Diadenosine Polyphosphate Analog Controls Postsynaptic Excitation in CA3-CA1 Synapses via a Nitric Oxide-Dependent Mechanism

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ABSTRACT

Previously, we have described the modulatory effect of diadenosine polyphosphates ApnA and ApnAs on synaptic transmission in the rat hippocampal slices mediated by presynaptic receptors (Klishin et al., 1994). In contrast, we now describe how nonhydrolyzable ApnA analog diadenosine-5′,5′-P1, P4-\([β,β′\text{-methylene}]\)tetraphosphate (AppCH2ppA) at low micromolar concentrations exerts strong nondesensitizing inhibition of orthodromically evoked field potentials (OFPs) without affecting the amplitude of excitatory postsynaptic currents and antidromically evoked field potentials, as recorded in hippocampal CA1 zone. The effects of AppCH2ppA on OFPs are eliminated by a P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) but not mimicked by purinoceptor agonist \(\alpha,\beta\text{-methylene-}ATP\) and adenosine 5′-O-(3-thio)triphosphate, indicating that a P2-like receptor is involved but not one belonging to the conventional P2X/P2Y receptor classes. Diadenosine polyphosphate receptor (P4) antagonist Ip4I (diinosine tetraphosphate) was unable to modulate AppCH2ppA effects. Thus, the PPADS-sensitive P2-like receptor for AppCH2ppA seems to control selectively dendritic excitation of the CA1 neurons. The specific nitric oxide (NO)-scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide is shown to significantly attenuate AppCH2ppA-mediated inhibitory effects, indicating that NO is involved in the cascade of events initiated by AppCH2ppA. Further downstream mediation by adenosine A1 receptors is also demonstrated. Hence, AppCH2ppA-mediated effects involve PPADS-sensitive P2-like receptor activation leading to the production of NO that stimulates intracellular synthesis of adenosine, causing in turn postsynaptic A1 receptor activation and subsequent postsynaptic CA1 dendritic inhibition. Such spatially selective postsynaptic dendritic inhibition may influence dendritic electrogensis in pyramidal neurons and consequently mediate control of neuronal network activity.

Diadenosine polyphosphates (ApnAs), where \(n\) equals 2 through 6, are ubiquitous in nature and found in both intracellular and extracellular locations. ApnAs have been identified as constituents of synaptic vesicles in the PNS and CNS, and previous evidence has suggested that ApnAs may exercise a variety of effects, such as increasing the spontaneous action potentials in locus coeruleus neurons of pontine slices, raising the level of excitation in nodose ganglion neurons, and depressing extracellular postsynaptic field potentials and excitary postsynaptic current (EPSCs) to a similar extent (Klishin et al., 1994; Pintor et al., 2000). Recent studies have indicated that high-affinity binding sites for ApnA (Pintor and Miras-Portugal, 1995), as differentiated from P1 or P2 receptors, are found in the olfactory bulb, cerebral cortex, and striatum, as well as several other brain areas (Rodriguez-Pascual et al., 1997). These receptors have been identified as purinergic P4 receptors (Rodriguez-Pascual et al., 1997). Taken together, these data indicated that diape...
nonsine polyphosphates may use unique signal transduction pathways and may activate physiological responses different from those caused by ATP or adenosine. However, in spite of being well known for many years (McLennan, 1992), pure functions of diadenosine polyphosphates have been difficult to define because of both specific enzymic and nonspecific hydrolysis in the presence of biological fluids and tissue samples. Here we report that a hydrolysis-resistant analog of Ap4A is capable of the selective inhibition of dendritic excitation in the hippocampus in nitric oxide (NO)-dependent manner via a P2-like receptor with atypical pharmacological properties.

Materials and Methods

Preparation of Ap4A Analogs. Diadenosine-5’,5”-P1,5P2-[β,γ-methylene]-tetraphosphate (AppCH2ppA) was synthesized using protocols based on the use of LysU. Ip,I was synthesized from Ap4A by means of 5'-adenylidene adaminase. LysU is a heat-stable lysyl-tRNA synthetase from Escherichia coli and is capable of efficiently synthesizing Ap4A from ATP (Wright et al., 2003). This process involves the formation of enzyme-bound lysyl adenylate followed by coupling to the second nucleotide. Because the enzyme is only ATP-specific for the first step, various second nucleotides or oligophosphates can be used to produce a variety of Ap4A analogs. LysU was overexpressed and purified for use according to procedures described previously (Theoclitou et al., 1996; Wright et al., 2003). After preparation, samples of AppCH2ppA and Ip,I were prepared for use by lyophilization and characterized by electrospray ionization spectrometry (Bruker Esquire 3000; Bruker Daltonics, Billerica, MA) and NMR (Bruker Ultra shield 400 MHz; Bruker Biospin, Billerica, MA): AppCH2ppA: electrospray ionization-mass spectroscopy (100%) [M-H] 832.9 m/z, δ4 (400 MHz, D2O) 8.5 (1H, s, 8-H-Ad), 8.1 (1H, s, 2-H-Ad), 5.9 (1H, m, 1’-H-rib), and 2.6 to 2.4 (1H, t, O-CH2-O) and δp (D2O, pH 7) 7.1 (2P, d, P2,Pβ'), 7.9 (2Jα, 6Jα, 56 Hz), ~11.2 ppm (2P, d, P2,Pα', 3Jα, 65 Hz), yield 63%; Ip,I: electrospray ionization-mass spectroscopy (100%) [M-H] 836.9 m/z, δ4 (400 MHz, D2O) 8.4 (1H, s, 2-H-In), 8.1 (1H, s, 8-H-In), 6.0 (1H, d, 1’-H-rib, J1,3,3’,3’-H 5.8 Hz), 4.5 (1H, t, 5’-H-rib), 4.3 (1H, m, 5’-H-rib), and 4.2 (2H, d, 2’/3’/4’-H-rib [unclear]) and δp (D2O, pH 7) ~11.3 (2P, m, P2,Pα') and ~23.1 (2P, m, P2,Pβ'), yield 84%. Compounds were estimated by ion-exchange HPLC (Agilent Technologies 11000; Agilent Technologies, Palo Alto, CA) to be ~99.5% pure.

Hippocampal Slice Preparation. This study was carried out on 21-day-old Wistar rats (WAG/GstO, Moscow, Russia). After decapitation, rat brains were immediately transferred to a Petri dish with chilled (4°C) solution of the following composition: 120 mM NaCl, 5 mM KCl, 26 mM NaHCO3, 1 mM MnCl2, and 20 mM glucose. Calcium salts were omitted to reduce possible neuronal damage. The solution was constantly bubbled with 95% O2/5% CO2 to maintain pH 7.4. During preincubation and experiments, the hippocampal slices (300–400 µM thick) were kept fully submerged in the extracellular solution, pH 7.4, comprising the solution 135 mM NaCl, 5 mM KCl, 26 mM NaHCO3, 1 mM NaHPO4, 1.5 mM CaCl2, 1.5 mM MgCl2, and 20 mM glucose (subjected to continuous bubbling with 95% O2/5% CO2) at 30–31°C. Picrotoxin (25–50 µM, Sigma/RBI, Natick, MA) was also included into the extracellular solution during preincubation.

Electrophysiological measurements were recorded after at least 2 h of preincubation.

Electrophysiological Measurements. Excitatory postsynaptic currents were recorded by a standard whole-cell patch clamp technique in the CA1 subfield of the hippocampal slices in response to stimulation of the Schaffer collateral/commissural pathway. To prevent the spread of electrical activity from area CA3, mini-slices were prepared by making a cut orthogonal to the stratum pyramidale extending to the mossy fiber layer. The intracellular solution, pH 7.2, for patch pipettes was composed of 100 mM CsF (Merck, Darmstadt, Germany), 40 mM NaH2PO4, 10 mM HEPES-CsOH, and 10 mM Tris-HCl. N-(2,6-Dimethyl-phenylcarbamoylmethyl)-triethylammonium bromide (QX-314; 2–3 mM; Tocris Cookson, Bristol, UK) was routinely added to the intracellular solution to block voltage-gated sodium conductances. Patch pipettes were pulled from soft borosilicate glass on a multi-stage horizontal puller. When fire-polished and filled with the intracellular solution, they had a resistance of 2 to 3 MΩ. To visualize cell bodies of CA1 pyramidal neurons, the stratum oriens and alveus were removed with a saline jet from a micropipette. Currents were digitally sampled at 400-µs intervals by a 12-digit analog-to-digital converter board and filtered at 3 kHz, and data were stored on a hard disk for further analysis. Access resistance was monitored throughout the experiments and ranged typically from 6 to 9 MΩ. Data from cells, where access resistance changed by more than 25% during the experiment, were discarded. Extracellular field potentials were recorded using nickel/chromium electrodes. The population spikes were digitized and stored on a computer disk. The effects of receptor agonists and antagonists were measured as the mean ratio I/I0, where I was the current under the substances action and I0 was the current in control saline. To stimulate the Schaffer collateral/commissural pathway input, a bipolar nickel/chromium electrode was positioned on the surface of the slice. Current pulses (10–100 µA) of 0.1 to 1-ms duration were delivered through the isolated stimulator HG 203 (Hi-Med, London, UK) at 0.066 to 0.2 Hz. The intensity of stimulus was adjusted to evoke field-potential amplitudes 50% of maximum.

Results

The ambiguities that surround Ap4A function derive in part from the difficulty of handling Ap4A in that they are unstable to specific enzymic and nonspecific hydrolysis in the presence of biological fluids and tissue samples (McLennan, 1992; Guranowski, 2000). For this reason, we elected to visit the role of Ap4A in neurotransmission using the nonhydrolyzable analog AppCH2ppA. This was prepared from ATP and β,γ-methylene-ATP by means of our previously described enzymic procedure that makes use of the E. coli lysyl tRNA synthetase isozyme LysU and inorganic pyrophosphatase to prepare Ap4As in high-quality yield and purity (Theoclitou et al., 1996; Wright et al., 2003). AppCH2ppA was isolated and characterized as pure and then was found to be completely stable for over 5 h in the presence of rat brain hippocampal slices under conditions appropriate for electrophysiology (Fig. 1). Having demonstrated adequate compound stability, AppCH2ppA was then applied to rat brain hippocampal slices for the analysis of orthodromically and antidromically evoked field potentials (OFPs and AFBs, respectively) or EPSCs. AppCH2ppA (2–10 µM) was found to produce reproducibly fast and reversible inhibition of OFPs in all of the tested synaptic pathways in the hippocampus, including CA3-CA1 synapses (Figs. 2 and 3).

OFPs induced in the CA1 field by stimulation of Schaffer collateral/commissural pathways were reduced in the presence of 8 µM AppCH2ppA to 12 ± 6% of control, p < 0.02, n = 7 (Fig. 3A), whereas amplitudes of AFBs (Fig. 3B) as well as EPSCs (Fig. 3C) remained unchanged (IAppCH2ppA/ICTRL × 100%) was 96 ± 6% of control, n = 6). These latter observations suggested that AppCH2ppA was not modulating either presynaptic glutamate release or the sensitivity of the glutamate receptors in postsynaptic density. Furthermore, because AppCH2ppA also did not modulate AFBs, axonal-axonal excitatory stability should remain unchanged in the presence
of AppCH₂ppA. Therefore, we concluded that inhibition of OFPs can arise from local modulation of apical dendritic excitability.

Having observed this highly spatially selective AppCH₂ppA effect, a series of P2 receptor antagonist and agonist experiments were then conducted to try and pinpoint and confirm the origins of the effect. Using PPADS (20 μM), a well-known broadband P2-receptor family antagonist (Bianchi et al., 1999; Pintor et al., 2000) (also see supplemental data), we found that the blocking effect of AppCH₂ppA on OFPs was significantly decreased (inhibition of OFPs by 4 μM AppCH₂ppA was 28 ± 8% of control; in the presence of PPADS, inhibition was reduced to 73 ± 13% of control, p < 0.02, n = 4) (Fig. 4A). Experiments were then performed using P2 receptor family agonists α,β-methylene-ATP or ATPγS (Fig. 5, A and B). Both are known to be P2X and/or P2Y family agonists (Wilkinson et al., 1994; Webb et al., 1996; Idzko et al., 2001) (also see supplemental data). However, neither was able to inhibit OFPs in the same manner as AppCH₂ppA, except for weak, slowly developing inhibition at very high nucleotide concentrations (100 μM), effects that are at best nonspecific given the concentrations of agonists used. In contrast to AppCH₂ppA data, the application of ATP (10 μM) caused extensive reversible inhibition of EPSCs (67 ± 2%, n = 3, p < 0.02, data not shown). Therefore, we concluded that AppCH₂ppA effects were indeed unlikely to be mediated by the main P2X or P2Y family receptors. This is perhaps surprising in view of the known capacity of Ap₄As to act as agonists of P2X₁–₄, P2Y₁, P2Y₂, and P2Y₄ receptors in neurological tissue (Pintor et al., 2000). As an alternative possibility, we evaluated the likelihood that AppCH₂ppA effects could be mediated by the Ap₄A-specific P4-dinucleotide receptor previously identified on rat brain synaptic terminals (Pintor and Miras-Portugal, 1995). In this event, AppCH₂ppA effects proved refractory to coadministration even at high concentrations of the known P4 antagonist Ip₄I.
(20 μM; IC_{50} = 8.3 μM) (Pintor et al., 2000), hence ruling out the possibility of P4-dinucleotide receptor involvement (Fig. 4B). This result then led us to the interesting possibility that an atypical P2-like receptor could be mediating our observed AppCH₂ppA effects.

The dendrites of CA1 pyramidal neurons are well known as an important target for cortical modulation mediated via numerous receptors, including A1 adenosine and GABA receptors. Therefore, experiments were conducted with antagonists of GABA_A, GABA_B, and glycine receptors. In these cases, AppCH₂ppA-induced inhibition of OFPs was unaffected by the presence of bicuculline (50 μM), SCH50911 (10 μM), or strychnine (500 nM), known antagonists of GABA_A, GABA_B, and glycine receptors, respectively. By contrast, the administration of cyclopentyl theophylline (CPT) (1 μM), an A1 adenosine receptor antagonist, was seen to eliminate the effect of AppCH₂ppA (inhibition of OFPs by 4 μM AppCH₂ppA was 28 ± 8% of control; in the presence of CPT, inhibition was reduced to 84 ± 8% of control, p < 0.01, n = 4) (Fig. 6A), thereby suggesting that AppCH₂ppA effects were mediated instead by A1 adenosine receptor activation downstream of PPADS-sensitive P2 receptor activation. Consistent with this observation, the administration of adenosine (5 μM) to hippocampal slices has been shown previously to produce effects that mimic our observed AppCH₂ppA effects with respect to inhibition of OFPs (Greene and Haas, 1991), although adenosine administration also diminished the amplitudes of EPSCs (Klishin et al., 1995), in direct contrast with our observed effects after AppCH₂ppA administration (Fig. 3C). Therefore, our observed AppCH₂ppA-mediated effects seem to be more spatially selective than those of adenosine alone.

Previously, a novel adenosine-binding protein that binds both nucleosides and nucleotides and is sensitive to CPT has been found in rat brain membranes. Based on the ligand binding specificities, this protein has not been classified as a member of the P1 or P2 purinoceptor families but is likely to be classified as a putative P3 purinoceptor (Saitoh and Nakata, 1996; Matsuoka et al., 1998; Yoshioka et al., 2001a). Activation of such P3 receptors has been observed to bring about presynaptic inhibi-
tion of glutamate release in hippocampal neurons (Mendoza-Fernandez et al., 2000). Therefore, it is conceivable that AppCH2ppA effects could be mediated through this P3 receptor. However, AppCH2ppA-induced effects were eliminated by adenosine deaminase (ADA) (2 U/ml), a fact that is not only inconsistent with a P3 purinoceptor-mediated mechanism but indicates that AppCH2ppA-mediated effects should be mediated by adenosine produced endogenously (Fig. 6B). Other recent research has demonstrated that P2 receptor activation triggers adenosine release, leading to adenosine receptor activation (Juranyi et al., 1999; Almeida et al., 2003). Furthermore, NO has been demonstrated to mediate adenosine outflow in response to P2 receptor activation (Juranyi et al., 1999). Therefore, we tested the possibility of NO production in our system. Accordingly, we were gratified to observe that the NO-specific scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO) (1 mM) was able to reduce the extent of inhibition of OFPs by 4 μM AppCH2ppA from 28 ± 8 to 51 ± 7% of control (p < 0.05, n = 4) (Fig. 7A), consistent with such a direct role for NO in our case as well. These data were also supported by the fact that the nitric oxide trappe hemoglobin (1 mM) also significantly attenuated the inhibitory effect of AppCH2ppA (inhibition of OFPs by 4 μM AppCH2ppA was 28 ± 8% of control; in the presence of hemoglobin, inhibition was reduced to 73 ± 6% of control, p < 0.05, n = 3) (Fig. 7B). Hence, we came to the conclusion that AppCH2ppA-mediated effects involve PPADS-sensitive P2-like receptor activation resulting from the binding of AppCH2ppA, leading to the production of NO that in turn stimulates the intracellular synthesis of adenosine leading to exclusive postsynaptic A1 receptor activation.

This putative pathway was tested with the following set of control experiments (Fig. 8). To demonstrate that our PPADS-sensitive P2-like receptor was upstream of NO-mediated adenosine generation, which leads to A1 receptor activation and a reduction in OFPs, we performed the following three sets of experiments. In the first experiment (Fig. 8A), the inhibitory effects of adenosine (A1 receptor agonist) were found to be almost independent of PPADS administration. In the second experiment (Fig. 8B), the inhibitory effects of nitroglycerin (NO-donor) were also found to be independent of PPADS administration. Therefore, both NO and A1 receptors do indeed appear to be downstream of our PPADS-sensitive P2-like receptor. In the third set (Fig. 8C), the inhibitory effects of adenosine were found to be independent of PTIO administration as well, a result that is entirely consistent with a cascade in which NO generation precedes adenosine synthesis and A1 receptor stimulation. These con-

Fig. 5. Agonists of purinoceptors do not mimic the effects of AppCH2ppA on synaptic transmission. A, left, time course of the change of amplitude of OFPs in CA1 under the influence of a,β-methylene-ATP. Right, original traces of field potentials (5-fold averaged) corresponding with points 1 (control) and 2 (in the presence of a,β-methylene-ATP) in the time course. B, left, time course of the change of amplitude of OFPs in CA1 in the presence of ATPγS. Right, original traces of field potentials (5-fold averaged) corresponding with points 1 (control) and 2 (in the presence of ATPγS) in the time course.
There is a decrease in overall transmitter release (Prince and Stevens, 1992). Adenosine in particular has been found to attenuate the liberation of several neurotransmitters by this mechanism, including glutamate, acetylcholine, dopamine, noradrenaline, and serotonin (Fredholm and Dunwiddie, 1988). A highly localized distribution of A1 receptors in the active zone and postsynaptic density of CNS synapses have been recently demonstrated in the rat hippocampus (Rebola et al., 2003). Subcellular fractionation of hippocampal nerve terminals revealed that postsynaptic A1 receptor immunoreactivity was strategically located in the postsynaptic density together with N-methyl-d-aspartate receptor and N- and P/Q-type calcium channel immunoreactivity, emphasizing the importance of A1 receptors in the control of dendritic integration (Rebola et al., 2003). Activation of the postsynaptic A1 receptors chiefly enhances rectifying K⁺ influx via G protein-coupled inwardly rectifying potassium channels (Sig- gins and Schubert, 1981; Segal, 1982; Gerber et al., 1989; Alzheimer and ten Bruggencate, 1991) and voltage-dependent, GABA-independent Cl⁻ conductances (Mager et al., 1990), leading to membrane hyperpolarization and subsequent inhibition. Furthermore, it has been reported that A1 receptors not only inhibit presynaptic N-type calcium channels but also control postsynaptic N-type calcium channels (Mogul et al., 1993; McCool and Farroni, 2001). In our case, our reported data interlock to support the view that AppCH₂ppA administration leads to the activation of A1 receptors localized in postsynaptic apical dendrites of hippocampal pyramidal neurons are, in line with the observations described above.

Recently, biochemical, pharmacological, and functional evidence for the existence of a heteromeric complex between P1 and P2 receptors have been provided. In particular, the oligomeric association of A1 receptors with P2Y1 receptors (A1/P2Y1 receptors) has been reported to generate A1 with P2Y1 receptor-like agonistic pharmacology (Yoshioka et al., 2001b). A high degree of such colocalization of A1 and P2Y1 receptors has been demonstrated by double immunofluorescence experiments in rat cortex, hippocampus, and cerebel-
lum (Yoshioka et al., 2002). The existence of diverse hetero-
meric assemblies of purine receptors subtypes suggests a
greater diversity of purine receptor pharmacology and puri-
nergic functions than might be expected from cloning studies
(Barajas-Lopez et al., 1995; Ikeuchi et al., 1996; Saitoh and
Nakata, 1996; Song and Chueh, 1996; Mendoza-Fernandez et
al., 2000). For example, it has been demonstrated that ATP
can inhibit the synaptic release of glutamate by direct acti-
vation of P2Y receptors that are sensitive to P1 receptor
antagonists (Mendoza-Fernandez et al., 2000). For example, it has been demonstrated that ATP can inhibit the synaptic release of glutamate by direct activation of P2Y receptors that are sensitive to P1 receptor antagonists (Mendoza-Fernandez et al., 2000). It is quite conceivable that our observed AppCH2ppA effects could be mediated by direct activation of some such heteromeric pu-
rine receptor(s). However, according to our data, AppCH2ppA
influences hippocampal excitability (if only partially) via en-
dogenous production of adenosine (Fig. 6), because enzymatic
degradation of adenosine by ADA decreases the effects of
AppCH2ppA.

Considering that AppCH2ppA is highly unlikely to undergo enzynatic conversion to adenosine (Fig. 1), some other en-
dogenous mechanism(s) should be responsible for adenosine
production in the hippocampus. Literature precedent indi-
cates that NO can mediate adenosine outflow in response to
P2 receptor activation (Juranyi et al., 1999; Almeida et al.,
2003). Therefore, we considered the possibility that
AppCH2ppA-induced NO-mediated adenosine release could
mediate inhibition of excitability of CA1 neurons. Entirely
consistent with this suggestion, NO scavengers, such as
PTIO and hemoglobin, were found to significantly decrease
the observed effects of AppCH2ppA (Fig. 7). Therefore, in
the light of these data, we appreciate that AppCH2ppA-mediated
activation of P2-like receptor should lead to direct stimula-
tion of an NO synthase (NOS). Hence, what are the likely
origins of this NO production?

NO is known to be synthesized from L-arginine and oxygen
by NOS in the presence of NADPH and plays a role in various
signal transduction processes in the CNS. Data from previ-
ous studies suggest that extracellular ATP induces Ca\textsuperscript{2+}
-activated formation of NOS (Reiser, 1995). After stimulation
of P2Y1 receptors by physiological agonists, an enhanced
formation of NO can be observed. The relationship between
P2 receptors expressed in astrocytes and NO production is
close (Liu et al., 2000), such that pretreatment of astrocytes
with P2 receptor antagonists, including PPADS, results in a
down-regulation of intercellular-1-stimulated NOS expres-
sion. Interestingly, the cardiac electrophysiological and cor-
nary vasomotor effects of Ap\textsubscript{4}As are also known to be me-
diated by NOS-dependent mechanisms (Stavrou et al., 2001). There are a number of possible cellular sources of constitutive NOS activity in the hippocampus, including nNOS from neurons and interneurons, eNOS from endothelial cells in blood vessels (Dinerman et al., 1994; Kantor et al., 1996; Topel et al., 1998; Downen et al., 1999; Blackshaw et al., 2003; Liu et al., 2003), and inducible NOS activity within astrocytes (Murphy et al., 1993). Hence, although the nNOS is the predominant isoenzyme of NOS in the neuronal tissue, a contribution from eNOS or inducible NOS to AppCH$_2$ppA-induced NO release cannot be excluded. For example, it has been reported recently that 7-NI, a specific inhibitor of nNOS, does not effect a significant change in adenine outflows in response to ischemic conditions. Furthermore, ischemia-induced activation of nNOS seems to be delayed in comparison with the activation of eNOS. Thus, at least under ischemic conditions, eNOS probably contributes toward the release of purines (Juranyi et al., 1999).

A number of studies have uncovered factors that influence the spatial and temporal properties of the NO signal (Gally et al., 1990; Lancaster, 1997; O'Shea et al., 1998; Philippides et al., 2005). nNOS has been found to localize specifically to synaptic spines in the dendrites of CA1 pyramidal neurons (Buret et al., 2002). Interestingly, eNOS is also known to be present specifically in the dendrites of CA1 pyramidal neurons (O'Dell et al., 1994; Kantor et al., 1996). Several protein-protein interactions that regulate the localization of nNOS within postsynaptic density have been described previously (Brennan et al., 1996; Jaffrey and Snyder, 1996; Jaffrey et al., 1998; Christopherson et al., 1999; Fang et al., 2000; Alderton et al., 2001; Dreyer et al., 2004). For instance, the postynaptic density protein PSD-95 binds nNOS via its N-terminal PDZ domain and holds nNOS in a functional complex with the N-methyl-D-aspartate subtype of glutamate receptors (Christopherson et al., 1999). These interactions are increased in the postsynaptic density of CA1 pyramidal neurons (Alderton et al., 2001). With respect to eNOS, several other recent studies have addressed the complex machinery that regulates eNOS-mediated NO production with respect to time and space, substrate and cofactor availability, protein-protein interactions, phosphorylation, acylation, and cellular localization (Govers and Oess, 2004). The tendency of nNOS to highly localize around synaptic spines in the dendrites of CA1 pyramidal neurons suggests that nNOS activation in the highly localized cellular milieu of synaptic spines on terminal dendrites should have a critical effect in this immediate vicinity.

We seem to have an integrated picture of the pathway from AppCH$_2$ppA activation to modulation of excitability in postsynaptic CA1 dendrites (Fig. 9). However, the scenario presented here does not specifically exclude the involvement of some alternative mechanisms. For instance, AppCH$_2$ppA may be acting through control of the ectoprotein kinase (Ehrlich et al., 1986; Chen et al., 1996). Another mechanism could be the modulation of hippocampal dinucleotide receptors by activation of adenosine receptors, as recently demonstrated in the rat hippocampal synaptosomes (Diaz-Hernandez et al., 2002). Such modulation, demonstrated for diadenosine polyphosphate and adenosine receptors, could reinforce effects of AppCH$_2$ppA operating through our suggested pathway. Furthermore, we would like to note for the sake of completeness that there is the possibility that pharmacological characteristics of AppCH$_2$ppA could be quite distinct from those of other endogenous dinucleotide polyphosphates.

In any event, taking these caveats aside, the effect of AppCH$_2$ppA and its probable mechanism described in this article may have important medical implications. Indeed, previous studies have indicated that Ap$_4$A reduces ischemic injury in the CNS and heart (Khattab et al., 1998; Ahmet et al., 2000a,b; Wang et al., 2003). Recently, Ap$_4$A has been shown to have strong neuroprotective properties mediated by P1 and P4 receptors in primary neuronal culture used as models for stroke and Parkinson’s disease (Wang et al., 2003). Here we have demonstrated a new mechanism of Ap$_4$A analog activity mediated by A1 adenosine receptors. The wide range of hyperpolarizing effects induced by stimulation of A1 adenosine receptors indicates the possibility (Bischofberger et al., 1997) that intraischemic activation of these receptors may constitute an important mechanism to delay the onset of hypoxic depolarization stage when ischemic events are initiated (Hansen and Nedergaard, 1988). Furthermore, this new mechanism may be linked to the phenomenon of neuroprotection induced by ischemic preconditioning in which nonlethal oxygen-glucose deprivation induces tolerance to a later potentially lethal level of oxygen-glucose deprivation. Recent data suggest that generation of NO and activation of A1 adenosine receptors may be important for the induction of such tolerance (Heurteaux et al., 1995; Nandagopal et al., 2001). Ap$_4$A has been recently shown to mimic cardioprotective effects of ischemic preconditioning in the rat heart (Ahmet et al., 2000a,b). Therefore, in our view, AppCH$_2$ppA administration could potentially represent a highly selective means to achieve ischemic preconditioning/neurological protection. This possibility will now be the subject of intensive future studies going forward.

We are of the overall opinion that our data with AppCH$_2$ppA yield a number of very significant conclusions concerning Ap$_4$A biology and applications. Firstly, the identification of highly specific AppCH$_2$ppA effects on dendritic excitation mediated by A1 receptors suggests that Ap$_n$As or
their analogs could be potent neuroprotective compounds in their own right. With regard to ApAβ biology, we seem to have identified for the first time a pure, highly selective ApAβ effect using a nonhydrolyzable ApA analog that was prepared in high-quality yield and in purity. The importance of working with such a high-quality chemical entity cannot be overestimated in view of the instability of ApAs to specific enzyme and nonspecific hydrolysis in the presence of biological fluids and tissue samples, leading to so many contradictory reports on the roles and functions of ApAs in living systems (McLennan, 1992; Guranowski, 2000; McLennan, 2000). Finally, these AppChP analogs seem to be mediated by a novel PPADS-sensitive P2-like receptor not previously characterized in connection with ApAβ effects. The characterization of this receptor will also be the subject of more intensive future investigations going forward as a potentially important new target for neuroprotective compounds.

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References
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### Agonists (\(\alpha,\beta\text{-meATP, ATP}_{\gamma}S\)) and Antagonist (PPADS) Selectivities for Cloned and Native P2 Receptors

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<tr>
<th>Receptor Subtype</th>
<th>(\alpha,\beta\text{-meATP} ) Agonist activity and functional potencies</th>
<th>Region of cells isolation /recording</th>
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<td><strong>P2X₁</strong></td>
<td>1-3 (\mu\text{M} )</td>
<td>Recombinant rat</td>
<td>(Dunn et al., 2001; North &amp; Surprenant, 2000; Khakh et al., 2001)</td>
<td>(pEC_{50} = 5.64 \pm 0.05 )</td>
<td>Recombinant human 1321hX₁ - 39</td>
<td>(Bianchi et al., 1999)</td>
<td>(IC_{50} = 1 )</td>
<td>Cloned P2 receptors</td>
<td>For review see (Ralevic &amp; Burnstock, 2003; Khakh et al., 2001)</td>
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<td>(EC_{50} = 0.2 \mu\text{M} )</td>
<td>Human cloned receptor</td>
<td>(Jacobson et al., 2000)</td>
<td>(EC_{50} = 2.3 )</td>
<td>Human cloned receptor</td>
<td>(Jacobson et al., 2000)</td>
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<td>(IC_{50} = 2 )</td>
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