Adenosine Induces Inositol 1,4,5-Trisphosphate Receptor-Mediated Mobilization of Intracellular Calcium Stores in Basal Forebrain Cholinergic Neurons

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In the cholinergic basal forebrain, we found previously that the extracellular adenosine concentration increase that accompanies sleep deprivation, acting via the A1 receptor, led to activation of the transcription factor nuclear factor-kB and to the upregulation of A1 adenosine receptor mRNA. We thus began to examine intracellular signaling mechanisms. We report here that adenosine, acting in a dose-dependent manner and predominantly via A1 receptors, stimulated IP3 receptor-regulated calcium release from intracellular stores. To the best of our knowledge, this calcium signaling pathway effect is a novel action of the Gq-coupled A1 adenosine receptor in neurons. Moreover, this calcium mobilization was not seen at all in noncholinergic neurons but was present in a large proportion of cholinergic neurons. These data suggest a potential role for a calcium-signaling pathway in adenosine-induced long-term effects of sleep deprivation and a key role for cholinergic neurons in this process.

Key words: adenosine; cholinergic basal forebrain; A1 adenosine receptor; intracellular calcium mobilization; inositol 1,4,5-trisphosphate receptor activation; sleep deprivation

There is now considerable evidence that adenosine, acting in the basal forebrain (BF), is a homeostatic sleep factor regulating the increased propensity to sleep after an increased duration of wakefulness (Porkka-Heiskanen et al., 1997; Basheer et al., 1999, 2000). There is also a considerable body of evidence about the immediate effects of adenosine on postsynaptic membrane ionic mechanisms. Adenosine, via the A1 receptor, activates an inwardly rectifying potassium current and blocks the hyperpolarizing current, thereby causing hyperpolarization of neurons (Rainnie et al., 1994; for review, see Haas and Selbach, 2000).

In contrast, very little is known in the brain, and nothing in the BF, about the long-term effects of adenosine that may be mediated by prolonged receptor activation and second messenger-mediated intracellular mechanisms. This may be important not only from the standpoint of the intrinsic value of increased cellular and biological knowledge but also for the possible behavioral consequences of such actions. For example, sleep restriction over several days produces progressive, additive effects such as decreased neurobehavioral alertness, decreased verbal learning, and changes in metabolic, endocrine, and immune functions, often referred to as “sleep debt” (Dinges et al., 1995, 1997; Spiegel et al., 1999; Drummond et al., 2000). Such effects might stem from adenosine receptor-mediated activation of second-messenger pathways that ultimately lead to altered transcription of genes coding for proteins that are important in the long-term effects of sleep deprivation.

The BF cholinergic zone has cells with several neurotransmitter phenotypes, including cholinergic, GABAergic, glutamatergic, and peptidergic (Gritti et al., 1993; Zaborszky et al., 1999; Semba, 2000). It has not been clear whether one or more of these cell types is associated with a distinctive second-messenger profile and hence might be associated with a distinctive functional role. The A1 receptor-mediated immediate ionic effects do not appear to discriminate between cell types, because they occur in cholinergic and noncholinergic neurons, at least in the laterodorsal tegmental nucleus (Rainnie et al., 1994).

Our previous identification of increased nuclear factor (NF)-kB DNA binding as a consequence of sleep deprivation and an effect mediated by the A1 receptor (Basheer et al., 2001a) suggested that we should first look at the possible signaling pathways coupling the A1 receptor to activation of this transcription factor. Several G12-coupled receptors have been shown to be capable of “dual signaling” [i.e., inhibition of adenylate cyclase and stimulation of phospholipase C (PLC)] (Gudermann et al., 1996, 1997). In smooth muscle cells and astrocytes, the A1 adenosine receptor has been shown to be capable of dual signaling (Gerwins and Fredholm, 1992; Biber et al., 1997). Activation of the PLC pathway is capable of activating protein kinase C (PKC) by IP3-mediated mobilization of internal calcium (Nishizuka, 1992; Berridge, 1998). Finally, PKC-mediated phosphorylation of the inhibitor I-kB and subsequent release and nuclear translocation of NF-kB are very well characterized in T lymphocytes (McKinsey et al., 1997). However, A1 adenosine receptor-
mediated activation of the PLC pathway and mobilization of intracellular calcium have not been reported in neurons, although there are reports of transient changes in intracellular calcium that can act as a part of a signal cascade coupling receptor activation to the nuclear events regulating transcription (Hardingham and Bading, 1999).

We thus chose to examine the intracellular signal transduction cascade activated by adenosine. More specifically, we investigated the following possible cascade components: whether adenosine was capable of inducing an increase in cytosolic calcium; the intracellular or extracellular source of this calcium; the involvement of the endoplasmic reticulum (ER) IP₃ receptor (IP₃R) versus the ryanodine receptor (RyR); and the adenosine receptor type(s) mediating these effects.

We report here that in the BF, adenosine acts to mobilize cytosolic calcium in a dose-dependent manner, that this mobilization is mediated by the ER IP₃R, and that these actions occur primarily through the A₁ adenosine receptor. Moreover, these events occur only in cholinergic neurons.

MATERIALS AND METHODS

**Experimental animals.** Male Long–Evans rats (350–300 g) were housed in a 12 hr light/dark cycle (lights on 7:00 A.M. to 7:00 P.M.) at a constant temperature (23°C) with access to food and water ad libitum.

**Acute brain slice preparation and loading of calcium orange dye.** The animals were decapitated after isoflurane-induced anesthesia, and their brains were rapidly removed. Coronal sections (200 μm thick) were cut with a vibratome (TPI series 3000; St. Louis, MO) at 4°C in artificial CSF (aCSF; in mm: 124 NaCl, 2 KCl, 3 KH₂PO₄, 1.3 MgCl₂, 2.5 CaCl₂, 26 NaHCO₃, and 10 glucose, pH 7.35; 315–320 mOsm when gassed with 95% O₂ and 5% CO₂). Three slices per animal [bregma coordinates from 0 to 0.6 (Paxinos and Watson, 1998), including the horizontal and diagonal band (HDB)–substantia innominata (SI)–magnocellular preoptic area (MCPO) region of the BF] were collected. The slices were hemisected and mounted on Parafilm. Calcium orange dye in aCSF (1 ml; 10 μM final concentration) was layered over the brain slice and incubated for 1 hr at 21°C; slices were washed and used for drug treatment and imaging in carboxygenated buffer. Uniform loading of the dye was evident because of the visible levels of basal fluorescence of calcium orange in resting (unstimulated) neurons. Tetrodotoxin (1 μM) in the buffer was used for all of the experiments to ensure the postsynaptic nature of the effects. The real-time change in intracellular calcium fluorescence was measured every 1.37 sec to determine the time needed for maximum effect.

To test the effect of fixing the slices in neutralized formalin on the intensity of calcium orange, we performed a time-course experiment in which calcium orange-loaded slices (four slices per time point from four rats) were treated with 100 μM adenosine for 0, 10, 20, 30, 40, 60, and 100 sec and fixed in formalin at the end of each time point before the fluorescence intensity was measured. The process of fixing did not have any effect on the increase in fluorescence intensity in response to adenosine. The time course of fluorescence intensity increase in fixed slices followed the same pattern that was observed in real-time measurements (Fig. 1 A,B).

**Drugs.** The sources of the drugs used were as follows: adenosine, the RyR blocker 1,1’-diethyl-4’-4’-bipyrindinum (DHBB), the IP₃R blocker xestospongin C, and 2-aminoethoxydiphenylborane (2APB) were obtained from Calbiochem (La Jolla, CA). The A₁ agonist N⁶-cyclo-hexyl-adenosine (CHA), A₂ agonist 6-cyclopentyl-1,3-dimethylxanthine (CPT), A₃ agonist N⁶-[2-(3,5-dimethoxyphenyl)-2-(methylphenyl)ethyl]adenosine (DPMA), A₅ agonist N⁶-[4-(aminobenzyl)-9-[5-(methylcarbonyl)-β-d-ribofuranosyl]adenine (AB-MEA), and A₇ agonist 3-ethyl-5-benzyl-2-methyl-4-phenylethyl-dihydropriridine-dicarboxylate (MRS1191) were obtained from Sigma-RBI (St. Louis, MO).

**Immunofluorescence labeling** Coronal sections that were used for calcium imaging were fixed in buffered formalin, pH 7.2, permeabilized with 0.5% TritonX-100 PBS under constant shaking for 2 hr, and subsequently treated with specific ChAT antibody (AB 144; goat antibody; Chemicon, Temecula, CA) used at a dilution of 1:4000. FITC-conjugated secondary antibody (anti-goat antibody; 1:100 dilution; Chemicon) was used to visualize ChAT-positive neurons. Samples were imaged by multiphoton microscopy.

**Multiphoton microscopy for intracellular calcium imaging.** A Bio-Rad (Hercules, CA) MRC 1024ES Multiphoton Imaging system coupled with a mode-locked Spectra-Physics (Fremont, CA) tunable Tsunami-sapphire laser system (pulse duration, <80 fs; repetition rate, 82 MHz) and a Zeiss (Oberkochen, Germany) Axiosvert S100 inverted microscope equipped with a high-quality water immersion objective (40×; 1.2 numerical aperture) was used for quantitative fluorescence imaging of

![Figure 1](image)
samples in epifluorescence mode. Multiphoton excitation was based on the principle that a fluorophore can absorb two or more photons essentially simultaneously and thereby undergo a transition to an excited state (Denk et al., 1995). Labeled neurons in the brain sections were identified by XYZ scanning, generally at depths of 30–170 μm. The 512 × 512 pixel images were collected in a direct detection configuration at a pixel resolution of 0.484 μm with a Kalman 3 collection filter. Multiple labeled images were acquired in separate channels using narrow bandpass filters to restrict the emission wavelength and thus avoid bleed-through of aberrant fluorescence. Images were reconstructed and processed using Bio-Rad LaserSharp and Metamorph (Universal Imaging, West Chester, PA) software. The data are presented as the average of at least three blinded experiments performed on different days.

RESULTS
Adenosine mediates cytosolic calcium increase in a time- and concentration-dependent manner
To investigate the effect of adenosine on the intracellular calcium response, brain slices were loaded with calcium orange, a calcium-sensitive fluorescent dye used previously in slices (Duffy and MacVicar, 1996). The temporal kinetics of the adenosine-mediated calcium response was examined in 18 individual neurons (n = 3 rats; three slices per rat) in real time (Fig. 1A). The temporal profile of adenosine-mediated calcium mobilization after 0, 10, 20, 30, 40, 60, and 100 sec of adenosine treatment (11–15 neurons per time point) was examined by measuring the fluorescence intensity after fixing the slices (four slices per time point from four rats) at the end of each time point (see Materials and Methods) (Fig. 1B). Although fixing with neutralized formalin increased the overall intensity of the fluorescence in slices, the net change (cytosolic minus background fluorescence) was found to be comparable with that observed in living neurons in real time. Similar results were obtained in calcium orange-loaded, cultured human embryonic kidney (HEK) 293 cells, in which cytosolic calcium fluorescence intensity remained stable without any leak into the medium after fixation with formalin (data not shown). In both cases (i.e., with or without fixing), the maximal (fourfold to sixfold) increase in calcium orange fluorescence was attained by 45–60 sec after adenosine treatment (Fig. 1A,B). Consequently, this time period was chosen for adenosine treatment in the rest of our experiments. As shown in Figure 1C, the intracellular calcium increased in a dose-dependent manner with increasing concentrations of adenosine, reaching a maximal effect at 100 μM. Consequently, all of the experiments described below were performed using 100 μM adenosine for 60 sec.

Adenosine-mediated cytosolic calcium increase was selective to cholinergic neurons
Interestingly, the calcium mobilization induced by adenosine in brain slices was limited to a select subpopulation of neurons that were 25–35 μm in size and located in the cholinergic portion of the BF region, which includes the HDB, the SI, and the MCPO. The observation that only a limited population of cells showed calcium mobilization with adenosine treatment was intriguing. To rule out potential artifacts caused by differential loading of the dye calcium orange in a specific cell type, we examined mobilization of intracellular calcium with thapsigargin (50 μM for 60 sec), an intracellular calcium pump inhibitor that is known to release calcium from internal stores in all cell types. Subsequent immunolabeling for ChAT showed that the thapsigargin-mediated increase in calcium orange fluorescence was seen in both cholinergic and noncholinergic cells (Fig. 2, right). In contrast, only those neurons with an increase in calcium orange fluorescence in response to adenosine were ChAT-positive (Fig. 2, left). In this analysis, a total of 129 ChAT-positive neurons (average size, 31.7 ± 2.9 μm) in the HDB–SI–MCPO area of the BF were examined (seven slices). Of these, 83 neurons (64.3%) showed an adenosine-mediated increase in fluorescence, whereas the remaining 46 (35.7%) did not show an increase in fluorescence in response to adenosine. The latter appeared similar in fluorescence to ChAT-positive neurons from control slices that were loaded with the fluorescent dye but not treated with adenosine.

An A1-selective agonist largely mimicked the pattern of adenosine response
The increase in intracellular calcium in response to adenosine (100 μM; n = 51) was measured in individual neurons in slices, as were the responses to the A1 receptor-selective agonist CHA (100 nM; n = 22), the A2-selective agonist DPMA (100 nM; n = 11), and the A3-selective agonist AB-MECA (10 nM; n = 33) (Fig.
However, combined use of CPT and the A<sub>3</sub>-selective antagonist MRS1191 rendered the adenosine response not significantly different from controls. The asterisks describe a significant difference when compared with controls.

At these concentrations, these agonists have been shown to have relatively high selectivity for their respective receptor subtypes (Klotz, 2000). A Kruskal--Wallis one-way ANOVA showed significant differences among the treatment groups (H = 115.054; df = 4; p < 0.001). Post hoc analysis (Dunn’s method) showed a significant increase (p < 0.05) in cytosolic calcium with adenosine, CHA, or AB-MECA treatment compared with controls (nonstimulated basal-level fluorescence; n = 45). The observed fivefold increase in fluorescence with adenosine treatment was closely matched by application of the A<sub>1</sub>-selective agonist CHA. The A<sub>1</sub>-selective agonist induced only a twofold increase in fluorescence compared with controls. The fluorescence in the cells treated with the A<sub>2</sub>-selective agonist DPMA was not significantly different from controls.

Figure 3B shows that although the effect of adenosine was not blocked completely, it was primarily blocked by pretreatment of the slices with the A<sub>1</sub>-selective antagonist CPT (1 μM; n = 58). The effect was blocked completely when both CPT (1 μM) and the A<sub>2</sub>-selective antagonist MRS1191 (100 nM; n = 58) were used (differences between the treatment groups were statistically significant as determined by Kruskal--Wallis nonparametric ANOVA; H = 97.252; df = 3; p < 0.001).

**Adenosine-mediated cytosolic calcium increase is caused by release from an intracellular thapsigargin-sensitive calcium store**

Increases in cytoplasmic calcium can be a result of influx of external calcium or release from intracellular stores. To determine whether the increase in cytosolic calcium was a result of influx from the external medium, the calcium orange-loaded slices were treated with adenosine in calcium-free buffer. The increases in cytosolic calcium in response to adenosine, CHA, and AB-MECA in cells stimulated in calcium-free medium were not significantly different from that seen in the presence of calcium in the external medium (illustrated in Fig. 4A; a Kruskal--Wallis one-way ANOVA confirmed the visual impression by a statistically significant difference between the calcium and the no-
calcium treatment groups: H = 173.777; df = 9; p < 0.001). This suggested that the source of calcium was from internal stores.

In a separate experiment, depletion of the internal stores by pretreatment of slices with thapsigargin failed to show a calcium response measured after 60 sec of adenosine treatment (H = 45.987; df = 2; p < 0.001) (Fig. 4B). This also confirmed that the adenosine-mediated increase in cytosolic calcium was a result of mobilization from an internal source.

**Adenosine-mediated calcium release from IP$_3$R but not RyR regulated intracellular stores**

In neurons, a major source of internal calcium is the stores present in the elaborately distributed network of the ER. Both IP$_3$Rs and RyRs distributed throughout the ER are responsible for releasing calcium from this internal source (Kostyuk and Verkhratsky, 1994; Simpson et al., 1995). To determine whether both or either one of these was responsible for the release of internal calcium, we examined the effect of blocking each of those receptors on cytosolic calcium increase in response to adenosine. Three slices per pharmacological agent were pretreated with DHPB (30 µg/ml), a potent antagonist of RyRs (Kang et al., 1994); xestospongin C (20 µM), a potent cell-permeable blocker of IP$_3$R (Gafni et al., 1997); and 2APB (50 µM), a functional and membrane-permeable IP$_3$R antagonist (Hamada et al., 1999) for 10 min. The drug treatment alone did not produce any increase in the fluorescence. Blocking of RyR with DHPB (n = 15) did not prevent an adenosine-mediated increase in fluorescence, whereas treatment of the slice with either of the IP$_3$R blockers (xestospongin C, n = 26; 2APB, n = 18) significantly reduced the increase in fluorescence seen with adenosine (H = 55.864; df = 4; p < 0.001) (Fig. 5). These results suggested that adenosine mobilizes intracellular calcium primarily via IP$_3$Rs and not via RyRs.

**DISCUSSION**

The present study demonstrated that adenosine stimulated cytosolic calcium increases in a subpopulation of cholinergic neurons in the BF of rats. This intracellular calcium increase was a result of internal release, primarily via IP$_3$Rs, in the absence of calcium influx. The response to adenosine was mediated predominantly by A$_1$ receptors, with a smaller but significant contribution by A$_3$ adenosine receptors. Both A$_1$ and A$_3$ receptors are coupled to inhibitory G-proteins. Although there is evidence in the literature for A$_3$ receptor-mediated activation of IP$_3$ production and calcium mobilization in neurons (Abbracchio et al., 1995) and for A$_1$ receptor-mediated calcium signaling in smooth muscle cells and astrocytes (Gerwins and Fredholm, 1992; Biber et al., 1997), this report is the first, to the best of our knowledge, to show A$_1$ receptor-mediated mobilization of IP$_3$-regulated intracellular calcium in a subpopulation of cholinergic neurons in the BF.

Our data demonstrated that adenosine induced a relatively slow release of intracellular calcium from internal calcium stores and that it occurred at higher concentrations of adenosine. The time course of calcium increase was similar to that in other reports on PLC-linked G-protein-coupled receptors, such as metabotropic glutamate receptor 5 and the angiotensin II receptor (Cecotto et al., 2001; Nash et al., 2001). The fact that IP$_3$-dependent mobilization of intracellular calcium is dependent on a high concentration of adenosine, similar to what has been described in astrocytes by Biber et al. (1997), supports the notion that increased agonist, and consequently a higher number of stimulated receptors, would release a larger number of G-protein βγ subunits and thus potentiate the activation of PLC (Camps et al., 1992). Recently, G-protein βγ subunit-mediated activation of PLC and a subsequent increase in cytosolic calcium caused by release from internal stores has been reported for G$_1$-coupled dopamine D$_2$ receptors in the striatum (Hernández-López et al., 2000). In neurons, the ER contributes significantly to the dynamics of calcium signaling by acting as either a source or a sink of calcium (Simpson et al., 1995; Berridge, 1998, 2000). Both IP$_3$Rs and RyRs are widely distributed in brain ER and can influence the release of internal calcium (McPherson et al., 1991; Seymour-Laurent and Barish, 1995). Our results strongly suggest that adenosine-dependent calcium increase in cholinergic neurons is mediated via IP$_3$Rs.

**Calcium mobilization in cholinergic neurons of the BF**

The selectivity of adenosine-induced calcium mobilization in cholinergic neurons is of interest in disambiguating the roles of different neurotransmitter phenotypes in the BF. That the increase in intracellular calcium fluorescence was always in cholinergic neurons suggested a selective functional recruitment of a subpopulation of cholinergic neurons in the BF. Recently, selective actions of galanin on cholinergic versus noncholinergic neurons in the HDB of the BF have been noted (Jhamandas et al., 2002). The cholinergic neurons examined in this study belong to part of the HDB, SI, and MCPO. These cholinergic neurons target both the neocortex and amygdala and regulate aspects of arousal, cognition, attention, and emotion (Szymusiak, 1995; Semb, 2000). Several approaches toward disentangling the anatomy of the cholinergic subpopulations have been adopted; for example, neurochemical distinctions may be based on the immunohistochemical studies for the coexpression of several neuropeptides (for review, see Semb, 2000). Recently, a functional correlation based on the firing pattern of cholinergic neurons was used to determine the projections to the retrosplenial or prefron-
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...cal cortical in separate sets of cholinergic neurons in the BF (Manns et al., 2000). Our data point to a large population (65%) of cholinergic neurons with a unique biochemical response to adenosine of calcium mobilization from internal stores.

An interesting question is the possible mechanism(s) by which the adenosine receptor, particularly the A\(_1\) receptor, known for adenosine of calcium mobilization from internal stores. (Manns et al., 2000). Our data point to a large population (65%) which is shown to induce nuclear translocation of NF-B.

We suggest that it is unlikely that the selectivity to cholinergic neurons is artifactual. The fact that tachyphagia treatment could elicit similar responses in all types of cells strongly supports our suggestion. Notably, our data also show that the dye calcium orange might be particularly useful for subsequent immunohistochemistry to identify the responding cholinergic neurons. This feature of calcium orange might most likely relates to its high photostability and membrane impermeability after de-esterification in cytoplasm (Eberhard and Erne, 1991; Thomas et al., 2000). Our results suggested that calcium orange retains its place within the neuron even after fixation with formalin as a fixative. We observed that calcium orange-loaded neurons could be fixed without apparent leak of fluorescence outside the cells, because there was no “aura” around neurons and no increase in background after 1 and 24 hr of formalin treatment. Similar observations have been reported for another fluorescent dye, Lucifer yellow (Stewart, 1978). Moreover, fixation of the slices also did not alter the fluorescence inside the cells, because the adenosine-induced increase in fluorescence after fixation was the same as that observed in real time. Similar results were obtained in calcium orange-loaded cultured HEK 293 cells, in which cytosolic calcium fluorescence intensity remained stable, without any leak into the medium, after fixation with formalin (data not shown).

**Calcium signaling and transcription**

Calcium signals induce gene expression that may be important for long-lasting adaptations (Bading, 2000; Mattson et al., 2000; Mellström and Naranjo, 2001). Such a role for calcium is well described in the nervous system, in which transient changes in intracellular calcium can produce distinct transcriptional responses (Bading et al., 1993; Ghosh and Greenberg, 1995). Calcium changes also regulate the transcription factor NF-B (Dolmetsch et al., 1998). We have reported previously that adenosine treatment of brain slices resulted in nuclear translocation of NF-B in the BF, a phenomenon also observed after 3 hr of sleep deprivation (Basheer et al., 2001a). Together, these observations support the idea that increased levels of extracellular adenosine (as present with sleep deprivation) may preferentially activate the PLC pathway to mobilize internal calcium, then activate PKC, which is shown to induce nuclear translocation of NF-B in lymphocytes (Nishizuka, 1992; Fino and Baldwin, 1995).

**A link between sleep deprivation-induced adenosine increase and NF-B activation**

The behavioral significance of these results may be best understood in the context of sleep deprivation and its long-term effects. Our previous reports showed a unique pattern of sleep deprivation-induced increases in extracellular adenosine as well as its effect on A\(_1\) adenosine receptor activation of transcription factor NF-B in the BF (Porkka-Heiskanen et al., 1997, 2000; Basheer et al., 2000, 2001a). One of the questions generated from those studies was the identity of the signal transduction pathway linking the increased levels of extracellular adenosine with the PKC-dependent induction of NF-B (Finco and Baldwin, 1995). Our results have provided evidence that adenosine can mobilize IP\(_3\)-regulated intracellular calcium, which may help the activation of NF-B. NF-B, in turn, may be involved in the expression of genes, including that of the A\(_1\) receptor, which is upregulated with sleep deprivation (Basheer et al., 2001b) and which may play a role in mediating the longer-term effects of sleep deprivation.

Finally, we believe that these observations help in understanding the complex organization of the BF by providing a biochemical distinction for a major subgroup of cholinergic neurons; these neurons may play a specific role in mediating some of the longer-term effects of sleep deprivation-induced adenosine in the BF.

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