PHOTOLABILE A₁-ADENOSINE RECEPTOR AGONISTS AS "CAGED" ELECTROPHYSIOLOGICAL PROBES[+]

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Abstract

5'-Ether derivatives of the potent adenosine agonist N^6-cyclopentyladenosine (CPA) were designed as "caged" ligands for the activation of A₁-adenosine receptors following *in situ* photolysis. The synthesis involved a 2',3'-diol protection scheme using the acid labile ethoxymethynyl group. Generation of CPA was demonstrated chromatographically and in a bioassay measuring the inhibition of synaptic potentials in the rat hippocampus.

Introduction

Adenosine acts as a neuromodulator in the CNS[1] and as a regulator of the activity of other organs, such as the heart[2] and kidneys,[3] generally as a protective depressant of the organ's activity in response to excessive oxygen demand and/or diminished oxygen supply.[4] In the central nervous system, adenosine agonists act to depress neuronal firing and the release of stimulatory neurotransmitters, such as acetylcholine[5] and glutamic acid.[6] The anticonvulsant effects of adenosine agonists in the hippocampus have been described.[7] A difficulty in the use of adenosine agonists in physiological systems is the apparent lag time between the application of the drug and the effect observed. This phenomenon has been ascribed to limited diffusion[8,9] of the normally hydrophilic adenosine derivative to the receptor site or to a mechanistic delay, in which adenosine receptors require a postulated biochemical activation.[10]

A technique for examining the fast kinetics of neurotransmitter action is that of "caged" probes,[11] in which the biologically active form of a molecule is generated in situ from an inactive precursor through a photochemical deblocking process. This technique has been developed for nucleotides and other neurotransmitters and biochemical messengers,[11] such as ATP, 3',5'-cyclic AMP, inositol-1,4,5-triphosphate, γ-aminobutyric acid,[12] and glutamic acid.[12] The photolabile linkages used previously and in this study are based on the 2-nitrobenzyloxy group, introduced by Patchornik and coworkers.[13,14]

[+]Dedicated to Prof. A. Patchornik on the occasion of his 65th birthday.

Materials and Methods

Chemistry

2-Nitrobenzylbromide was purchased from Lancaster Synthesis (Windham, NH). N^6-Cyclopentyladenosine (CPA) and 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene (NV-Br) were synthesized as reported.[13b, 17] Analytical TLC plates and silica gel (230–400 mesh) were purchased from VWR. [1]NMR spectra in $CDCI_3$ or DMSO-d_6 were obtained at 300 MHz on a VARIAN GEMINI-300. Mass Spectra were obtained using a Finnigan 10150 for CI and using a Jeol JMS-SX102 for FAB ionization. Elemental analyses were by Galbraith Labs (Knoxville, TN).

N^6-Cyclopentyl-2'-3'-*O*-ethoxymethylene-adenosine. 2—CPA, **1**, (500 mg, 1.5 mmol) and trichloroacetic acid (500 mg, 3 mmol) were dissolved in dioxane (25 ml), and the mixture was stirred at room temperature over 4Å molecular sieves. Triethylorthoformate was added (2 ml, 12 mmol), and the solution heated at 60°C for 45 min under a nitrogen atmosphere. Sodium bicarbonate (1 g, 12 mmol) was added at 0° C, and after 1.5 h of vigorous shaking the mixture was filtered and the solvent evaporated. The residue was purified by flash column chromatography on silica gel with chloroform:methanol, 98:2, by vol, as eluent. Evaporation of the solvent from the pure fraction afforded 480 mg of **2** as an oil in quantitative yield.

^{1}H-NMR δ (DMSO-d_6): 1.1 & 1.2 (t, 3H, CH_3 endo/exo, J = 7Hz), 1.6, 1.7 & 1.95 (m, 8H, Cp), 3.5–3.7 (m, 4H, CH_2O & 5'-H), 4.2 & 4.3 (m, 1H, 4'-H), 4.5 (m, 1H, C<u>H</u>N), 4.95 & 5.05 (dd, 1H, 3'-H endo/exo, $J_{3', 4'}$ = 3.5 Hz, $J_{3', 2'}$ = 7.1 Hz), 5.40 & 5.46 (dd, 1H, 2'-H endo/exo, $J_{2', 1'}$ = 3.1 Hz), 6.17 & 6.21 (s, 1H, O_2CH endo/exo), 6.28 (d, 1H, 1'-H), 7.78 & 8.2 (Is, 1H endo/exo, NH), 8.32 & 8.4 (s & d, 2H, 2-H, 8-H). Anal. calcd. for $C_{18}H_{25}N_5O_5 \cdot 0.5CHCl_3$: C, 49.17; H,.5.64; N, 15.05. Found: C, 49.26; H,.5.70; N, 15.52. MS (CI-NH_3) 392 M+1, 204 Base+2. High resolution MS (FAB/ NOBA matrix): m/e 392.1945, M+1, 392.1934 calcd. for $C_{18}H_{26}N_5O_5$

N^6-Cyclopentyl-5'-*O*-(2-nitrobenzyl)-2'-3'-O-ethoxymethylene-adenosine, 3a—Sodium hydride (15 mg of 50% oil dispersion, 0.26 mmol) was washed with pentane and was dispersed in 2 ml of dry DMF. After cooling the suspension to 0°C compound **2** (50 mg, 0.13 mmol) in 2.5 ml of DMF was added. After gas generation had ceased (45 min), 2-nitrobenzylbromide (70 mg, 0.26 mmol) was added in 0.5 ml of DMF at 0° C in the dark, and the mixture was stirred at room temperature overnight. The reaction was quenched with ethanol, and diluted with H_2O and then extracted 3 times with Et_2O. The combined Et_2O layers were dried and after evaporation the residue was purified by flash chromatography with hexane:EtOAc, 1:1 to 1:0, by vol, to give 19 mg of **3a** (28%) as an oil, and 25 mg of starting material **2** (50%) was recovered.

^{1}H-NMR δ ($CDCI_3$): 1.3 (m, 3H, CH_3), 1.5–1.8 & 2.(m, 8H, Cp), 3.7 (m, 2H, $CH_3C\underline{H}_2O$), 4.39 (dd, 1H, 5'-H, J gem = 11.5 Hz, $J_{4'-5'}$ = 7 Hz,) 4.47 (dd, 1H, 5'-H, $J_{4'-5'}$ = 4.5 Hz), 4.62 (m, 1H, 4'-H), 5.10 (m, 3H, 3'-H & PhC<u>H</u>$_2$O), 5.59 (dd, 1H, 2'H, $J_{2', 3'}$ = 7.1 Hz, $J_{2', 1'}$ = 2.3 Hz), 5.70 (m, 1H, C<u>H</u>N), 6.04 & 6.06 (s, 1H, O_2CH endo/exo), 6.20 & 6.22 (d, 1H, 1'-H endo/exo), 7.19 (d, 1H, o-H-phenyl, J = 8 Hz), 7.44 (t, 1H, p-H-phenyl), 7.56 (dt, 1H, m-H-

phenyl, J = 1 Hz), 7.80 & 7.84 (s, 1H, 8-H exo/endo), 8.45 (dd, 1H, m-H-phenyl) 8.30 & 8.34 (br s, 1H, 2-H exo/endo). Anal. calcd. for $C_{25}H_{30}N_6O_7$: C, 57.03; H,.5.74. Found: C, 57.18; H,.5.65. MS (CI-NH$_3$) 527 M+1, 497 MH-ethane, 434 MH$_2$-N0$_2$Benzyl, 204 Base+2.

N[6]-Cyclopentyl-5'-*O*-(2-nitrobenzyl)-adenosina. 4a—Compound **3a** (19 mg, 0.04 mmol) was dissolved in 5 ml of chloroform. Trifluoroacetic acid (100 μl) was added at 0°C. After stirring overnight at 8°C an equal amount of TFA (100 μl) was added and the mixture was stirred for an additional 12 h. The solvent was then coevaporated several times with ethanol and the residue was purified by flash chromatography using ethyl acetate followed by ethyl acetate:methanol, 98:2, by vol. After concentration of the desired fraction, 6 mg of the product **4a** was isolated as an oil (56%). This compound applied t a TLC plate upon irradiation with 254 nm light for 1 hour was totally converted to CPA. ^{1}H-NMR δ (CDCl$_3$): 1.5 –1.8 & 2.1 (m, 8H, Cp), 3.84 (m, 2H, 5'-H), 4.48 (m, 1H, 3'-H), 4.50 (m, 1H, 4'-H), 4.56 (m, 1H, 2'-H), 5.94 (d, 1H, J$_{1',2'}$ = 5.3 Hz), 7.44 (m, 3H, H$_{arom}$), 7.98 (s, 1H, 8-H), 8.2 (d, 1H, m-H-phenyl), 8.30 (br s, 1H, 2-H). High resolution MS (FAB/ NOBA matrix): m/e 471.1982, M+1, 471.1992 calcd. for $C_{22}H_{27}N_6O_6$.

N[6]-Cyclopentyl-5'-*O*-(6-nitroveratryl)-adenosine. 4b—The procedures described above for **3a** and **4a** were followed starting from NV-Br. The overall yield starting from CPA was 9%, and the product was obtained as a yellow oil. NMR were consistent for **3b** and **4b**. High resolution MS (FAB/ NOBA matrix) **3b** : m/e 587.2478, M+1, 587.2478 calcd. for $C_{27}H_{35}N_6O_9$, **4b** : m/e 531.2170, M+1, 531.2203 calcd. for $C_{24}H_{31}N_6O_8$.

Biological assays—Hippocampal slices were prepared from adults rats (200–300 g) as previously described.[15] Field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of the CA1 region in response to stimulation of Schaffer collateral / commisural afferents. Slices were superfused continuously with artificial cerebrospinal fluid (ACSF)[15] and fEPSPs were sampled every 15 seconds.

The amplitude of fEPSPs responses was measured on-line and plotted over the time course of each experiment. The magnitude of the inhibitory effect of a compound on fEPSP amplitude was expressed as the percentage inhibition, according to the formula: Percentage inhibition = [(Amplitude in ACSF only - Amplitude in drug) ÷ Amplitude in ACSF only] × 100.

The following compounds were added, alone or in combination, to the ACSF, and the samples were superfused for 30–40 minutes over the slices: CPA (10, 50 and 100 nM), non irradiated **4a** (50 and 100 nM), irradiated **4a** (10, 50 and 100 nM), non irradiated **4b** (50 nM), irradiated **4b** (10, 20 and 50 nM), and theophylline (200 μM). For the irradiation of compounds **4a** and **4b**, a 10 μM stock solution was placed under a Spectroline UV light source (254 nm) for 10 min. The 10 min period was selected because of the relatively weak source of UV irradiation. The stock solution was then added to the ACSF to achieve the desired concentration, and inhibition was measured.

Results

The structure activity relationships for adenosine analogs have been explored in great detail.[16] In general, monosubstitution by alkyl,[17] cycloalkyl,[18] or aryl[19] groups as N^6-amino substituents has been associated with agonists of high potency and selectivity for A_1-receptors. A_2-selective agonists[20] have been synthesized through substitutions at the 2-position of the purine ring and/or with N-(small alkyl)carboxamide substitution at the 5'-position. For A_1-selective compounds, such as N^6-cyclopentyladenosine (CPA), the integrity of the ribose ring is generally important for agonist activity, and certainly at the 5'-position there are severe steric constraints. Thus, the 5'-hydroxyl was selected as the site for the incorporation of a photolabile blocking group.

A. Synthesis

The photolabile groups used included the 2-nitrobenzyl group[13] (NB) and the 6-nitroveratryl group[21] (NV), shown previously to cleave upon ultraviolet irradiation. 5'-NB-CPA, **4a**, and 5'-NV-CPA, **4b**, were synthesized using an alcohol protection scheme involving the acid labile 2,'3'-ethoxymethynyl group introduced by reaction of 1 with triethylorthoformate. The large excess of reagent required to drive the reaction to completion also caused the formation of an undesired product bearing a 5'-orthoester. This 5'-blocked by-product was reconverted to the desired compound during the purification on silica gel, giving the protected derivative, **2**, in one step with an 86% yield (Scheme 1). Attachment of the photolabile blocking group required O-alkylation of **2** which was achieved through treatment with 2-fold excess of sodium hydride in dimethylformamide at 0° C followed by reaction with an excess of the corresponding 2-nitrobenzyl bromide. The modest yield generally obtained[21] (~30%) in this step was not improved by changing the nature of the leaving group (-Cl, -I, -OTf). Isolation of the stilbene derivative, **5**, in both cases may suggest that a carbene formed by α-elimination is involved in competing side reactions.

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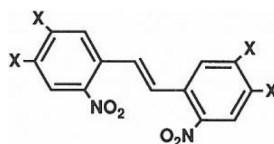
The final deprotection to give **4a–b** was carried out under mild conditions (4% trifluoroacetic acid in $CHCl_3$). The final product is isolated by flash chromatography on silica gei in yields of 50–60 % (Scheme).

B. Bioassay for activity

Adenosine derivatives are potent inhibitors of synaptic potentials in the hippocampal formation, an action mediated by A_1-receptors.[22] Synaptic responses (fEPSPs) were recorded in the CA1 region of *in vitro* hippocampal slices, and the actions of the following adenosine derivatives were tested: CPA, **4a** (irradiated and non-irradiated), and **4b** (irradiated and non-irradiated). The adenosine antagonist theophylline blocked the inhibitory effects of these derivatives (shown in Figure, for **4a**). All data shown here for irradiated

compounds are from a ten minute period of irradiation. Preliminary data with the the same, simple light source source indicate that a 1 min period of irradiation of compound 4a (50 nM) resulted in an approx. 50% inhibition of fEPSPs. Non-irradiated **4a** and **4b** elicited less than 10% inhibition of fEPSPs at concentrations up to 50 nM. At a concentration of 100 nM non-irradiated **4a** inhibited by approximately 10% (Figure).

Discussion

Most of the previously reported "caged" probes were for receptors that govern ion channels directly. The linkage between adenosine receptors and ion channels is indirect, but the outstanding problems in this field warrant the development of "caged" adenosine agonists. Adenosine is a powerful modulator of the biochemical and electrophysiological events in a variety of tissues. These effects are mediated by multiple categories of specific membrane receptors which exhibit restricted regional and cellular distributions. The availability of highly selective agonist and antagonist tigands for the A_1-adenosine receptor has facilitated the identification and partial characterization of this receptor subtype. The present study demonstrates that the strategy of utilizing photolabile "caged" receptor agonists for the study of A_1-receptors is a viable new approach to further characterize the physiological actions of adenosine.

N^6-Cyclopentyiadenosine (CPA) is among the most potent of the A_1-selective agonists. CPA inhibits synaptic transmission at nanomolar concentrations, thus it is approximately three orders of magnitude more potent than adenosine itself.[22] This neuromodulatory activity can be inhibited by placing a 2-nitrobenzyl group at the S′-position of CPA (compound **4a**). The 2-nitrobenzyl group is stable under physiological conditions, superfusion of brain slices with compound **4a** for up to one hour did not mimic the inhibitory effect of similar concentrations of CPA. When subjected to photoactivation, compound **4a** behaved in a manner similar to native CPA. Photoactivated **4a** inhibited synaptic potentials in a dose-dependent manner at nanomolar concentrations, and this effect was blocked by the adenosine receptor antagonist theophylline. Compound **4a** thus represents a caged adenosine agonist with potent activity at A_1-receptors.

Caged adenosine receptor agonists represent a promising tool for the characterization of the receptors and physiological actions of adenosine. Several applications for these compounds can be anticipated. The rapid kinetics of adenosine′s physiological effects should now be accessible for analysis. One of the main problems associated with the high-affinity A_1-receptor agonists is their characteristic limited diffusion into a tissue. For instance, stable responses to CPA require at least 20 min of continuous application in superfused slices of the brain. The slow onset of the inhibitory action of CPA likely reflects its limited penetration through the tissue. Utilizing caged compounds it should be possible to control precisely the biological availability (both timing and concentration) of adenosine agonists. Another potential use of these compounds is the controlled delivery of adenosine agonists to a restricted region of interest. The use of focal irradiation, e.g. with an excimer laser,[12] in conjunction with **4a** or **4b** will allow the presentation of an adenosine agonist to an activated region while transiently maintaining other regions relatively free of irradiated agonist. This

might be beneficial in a surgical context in which A_1-receptors could be rapidly activated in a restricted region of interest.
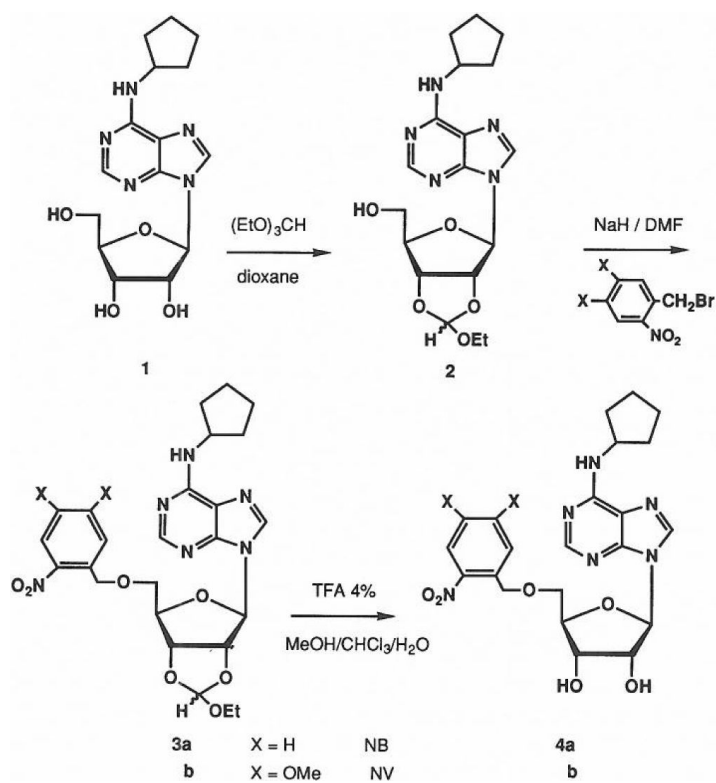
Acknowledgements

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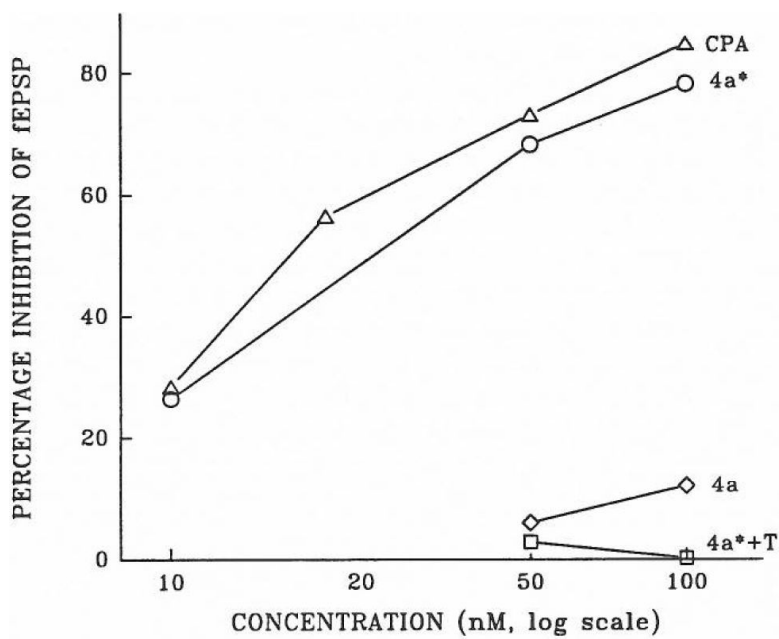
Figure.
The effect of adenosine derivatives on synaptic potentials in the rat hippocampus. The percentage inhibition of fEPSP responses is shown for CPA, irradiated **4a*** (irradiated **4a**) and non-irradiated **4a**. The effect of irradiated **4a*** is also shown when superfused together with 200 μM theophylline (**4a* + T**). Each point on the graph represents an average of two or three observations.

SCHEME 1.