5.6 ± 0.7 (n = 5) and 6.5 ± 0.3% (n = 3), respectively, of the levels of expression of β-actin $\alpha_1$ in each tissue ($p > 0.05$).

**Results**

To confirm that A$_1$AdoR are present in both epididymal and inguinal adipose tissues of the rat under the conditions of our experiments, both radioligand binding and mRNA expression studies were done. The antagonist radioligand [$^3$H]CPX bound to membranes prepared from epididymal adipocytes with $B_{max}$ and $K_a$ values of 299 ± 47 (95% confidence limits, 202–396) fmol/mg protein and 0.8 (0.1–1.5) nM ($n = 4$), respectively. [$^3$H]CPX bound to membranes prepared from inguinal adipocytes with $B_{max}$ and $K_a$ values of 32 (287–423) fmol/mg and 1.7 (1.0–2.4) nM ($n = 2$), respectively. The affinities of CPA for the low-affinity state of the A1AdoR ($i.e.$, A1AdoR) in DDT1MF-2 and CHO cells expressing the human A$_1$AdoR were 113 (76–167) nM ($n = 4$) and 1.1 (0.8–1.5) μM ($n = 7$), respectively. The values of $K_a$ for CPA in the same assays were 10 (5–17) nM ($n = 4$) and 0.3 (0.2–0.3) μM ($n = 3$), respectively. Neither CVT-3619 (0.1 nM-10 μM) nor CPA (1 nM-100 μM) displaced the binding of [$^3$H]ZM241385 (2 nM) to human A$_2a$AdoR or the binding of [$^3$H]ZM241385 (14 nM) to human A$_2b$AdoR expressed in HEK-293 cells ($n = 3$). Neither CVT-3619 nor CPA displaced the binding of the A$_3$AdoR ligand [$^3$H]MRE3008F20 (0.7 nM) at concentrations up to 3 μM, and at a concentration of 10 μM (the highest concentration tested), each displaced 25% of [$^3$H]MRE3008F20 binding.

**Reductions by CVT-3619 and CPA of Cyclic AMP Content of Adipocytes.** CVT-3619 and CPA each reduced the content of cyclic AMP in both epididymal and inguinal adipocytes incubated with 3 μM forskolin (Fig. 2). Values of IC$_{50}$ for CVT-3619 and CPA to reduce the cyclic AMP content of inguinal adipocytes were 44.3 (11.6–168) and 0.011 (0.004–0.029) nM, respectively ($p < 0.05$). Values of IC$_{50}$ for CVT-3619 and CPA to reduce the cyclic AMP content of inguinal adipocytes were 44.3 (11.6–168) and 0.011 (0.004–0.029) nM, respectively ($p < 0.05$). Maximal reductions by CVT-3619 and CPA of forskolin-stimulated cyclic AMP content were 87 and 90% in epididymal and 76 and 84% in inguinal adipocyte preparations, respectively (Fig. 2). The

Fig. 2. Attenuation by CVT-3619 and CPA of stimulation by 3 μM forskolin of cyclic AMP accumulation in rat epididymal (A) and inguinal (B) adipocytes. Forskolin (3 μM) alone increased the cyclic AMP contents of epididymal and inguinal adipocytes by 15 ± 2- and 10 ± 1-fold, respectively, above basal (no drug). Cilostamide (1 μM) and rolipram (10 μM) were present in the incubation media to inhibit cyclic AMP phosphodiesterase activity. Values of IC$_{50}$ for CVT-3619 to reduce cAMP contents of epididymal and inguinal cells were 6 and 44 nM, respectively; the same values of IC$_{50}$ for CPA were 14 and 11 pM. Each symbol represents a mean ± S.E.M. of data from seven experiments, each with four replicate determinations. See Materials and Methods for experimental conditions.

Fig. 1. Displacement by CVT-3619 and CPA of the binding of [$^3$H]CPX (2 nM) to membranes prepared from rat epididymal adipocytes. Each symbol represents a mean ± S.E.M. of data from three experiments, each with three replicate determinations. The specific binding of [$^3$H]CPX (2 nM) in the absence of competing ligand was approximately 2000 dpm per assay well (total binding averaged 2376 dpm; nonspecific binding averaged 294 dpm). Values of $K_i$ and $K_i$ for CPA-3619 were 14 nM and 5.4 μM, respectively; for CPA, these same values were 0.3 nM and 0.14 μM. See Materials and Methods for assay conditions.
maximal effects of CVT-3619 and CPA to reduce the cyclic AMP contents of epididymal and inguinal adipocytes were not significantly different. Responses of epididymal and inguinal adipocytes were also not significantly different (Fig. 2).

**Reductions by CVT-3619 and CPA of the Release of NEFA from Adipocytes.** CVT-3619 and CPA each reduced the release of NEFA from both epididymal and inguinal adipocytes in the presence of 1 μM forskolin (Fig. 3). The responses of epididymal and inguinal adipocytes (Fig. 3) either to CVT-3619 or to CPA were not significantly different (p > 0.05 by two-tailed t test). Forskolin (1 μM) increased NEFA release by 13-fold above control (absence of drug). Values of IC50 for CVT-3619 and CPA to reduce the release of NEFA from epididymal adipocytes were 47 (9–240) and 0.5 (0.2–1.3) nM, respectively (p < 0.05). Reduction by CVT-3619 (10 μM) of the release of NEFA from epididymal adipocytes was attenuated by the selective A1AdoR antagonist CPX with an EC50 value of 0.10 (0.06–0.16) μM (data not shown). CPX (10 μM) abolished the effect of 10 μM CVT-3619 to reduce the release of NEFA. Values of IC50 for CVT-3619 and CPA to reduce the release of NEFA from inguinal adipocytes were 170 (86–370) and 0.19 (0.08–0.48) nM, respectively (p < 0.05). Relative to CPA, CVT-3619 was a partial agonist to reduce the release of NEFA from both epididymal and inguinal adipocytes (Fig. 3). CPA reduced the forskolin (1 μM)-stimulated release of NEFA from epididymal and inguinal adipocytes by ≥100% (i.e., to control levels or slightly below), respectively (Fig. 3). Maximal reductions by CVT-3619 of the release of NEFA from epididymal and inguinal adipocytes were only 42 and 58%, respectively, of those caused by CPA. However, when the concentration of forskolin used to stimulate NEFA release from epididymal adipocytes was reduced 10-fold (from 1 to 0.1 μM), both CVT-3619 and CPA were able to fully attenuate the response. Forskolin (0.1 μM) increased NEFA release by 3.5-fold (Fig. 3C). CVT-3619 (10 μM) and CPA (0.1 μM) reduced the forskolin-induced stimulation of NEFA release by 102 ± 1 and 104 ± 2%, respectively (p < 0.01 for both, compared with forskolin; Fig. 3C).

**Assessing Heterogeneity of Rat Epididymal A1AdoR.** To investigate the possibility that the population of A1AdoR in intact epididymal adipocytes is heterogeneous with respect to affinities for agonists and antagonists, two types of experiments were done. First, concentration-response relationships for the A1AdoR antagonist CPX to increase the adenosine content of cyclic AMP in the absence and presence of three different concentrations of adenosine were determined (Fig. 4). CPX alone, by antagonizing the inhibitory action of endogenous adenosine in the presence of 0.1 μM isoproterenol, increased the cyclic AMP content of adipocytes. The EC50 value for CPX to increase cyclic AMP content was 7.1 (6.3–8.0) nM and the Hill slope of the concentration-response relationship was 1.1 (1.0–1.2). In the presence of 0.1, 1, and 10 μM adenosine, the EC50 values for CPX to increase cyclic AMP were 41 (33–52), 210 (178–249), and 1162 (921–1467) nM, respectively, and the Hill slopes of the CPX concentration-response relationships were 1.3 (1.0–1.6), 1.3 (1.0–1.5), and 2.3 (1.0–3.6), respectively. Increasing the concentration of adenosine from 0.1 to 1 and from 1 to 10 μM caused 5.1- and 5.5-fold increases (p > 0.05), respectively, in the EC50 values for CPX to increase adenosine cyclic AMP. The results are consistent with the presence of a single receptor site (i.e., A1AdoR) for adenosine.

To further confirm the hypothesis that A1AdoR of epididymal adipocytes are homogeneous with regard to ligand affinity, we determined the values of Kd for CPX to antagonize the actions of three structurally different A1AdoR agonists (CCPA, NECA, and SPA) (Fig. 5). CCPA is a 2-Cl, 6 analog that presumably does not activate A1AdoR, while NECA is a 5′-substituted analog. SPA is a charged 6 analog that presumably does not activate A1AdoR. We observed affinities for agonists and antagonists at epididymal and inguinal adipocytes. The EC50 value for CPX to increase adipocyte cyclic AMP was 7.1 (6.3–8.0) nM and the Hill slope of the concentration-response relationship was 1.1 (1.0–1.2). In the presence of 0.1, 1, and 10 μM adenosine, the EC50 values for CPX to increase cyclic AMP were 41 (33–52), 210 (178–249), and 1162 (921–1467) nM, respectively, and the Hill slopes of the CPX concentration-response relationships were 1.3 (1.0–1.6), 1.3 (1.0–1.5), and 2.3 (1.0–3.6), respectively. Increasing the concentration of adenosine from 0.1 to 1 and from 1 to 10 μM caused 5.1- and 5.5-fold increases (p > 0.05), respectively, in the EC50 values for CPX to increase adenosine cyclic AMP. The results are consistent with the presence of a single receptor site (i.e., A1AdoR) for adenosine.
cross the cell membrane. Each agonist alone reduced cyclic AMP content of epididymal adipocytes in the presence of 0.1 μM isoproterenol by up to 95%. The values of $pK_B$ (determined by analysis of Schild plots) for CPX to antagonize the actions of CCPA, NECA, and SPA were 8.45 (7.88–9.84), 8.41 (8.22–8.65), and 8.64 (7.98–10.55), respectively (Fig. 5). The Hill slopes of the Schild regressions for CCPA, NECA, and SPA were 1.18, 1.17, and 1.15, respectively; these values of Hill slopes were not significantly different from unity ($p > 0.05$). Neither the values of $K_B$ nor the values of Hill slopes of the Schild plots for CPX to antagonize responses to the different agonists were significantly different ($p > 0.05$).

Effects of CVT-3619 on Electrophysiologic and Hemodynamic Parameters in Isolated Rat and Guinea Pig Hearts. The effects of CVT-3619 and CPA on spontaneous atrial rate in the rat heart were determined to allow calculation of the functional selectivity of each compound for A1AdoR-mediated responses in adipose versus heart tissue. CVT-3619 (0.1–30 μM) decreased spontaneous atrial rate of the isolated rat heart by 12 ± 2% ($n = 6$) at a concentration of 30 μM (data not shown). The estimated values of $EC_{15}$ for CVT-3619 to decrease spontaneous atrial rate and adipocyte lipolysis (i.e., 1 μM forskolin-stimulated NEFA release) were 30 μM and 30 nM, respectively, and therefore, the selectivity (i.e., the ratio of values of $EC_{15}$) of CVT-3619 to reduce lipolysis was 1000-fold. In contrast, CPA (0.1–1 μM) decreased the spontaneous atrial rate by 100% ($n = 3$) with an $EC_{50}$ value of 50 nM (data not shown). Thus, the values of $EC_{50}$ for CPA to decrease spontaneous atrial rate and adipocyte lipolysis were 50 and 0.5 nM, respectively, and the selectivity of CPA to reduce lipolysis was 100-fold. CVT-3619 was a partial agonist relative to CPA to reduce both atrial rate and lipolysis.

The functional selectivity of CVT-3619 for A1 versus A2AAdoR was determined using the guinea pig rather than the rat heart, because the guinea pig heart responds better to A1AdoR agonists than the rat heart (Froldi and Belardinelli, 1990). Drug effects to prolong the S-H interval and to slow the spontaneous atrial rate (actions mediated by the A1AdoR) and to increase coronary conductance (an action mediated by the A2AAdoR) (Belardinelli et al., 1998; Shryock et al., 1998) were measured. CVT-3619 (10 nM–30 μM) caused a small but significant increase of the S-H interval by 6 ± 1 ms ($n = 7$, $p < 0.01$ above baseline) without causing second or higher degree atrioventricular block (Fig. 6). In contrast, CPA significantly prolonged the S-H interval by as much as 38 ms ($n = 5$, $p < 0.001$) and caused second or higher degree atrioventricular block at concentrations >30 nM (Fig. 6).
CVT-3619 (10 μM) shifted the concentration-response relationship for CPA to increase the S-H interval to the right (data not shown; n = 5 hearts). Concentrations of CPA that prolonged the S-H interval to 60 ms were 22 and 158 nM in the absence and presence of CVT-3619 (10 μM), respectively. This result suggests that CVT-3619 is a partial agonist of the A1AdoR, which mediates prolongation of the S-H interval. CVT-3619 (10 nM-30 μM) caused no significant change in atrial rate (n = 4, p > 0.05) (data not shown). CVT-3619 (1–10 μM) caused a relatively small increase of coronary conductance (±29 ± 4%).

Discussion

The present study investigated the actions of the novel A1AdoR agonist CVT-3619 on adipocytes from rat white adipose tissue. Receptor binding and functional effects of CVT-3619 and CPA (the reference full A1AdoR agonist) were characterized. The results indicated that 1) CVT-3619 bound to two states (i.e., high and low affinity) of adipocyte A1AdoR with affinities that were approximately 40-fold lower than that of the reference agonist CPA; 2) CVT-3619 decreased both cellular cAMP content and release of NEFA from both epididymal and inguinal adipocytes with potencies that were at least 100-fold lower than those of CPA; 3) CVT-3619 was a full agonist to decrease adipocyte cyclic AMP content but a partial agonist to decrease the release of NEFA relative to CPA to the full agonist CPA; and 4) the expression of A1AdoR in and the effects of both CVT-3619 and CPA on adipocytes from epididymal (abdominal) and inguinal (subcutaneous) fat tissues were not different. Investigation of the binding of CVT-3619 to human AdoR that was expressed in cultured cell lines revealed that CVT-3619 was at least 10–100-fold selective for A1 versus either A2A or A2BAdoR and at least 10-fold selective for A1 versus A3AdoR. In addition, results of investigations of the actions of CVT-3619 and CPA on electrophysiologic (atrial rate, S-H interval) and hemodynamic coronary perfusion pressure parameters in isolated hearts of rats and guinea pigs indicated that CVT-3619 was a partial agonist (relative to CPA) to cause responses mediated by either A1 or A2AAdoR and seemed to be relatively selective for the A1AdoR. Lastly, it was shown by rigorous pharmacologic criteria (Kenakin, 1992, 1997) that rat epididymal adipocyte A1AdoR are a homogeneous receptor population with regard to responses to agonist ligands.

Both CVT-3619 and CPA seemed to be selective for the A1AdoR when assessed in radioligand binding assays using membranes prepared from cells in which a single AdoR subtype was overexpressed. Thus, neither CVT-3619 nor CPA, at the concentrations tested, displaced the binding of receptor subtype-selective antagonists to A2A or A2BAdoR but displaced binding of radioligand to A1AdoR only at a concentration of 10 μM. In these assays, guanosine 5′-[γ-thio]-triphosphate was present during the incubations to uncouple AdoR from G proteins. This was done to facilitate comparisons for each agonist (CPA and CVT-3619) to bind to a single state of each AdoR (i.e., the low-affinity agonist-binding state), because the fraction of receptors in the high-affinity state in each assay/cell line may be different. However, a limitation of the assays used is that the selectivities of CVT-3619 and CPA for AdoR, when these receptors are in low- and high-affinity agonist binding states, may be different.

Values of IC50 for both CPA and CVT-3619 to reduce the cyclic AMP content of epididymal adipocytes were lower than the values of IC50 for CPA to release NEFA. Although comparison of these values must be done with caution because the conditions of each assay are different, these results are not unexpected. The ratios of values of IC50 for CPA to CVT-3619 to reduce cyclic AMP were 21 and 2.3, respectively. These data are consistent with the report (Liang et al., 2002) that activation of a small percentage of the adipocyte A1AdoR population elicits a disproportionately larger functional response (expressed as percentage of maximum), indicative of a large receptor reserve. The data also suggest that the intrinsic efficacy (Stevenson, 1956) of CPA to activate the adipocyte A1AdoR is greater than that of CVT-3619. This interpretation is consistent with the finding that CVT-3619 is an A1AdoR partial agonist relative to CPA, both to reduce lipolysis in adipocytes and to prolong the S-H interval in the isolated heart. Both CPA and CVT-3619 reduced the cyclic AMP content of adipocytes, with greater potencies than they reduced NEFA release. A possible explanation of this finding is that the relationship between cyclic AMP content and lipolysis is not linear. Rather, the rate of lipolysis apparently reaches a maximum at relatively low levels of cAMP (Honnor et al., 1985). Thus, reductions of either isoproterenol- or forskolin-induced elevations of cyclic AMP content may not result in reduction of lipolysis until cyclic AMP is substantially reduced. In addition, because the durations of the lipolysis and cyclic AMP assays were 60 and 6 min, respectively, desensitization of A1AdoR (Green, 1987) in the longer assay may be an explanation of differences in agonist potency in the two assays. In this situation, desensitization to the full agonist CPA may be expected to have been greater than desensitization to the partial agonist CVT-3619 (Clark et al., 1999), thus potentially causing a greater underestimation of the potency of the CPA than of CVT-3619 to reduce lipolysis.

The functional selectivities of CVT-3619 and CPA in the rat to decrease epididymal adipose tissue lipolysis relative to atrial rate (both A1AdoR-mediates effects) were approximately 1000- and 100-fold, respectively. The fact that A1AdoR reserve is significantly higher in adipose tissue compared with heart (Srinivas et al., 1997; Liang et al., 2002) may explain the functional selectivities of CVT-3619 and...
CPA for adipose tissue. The partial agonist CVT-3619 was more selective than the full agonist CPA to inhibit lipolysis in the rat. In the guinea pig isolated heart, CVT-3619 caused only a small increase in the S-H interval and no significant change in atrial rate. In contrast, CPA caused A-V block in this study and has been shown to cause a near-complete reduction of atrial electrical activity (i.e., decrease of atrial rate) in the guinea pig isolated heart (Wu et al., 2001). Partial agonists of the A₁AdoR have previously been shown to have selectivity of action to reduce lipolysis (van Schaick et al., 1998). The potencies and maximal effects of low-efficiency analogs of CPA were found to be greater for reduction of lipolysis than for reduction of heart rate in the rat (van Schaick et al., 1998). By use of pharmacokinetic-pharmacodynamic modeling of the in vivo antilipolytic and bradycardic effects of A₁AdoR agonists in rats, it was found that the density and/or efficiency of coupling of A₁AdoR is 38-fold higher for mediation of the antilipolytic than for the bradycardic (i.e., heart rate slowing) effect (van der Graaf et al., 1999). Thus, the results of the study of van Schaick et al. (1998), the modeling study of van der Graaf et al. (1999), and the present study support the idea that partial A₁AdoR agonists can reduce lipolysis without causing cardiodepressant side effects, within an appropriate concentration range (e.g., up to 10 μM for CVT-3619).

CVT-3619 caused a small (up to 29%) decrease of coronary perfusion pressure (an action mediated by the A₂₅AdoR; Belardinelli et al., 1998), in spite of the fact that receptor reserve for an A₂₅AdoR-mediated increase of coronary conductance is very high (Shryock et al., 1998). In contrast, CPA has been shown to increase coronary conductance in the guinea pig isolated heart by 106% (Wu et al., 2001). Thus, the partial agonist CVT-3619 was more selective than the full agonist CPA for A₁ versus A₂₅AdoR. On the other hand, neither CVT-3619 nor CPA was selective to reduce either lipolysis or cyclic AMP in epididymal versus inguinal adipocytes. There was also no significant difference in the level of expression of A₁AdoR in epididymal versus inguinal fat tissues.

This study is the first to determine whether the population of A₁AdoR in adipocytes is heterogeneous. The results suggest that a single population of A₁AdoR mediates an inhibition by agonists of the activity of adenylyl cyclase. Two approaches were used to detect a possible heterogeneity in A₁AdoR in adipocytes. First, concentration-response relationships for the A₁AdoR antagonist CPX to increase adipo- cyte content of cyclic AMP in the absence and presence of three different concentrations of adenosine were determined. The presence of two or more populations of receptors with differing affinities for adenosine might be expected to lead to biphasic or nonparallel CPX concentration-response curves as the concentration of adenosine was increased. This was not observed (Fig. 4). Second, the Kᵥ values and Hill slopes for CPX to antagonize the actions of three different A₁AdoR agonists (CCPA, NECA, and SPA) on cyclic AMP content of adipocytes were determined. These three agonists were selected because of their dissimilarities in structure and lipid solubility. The values of Kᵥ and Hill slope for CPX to antagonist the actions of CCPA, NECA, and SPA were not significantly different (Fig. 5). The results suggest that the adipocyte A₁AdoR that mediate inhibitions of adenylyl cyclase activity by CCPA, NECA, and SPA are pharmacologically homogenous.

In conclusion, this study was demonstrated that CVT-3619 is a relatively selective and partial A₁AdoR agonist with minimal effects on cardiac function. CVT-3619 inhibited cyclic accumulation and NEFA release from both epididymal and inguinal adipose tissues. It was found that A₁AdoR in rat epididymal adipose tissue can be described as a single homogenous population with regard to affinities for agonist and antagonist ligands.

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References


Address correspondence to: Dr. John C. Shryock, 3172 Porter Drive, Palo Alto, CA 94304. E-mail: john.shryock@cvrt.com