

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

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List of nonstandard abbreviations:

ADA, adenosine deaminase; BHA, butylated hydroxyanisole; BSA, bovine serum albumin; CCP, coronary perfusion pressure; CCPA, 2-chloro-N⁶-cyclopentyladenosine; CPA, N⁶-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; CVT-3619, 2-{6-[(1R,2R)-2-hydroxycyclopentyl]amino}purin-9-yl}(4S,5S,2R,3R)-5-[(2-fluorophenylthio)methyl]oxolane-3,4-diol; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-

adenine; HE, HEPES-EDTA; GTP γ S, guanosine 5'-[γ -thio]triphosphate; K-H, Krebs-Henseleit; KRH, Krebs-Ringer-HEPES; MRE3008F20, 5*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; NECA, 5'-N-ethylcarboxamidoadenosine; NEFA, non-esterified fatty acid; S-H, stimulus to His bundle; SPA, N⁶-sulfophenyladenosine; TE, Tris-EDTA; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

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ABSTRACT

This study characterizes the receptor binding and functional effects of CVT-3619, a novel N⁶, 5'-substituted adenosine analogue and A₁-adenosine receptor (A₁AdoR) agonist, on rat epididymal and inguinal adipocytes and on the isolated heart, and compares these effects to those caused by the full agonist N⁶-cyclopentyladenosine (CPA). In addition, the hypothesis that adipocyte A₁AdoR are a heterogeneous population as regards their affinities for ligands was tested. CVT-3619 was 10-100-fold selective for A₁- versus other AdoR and bound to adipocyte membranes with high (K_H = 14 nmol/L) and low (K_L = 5.4 μmol/L) affinities. CVT-3619 reduced cyclic AMP content and release of nonesterified fatty acids from epididymal adipocytes with IC₅₀ values of 6 and 44 nmol/L, 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-msec) prolongation of the S-H 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 A_{2A}AdoR) only at concentrations ≥ 10 μmol/L. 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 as regards affinities for ligands, and that CVT-3619 is a partial agonist with selectivity for A₁AdoR and inhibition of lipolysis.

INTRODUCTION

A₁-Adenosine receptors (A₁AdoR) are highly expressed in white adipose tissue (Ukena et al., 1984; Dong et al., 1994; Dhalla et al., 2003). A₁AdoR mediate inhibition by adenosine and adenosine analogs of adenylyl cyclase activity, reduction of cyclic AMP formation, and inhibition of lipolysis (Fain et al., 1972; Schwabe et al., 1973). A reduction of lipolysis in adipocytes is of potential benefit in treatments of dyslipidemia, type II diabetes, and metabolic syndrome (Boden and Laakso, 2004; DeFronzo, 2004; Bergman et al., 2001; Lebovitz and Banerji, 2004). However, A₁AdoR agonists have potential unintended side effects as a result of the presence of A₁AdoR in many other tissues (Merkel et al., 1995).

The undesired effects of A₁AdoR in non-adipose tissues can theoretically be minimized by use of low-efficacy agonists (also called partial agonists), to achieve functional selectivity of drug action (van Schaick et al., 1998). A partial agonist may either stabilize a less active receptor conformation or activate a smaller portion of a dichotomous receptor population than a full agonist. A partial agonist may thus elicit a maximal response only in tissues in which there is receptor reserve (i.e., tissues wherein sub maximal receptor activation nevertheless leads to a maximal functional effect), even when the concentration of agonist is sufficiently high to occupy all receptors. In adipose tissue a partial agonist can provoke a 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 A₁AdoR 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, and demonstrate that the compound is a partial agonist relative to the reference full agonist N⁶-cyclopentyladenosine (CPA), and 2) to investigate the possibility that A₁AdoR in adipocytes are a heterogeneous population with subclasses that can be distinguished by different agonist ligands. The effects of CVT-3619 on cyclic AMP content and lipolysis in both epididymal and inguinal adipose tissues from the rat are described. Two white adipose tissues were studied because it is clear that the regulation and function of subcutaneous (e.g., inguinal) and visceral (e.g., epididymal) adipose tissues are often distinct (Ostman et al., 1979; Wajchenberg, 2000; Kabir et al., 2004). To detect the potential presence of two (or more) populations of A₁AdoR we used a pharmacologic method described by Kenakin (1992, 1997). In this method the presence

of two-receptor systems is detected as a dependence of antagonist potency on the type of agonist used to produce an effect on the tissue; thus, different Schild regressions (Arunlakshana and Schild, 1959) for a given antagonist to reduce the effects of different agonists can indicate that those agonists produce responses by activation of a mixed receptor population (Kenakin, 1992). Therefore we analyzed Schild regressions for the A₁AdoR antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX) to block the effects of three structurally diverse A₁AdoR agonists, 2-chloro-N⁶-cyclopentyladenosine (CCPA), N⁶-sulfophenyladenosine (SPA), and 5'-N-ethylcarboxamidoadenosine (NECA) on intact adipocytes.

METHODS

Chemical and biological reagents. 2-{6-[(1R,2R)-2-hydroxycyclopentyl]amino]purin-9-yl}(4S,5S,2R,3R)-5-[(2-fluorophenylthio)methyl]oxolane-3,4-diol (CVT-3619, Lot # 736-87) was synthesized by the Department of Medicinal and Bio-Organic Chemistry of CV Therapeutics, Inc. (Palo Alto, CA). Other agents were purchased from the following sources: collagenase types I and IV from Worthington Biochemicals (Lakewood, NJ); adenosine deaminase (ADA) and protease inhibitor cocktail tablets (Complete) from Roche Diagnostics (Mannheim, Germany); [³H]-8-cyclopentyl-1,3-dipropylxanthine ([³H]CPX) from Perkin Elmer Life and Analytical (Boston, MA); [³H]ZM241385 from Tocris-Cookson (Bristol, UK); [³H]MRE3008F20 from Amersham (Buckinghamshire, UK); DDT₁MF-2, CHO, and HEK cell lines, F-12K media, and fetal bovine serum from ATCC (Manassas, VA); trypsin-EDTA from Clonetics (Baltimore, MD); G-418 from US Biological (Swampscott, MA). All other chemicals were purchased from Sigma. Adenosine receptor agonists and antagonists were dissolved in dimethyl sulfoxide (DMSO) as 10 mmol/L stock solutions and diluted in physiological saline for use. The maximum percentage of DMSO in any cell or membrane incubation medium was 0.15%.

Animals. Animals received humane care according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication 86-23, revised 1996). Animal use was reviewed and approved by the Institutional Animal Care and Use Committee at CV Therapeutics. Sprague-Dawley male rats (350-430 g) were purchased from Charles River (Hollister, CA) and Hartley guinea pigs of either sex (300-350 g)

were purchased from Harlan (Indianapolis, IN). All animals had access to food and water *ad libitum* and were maintained on a 12:12-h light:dark cycle.

Isolation of adipocytes. Adipocytes were isolated by collagenase digestion of epididymal (abdominal) and/or inguinal (subcutaneous) fat pads of rats following the method of Rodbell (Rodbell, 1964) with modifications as previously described (Liang et al., 2002). Briefly, rats were anesthetized with isoflurane and fat tissues were isolated then digested with either Type I or Type IV collagenase (1 mg/mL) for 50 min at 34-36° C in Krebs-Ringer-HEPES (KRH) buffer containing 1% defatted bovine serum albumin (BSA), 2 μmol/L nicotinic acid (to inhibit lipolysis), and 10 μmol/L butylated hydroxyanisole (BHA, an anti-oxidant). Adipocytes were washed with fresh buffer without either nicotinic acid or BHA and collected by flotation. Aliquots of adipocyte preparations were placed into wells of either 48- or 96-well plastic microtiter plates for experimentation.

Assay of cyclic AMP content of 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 μmol/L forskolin, ADA (1 U/ml), and 1% defatted BSA in KRH buffer. Cyclic AMP phosphodiesterase inhibitors (10 μmol/L rolipram and 1 μmol/L cilostamide) were used when indicated to increase the accumulation of cyclic AMP. The effect of forskolin to increase cAMP content of rat adipocytes was near-maximal at 3 and maximal at a concentration of 30 μmol/L.

The assay of cyclic AMP in experiments to investigate the heterogeneity of adipocyte A₁AdoR was done as previously described (Liang et al., 2002) using the

radioimmunoassay method of Brooker (Brooker et al., 1979). For all other experiments wherein cyclic AMP was measured, incubations were terminated and cells were lysed using a commercial lysis buffer and a chemiluminescent competitive immunoassay kit (BioApplied Systems, Foster City, CA). Luminescence of samples was quantified using a Packard TopCount NXT microplate luminescence counter (Perkin Elmer Life Sciences, Downers Grove, IL).

NEFA release from rat adipocytes. To determine the effects of A₁AdoR agonists on NEFA release from adipocytes, adipocytes in 200- μ L aliquots (200,000-400,000 cells) in KRH buffer containing 0.1-1 μ M forskolin, ADA (1 U/mL) and 1% defatted BSA were placed into the wells of plastic microtiter plates and incubated for 60 min at 36^o C in the absence or presence of appropriate drugs. Incubations of adipocytes with drugs were terminated by filtration (Millipore, Bedford, MA). Incubation media were assayed for NEFA content. NEFA content was determined by use of a colorimetric NEFA C kit (Wako Chemicals, Richmond, VA) and a spectrophotometer.

Rat adipocyte membrane preparation. Isolated cells were washed once with fresh KRH buffer (without nicotinic acid), then collected and added to a chilled (4^o C) Tris-EDTA buffer containing (in mmol/L): 250 sucrose, 10 Tris, 1 EDTA, and protease inhibitor cocktail (pH 7.4). The cell suspension was homogenized using a Wheaton Potter-Elvehjam tissue grinder. The homogenate was centrifuged at 450 x g for 10 min at 4^o C. The supernatant fatty cake was discarded, and the infranate was removed to fresh tubes. Membranes were collected by centrifugation of the infranate at 16,000 x g for 15 min at 4^o C. The membrane pellet was resuspended and the centrifugation process was repeated. The final pellet was resuspended in a small volume of 250 mmol/L sucrose

with 10 mmol/L Tris (pH 7.4) and frozen in liquid nitrogen. The protein content of membrane preparations was determined using a BioRad D_c protein assay kit (BioRad 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. To determine the values of K_d and B_{max} for binding of the A₁AdoR antagonist [³H]CPX to adipocyte membranes, increasing concentrations [³H]CPX (0.075-9.6 nmol/L) were incubated with adipocyte membranes (25 μg) for 90 min at 25° C in a 150-μL volume of HEPES-EDTA (HE) buffer (pH 7.4) containing (in mmol/L): 10 HEPES, 1 EDTA, 5 MgCl₂, 0.1 benzamidine chloride, ADA (1 U/mL), 0.02% sodium azide, and protease inhibitor cocktail. At the end of the incubation, bound and free radioligand were separated by rapid filtration of the membrane suspension using a cell harvester. Collected membranes (with bound radioactivity) were washed 3 times with ice-cold buffer containing 10 mmol/L TrisHCl and 1 mmol/L 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 μmol/L 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 HE buffer + ADA (1 U/ml) (pH 7.4), increasing concentrations of agonist, and 2 nmol/L [³H]CPX. All assays were done in triplicate.

Binding of CVT-3619 to AdoR subtypes in cell lines. The stable cell lines DDT₁MF-2, CHO cells overexpressing either human A₁ or A₃AdoR, or HEK cells overexpressing either human A_{2A} or A_{2B}AdoR were used to assess the relative selectivity of CVT-3619 for the A₁AdoR. Cell membranes were prepared and competition binding assays were conducted by incubation of 75 μg of cell membrane at 25° C for 90 min with increasing concentrations of radioligand in 150 μL of incubation medium containing 10 mmol/L Tris (pH 7.4), 5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 U ADA/mL, 20 μmol/L GTPγS, and protease inhibitor cocktail. Radioligands for A₁, A₃, and A_{2A}/A_{2B} AdoR were [³H]CPX (1.5 nmol/L), [³H]MRE3008F20 (0.7 nmol/L), and [³H]ZM241385 (2 nmol/L for A_{2A} and 14 nmol/L for A_{2B}AdoR assays), respectively. Determinations in each assay were done in triplicate or quadruplicate.

Expression of A₁AdoR in rat epididymal and inguinal adipose tissues. RNA was isolated from freshly prepared adipocytes using an Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA). cDNA was transcribed by reverse transcription (RT) using a Taqman kit (Applied Biosystems, CA) and a Gene Amp PCR System 9700 (Applied Biosystems, CA). To quantify A₁AdoR expression levels, 2 μl of the cDNA was amplified (in triplicate) by polymerase chain reaction (PCR) using an SyBR Green kit (PE Biosystems, Warrington, UK) and a Gene Amp 5700 Sequence Detector (Applied Biosystems, CA). PCR primers were designed based on the published rat brain A₁AdoR cDNA sequence (accession no. M64299) (Mahan et al., 1991) and using Primer Express (Applied Biosystems, CA) software. Forward and reverse primers (5'TCCTCACCCAGAGCTCCATT3' and 3'GAGGGATCTTGACTCGGAGGTAT5', respectively) for the A₁AdoR and forward and reverse primers

(5'TTCAACACCCCAGCCATGT3' and 3'AGTGGTACGACCAGAGGCATACA5', respectively) for the housekeeping gene β -ActinA1 were obtained from Operon (Germantown, MD). Rat genomic DNA (BD Biosciences, San Diego, CA) and autoclaved distilled water were used as positive and negative controls, respectively.

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 μ mol/L isoproterenol (to stimulate activity of adenylyl cyclase), 1 mmol/L ascorbic acid, 10 μ mol/L *erythro*-9-(2-hydroxy-3-nonyl)-adenine (EHNA, an inhibitor of ADA), and 0.1 nmol/L-10 μ mol/L CPX in the absence or presence of 0.1, 1, and 10 μ mol/L adenosine. Incubations were terminated by addition of HCl, and lysates were assayed 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 μ mol/L isoproterenol, 1 mmol/L ascorbic acid, 10 μ mol/L EHNA, and CPX (0.01, 0.1, or 1 μ mol/L) in the presence of either CCPA (0.01 nmol/L-10 μ mol/L), NECA (0.01 nmol/L-30 μ mol/L), or SPA (0.3 nmol/L-100 μ mol/L). The effect of isoproterenol to increase cAMP content of adipocytes was near-maximal at 0.1 and maximal at 1 μ mol/L. Concentration-response relationships for agonists to decrease adipocyte cyclic AMP content, and values of K_B for CPX to antagonize agonist-mediated responses (from Schild plots) were determined using GraphPad Prism version 3.0 (GraphPad, 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 (K-H) solution at a temperature of $36.0 \pm 0.5^\circ \text{C}$ as previously described (Wu et al., 2001).

The effects of CVT-3619 and CPA to decrease spontaneous atrial rate of rat hearts were measured as previously described (Froldi and Belardinelli, 1990) to enable the determination of agonist functional selectivity for adipose tissue versus heart. Average atrial rate (depolarizations/min) 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 A_1 versus A_{2A} AdoR 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 (Froldi 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 time from the first pacing artifact to the maximum deflection of the His bundle signal was used as the S-H interval. To measure the effects of drug on the coronary perfusion pressure (CPP), a pressure transducer was connected to a port on the aortic perfusion cannula and pressure signals were analyzed using a Power Lab acquisition system (AD Instruments, Australia) and a computer. Drugs were infused via the aortic cannula and steady-state responses were recorded for analysis. Coronary conductance (in $\text{mL} \cdot \text{min}^{-1} \cdot \text{mm Hg}^{-1}$) was calculated as the ratio of coronary flow (10 mL/min) to CPP (in mm Hg).

Data Analysis: All data are reported either as mean \pm SEM 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 K_d and maximal binding (B_{max}) of [³H]CPX in saturation binding assays, and values of K_d, K_H and K_L in competitive radioligand binding assays, were determined by nonlinear regression analysis using Prism.

Statistical analysis of data from experiments with 2 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 (ANOVA) followed by Student-Newman-Keuls test was used to compare values of measurements obtained from experiments with more than 2 treatment or time groups. Differences between/among treatment groups were considered to be significant when the probability of their occurrence by chance alone was < 0.05.

RESULTS

To confirm that A₁AdoR 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 [³H]CPX bound to membranes prepared from epididymal adipocytes with B_{max} and K_d values of 299 ± 47 (95% confidence limits, 202-396) fmol/mg protein and 0.8 (0.1-1.5) nmol/L (*n* = 4), respectively. [³H]CPX bound to membranes prepared from inguinal adipocytes with B_{max} and K_d values of 355 ± 32 (287-423) fmol/mg and 1.7 (1.0-2.4) nmol/L (*n* = 2), respectively. The differences between values of B_{max} and K_d for [³H]CPX 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-actinA1 in each tissue (*p* > 0.05).

Binding of CVT-3619 and CPA to A₁AdoR in rat adipocytes. The binding of CVT-3619, and for comparison, CPA (a prototype A₁AdoR agonist), to membranes prepared from rat epididymal adipocytes was determined by analysis of displacement by each agonist of the binding of [³H]CPX. Both CVT-3146 and CPA displaced the binding of [³H]CPX in a concentration-dependent and biphasic manner (Fig. 1). The affinities of CVT-3619 and CPA for the high-affinity state of the A₁AdoR (i.e., K_H) were 14 (8-22) and 0.30 (0.21-0.42) nmol/L, respectively (*p* < 0.05). The affinities of CVT-3619 and CPA for the low-affinity state of the A₁AdoR (K_L) were 5.4 (1.4-20.4) and 0.14 (0.11-0.19) μmol/L, respectively (*p* < 0.05). The percentages of receptors with high affinity for CVT-3619 and for CPA were 50 ± 5 and 36 ± 2%, respectively (*p* > 0.05). The affinities

of CPA for high and low affinity agonist-binding states of the adipocyte A₁AdoR were 47- and 39-fold higher, respectively, than the affinities of CVT-3619 for the same states.

Binding of CVT-3619 to A₁, A_{2A}, A_{2B}, and A₃AdoR. The binding of CVT-3619 to subtypes of AdoR was measured to assess the selectivity of the compound for the A₁AdoR. The values of K_i for CVT-3619 to bind to [³H]CPX binding sites (i.e., A₁AdoR) in DDT₁MF-2 and CHO cells expressing the human A₁AdoR were 113 (76-167) nmol/L (*n* = 4) and 1.1 (0.8-1.5) μmol/L (*n* = 7), respectively. The values of K_i for CPA in the same assays were 10 (5-17) nmol/L (*n* = 4) and 0.3 (0.2-0.3) μmol/L (*n* = 3), respectively. Neither CVT-3619 (0.1 nmol/L-10 μmol/L) nor CPA (1 nmol/L-100 μmol/L) displaced the binding of [³H]ZM241385 (2 nmol/L) to human A_{2A}AdoR or the binding of [³H]ZM241385 (14 nmol/L) to human A_{2B}AdoR expressed in HEK-293 cells (*n* = 3). Neither CVT-3619 nor CPA displaced the binding of the A₃AdoR ligand [³H]MRE3008F20 (0.7 nmol/L) at concentrations up to 3 μmol/L, and at a concentration of 10 μmol/L (the highest concentration tested), each displaced 25% of [³H]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 μmol/L forskolin (Fig. 2). Values of IC₅₀ for CVT-3619 and CPA to reduce the cyclic AMP content of epididymal adipocytes were 5.91 (1.73-20.2) and 0.014 (0.007-0.029) nmol/L, respectively (*p* < 0.05). Values of IC₅₀ 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) nmol/L, 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 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$) increased NEFA release by 13-fold above control (absence of drug). Values of IC_{50} for CVT-3619 and CPA to reduce the forskolin-stimulated release of NEFA from epididymal adipocytes were 47 (9-240) and 0.5 (0.2-1.3) nmol/L , respectively ($p < 0.05$). Reduction by CVT-3619 (10 $\mu\text{mol/L}$) of the release of NEFA from epididymal adipocytes was attenuated by the selective A_1AdoR antagonist CPX with an EC_{50} value of 0.10 (0.06-0.16) $\mu\text{mol/L}$ (not shown). CPX (10 $\mu\text{mol/L}$) abolished the effect of 10 $\mu\text{mol/L}$ CVT-3619 to reduce the release of NEFA. Values of IC_{50} 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) nmol/L , 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 $\mu\text{mol/L}$)-stimulated release of NEFA from epididymal and inguinal adipocytes by $\geq 100\%$ (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 $\mu\text{mol/L}$), both CVT-3619 and CPA were able to fully attenuate the response. Forskolin (0.1 $\mu\text{mol/L}$) increased NEFA release by 3.5-fold (Fig. 3, panel C). CVT-3619 (10 $\mu\text{mol/L}$) and CPA (0.1 $\mu\text{mol/L}$) reduced the forskolin-induced stimulation of NEFA release by 102 ± 1 and $104 \pm 2\%$, respectively ($p < 0.01$ for both, compared to forskolin; Fig. 3, panel C).

Assessing heterogeneity of rat epididymal $A_1\text{AdoR}$. To investigate the possibility that the population of $A_1\text{AdoR}$ 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 $A_1\text{AdoR}$ antagonist CPX to increase the adipocyte content of cyclic AMP in the absence and presence of 3 different concentrations of adenosine were determined (Fig. 4). CPX alone, by antagonizing the inhibitory action of endogenous adenosine in the presence of 0.1 $\mu\text{mol/L}$ isoproterenol, increased the cyclic AMP content of adipocytes. The EC_{50} value for CPX to increase adipocyte cyclic AMP content was 7.1 nmol/L (6.3-8.0) 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 EC_{50} values for CPX to increase cyclic AMP were 41 (33-52), 210 (178-249), and 1162 (921-1467) nmol/L, 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 $\mu\text{mol/L}$ caused 5.1 and 5.5-fold increases ($p > 0.05$), respectively, in the EC_{50} values for CPX to increase adipocyte cyclic AMP. The results are consistent with the presence of a single receptor site (i.e., $A_1\text{AdoR}$) for adenosine.

To further confirm the hypothesis that A₁AdoR of epididymal adipocytes are homogeneous with regard to ligand affinity, we determined the values of K_B for CPX to antagonize the actions of three structurally different A₁AdoR agonists (CCPA, NECA and SPA) (Fig. 5). CCPA is a 2-Cl, N⁶-substituted adenosine analog, NECA is a 5'-substituted analog (the NECA binding domain was determined by Olah et al., 1994), and SPA is a charged N⁶ analog that presumably does not cross the cell membrane. Each agonist alone reduced cyclic AMP content of epididymal adipocytes in the presence of 0.1 μmol/L 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 μmol/L) decreased spontaneous atrial rate of the isolated rat heart by 12 ± 2 % (n = 6) at a concentration of 30 μmol/L (not shown). The estimated values of EC₁₅ for CVT-3619 to decrease spontaneous atrial rate and adipocyte lipolysis (i.e., 1 μmol/L forskolin-stimulated NEFA release) were 30 μmol/L and 30 nmol/L, respectively, and therefore the selectivity (i.e., the ratio of values of EC₁₅) of CVT-3619

to reduce lipolysis was 1000-fold. In contrast, CPA (0.1-1 $\mu\text{mol/L}$) decreased the spontaneous atrial rate by 100 % ($n = 3$) with an EC_{50} value of 50 nmol/L (not shown). The values of EC_{50} for CPA to decrease spontaneous atrial rate and adipocyte lipolysis were thus 50 and 0.5 nmol/L , 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 A_1 versus A_{2A} AdoR was determined using the guinea pig rather than the rat heart, because the guinea pig heart responds better to A_1 AdoR 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 A_1 AdoR) and to increase coronary conductance (an action mediated by the A_{2A} AdoR) (Belardinelli et al., 1998; Shryock et al., 1998) were measured. CVT-3619 (10 nmol/L -30 $\mu\text{mol/L}$) caused a small but significant increase of the S-H interval by 6 ± 1 msec ($n = 7$, $p < 0.01$ above baseline) without causing second or higher degree atrioventricular (AV) block (Fig. 6). In contrast, CPA significantly prolonged the S-H interval by as much as 38 msec ($n = 5$, $p < 0.001$) and caused second or higher degree AV block at concentrations > 30 nmol/L (Fig. 6). CVT-3619 (10 $\mu\text{mol/L}$) shifted the concentration-response relationship for CPA to increase the S-H interval to the right (not shown; $n = 5$ hearts). Concentrations of CPA that prolonged the S-H interval to 60 msec were 22 and 158 nmol/L in the absence and presence of CVT-3619 (10 $\mu\text{mol/L}$), respectively. This result suggests that CVT-3619 is a partial agonist of the A_1 AdoR which mediates prolongation of the S-H interval. CVT-3619 (10 nmol/L -30 $\mu\text{mol/L}$) caused no

significant change in atrial rate ($n = 4$, $p > 0.05$) (not shown). CVT-3619 (1-10 $\mu\text{mol/L}$) caused a relatively small increase of coronary conductance ($\leq 29 \pm 4\%$).

DISCUSSION

The present study investigated the actions of the novel A_1 AdoR agonist CVT-3619 on adipocytes from rat white adipose tissue. Receptor binding and functional effects of CVT-3619 and CPA (the reference full A_1 AdoR agonist) were characterized. The results indicated that: a) CVT-3619 bound to two states (i.e., high and low affinity) of adipocyte A_1 AdoR with affinities that were approximately 40-fold lower than that of the reference agonist CPA; b) 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; c) CVT-3619 was a full agonist to decrease adipocyte cyclic AMP content, but a partial agonist to decrease the release of NEFA, relative to the full agonist CPA; and d) the expression of A_1 AdoR 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 were expressed in cultured cell lines revealed that CVT-3619 was at least 10- to 100-fold selective for A_1 versus either A_{2A} or A_{2B} AdoR, and at least 10-fold selective for A_1 versus A_3 AdoR. In addition, results of investigations of the actions of CVT-3619 and CPA on electrophysiologic (atrial rate, S-H interval) and hemodynamic (CPP) 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 A_1 or A_{2A} -AdoR, and appeared to be relatively selective for the A_1 AdoR. Lastly, it was shown by rigorous pharmacologic criteria (Kenakin, 1992; Kenakin, 1997) that rat epididymal adipocyte A_1 AdoR are a homogeneous receptor population with regard to responses to agonist ligands.

Both CVT-3619 and CPA appeared to be selective for the A_1 AdoR 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 tested concentrations, displaced the binding of receptor subtype-selective antagonists to A_{2A} or A_{2B} AdoR, and displaced binding of radioligand to A_3 AdoR only at a concentration of 10 μ mol/L. In these assays GTP γ S 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 IC_{50} for both CPA and CVT-3619 to reduce the cyclic AMP content of epididymal adipocytes were lower than values of K_H from binding assays, and much lower than values of IC_{50} for CPA and CVT-3619 to reduce the release of NEFA. Although comparison of these values must be done with caution because the conditions of each assay were different, these results are not unexpected. The ratios of values of K_H (from the binding assay) to values of IC_{50} for CPA and CVT-3619 to decrease 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 A_1 AdoR 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 A_1 AdoR 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-mediated effects) were approximately 1000- and 100-fold, respectively. The fact that A₁Ado receptor reserve is significantly higher in adipose tissue compared to heart (Liang et al., 2002; Srinivas et al., 1997) 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-efficacy analogues 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 μmol/L for CVT-3619).

CVT-3619 caused a small (up to 29%) decrease of coronary perfusion pressure (an action mediated by the A_{2A}AdoR; Belardinelli et al., 1998) in spite of the fact that receptor reserve for an A_{2A}AdoR-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 A_{2A}AdoR. 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 adipocyte 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 K_B 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 K_B and Hill slope for CPX to antagonize 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, in this study it 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 as regards affinities for agonist and antagonist ligands.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Displacement by CVT-3619 and CPA of the binding of [³H]CPX (2 nmol/L) to membranes prepared from rat epididymal adipocytes. Each symbol represents a mean ± SEM of data from 3 experiments, each with 3 replicate determinations. The specific binding of [³H]CPX (2 nmol/L) in the absence of competing ligand was approximately 2000 dpm per assay well (total binding averaged 2376 dpm; non-specific binding averaged 294 dpm). Values of K_H and K_L for CVT-3619 were 14 nmol/L and 5.4 μmol/L, respectively; for CPA these same values were 0.3 nmol/L and 0.14 μmol/L. See Methods for assay conditions.

Fig. 2. Attenuation by CVT-3619 and CPA of stimulation by 3 μmol/L forskolin of cyclic AMP accumulation in rat epididymal (panel A) and inguinal (panel B) adipocytes. Forskolin (3 μmol/L) alone increased the cyclic AMP contents of epididymal and inguinal adipocytes by 15 ± 2-fold 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 nmol/L, respectively; the same values of IC_{50} for CPA were 14 and 11 pmol/L. Each symbol represents a mean ± SEM of data from 7 experiments, each with 4 replicate determinations. See Methods for experimental conditions.

Fig. 3. Reduction by CVT-3619 and CPA of the forskolin (1 $\mu\text{mol/L}$)-stimulated release of NEFA from rat epididymal (panel A) and inguinal (panel B) adipocytes. Each symbol represents a mean \pm SEM of data from 5-6 experiments with 4 replicate determinations each. Values of IC_{50} for CVT-3619 to inhibit NEFA release from epididymal and inguinal cells were 47 and 170 nmol/L, respectively; for CPA these values were 0.5 and 0.19 nmol/L. Reduction by CVT-3619 and CPA of the forskolin (0.1 $\mu\text{mol/L}$)-stimulated release of NEFA from rat epididymal adipocytes (panel C). Each bar represents a mean \pm SEM of data from 3 experiments with 4 replicate determinations each. See Methods for experimental conditions.

Fig. 4. Concentration-response relationships for the A_1AdoR antagonist CPX to increase the adipocyte content of cyclic AMP in the absence and presence of adenosine 0.1, 1, and 10 $\mu\text{mol/L}$. Isoproterenol (0.1 $\mu\text{mol/L}$) and 10 $\mu\text{mol/L}$ *erythro*-9-(2-hydroxy-3-nonyl) adenine (EHNA) were present in all media to stimulate adenylyl cyclase activity and to inhibit the activity of adenosine deaminase, respectively. Each symbol represents a mean \pm SEM of data from 4 experiments, each with 6 replicate determinations. See Methods for experimental conditions.

Fig. 5. Concentration-response relationships for the A_1AdoR antagonist CPX to antagonize reductions of rat epididymal adipocyte cyclic AMP content caused by the A_1AdoR agonists CCPA (panel A), NECA (panel B), and SPA (panel C). A Schild plot of the data depicted in panels A-C is shown in panel D. Each symbol represents a mean

± SEM of data from 4 experiments, each with 6 replicate determinations. See Methods for experimental conditions.

Fig. 6. Concentration-response relationships for the negative dromotropic effect (i.e., increase of the S-H interval) of CVT-3619 and CPA. Each symbol represents a mean ± SEM of single S-H interval measurements in each of 5 (CPA) and 7 (CVT-3619) hearts before (0 drug) and after exposure to drug. An asterisk indicates that second or higher degree atrioventricular block occurred at concentrations of CPA higher than 30 nmol/L.

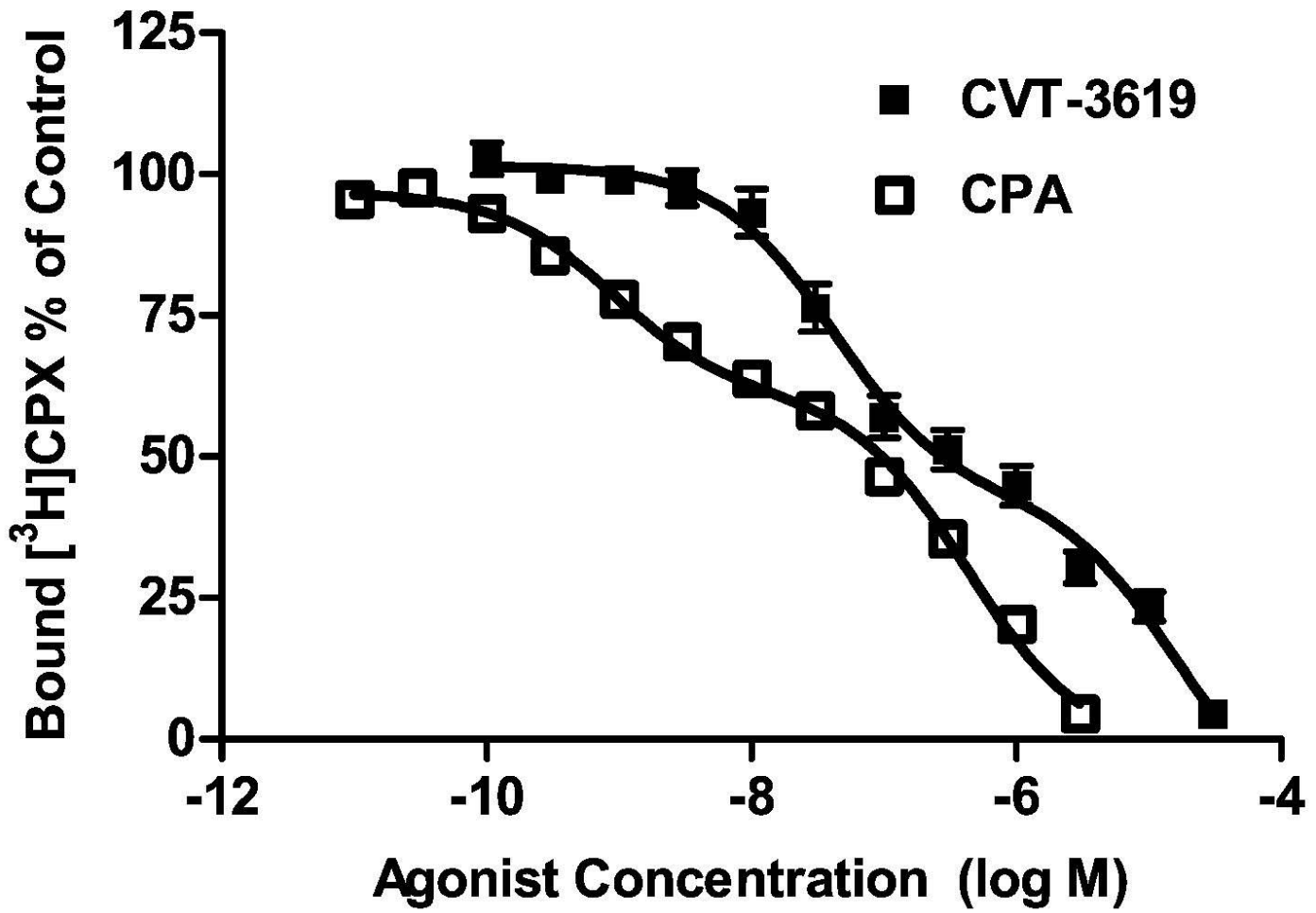


Figure 1

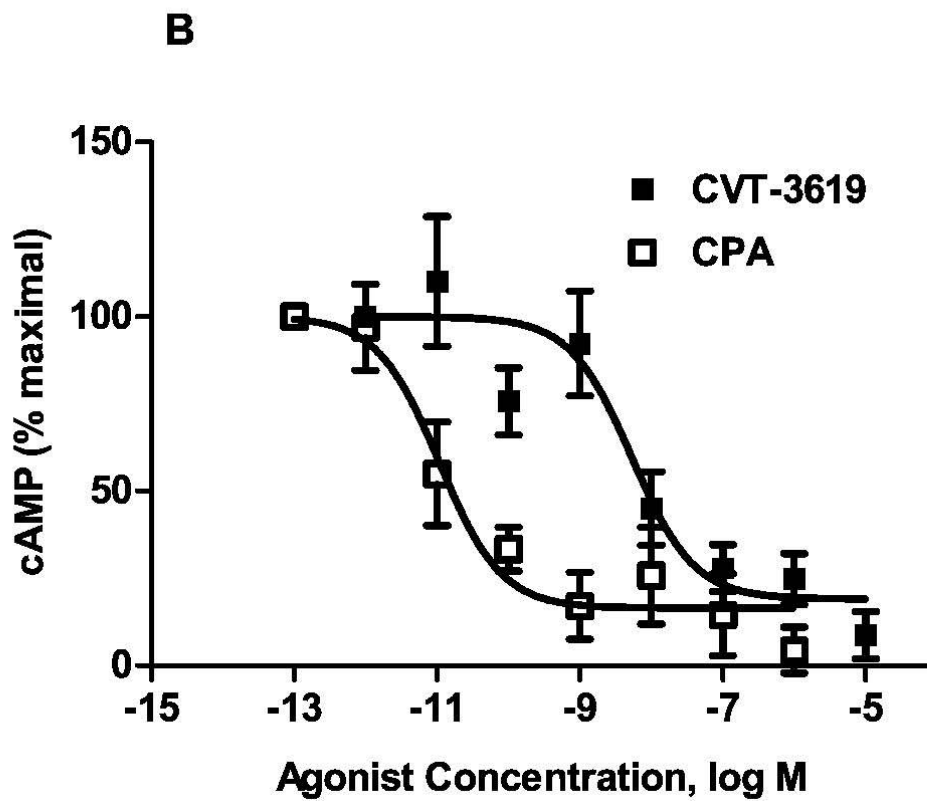
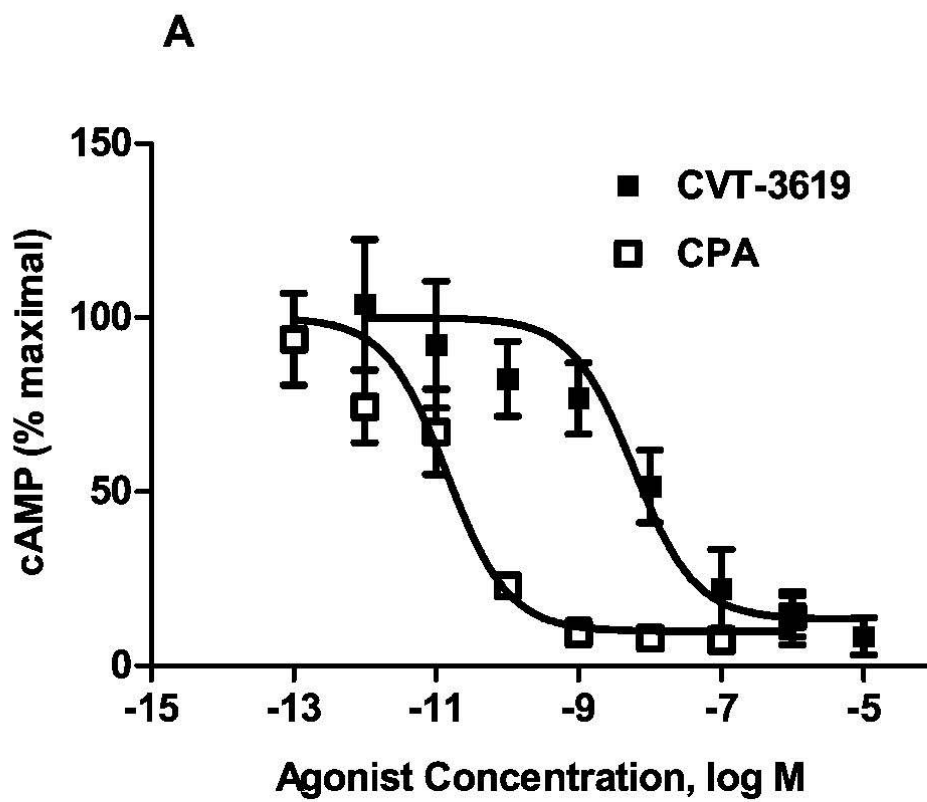
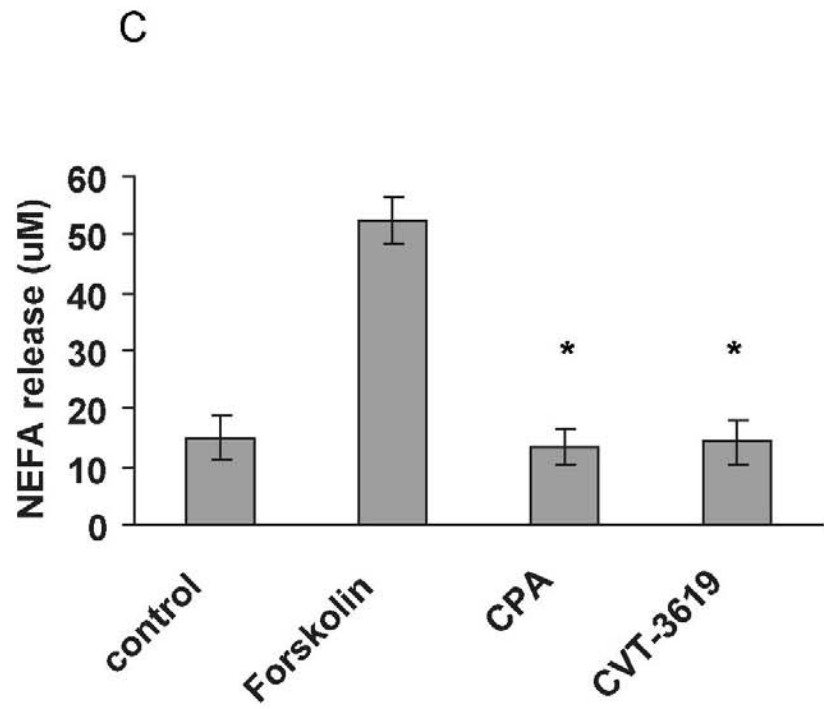
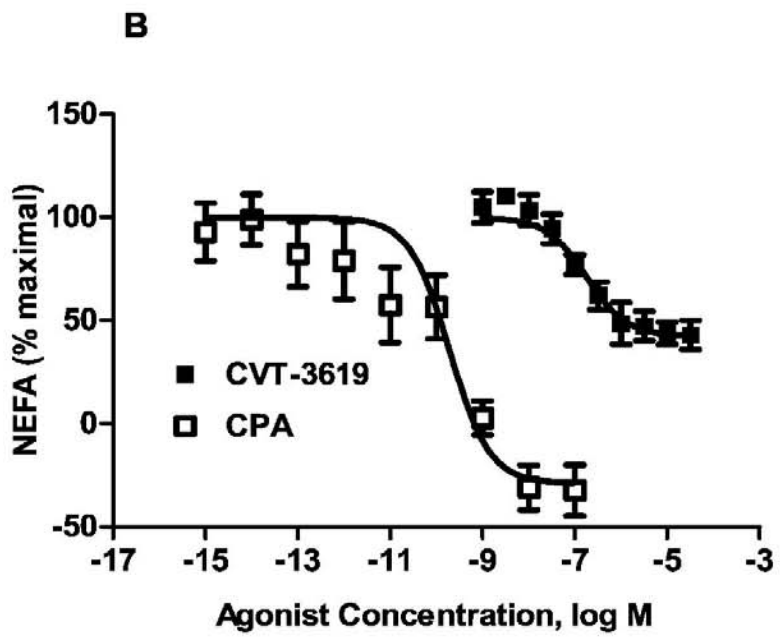
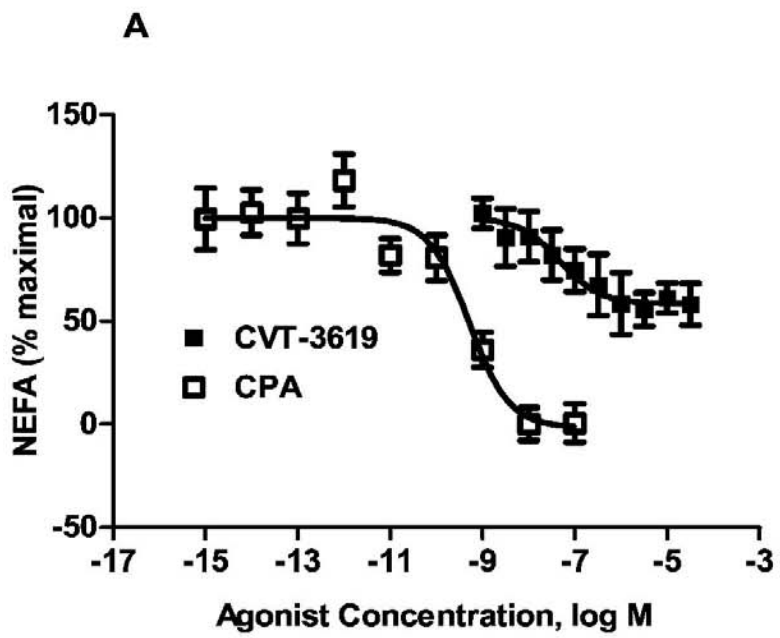


Figure 2



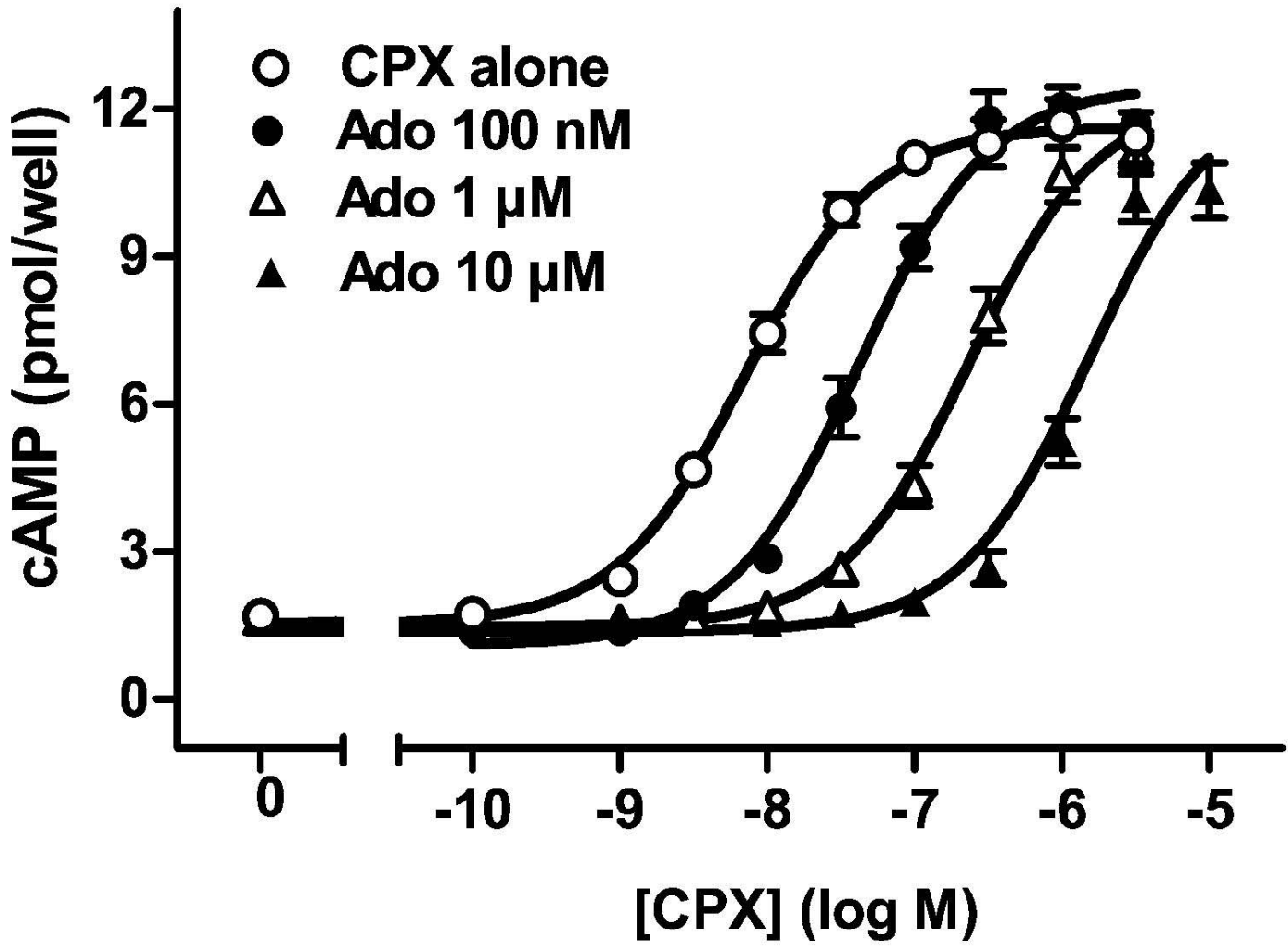


Figure 4

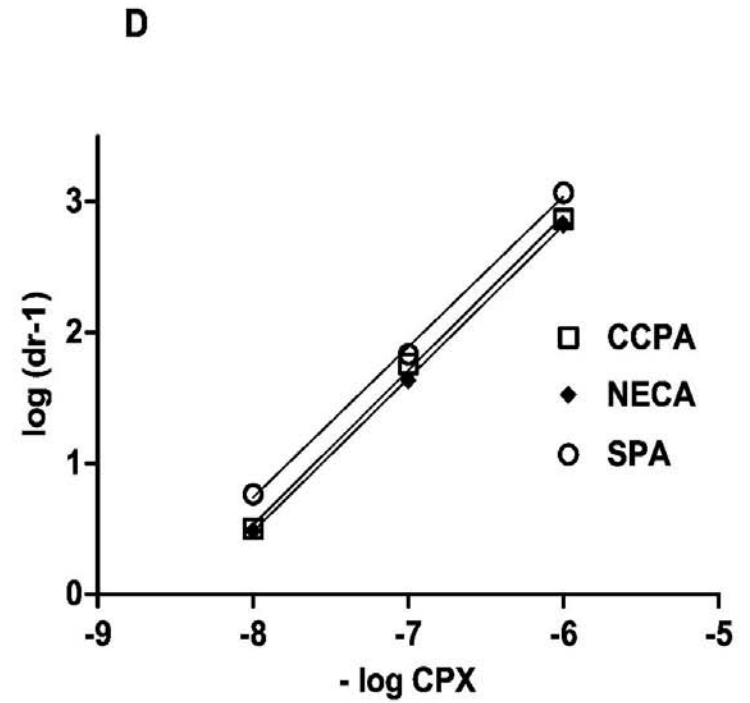
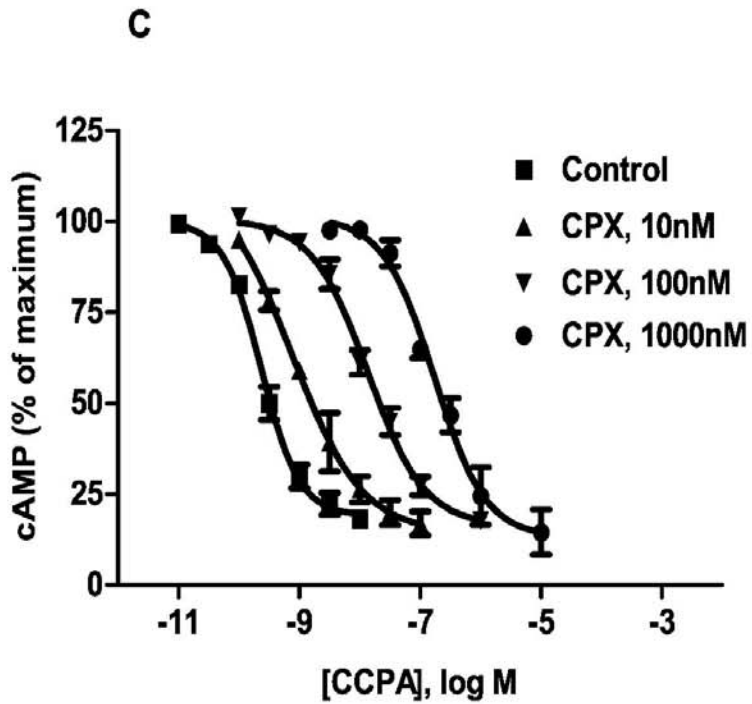
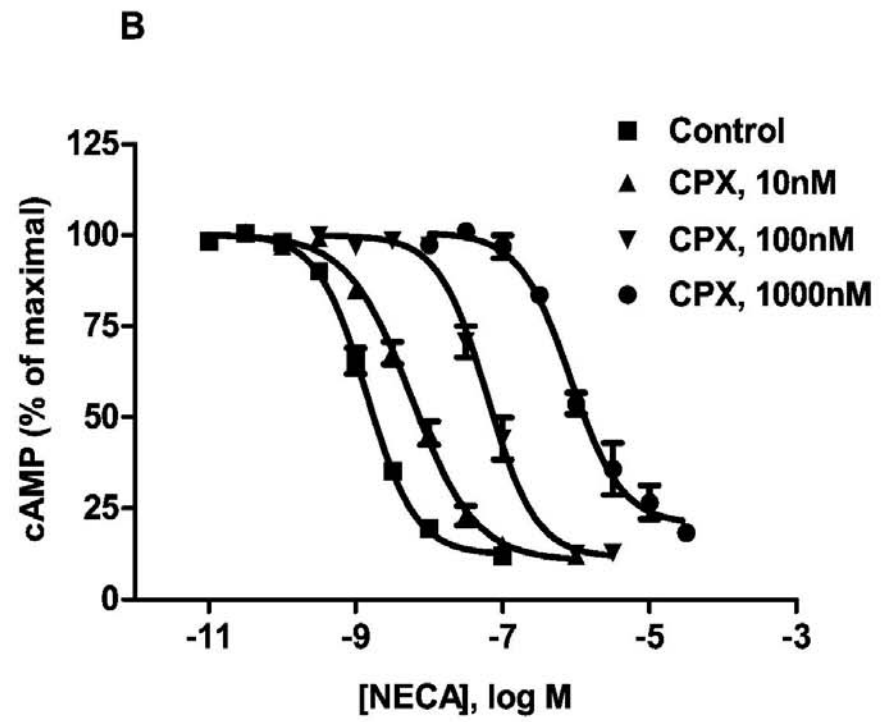
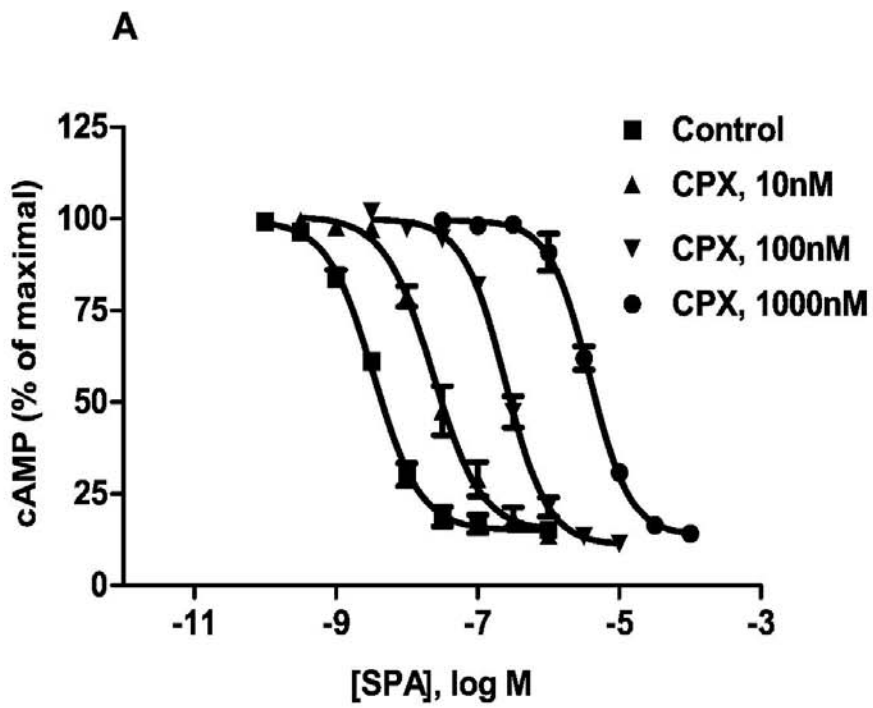


Figure 5

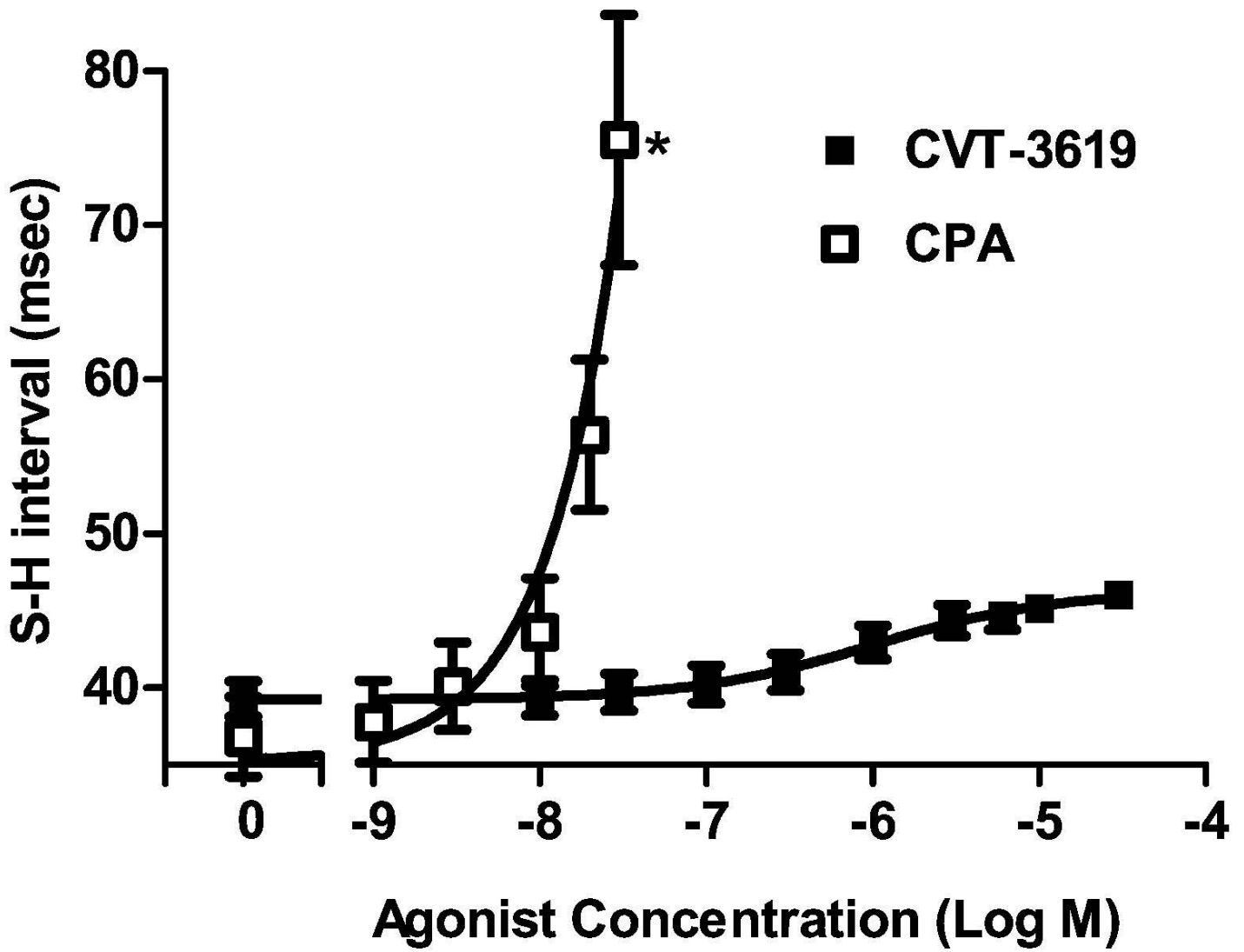


Figure 6