0026-895X/96/010196-10$3.00/0

MOLECULAR PHARMACOLOGY, 50:196–205 (1996).

A Novel Irreversible Antagonist of the A_1-Adenosine Receptor

MIDUTURU SRINIVAS, JOHN C. SHRYOCK, PETER J. SCAMMELLS, JACKIE RUBLE, STEPHEN P. BAKER, and LUIZ BELARDINELLI

Departments of Pharmacology (M.S., S.P.B., L.B.) and Medicine (J.C.S., J.R., L.B.), University of Florida, Gainesville, Florida, and School of Biological and Chemical Sciences, Deakin University, Geelong, Victoria 3217, Australia (P.J.S.)

Received February 9, 1996; Accepted March 14, 1996

SUMMARY

We determined the effects of 8-cyclopentyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]propyl]-1-propylxanthine (FSCPX), a putative irreversible antagonist of the A_1-adenosine receptor, on cardiac A_1-adenosine receptor-mediated responses and on the specific binding of [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]CPX) to guinea pig cardiac and brain membranes. FSCPX (5 μM) completely reversed the increase in K^+ current of guinea pig atrial myocytes caused by 100 μM adenosine (259 ± 30 to 20 ± 7 pA) but had no significant effect on K^+ currents caused by either 0.5 μM carbachol or 100 μM GTPγS. The attenuation of K^+ current by FSCPX was both time and concentration dependent and persisted after washout of the antagonist. Pretreatment of atrial myocytes with FSCPX (50 nM) markedly attenuated the activation of K^+ current and the inhibition of isoproterenol-stimulated $I_{Ca,L}$ caused by adenosine by 90.1% and 84.2%, respectively, but did not alter the responses of atrial myocytes to carbachol. FSCPX (1 μM) irreversibly antagonized the A_1-adenosine receptor-mediated increase in atrioventricular nodal conduction time of isolated perfused guinea pig hearts from 10.5 ± 0.5 to 0.7 ± 0.6 msec but did not significantly alter the A_2-adenosine receptor-mediated decrease in coronary resistance. Preincubation of guinea pig cardiac membranes with 0.1, 1.0, or 3.0 μM FSCPX for 30 min reduced the B_{max} of [^{3}H]CPX binding by 41 ± 10%, 67 ± 6%, and 80 ± 1% (mean ± standard error, three experiments), respectively, with no significant change in the K_d. Similarly, 0.1 and 1.0 μM FSCPX irreversibly reduced the binding of [^{3}H]CPX to guinea pig forebrain membranes by 65 ± 5% and 83 ± 2% (four experiments), respectively, but did not reduce the binding of [^{3}H]CGS 21680, an A_{2a}-adenosine receptor agonist, to striatal membranes. FSCPX did not affect the potency of 5′-guanylylimidodiphosphate to inhibit the binding of [^{3}H]CCPA, an A_1-adenosine receptor agonist, to brain membranes. The results indicate that FSCPX is a specific, irreversible, A_1-adenosine subtype-selective receptor antagonist.

The A_1-adenosine receptor is widely distributed in the body and mediates many of the actions of adenosine. Activation of A_1-adenosine receptors has been shown to slow heart rate and atrioventricular nodal conduction, inhibit lipolysis, inhibit synaptic activity in the central nervous system, and facilitate neutrophil chemotaxis (1–3). For assessment of the role of adenosine and of adenosine analogues in mediation of various responses and for measurement of the density and affinity of adenosine receptors by radioligand binding, reversible antagonists of A_1-adenosine receptors have been widely used. Reversible antagonists are especially suitable for experiments in which competitive equilibria or steady state conditions are desired. In contrast, irreversible antagonists are indispensable for measurement of receptor reserve and for localization of ligand binding sites; in these circumstances, irreversible occupancy by antagonist of the receptor binding site is necessary.

Both photoaffinity-labeled and chemoreactive irreversible antagonists possessing a high affinity for the A_1-adenosine receptor have been described previously and used to identify the ligand binding subunit of the A_1-adenosine receptor (4–7). Chemoreactive compounds were made through attachment of reactive electrophilic groups to derivatives of xanthine and adenosine (7). One such compound, *m*-DITC-XAC, a derivative of XAC, was shown to be a potent, irreversible inhibitor of the A_1-adenosine receptor in the rat brain and was subsequently used to estimate the extent of receptor reserve in the guinea pig atrioventricular node (6, 8). How-

This work was supported by a Multidisciplinary Project Grant from the American Heart Association, Florida Affiliate (91/MPG), and by National Institutes of Health Grant HL35272.

ABBREVIATIONS: *m*-DITC, *meta*-1,3-phenylenediisothiocyanate; XAC, xanthine amine congener; CPX, 8-cyclopentyl-1,3-dipropylxanthine; FSCPX, 8-cyclopentyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]propyl]-1-propylxanthine; Gpp(NH)p, guanosine-5′-(β,γ,-imido)triphosphate; GTPγS, guanosine-5′-*O*(3-thio)triphosphate; DMSO, dimethylsulfoxide; K-H, Krebs-Henseleit; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS 21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride; I_{KAdo}, adenosine-induced inwardly rectifying K^+ current; I_{KAch}, carbachol-induced inwardly rectifying K^+ current; $I_{Ca,L}$, L-type Ca^{2+} inward current; β-$I_{Ca,L}$, isoproterenol-stimulated Ca^{2+} current; I_H, holding current; S-H, stimulus-to-His bundle.

ever, even though antagonism of the negative dromotropic effect of adenosine in isolated hearts by *m*-DITC-XAC was not reversible, we were unable to correlate the degree of antagonism by *m*-DITC-XAC of functional responses with a reduction by *m*-DITC-XAC of A_1-adenosine receptor binding to cardiac membranes.[1] Thus, our unpublished results were that the binding of [^{3}H] CPX to membranes prepared from *m*-DITC-XAC-treated hearts was not reduced from control levels. It thus seemed that inactivation of receptor binding by *m*-DITC-XAC was reversed during the process of membrane preparation. The inability of *m*-DITC-XAC to provide reliable estimates of cardiac A_1-adenosine receptor inactivation motivated the synthesis of an additional series of irreversible ligands of the A_1-adenosine receptor.

Recently, Scammells *et al.* (9) synthesized a novel, irreversible antagonist of the A_1-adenosine receptor by attaching a reactive 4-(fluorosulfonyl)benzoyl group to the 3-propyl substituent of CPX; we refer to the new compound as FSCPX (Fig. 1). Results of preliminary radioligand binding studies of DDT_1MF-2 cells suggested that FSCPX bound irreversibly to the A_1-adenosine receptor. In this study, we demonstrate that FSCPX antagonizes cardiac A_1-adenosine receptor-mediated responses and that the antagonism by FSCPX is specific, irreversible, and selective for the A_1-adenosine receptor. This irreversible antagonist should be useful for the measurement of adenosine receptor reserve.

Materials and Methods

Chemicals. Adenosine, adenosine deaminase, carbachol, isoproterenol, Gpp(NH)p, and GTPγS were purchased from Sigma Chemical Co. (St. Louis, MO). CPX, 8-cyclopentyltheophylline, N^6-cyclohexyladenosine, and (*R*)-N^6-phenylisopropyladenosine were purchased from Research Biochemicals (Natick, MA). All radioligands were purchased from DuPont-New England Nuclear Research Products (Boston, MA). FSCPX was synthesized as described by Scammells *et al.* (10). Stock solutions of FSCPX (10 mM) and CPX (10 mM) were prepared in DMSO. The final concentration of DMSO in the perfusion fluid and incubation buffer in radioligand binding studies was ≤0.05% (v/v).

Isolated atrial myocytes. Atrial myocytes were freshly isolated from hearts of adult guinea pigs according to a method described previously (10). Briefly, guinea pigs were anesthetized with methoxyflurane, and the hearts were quickly excised and perfused in retrograde fashion through the aorta for 5–10 min with warm (35°) modified K-H solution, containing 127 mM NaCl, 4.6 mM KCl, 2 mM $CaCl_2$, 1.1 mM $MgSO_4$, 2 mM sodium pyruvate, 10 mM glucose, 10 mM creatine, 20 mM taurine, 5 ribose mM, 0.01 mM adenine, 0.1 mM allopurinol, and 5 mM HEPES, pH 7.4. The hearts were continuously perfused for another 10 min with Ca^{2+}-free modified K-H solution and then enzymatically digested through perfusion for 15–20 min with Ca^{2+}-free K-H solution containing 0.4 mg/ml collagenase type 2, 0.04 mg/ml dispase, 0.04 mg/ml trypsin, and 2 mg/ml albumin for 15–20 min. The atria were dissected, minced, and incubated at 35° with enzyme solution in a shaker bath. Dissociated atrial cells were collected and stored at room temperature in modified K-H solution containing 0.1 mM Ca^{2+} until further use.

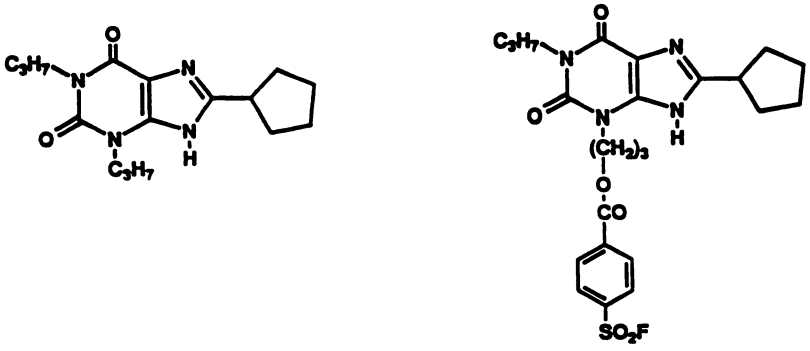
Electrophysiological measurements. Myocytes were transferred into a recording chamber and superfused at a rate of 2–3 ml/min and at a constant temperature of 35° with K-H solution containing 118 mM NaCl, 4.6 mM KCl, 1.2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Ionic currents were recorded with the use of glass suction pipettes (Kimble Glass Inc, Vineland, NJ) in a whole-cell patch-clamp configuration (11). The recording electrodes had resistances of 2–4 MΩ when filled with pipette solution containing 10 mM KCl, 130 mM K-aspartate, 4 mM Na_2ATP, 1 mM $MgCl_2$, 0.1 mM Na_3GTP, 10 mM KH_2PO_4, 10 mM glucose, 1 mM NaEGTA, and 10 mM HEPES, pH adjusted to 7.2 with KOH. Junction potentials between pipette and bath medium were nulled before seal formation. Recordings were made with an Axopatch-1B amplifier (Axon Instruments, Burlingame, CA) and filtered at a bandwidth of 1 kHz. Membrane currents were displayed on a storage oscilloscope and simultaneously recorded with a strip-chart recorder.

The activations of I_{KAdo} and I_{KAch} were measured as increases in the holding current at −40 mV. The activation of GTPγS-induced K^+ current was measured as the outward current caused by intracellular perfusion of pipette solution containing 100 μM GTPγS. To elicit $I_{Ca,L}$, we used 200-msec-long depolarizing pulses from a holding potential of −40 mV to a test potential of 10 mV. Voltage pulses were applied at a rate of 0.25 Hz. $I_{Ca,L}$ was defined as the difference between the peak inward current and the current at the end of a 200-msec pulse. The effects of adenosine (100 μM) and carbachol (0.5 μM) on isoproterenol (100 nM)-stimulated $I_{Ca,L}$ were calculated as the difference in responses (magnitudes of reduction of $I_{Ca,L}$) caused by either adenosine or carbachol in the presence and absence of isoproterenol. The small decreases in basal $I_{Ca,L}$ (absence of isoproterenol) caused by adenosine and carbachol were separately determined in five or six myocytes.

The effect of FSCPX to reduce I_{KAdo} was determined after a 5-min exposure of each cell to a single concentration of FSCPX and was calculated as a percentage of the steady state current observed in the absence of FSCPX. Different cells were studied to determine the effect of various concentrations of FSCPX. The effects of FSCPX in reducing increases in holding current caused by carbachol and by GTPγS were determined in a similar manner.

To demonstrate the irreversibility of the antagonism of A_1-aden-

[1] M. Srinivas, J. C. Shryock, P. J. Scammells, J. Ruble, S. P. Baker, and L. Belardinelli, unpublished observations.



CPX FSCPX

Fig. 1. Structures of CPX and FSCPX.

osine receptor-mediated responses by FSCPX, single guinea pig atrial myocytes were pretreated with either vehicle (DMSO plus K-H solution) or FSCPX (50 nM) for 30 min. After the incubation period, cells were repeatedly washed for ≤5 hr. The activations of I_{KAdo} and I_{KAch} and the inhibitions of β-$I_{Ca,L}$ caused by adenosine (100 μM) and carbachol (0.5 μM) were determined in vehicle (control)- and FSCPX-pretreated cells throughout the washout period. The responses to adenosine determined during the first 2 hr of the washout period (<2 hr after treatment) were compared with responses determined during the last 3 hr of the 5-hr washout period (>2 hr after treatment).

Isolated perfused hearts. Hearts of adult guinea pigs were excised and perfused as described above (Isolated atrial myocytes) with oxygenated (95% O_2/5% CO_2) K-H solution at 35 ± 0.5°. The sinoatrial nodal region and part of the right atrium were cut out to facilitate electrical pacing of the heart and to expose the region of the atrioventricular node. The hearts were paced via bipolar electrodes positioned on the left atrium or interatrial septum. An interval generator (model 1830, World Precision Instruments, Sarasota, FL) and a stimulus isolation unit (model 1880, World Precision Instruments) were used to deliver square wave stimulus pulses of 3-msec intensity. His-bundle electrograms were recorded with unipolar extracellular electrodes placed in the region of the atrioventricular node (12). The His-bundle electrogram was filtered and amplified with a differential amplifier (Tektronix, Beaverton, OR) and displayed on a dual-beam storage oscilloscope (Tektronix). Because the depressant effect of adenosine on atrioventricular nodal conduction (negative dromotropic effect) is confined to the proximal part of the atrioventricular node, the S-H interval was used as a measure of the negative dromotropic effect of adenosine (13). The S-H interval was obtained directly from the oscilloscope display of the His-bundle electrogram. Coronary perfusion pressure was measured with the use of a pressure transducer connected to the perfusion line via a T-connector. Pressures were recorded with an ink chart recorder. Because hearts were perfused at a constant flow, coronary resistance was calculated as the ratio of perfusion pressure (in mm Hg) to coronary perfusion rate (10 ml/min).

The experimental protocol for the determination of the effects of FSCPX on the adenosine-induced S-H interval prolongation and on the decrease in coronary resistance was as follows. The S-H interval and coronary perfusion pressure were measured simultaneously. In each heart, after an equilibration period of >30 min, the perfusate concentration of adenosine was gradually increased until a stable S-H interval prolongation of 10 msec was obtained. Hearts were then perfused with either FSCPX (1 μM) or K-H plus DMSO (vehicle) for 25 min, followed by a washout period of 75 min with K-H solution. During the washout period, hearts of either group were exposed to adenosine at 15-min intervals, and the S-H interval prolongation and coronary resistance were determined.

Membrane preparation. Guinea pig atrial, ventricular, and brain tissues were separately minced and then homogenized in ice-cold 50 mM Tris·HCl buffer, pH 7.4. Homogenates were filtered through cotton gauze and centrifuged at 48,000 × g for 15 min. The membrane pellets were washed twice by resuspension in fresh buffer and centrifugation. Final pellets were resuspended in 50 mM Tris·HCl buffer, pH 7.4, and frozen at −80° until use.

Radioligand binding protocols. To complement the functional studies described above, the effects of FSCPX on binding of adenosine receptor ligands to guinea pig cardiac and brain membranes were determined. Assays for A_1- and A_{2a}-adenosine receptors were carried out by using the A_1 receptor antagonist [^{3}H]CPX, the A_1 receptor agonist [^{3}H]CCPA, and the A_{2a} receptor agonist [^{3}H]CGS 21680. Membranes were treated with adenosine deaminase (2 units/ml) for 20 min at room temperature before and during radioligand binding assays. Membranes (0.2–0.7 mg), adenosine deaminase, and the indicated radioligand were incubated for 3 hr in a 300-μl volume of 50 mM Tris·HCl buffer, pH 7.4. Assays were carried out in triplicate at room temperature. After the incubation period, bound and free radioligands were diluted by the addition of 5 ml of ice-cold Tris·HCl buffer and immediately separated through vacuum filtration of assay contents onto Whatman GF/C filters and washing of trapped membranes with 20 ml of Tris·HCl buffer. Filter disks containing membrane-bound radioactivity were placed in 4 ml of Scintiverse (Fisher Scientific, Pittsburgh, PA), and the radioactivity was quantified with the use of a liquid scintillation counter. Specific binding of [^{3}H]CPX, [^{3}H]CCPA, and [^{3}H]CGS 21680 was defined as membrane binding displaced in the presence of 8-cyclopentyltheophylline (10 μM), N^6-cyclohexyladenosine (10 μM), and (*R*)-N^6-phenylisopropyladenosine (10 μM), respectively.

Determination of the potency of FSCPX to displace the specific binding of [^{3}H]CPX and [^{3}H]CGS 21680 to guinea pig cardiac and brain membranes. The apparent potencies of FSCPX (1 pM to 10 μM) to displace the specific binding of [^{3}H]CPX (1 nM) to brain membranes and of [^{3}H]CGS 21680 (2 nM) to striatal membranes were determined. Membranes were incubated with the indicated concentrations of radioligand and increasing concentrations of FSCPX for 3 hr in a total volume of 300 μl. Assays were terminated as described above. Apparent IC_{50} values were calculated as described in Data analysis.

Effect of FSCPX on maximum specific binding of A_1- and A_{2a}-adenosine receptor radioligands to guinea pig cardiac and brain membranes. The effects of FSCPX on the maximum specific binding (B_{max}) and the dissociation constant (K_d) of [^{3}H]CPX binding to cardiac and brain membranes and of [^{3}H]CGS 21680 binding to striatal membranes were determined. Membranes were prepared as described above and incubated with the indicated concentrations of FSCPX or buffer for 30 min at 25°. Membranes were then washed ≤12 times through centrifugation and resuspension with Tris·HCl buffer and were used in radioligand binding assays. Aliquots of membranes pretreated with FSCPX were incubated with adenosine deaminase (2 units/ml) and various concentrations of [^{3}H]CPX (0.32–20 nM) in a total volume of 300 μl for 3 hr. Similarly, various concentrations of [^{3}H]CGS 21680 (0.32–145 nM) were incubated with aliquots of striatal membranes and adenosine deaminase following an identical protocol. Assays were terminated as described above. The B_{max} and K_d values were calculated as described in Data analysis.

Effect of FSCPX on coupling of A_1-adenosine receptors to G proteins. To determine whether FSCPX affects receptor/G protein coupling, the inhibition of [^{3}H]CCPA binding by Gpp(NH)p was determined in guinea pig brain membranes. Membranes were pretreated with 0.1 μM FSCPX for 30 min and washed through resuspension and centrifugation 12 times before assays were carried out. Increasing concentrations of Gpp(NH)p (1 pM to 1 μM) were incubated with [^{3}H]CCPA (2 nM), adenosine deaminase (2 units/ml), and aliquots of membranes in a total volume of 300 μl for 30 min. Assays were terminated as described above. IC_{50} values for Gpp(NH)p to inhibit binding of [^{3}H]CCPA were calculated as described in Data analysis.

Data analysis. All values are expressed as mean ± standard error. Statistical significance of differences between individual mean values in an experiment with several treatment groups was determined through the use of analysis of variance. Differences between mean values were considered statistically significant at $p < 0.05$. Equilibrium binding parameters (i.e., B_{max}, K_d, and IC_{50}) were determined with the radioligand binding analysis program LIGAND 3.0 (Elsevier-Biosoft, Cambridge, UK).

Results

Results of functional studies to determine whether FSCPX is a specific, irreversible, and selective antagonist of the A_1-adenosine receptor are presented first, followed by results of radioligand binding assays.

Functional Studies

Antagonism by FSCPX of the A_1-adenosine receptor-mediated activation of I_{KAdo}. FSCPX significantly reduced the holding current in the presence of 100 μM adenosine. In a representative experiment, adenosine increased the holding current from 10 to 280 pA (Fig. 2A). The increase by adenosine of holding current has been shown to be due to the activation of a specific inwardly rectifying K^+ current, I_{KAdo} (1, 15). FSCPX (5 μM) reduced the adenosine-mediated increase of holding current from 280 to 20 pA (Fig. 2A). The inhibition of I_{KAdo} was concentration and time dependent. The concentration dependence of FSCPX action is illustrated in Fig. 2B. Exposure of cells to FSCPX at 0.5, 1, 4, and 5 μM for 5 min inhibited I_{KAdo} by 25%, 46%, 86%, and 90%, respectively.

A relatively low concentration of FSCPX did not cause the steady state submaximal response that is associated with the use of a reversible antagonist. Fig. 3 shows the time-dependent inhibition of steady state I_{KAdo} by two different concentrations of FSCPX (1 and 5 μM) and of CPX (0.1 and 1 μM). FSCPX at 5 μM reduced I_{KAdo} by 90 ± 3.4% (four experiments) in 5 min. In comparison, a 5-min exposure of cells to FSCPX at 1 μM inhibited I_{KAdo} by only 50 ± 3.4% (Fig. 3). However, the antagonism of I_{KAdo} caused by FSCPX at this concentration increased continuously with time. Thus, 89 ± 3.6% inhibition of I_{KAdo} by 1 μM FSCPX was achieved after 10 min of superfusion with the antagonist. In contrast, the effect of CPX (0.1 μM) reached a steady state at a submaximal level of inhibition of I_{KAdo} in 5 min and did not increase further.

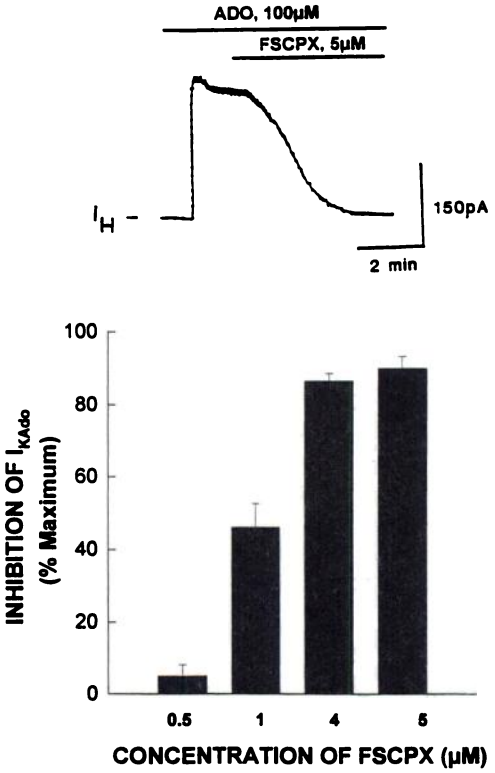


Fig. 2. Antagonism by FSCPX of adenosine-mediated activation of I_{KAdo} in guinea pig atrial cells. *Top*, Trace of the holding current (I_H; holding potential = −40 mV) recorded from a single atrial myocyte. The increase in I_H reflects the activation of I_{KAdo}. The adenosine (100 μM)-induced increase in I_H was completely reversed by 5 μM FSCPX. Similar results were obtained with six different cells from three hearts. *Bottom*, Summary of data depicting inhibition of I_{KAdo} after a 5-min exposure to each indicated concentration of FSCPX. *Bars*, mean ± standard error of values from five to seven experiments.

Irreversibility of FSCPX action. The antagonism by FSCPX of the action of adenosine was not reversible. The recovery of I_{KAdo} after washout of FSCPX (5 μM) was minimal, whereas recovery of I_{KAdo} after washout of CPX was nearly complete. As illustrated in Fig. 5, the activation of I_{KAdo} by adenosine recovered to 83 ± 4.4% (four experiments) of the steady state value of the current obtained in the presence of adenosine alone within 6 min of washout of CPX (1 μM). In contrast, the antagonism by FSCPX of I_{KAdo} persisted after 10 min of washout (12 ± 5.1% of control steady state current; five experiments).

Pretreatment of cells with FSCPX irreversibly reduced the response to adenosine but did not alter the response to carbachol. As illustrated in Fig. 6, the magnitudes of both the adenosine-induced activation of I_{KAdo} and the inhibition of β-$I_{Ca,L}$ by adenosine were markedly attenuated in cells pretreated with FSCPX, even after washout of the antagonist for ≤5 hr. In vehicle-treated cells (control), adenosine and carbachol increased the holding current by 8.54 ± 0.5 pA/pF (eight experiments) and 7.5 ± 0.6 pA/pF (six experiments), respectively. In cells pretreated with FSCPX, the increase in the holding current caused by adenosine was markedly attenuated to 0.78 ± 0.2 pA/pF, whereas the carbachol-induced increase in the holding current was not significantly different from control (7.9 ± 0.6 pA/pF; four experiments). Similarly, FSCPX pretreatment markedly attenuated the adenosine-induced but not the carbachol-induced inhibition of β-$I_{Ca,L}$ (Fig. 6). The magnitudes of attenuation by adenosine and carbachol of β-$I_{Ca,L}$ of FSCPX-pretreated cells were 15.8 ± 4.3% (five experiments) and 98.2 ± 7.2% (four experiments), respectively, of those of control cells.

Selectivity of FSCPX inhibition for A_1- versus A_2-adenosine receptors. FSCPX attenuated the A_1-adenosine receptor-mediated S-H interval prolongation but not the A_2-adenosine receptor-mediated decrease in coronary resistance. The selectivity of FSCPX for A_1- vs A_2-adenosine receptors was determined by treating guinea pig isolated hearts with either FSCPX (1 μM) or vehicle (DMSO plus K-H solution). As shown in Fig. 7A, the S-H interval prolongation of 10.5 ± 0.5 msec caused by 4.0 μM adenosine was significantly attenuated after FSCPX treatment to 0.7 ± 0.6 msec (five experiments). The magnitude of attenuation of the adenosine-induced S-H interval prolongation decreased slightly but not significantly, even after 75 min of washout of the antagonist. In contrast, the decreases in coronary resistance

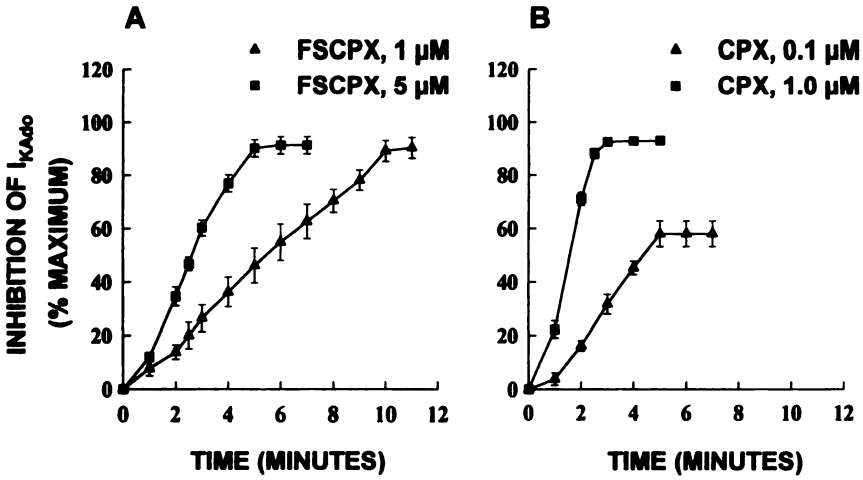


Fig. 3. The time courses of inhibition of I_{KAdo} caused by the irreversible A_1-adenosine receptor antagonist FSCPX (1 μM and 5 μM, A) and by the reversible A_1-adenosine receptor antagonist CPX (0.1 μM and 1 μM, B) in the presence of 100 μM adenosine. *Points*, mean ± standard error of determinations from four to six single atrial cells from three or four guinea pig hearts.

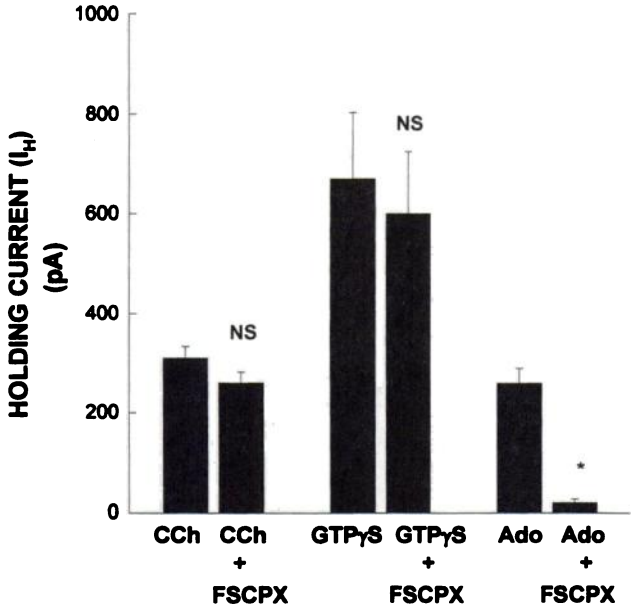


Fig. 4. Specificity of the antagonism by FSCPX of the adenosine (*Ado*)-mediated increase in the holding current (I_H; holding potential = −40 mV). Summary of data contrasting the lack of effect of FSCPX (5 μM) on the increase in I_H caused by either 0.5 μM carbachol (*CCh*) or 100 μM GTPγS with the inhibition by FSCPX of a 100 μM adenosine-induced increase in I_H. Each bar represents the mean ± standard error of data from five atrial cells from two or three hearts. *, Significantly different from adenosine ($p < 0.05$). *NS*, $p > 0.05$ compared with intervention in the absence of FSCPX.

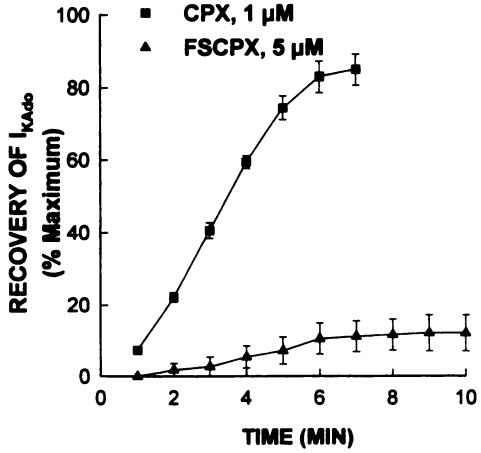


Fig. 5. Time course of recovery of I_{KAdo} of a single atrial myocyte after termination of exposure to FSCPX (irreversible antagonist) and to CPX (reversible antagonist). Recovery of I_{KAdo} (measured as the holding current at −40 mV) is expressed as a percentage of maximum current. The attenuation of I_{KAdo} caused by FSCPX (5 μM) persisted even after removal of this antagonist, whereas the effect of CPX (1 μM) was almost completely reversed after 6 min of washout. *Points*, mean ± standard error of data from four cells from two or three hearts.

caused by adenosine measured before and after treatment of hearts with FSCPX were not significantly different (3.31 ± 0.29 versus 2.86 ± 0.29 mm Hg/ml/min; five experiments, Fig. 7B). Thus, FSCPX selectively attenuated the A_1-adenosine receptor-mediated response in isolated guinea pig hearts. Treatments of hearts with vehicle alone caused no change in the responsiveness of hearts to either the A_1- or the A_2-adenosine receptor-mediated actions of adenosine (Fig. 7). In addition, the specific binding of [^{3}H]CPX to atrial and ventricular membranes prepared from hearts treated with FSCPX was reduced to 46 (two experiments) and 11 ± 0.5 fmol/mg protein (four experiments) from 72 (two experiments) and 33 ± 6 fmol/mg protein (four experiments), respectively, in vehicle-treated hearts (not shown).

Radioligand Binding Studies

Irreversibility of FSCPX binding. FSCPX significantly and irreversibly reduced the specific binding of the A_1-adenosine receptor antagonist [^{3}H]CPX to guinea pig atrial membranes in a concentration-dependent manner. Pretreatment of atrial membranes with 0.1, 0.3, and 3.0 μM FSCPX for 30 min, followed by extensive washing (12 times), reduced the calculated maximal specific binding (B_{max}) of [^{3}H]CPX by 41 ± 10%, 67 ± 6%, and 80 ± 1% (mean ± standard error, three experiments), respectively, compared with control. Results of a representative experiment are shown in Fig. 8. Note that pretreatment with FSCPX did not significantly affect the affinity of [^{3}H]CPX for the remaining (unmodified) adenosine receptors. The K_d of [^{3}H]CPX binding in the absence of FSCPX was 3.6 nM, whereas in membranes treated with 0.1, 0.3, and 3.0 μM FSCPX, the K_d values were 4.0, 2.8, and 3.9 nM, respectively.

To further demonstrate the irreversibility of the binding of FSCPX, the specific binding of [^{3}H]CPX (20 nM) to guinea pig forebrain membranes preincubated for 30 min with FSCPX (50 nM), CPX (50 nM), or vehicle (DMSO plus Tris buffer) was

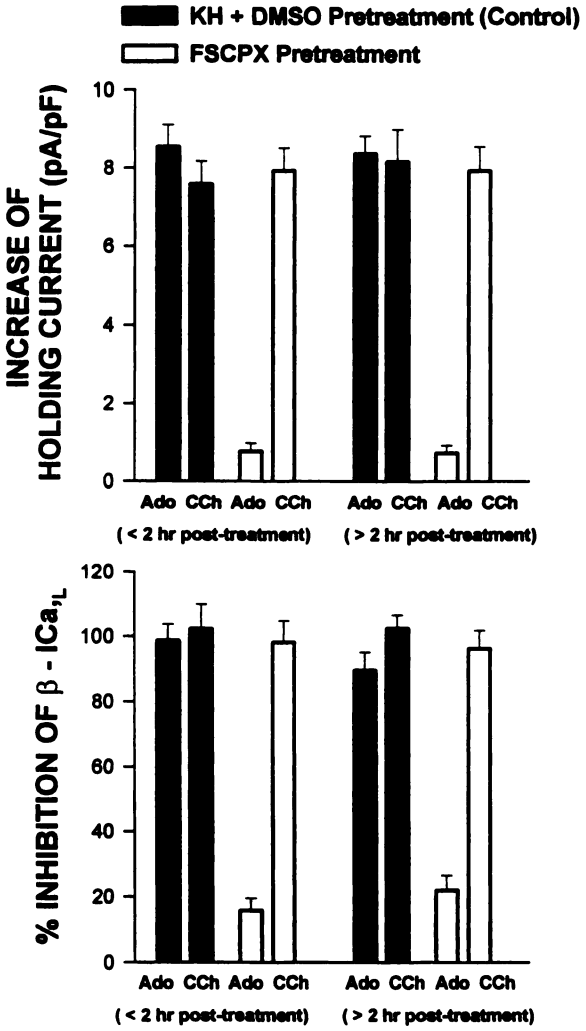


Fig. 6. Irreversible antagonism by FSCPX of the increase in holding current and inhibition of isoproterenol-stimulated $I_{Ca,L}$ (β-$I_{Ca,L}$) caused by adenosine (*Ado*) but not by carbachol (*CCh*) in guinea pig atrial myocytes. Cells were incubated with either FSCPX (50 nM) or vehicle (DMSO plus K-H solution) for 30 min and then washed through superfusion with fresh K-H solution containing 100 μM Ca^{2+}. Measurements were made between 10 min to 2 hr (<2 hr) and 2–5 hr (>2 hr) after washout of FSCPX or vehicle. *Top*, Activations of I_{KAdo} by adenosine (100 μM) and of I_{KAch} by carbachol (0.5 μM) were measured as an increase in holding current at −40 mV. *Bottom*, Inhibitions of isoproterenol (100 nM)-stimulated $I_{Ca,L}$ by adenosine and by carbachol were measured as a difference between the late outward current and the peak inward current elicited by 200-msec depolarizing pulses from −40 mV to +10 mV. *Bars*, mean ± standard error of data representing four to seven atrial cells from five to seven guinea pig hearts.

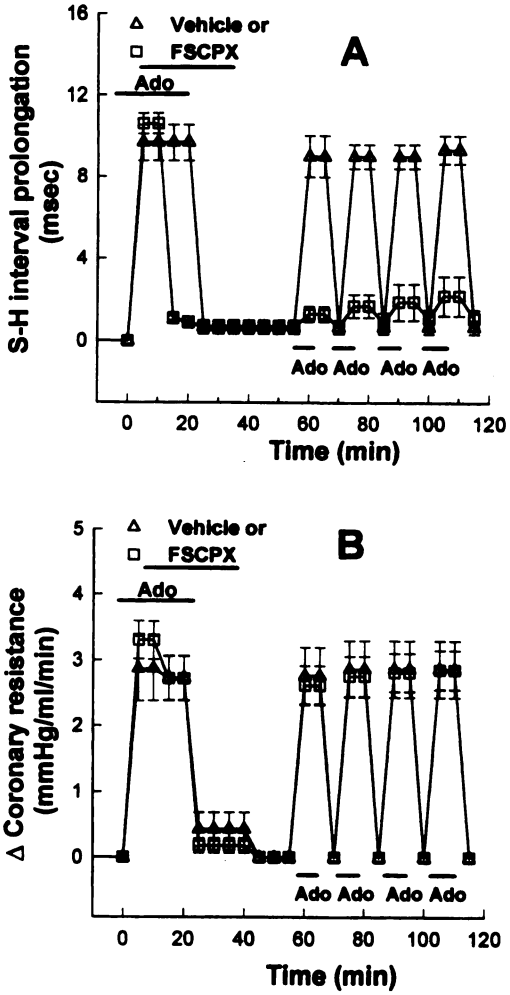


Fig. 7. Selective antagonism by FSCPX of the negative dromotropic (A_1-adenosine receptor-mediated, A) but not the coronary vasodilatory (A_2-adenosine receptor-mediated, B) effect of adenosine in isolated guinea pig hearts. Hearts were treated without (control) or with FSCPX (1 μM) for 30 min followed by 75 min of washout. The responses of hearts to adenosine were determined before treatment and at 15-min intervals after termination of treatment. FSCPX antagonized the increase in atrioventricular nodal conduction time without significantly inhibiting the coronary vasodilation caused by adenosine (4.0 ± 0.5 μM). The density of A_1-adenosine receptors measured in the cardiac membranes of the same hearts was reduced from control by 36% in atria and by 67% in ventricles. *Symbols*, mean and standard error of measured parameters in experiments with five guinea pig hearts.

determined after each of seven successive washes (Fig. 9). The specific binding of [^{3}H]CPX to membranes preincubated with CPX recovered to control levels after three washes. In contrast, the specific binding of [^{3}H]CPX to membranes preincubated with FSCPX did not recover despite undergoing seven washes (Fig. 9).

Selectivity of FSCPX binding. FSCPX displaced the specific binding of both the A_1-adenosine receptor antagonist [^{3}H]CPX and the A_{2a}-adenosine receptor agonist [^{3}H]CGS 21680 but with markedly different potencies (Table 1). The apparent IC_{50} values for the inhibition of [^{3}H]CPX binding to cardiac and brain membranes by FSCPX were in the low nanomolar range. In contrast, FSCPX was 15–100-fold less potent at inhibiting the binding of [^{3}H]CGS 21680 to striatal membranes. Furthermore, the binding of FSCPX to striatal A_{2a}-adenosine receptors was reversible on washout of the antagonist (Fig. 10). Pretreatment of membranes with FSCPX (0.1 or 1 μM) reduced the specific binding of [^{3}H]CPX to forebrain membranes by 65 ± 5% and 83 ± 2% (four experiments), respectively. Results of a representative experiment are shown in Fig. 10A. In this experiment, the K_d values for binding of [^{3}H]CPX to the A_1-adenosine receptor in 0.1 and 1 μM FSCPX-treated membranes were not significantly different from the control K_d values (2.4 and 3.1 nM versus 3.8 nM, respectively). In contrast, neither the specific binding of [^{3}H]CGS 21680 nor its affinity to the A_{2a}-adenosine receptor in control and FSCPX-treated membranes was significantly different (Fig. 10B).

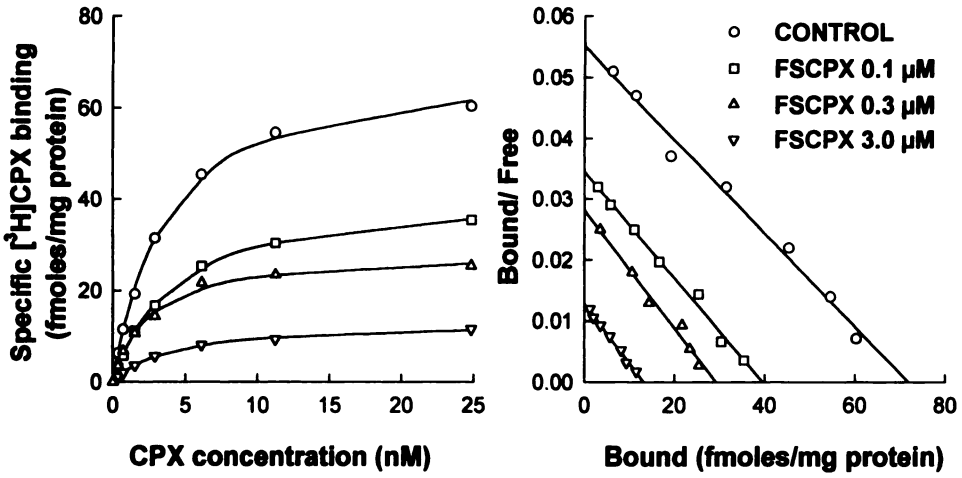


Fig. 8. Effect of FSCPX in reducing the specific binding of [^{3}H]CPX to guinea pig atrial membranes. Membranes were incubated without (control) or with FSCPX (0.1, 1.0, or 3.0 μM) for 30 min and extensively (12 times) washed with cold buffer solution. The saturation binding isotherms (*left*) and Scatchard plots (*right*) show a decrease in the maximal specific binding (B_{max}) of [^{3}H]CPX by 51%, 69%, and 76% after treatment with 0.1, 1, and 3.0 μM FSCPX, respectively. The slopes of the Scatchard plots of binding of [^{3}H]CPX to FSCPX-treated membranes were not significantly different from control. *Points*, mean of triplicate determinations in a single experiment.

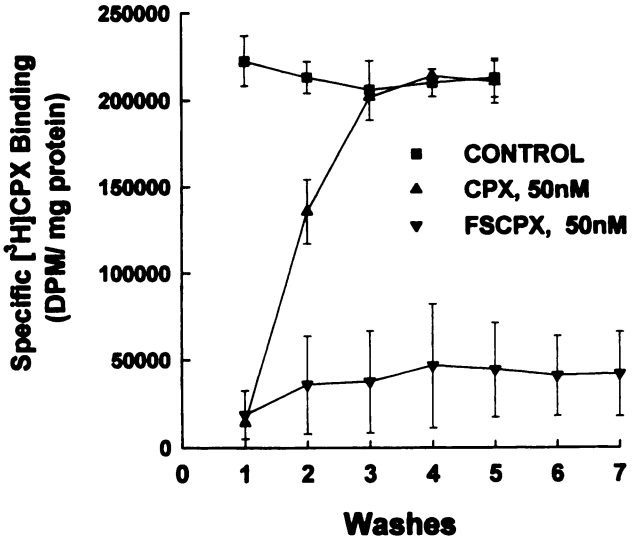


Fig. 9. Irreversibility of the binding of FSCPX to A_1-adenosine receptors in guinea pig brain membranes. Shown is the recovery of specific binding of [^{3}H]CPX to guinea pig brain membranes pretreated with either FSCPX (50 nM) or CPX (50 nM). After an incubation period of 60 min, membranes were washed seven times with cold buffer to remove free and reversibly bound antagonist. The specific binding of [^{3}H]CPX (20 nM) was determined after each wash. Control membranes were incubated with buffer solution in the absence of antagonist. Specific binding of [^{3}H]CPX to FSCPX-treated membranes did not recover to control levels despite seven washes, whereas the specific binding of [^{3}H]CPX to CPX-treated membranes recovered to control levels after two washes. *Points*, mean ± standard error of triplicate determinations in each of two experiments.

TABLE 1

Potency of FSCPX to displace the specific binding of [^{3}H]CPX and [^{3}H]CGS 21680 to cell membranes from guinea pig heart and brain

Tissue	IC_{50}	
	[^{3}H]CPX	[^{3}H]CGS 21680
	nM	
Brain	1.2 ± 0.3	135.8 ± 28.7
Ventricles	8.8 ± 1.6	ND[a]
Atria	8.6 ± 1.3	ND

[a] ND, not determined.

Lack of Effect of FSCPX on the Inhibition by Gpp(NH)p of Agonist Binding to the A_1-Adenosine Receptor

FSCPX did not significantly affect the potency of Gpp(NH)p in reducing the binding of [^{3}H]CCPA to guinea pig forebrain membranes (Fig. 11). Gpp(NH)p inhibited the specific binding of [^{3}H]CCPA with IC_{50} values of 5.56 ± 1.6 μM (three experiments) and 3.46 ± 1.7 μM (three experiments) in untreated (control)- and FSCPX-treated membranes, respectively.

Discussion

Results of this study demonstrate that FSCPX antagonized the A_1-adenosine receptor-mediated activation of I_{KAdo} and inhibition of β-$I_{Ca,L}$ in a specific and irreversible manner. Furthermore, FSCPX irreversibly attenuated the A_1-adenosine receptor-mediated negative dromotropic effect but did not significantly affect the A_2-adenosine receptor-mediated decrease in coronary resistance of isolated, perfused hearts. Consistent with these results, FSCPX displaced the specific binding of the A_1-adenosine receptor antagonist [^{3}H]CPX to guinea pig cardiac and brain membranes with apparent IC_{50} values in the low nanomolar range. Moreover, the binding of FSCPX to A_1-adenosine receptors was not reversible. Thus, preincubation of membranes with FSCPX followed by extensive washes reduced the maximum specific binding of [^{3}H]CPX but did not cause significant changes in either the affinity of [^{3}H]CPX to the remaining A_1-adenosine receptors or the coupling of these receptors to G proteins. In contrast, even though FSCPX displaced [^{3}H]CGS 21680 binding to striatal membranes (IC_{50} = 135 nM), the binding of FSCPX to A_{2a}-adenosine receptors was reversed on washout of the antagonist. These results suggest that FSCPX is a specific, selective, and irreversible antagonist of cardiac and brain A_1-adenosine receptors.

Irreversibility of FSCPX action. Four complementary lines of evidence demonstrate that the action of FSCPX is irreversible. First, FSCPX significantly attenuated both the activation of I_{KAdo} and the inhibition of β-$I_{Ca,L}$ caused by adenosine in atrial myocyte preparations, and the magnitude of attenuation of the adenosine-mediated responses was not diminished by washout of FSCPX (Figs. 5 and 6). Thus, the responses of cells to adenosine measured after <2 hr of washout of FSCPX and after >2 hr of washout were not significantly different (Fig. 6). Second, the response to adenosine of isolated hearts treated with FSCPX (1 μM) did not

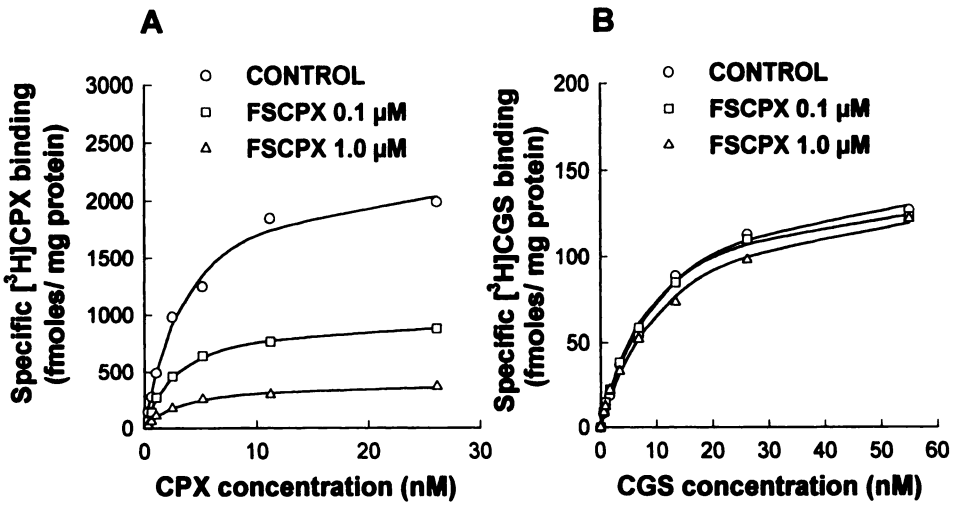


Fig. 10. Inhibition by FSCPX of the specific binding of the A_1-adenosine receptor antagonist [^{3}H]CPX (A) but not the A_{2a}-adenosine receptor agonist [^{3}H]CGS 21680 (B). Guinea pig brain membranes (A) or guinea pig striatal membranes (B) were pretreated without or with FSCPX (0.1 or 1.0 μM) for 30 min and washed 12 times before assay of radioligand binding. *Points*, mean of triplicate determinations in a single experiment.

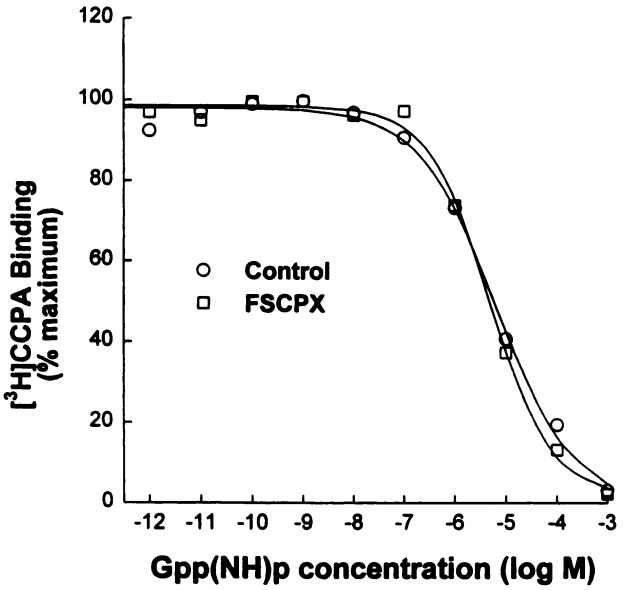


Fig. 11. Lack of effect of FSCPX on the inhibition by Gpp(NH)p of the specific binding of [^{3}H]CCPA, an A_1-adenosine receptor agonist, to guinea pig brain membranes. Membranes were incubated without or with FSCPX FSCPX (0.1 μM) for 30 min and washed 12 times with cold buffer solution before assay of [^{3}H]CCPA binding sites. FSCPX did not significantly affect the potency of Gpp(NH)p to inhibit the binding of [^{3}H]CCPA to guinea pig forebrain membranes. *Points*, mean of triplicate determinations from a single experiment.

recover significantly even after 75 min of washout of the antagonist (Fig. 7). Of equal significance is that the specific binding of [^{3}H]CPX to atrial and ventricular membranes prepared from the same hearts was reduced by 36 and 66%, respectively, compared with control. Thus, the attenuation of adenosine-mediated S-H interval prolongation by FSCPX was associated with a reduction in A_1-adenosine receptor density. Third, in keeping with the results of functional studies, FSCPX irreversibly reduced the specific binding of [^{3}H]CPX to cardiac and brain membranes (Figs. 8 and 10A) but did not significantly alter the affinity of [^{3}H]CPX binding to noninactivated A_1-adenosine receptors. The specific binding of [^{3}H]CPX to FSCPX-treated membranes did not recover even when membranes were washed up to seven times. In contrast, washing of membranes preincubated with CPX, the parent compound from which FSCPX was synthesized, resulted in a near-total recovery (90%) of specific binding of [^{3}H] CPX. Last, the effect of FSCPX was both time and concentration dependent. Thus, as illustrated in Figs. 1 and 2, the degree of attenuation of I_{KAdo} by FSCPX was dependent on both the duration of exposure to the antagonist and its concentration. This behavior is consistent with the fact that the binding of an irreversible antagonist to a receptor does not attain thermodynamic equilibrium.

Although the nature of the chemical interaction of FSCPX with A_1-adenosine receptor is unknown, the fluorosulfonyl moiety is known to be chemoreactive (9), and a covalent bonding of FSCPX with the receptor would account for our observations. The lack of recovery of A_1-adenosine receptor-mediated responses in single cells over a 5-hr washout period and the persistent inhibition of [^{3}H]CPX binding to A_1-adenosine receptors of cardiac and brain membranes despite extensive washing support this hypothesis. However, the most definitive criterion to demonstrate a covalent interaction is to radiolabel the probe and subsequently demonstrate incorporation of the labeled probe into the receptor protein (15, 16). Additional studies will therefore be required to determine whether FSCPX interacts covalently with the A_1-adenosine receptor.

Selectivity of FSCPX. FSCPX selectively and irreversibly inactivated A_1-adenosine but not A_{2a}-adenosine receptors of cardiac and brain membranes. The binding of [^{3}H]CGS 21680 to striatal membranes that had been preincubated with FSCPX and then washed was not reduced from control (Fig. 10B). Similarly, the coronary vasodilation caused by adenosine was not altered by pretreatment of hearts with FSCPX (Fig. 7B). FSCPX displaced binding of [^{3}H]CGS 21680 to striatal membranes with an IC_{50} value of 135 nM but did not significantly antagonize the adenosine-induced decrease in coronary resistance at nearly 10-fold higher concentrations. These results are in agreement with a similar lack of effect of other adenosine receptor antagonists on the coronary vasodilation caused by adenosine and may reflect the existence of different subtypes of A_2-adenosine receptors in striatum and coronary arteries (17). Nevertheless, these results conclusively demonstrate that FSCPX is a selective irreversible antagonist of the A_1-adenosine receptor.

FSCPX does not affect the function of postreceptor elements. Reactive functional groups, such as the fluorosul-

fonyl group, are capable of interacting with available nucleophiles on the cell surface, resulting in nonspecific effects. This phenomenon has been noted previously for irreversible ligands of adrenergic receptors (15, 16). Therefore, we sought to determine whether FSCPX altered components of the adenosine signal transduction pathway in addition to the receptor. As shown in Fig. 4, FSCPX significantly attenuated the increase in the holding current caused by adenosine but did not significantly antagonize either the carbachol- or the GTPγS-mediated increases in holding current. In addition, pretreatment of atrial myocytes with FSCPX did not cause a significant change in the responsiveness of cells to carbachol (Fig. 4). Both adenosine and carbachol share similar coupling mechanisms (1), activate a similar K^+ conductance, and inhibit adenylate cyclase activity in cardiac myocytes via a G protein-mediated mechanism (14). Thus, the lack of a significant effect of 50 nM FSCPX on the carbachol- and GTPγS-induced increases in the K^+ current suggests that FSCPX does not directly affect the muscarinic receptors, the K^+ channel, or the interaction between inhibitory receptor and G proteins. Similarly, the lack of an effect of FSCPX on the carbachol-induced inhibition of β-$I_{Ca,L}$ suggests that at the tested concentrations, FSCPX does not directly affect adenylate cyclase activity and L-type Ca^{2+} channels. To further determine whether FSCPX affects A_1-adenosine receptor/G protein coupling, the inhibition of agonist binding by Gpp(NH)p in the absence and presence of FSCPX was used as a functional assay (18). As shown in Fig. 11, FSCPX did not significantly alter the potency of Gpp(NH)p to inhibit the specific binding of [^{3}H]CCPA to guinea pig brain membranes. Taken together, these results suggest that FSCPX does not affect the function of postreceptor elements that are involved in A_1-adenosine receptor-mediated responses. However, these studies do not exclude the possibility of nonspecific interactions of FSCPX with other cellular proteins at higher concentrations and/or at longer durations of exposure to the antagonist.

Two irreversible antagonists of A_1-adenosine receptors have been described: *m*-DITC-XAC (6–8) and FSCPX (9). An assessment of the relative merits of the two compounds would be useful; however, available information allows only a tentative evaluation. The selectivity of *m*-DITC-XAC has been investigated only once. The ratio of A_1 to A_{2a} selectivity (K_i at A_2/K_i at A_1) for *m*-DITC-XAC in rat brain was reported to be 144 (7), a value that is similar to the ratio of A_1 to A_{2a} selectivity for FSCPX in guinea pig brain. However, the affinity of A_1-adenosine receptor antagonists, including CPX, is reported to be less in guinea pig than in rat brain (17, 19), suggesting that *m*-DITC-XAC would be less selective than FSCPX for A_1-adenosine receptors in the guinea pig. If the selectivity of the parent xanthine compounds (CPX and XAC) is an indication of the selectivity of the irreversible derivatives (FSCPX and *m*-DITC-XAC), then we would expect FSCPX to be more A_1 selective than *m*-DITC-XAC, based on reported affinities of CPX and XAC (20). The orientation of chemically reactive moieties is also different in the two irreversible antagonists. The fluorosulfonyl group is attached to the 3-propyl substituent of xanthine in CPX, whereas the DITC group is located at the end of the relatively long 8-phenyl substituent of xanthine in XAC. The molecular weights of *m*-DITC-XAC and FSCPX are 621 and 507, respectively. Thus, *m*-DITC-XAC and FSCPX may alkylate different amino acids in the A_1-adenosine receptor. Last, *m*-DITC-XAC and FSCPX have been used in a limited number of *in vitro* biological preparations, and selective irreversible antagonism of A_1-adenosine receptors by *m*-DITC-XAC in a physiological preparation has not been shown. Thus, *m*-DITC-XAC and FSCPX may not necessarily be selective, stable, or irreversible antagonists in all preparations.

Summary and implications. We describe the first adenosine receptor antagonist that causes both an irreversible attenuation of cardiac A_1-adenosine receptor-mediated responses and a demonstrable reduction in A_1-adenosine receptor density of cardiac membranes as measured in radioligand binding studies. Furthermore, FSCPX seems to possess a high affinity for the A_1-adenosine receptor, does not affect the function of postreceptor elements, and can be used to cause various levels of A_1-adenosine receptor inactivation. Binding of FSCPX to A_2-adenosine receptors seems to be of low affinity and is reversible. Thus, to estimate the A_1-adenosine receptor reserve, FSCPX has considerable advantages over both photoaffinity ligands and other known irreversible ligands of the A_1-adenosine receptor.

The pleiotropic effects of exogenously administered A_1-adenosine receptor agonists make it necessary to design approaches that confer organ and response selectivity to these agents. Toward this goal, researchers at our laboratory recently demonstrated the use of allosteric enhancers to selectively augment the response to adenosine in organs in which the production of adenosine is increased (21). A second approach to achieve organ selectivity is to exploit tissue differences in receptor reserve (22). If differences in receptor reserve are large, tissue selectivity can be achieved by using low concentrations of an agonist (23). That is, lower concentrations of agonist can elicit responses from tissues with large receptor reserves. This relationship between receptor reserve and potency of an agonist to elicit a response has been demonstrated for a number of receptor systems (24, 25). The availability of FSCPX will make it possible to use Furchgott's method of irreversible receptor inactivation (26) to estimate the A_1-adenosine receptor reserve in various organ systems and determine whether tissue-selective responses to A_1-adenosine agonists are likely to be attained.

Acknowledgments

We thank Yi Zhang for technical assistance and Dr. Donn Dennis for fruitful discussions throughout the course of this study.

References

1. Belardinelli, L., J. Linden, and R. M. Berne. The cardiac effects of adenosine. *Prog. Cardiovasc. Dis.* **32**:73–97 (1987).
2. Gerlach, E., and B. F. Becker, eds. *Topics and Perspectives in Adenosine Research*. Springer-Verlag, Berlin (1987).
3. Belardinelli, L., and A. Pelleg, eds. *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology*. Kluwer Academic Publishers, Norwell, MA (1995).
4. Stiles G. L, D. T. Daly, and R. A. Olsson. The A_1 adenosine receptor: identification of the binding subunit by photoaffinity crosslinking. *J. Biol. Chem.* **260**:10806–10811 (1985).
5. Lohse, M. J., K. Klotz, and U. Schwabe. Agonist photoaffinity labeling of A_1 adenosine receptors: persistent activation reveals spare receptors. *Mol. Pharmacol.* **30**:403–409 (1986).
6. Stiles, G. L., and K. A. Jacobson. High affinity acylating antagonists for the A_1 adenosine receptor: identification of binding subunit. *Mol. Pharmacol.* **34**:724–728 (1988).
7. Jacobson, K. A., S. Barone, U. Kammula, and G. L. Stiles. Electrophilic derivatives of purines as irreversible inhibitors of A_1-adenosine receptors. *J. Med. Chem.* **32**:1043–1051 (1989).
8. Dennis, D., K. A. Jacobson, and L. Belardinelli. Evidence of spare A_1-

adenosine receptors in guinea pig atrioventricular node. *Am. J. Physiol.* **262**:H1070–H1078 (1992).
9. Scammells, P. J., S. P. Baker, L. Belardinelli, and R. A. Olsson. Substituted 1:3-dipropylxanthines as irreversible antagonists of A_1-adenosine receptors. *J. Med. Chem.* **37**:2704–2712 (1994).
10. Isenberg, G., and U. Klokner. Calcium tolerant myocytes prepared by preincubation in a "KB medium." *Pfluegers Arch.* **395**:6–18 (1982).
11. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch clamp techniques for high resolution current recordings from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
12. Jenkins, J., and L. Belardinelli. Atrioventricular nodal accommodation in isolated guinea pig hearts: physiological significance and role of adenosine. *Circ. Res.* **63**:97–116 (1988).
13. Clemo, H. F., and L. Belardinelli. Effect of adenosine on atrioventricular conduction. I. Site and characterization of adenosine action in the guinea pig atrioventricular node. *Circ. Res.* **59**:427–436 (1986).
14. Kurachi, Y., T. Nakajima, and T. Sugimoto. On the mechanism of activation of muscarinic K^+ channels by adenosine in isolated atrial myocytes: involvement of GTP-binding proteins. *Pfluegers Arch.* **407**:264–274 (1986).
15. Dickinson, K. E., S. L. Heald, P. W. Jeffs, R. J. Lefkowitz, and M. G. Caron. Covalent labeling of the β-adrenergic ligand binding site with *para*-(bromoacteamidyl)benzylcarazolol: a highly potent β-adrenergic affinity label. *Mol. Pharmacol.* **27**:499–506 (1985).
16. Regan, J. W., R. M. DeMarinis, M. G. Caron, and R. J. Lefkowitz. Identification of the subunit binding site of α_2-adrenergic receptors using [^{3}H] phenoxybenzamine. *J. Biol. Chem.* **259**:7864–7869 (1984).
17. Belardinelli, L., J. C. Shryock, Y. Zhang, P. J. Scammells, R. Olsson, D. Dennis, P. Milner, J. Pfister, and S. P. Baker. 1:3-Dipropyl-8-[2-(5,6-epoxy)norbornyl]xanthine, a potent, specific and selective A_1 adenosine receptor antagonist in guinea pig heart and brain and in DDT_1MF-2 cells. *J. Pharmacol. Exp. Ther.* **275**:1167–1176 (1995).
18. Green, A., and J. L. Johnson. Evidence for impaired coupling of receptors to G_i protein in adipocytes from streptozocin-induced diabetic rats. *Diabetes* **40**:88–94 (1991).
19. Suzuki, F., J. Shimada, S. Shiozaki, S. Ichikawa, A. Ishii, J. Nakamura, H. Nonaki, H. Kobayashi, and E. Fuse. Adenosine A_1 antagonists. 3. Structure-activity relationships on amelioration against scopolamine- or N^6-((*R*)-phenylisopropyl)adenosine-induced cognitive disturbance. *J. Med. Chem.* **36**:2508–2518 (1993).
20. Daly, J. W., and K. A. Jacobson. Adenosine receptors: selective agonists and antagonists, in *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology*. (L. Belardinelli and A. Pelleg, eds.). Kluwer Academic Publishers, Norwell, MA (1995).
21. Kollias-Baker, C., J. Xu, A. Pelleg, and L. Belardinelli. Novel approach for enhancing atrioventricular nodal conduction delay mediated by endogenous adenosine. *Circ. Res.* **75**:972–980 (1994).
22. Kenakin, T. P. Drugs and receptors: an overview of the current state of knowledge. *Drugs* **40**:666–687 (1991).
23. Jenkinson, D., and A. Abbott. Are molecular and electrophysiological data refining models of agonism? *Trends Pharmacol. Sci.* **11**:91–94 (1990).
24. Brown, J. H., and D. Goldstein. Differences in muscarinic receptor reserve for inhibition of adenylate cyclase and stimulation of phosphoinositide hydrolysis in chick heart cells. *Mol. Pharmacol.* **30**:466–470 (1986).
25. Meller, E., K. Bohmaker, Y. Namba, A. J. Friedhoff, and M. Goldstein. Relationship between receptor occupancy and response at striatal dopamine autoreceptors. *Mol. Pharmacol.* **31**:592–598 (1987).
26. Furchgott, R. F. The use of haloalkylamines in the differentiation of receptors and the determination of dissociation constants of receptor-agonist complexes. *Adv. Drug. Res.* **3**:21–55 (1966).

Send reprint requests to: L. Belardinelli, M.D., University of Florida, Department of Medicine, P.O. Box 100277, Gainesville, FL 32610. E-mail: kathi.med@shands.ufl.edu