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The A₃ adenosine receptor attenuates the calcium rise triggered by NMDA receptors in retinal ganglion cells

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Abstract

The A₃ adenosine receptor is emerging as an important regulator of neuronal signaling, and in some situations receptor stimulation can limit excitability. As the NMDA receptor frequently contributes to neuronal excitability, this study examined whether A₃ receptor activation could alter the calcium rise accompanying NMDA receptor stimulation. Calcium levels were determined from fura-2 imaging of isolated rat retinal ganglion cells as these neurons possess both receptor types. Brief application of glutamate or NMDA led to repeatable and reversible elevations of intracellular calcium. The A₃ agonist CI-IB-MECA reduced the response to both glutamate and NMDA. While adenosine mimicked the effect of CI-IB-MECA, the A₃ receptor antagonist MRS 1191 impeded the block by adenosine, implicating a role for the A₃ receptor in the response to the natural agonist. The A₁ receptor antagonist DPCPX provided additional inhibition, implying a contribution from both A₁ and A₃ adenosine receptors. The novel A₃ agonist MRS 3558 (1'S,2'R,3'S,4'R,5'S)-4-(2-chloro-6-(3-chlorobenzylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide and mixed A₁/A₃ agonist MRS 3630 (1'S,2'R,3'S,4'R,5'S)-4-(2-chloro-6-(cyclopentylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide also inhibited the calcium rise induced by NMDA. Low levels of MRS 3558 were particularly effective, with an IC₅₀ of 400 pM. In all cases, A₃ receptor stimulation inhibited only 30-50% of the calcium rise. In summary, stimulation of the A₃ adenosine receptor by either endogenous or synthesized agonists can limit the calcium rise accompanying NMDA receptor activation. It remains to be determined if partial block of the calcium rise by A₃ agonists can modify downstream responses to NMDA receptor stimulation.

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Keywords

A₃ adenosine receptor; NMDA receptor; glutamate; ATP; purinergic signaling; neuroprotection; excitotoxicity; retinal ganglion cells

Adenosine acts as a transmitter throughout the nervous system, stimulating each of four receptors, termed A₁, A_{2a}, A_{2b} and A₃ (Fredholm *et al.*, 2001a). While roles for the A₁ and A_{2a} receptors are well established, and that of the A_{2b} receptor is emerging, the contribution of A₃ receptors to neural signaling remains only partially elucidated. Both the restricted distribution of A₃ receptors and the delayed availability of specific agonists and antagonists have hindered investigation (Rivkees *et al.*, 2000; Jacobson & Knutsen, 2001).

In spite of these restrictions, evidence is accumulating that the A₃ receptor exerts an inhibitory influence on at least some neuronal systems. Adenosine acting at cerebral A₃ receptors is neuroprotective in stroke models (Shen *et al.*, 2005), while A₃ agonists can limit ischemic damage (Von Lubitz *et al.*, 1999). Moreover, A₃ receptor knockout mice are more susceptible to neurodegeneration after hypoxic challenge (Fedorova *et al.*, 2003). Stimulation of the A₃ receptor prevents apoptosis of cultured cortical neurons exposed to extended chemical hypoxia in vitro, and repeated injections of A₃ agonists confer prolonged protection from the effects of middle cerebral artery ligation (Chen *et al.*, 2006). A₃ receptors contribute to the inhibition of cortical synaptic potentials during hypoxia (Hentschel *et al.*, 2003) and inhibit synaptic transmission in the enteric nervous system (Wunderlich *et al.*, 2008). A₃ receptors have also been shown to protect retinal ganglion cells following the Ca²⁺ elevation and cell death accompanying stimulation of the P2X₇ receptor (Zhang *et al.*, 2006c). The neuronal actions of the A₃ receptor are varied, and receptor stimulation can also damage neurons under some circumstances (Pugliese *et al.*, 2003), with the timing and concentration of drugs likely to determine the overall effect (Abbracchio & Cattabeni, 1999; Von Lubitz *et al.*, 2001; Pugliese *et al.*, 2007). However, the evidence above suggests that A₃ receptors limit neuronal excitation in at least some situations.

Glutamate is the primary excitatory transmitter in the nervous system, mediating its effects through ionotropic and metabotropic receptors. The NMDA, AMPA, and kainate receptors comprise the ionotropic group, with activation leading to channel opening and the influx of sodium and calcium (Dingledine *et al.*, 1999). The corresponding depolarization can open voltage-dependent Ca²⁺ channels, leading to a secondary pathway of Ca²⁺ influx. While the kainate and AMPA receptors inactivate rapidly, NMDA receptors inactivate much more slowly; the sustained elevation in Ca²⁺ levels accompanying NMDA receptor stimulation ensures that the receptor makes a major contribution to neuronal excitability. Modulating the rise in Ca²⁺ that accompanies NMDA receptor activation is a key pathway to control neuronal excitability and thus a likely target for the inhibitory actions of A₃ receptors. NMDA-dependent synaptic transmission is depressed by A₃ receptor stimulation (Rubaj *et al.*, 2003), suggesting the receptors can indeed modulate excitatory glutaminergic signaling. However, it is not presently known whether the mechanism underlying this inhibition involves attenuation of the Ca²⁺ rise.

This study specifically asked whether stimulation of the A₃ adenosine receptor could limit the Ca²⁺ rise that accompanies NMDA stimulation in neurons. Isolated rat retinal ganglion cells were chosen as the assay system because they have been shown to express A₃ receptors on a molecular (Zhang *et al.*, 2006a) and functional (Zhang *et al.*, 2006c) level, in addition to possessing well characterized NMDA receptors (Sucher *et al.*, 1991; Sucher *et al.*, 2003; Hartwick *et al.*, 2004; Goebel & Winkler, 2006; Gustafson *et al.*, 2007). Freshly dissociated ganglion cells can be isolated from other retinal cells using a two-step immunopanning

procedure, ensuring that neurochemical interactions are not confounded by interference from glial cells (Barres *et al.*, 1988; Hartwick *et al.*, 2004). This preparation is also amendable to Ca^{2+} measurements using the ratiometric dye fura-2, providing a quantifiable measurement of intracellular levels. Isolated ganglion cells were thus used to examine the effect of A_3 adenosine receptors on the Ca^{2+} rise triggered by NMDA receptors.

Preliminary versions of some of the data have been presented in abstract form (Zhang *et al.*, 2006b).

2. Methods

2.1. Immunopanning isolation of retinal ganglion cells

Purification of ganglion cells using the immunopanning procedure was performed as described previously (Zhang *et al.*, 2005) based upon standard protocols (Barres *et al.*, 1988; Hartwick *et al.*, 2004). Retinas were removed from rat pups on post-natal day (PD) 5-11 from untimed pregnant Long-Evan rats (Jackson Laboratory Inc., Bar Harbor, ME). All animals were treated in accordance with University of Pennsylvania IACUC approved protocols and the National Institutes of Health guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Retinas were enzymatically dissociated for 30 min with 15 U/mL papain, 0.2 mg/mL DL-cysteine and 0.004% DNase I (Worthington/Cooper, Lakewood, NJ). The tissue was washed, centrifuged, resuspended and filtered. Cells were incubated with rabbit antimacrophage antibody (1:75, Accurate Chemical, Westbury, NY), then incubated in a 100 mm dish coated with goat anti-rabbit IgG antibody (1:400, Jackson ImmunoResearch Laboratories Inc, West Grove, PA). Non-adherent cells were removed to a second petri-dish coated with goat anti-mouse IgM (1:300, Jackson ImmunoResearch Laboratories Inc) and anti-Thy 1.1 antibody (from hybridoma T11D7e2; American Type Culture Collection, Rockville, MD). After 30 min, non-adherent cells were washed off and adherent ganglion cells were incubated with 0.125% trypsin for 8 min at 37°C. Digestion was stopped with fetal bovine serum (30%) in Neurobasal medium and cells were centrifuged and plated on coverslips coated with poly-L-lysine and laminin. The basic growth medium contained Neurobasal medium with 2 mM glutamine, 100 µg/ml gentamicin, 0.025ml/ml B27 supplement (all Invitrogen Inc., Carlsbad, CA), 0.7% methylcellulose (Stemcell Technologies Inc., Vancouver, BC, Canada) and 2.5% rat serum (Cocalico Biologicals Inc., Reamstown, PA). Retinal ganglion cells were incubated at 37°C with 5% CO_2 . Quantification with labeled cells indicated that the immunopurified preparation contained at least 98% ganglion cells.

2.2. Intracellular Ca^{2+} measurements

Unlabeled RGCs from isolated ganglion cell preparations attached to coverslips for 24 hrs were loaded with 10 µM fura-2 and 2% Pluronic F-127 (Invitrogen, Inc.) for 60-90 min at room temperature, rinsed and maintained in fura-2-free solution for 30 min before data acquisition began. The coverslips were mounted on a Nikon Diaphot inverted microscope and visualized with a 40x objective. To obtain Ca^{2+} measurements, the field was alternatively excited at 340nm and 380nm with a scanning monochromator and the light emitted at >520 nm from a region of interest surrounding individual retinal ganglion cells was imaged with a CCD camera and analyzed (all Photon Technologies International, Inc., Lawrenceville, N.J.). Cells were perfused with a control solution at the start of Ca^{2+} imaging experiments containing (in mM) 105 NaCl, 5 KCl, 4 NaHepes, 6 Hepes acid, 5 NaHCO_3 , 1.3 CaCl_2 , 5 glucose, 60 mannitol, pH 7.4. Drugs were dissolved in the control solution. Calibration was performed separately on each cell after the experiment by perfusing cells in the presence of 5 µM ionomycin and control solution (with 1.3 mM Ca^{2+}) followed by ionomycin in the base solution without Ca^{2+} and

with the addition of 5 mM EGTA (pH 8.0). The 340/380 ratio was converted to Ca^{2+} concentration as previously described (Zhang *et al.*, 2005). In some experiments, data are expressed as this ratio as the large number of solutions precluded conversion. Ca^{2+} measurements were performed at room temperature.

2.3 Materials and data analysis

MRS 3558 (1'S,2'R,3'S,4'R,5'S)-4-(2-chloro-6-(3-chlorobenzylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide and MRS 3630 (1'S,2'R,3'S,4'R,5'S)-4-(2-chloro-6-(cyclopentylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide were developed at the Laboratory of Bioorganic Chemistry NIDDK, National Institutes of Health (Tchilibon *et al.*, 2005; Jacobson *et al.*, 2005). All other materials were obtained from Sigma Chemical Corp. (St. Louis, MO) unless otherwise indicated. Data are presented as mean $\pm$ standard error of the mean. Significance was generally evaluated using an appropriate Student's *t* test for comparison between two variables or a one-way ANOVA with post-hoc test for more than two variables using SigmaStat 3.5 (Systat Software, Inc., San Jose, CA), with $p < 0.05$ taken as a significant difference. "n" is defined as the number of individual ganglion cells from which measurements were made. The Ca^{2+} response was defined as the difference between peak and baseline for each application of agonist. Block by MRS 3558 was characterized by fitting data to an exponential decay with three parameters using a least-squared analysis with Sigmaplot 9 (Systat Software, Inc).

3. Results

3.1. The A_3 receptor inhibits the calcium rise triggered by NMDA in rat retinal ganglion cells

Initial experiments tested whether the relatively specific adenosine A_3 receptor agonist 2-chloro- N^6 -(3-iodobenzyl)-adenosine-5'-*N*-methyluronimide (CI-IB-MECA) could reduce the calcium elevation induced by NMDA in rat retinal ganglion cells. Neurons loaded with fura-2 were exposed to recurring 15-second applications of 10 μM NMDA followed by a washout. As NMDA receptors require glycine as a co-agonist (Danysz & Parsons, 1998), 10 μM glycine was always included in the mixture. All experiments were done in the absence of Mg^{2+} to limit the Mg^{2+} -dependent block of the NMDA receptor (Nowak *et al.*, 1984). This brief exposure to 100 μM NMDA + 10 μM glycine led to clear elevations in calcium levels that promptly returned to baseline upon the removal of agonist (Fig. 1A). The absolute increase in Ca^{2+} triggered by this level of NMDA was $658 \pm 124 \text{ nM}$ ($n=44$). The elevations were recurring upon reapplication of NMDA, with the magnitude of the Ca^{2+} elevation relatively consistent throughout multiple applications. Application of agents to alternate applications thus provided an ideal way to test for inhibition within a single cell, consistent with Hartwick *et al.*, (2004).

To determine whether A_3 receptor stimulation could modulate the response to NMDA, the A_3 agonist CI-IB-MECA was added to the bath and the response to NMDA was determined. CI-IB-MECA led to a clear reduction in the calcium rise triggered by NMDA (Fig. 1B). The block by CI-IB-MECA was reversible and repeatable within a given cell and led to a significant reduction of the mean calcium response (Fig. 1C).

3.2. The A_3 receptor inhibits the calcium rise triggered by glutamate

While glutamate is the endogenous agonist at NMDA receptors, it can also stimulate other metabotropic and ionotropic receptors. To determine if the A_3 receptor also attenuated the response to glutamate, isolated ganglion cells were exposed to brief, recurrent applications of 10 μM glutamate + 10 μM glycine. The responses were very similar to those induced by NMDA; Ca^{2+} levels rose sharply, and then returned to baseline promptly after agonist removal (Fig. 2A). The mean rise in calcium induced by glutamate was $612 \pm 105 \text{ nM}$ ($n=24$). This

increase was not significantly different from that induced by NMDA, consistent with reports of the relative potency of the two agonists at NMDA receptors (Moriyoshi *et al.*, 1991).

Stimulation of the A₃ receptor with CI-IB-MECA also inhibited the Ca²⁺ rise evoked by glutamate (Fig. 2B.). This inhibition was readily reversible, with glutamate producing a robust response upon removal of CI-IB-MECA. Overall, CI-IB-MECA led to a significant reduction in the Ca²⁺ elevation induced by glutamate (Fig. 2C).

3.3. The A₃ and A₁ receptors contribute to the block by adenosine

Rat retinal ganglion cells express multiple adenosine receptors (Kvanta *et al.*, 1997), and the A₁ receptor is known to attenuate the response to NMDA (Hartwick *et al.*, 2004). As adenosine is the endogenous agonist at all 4 receptor subtypes, it was important to determine whether the A₃ receptor contributed to the block by adenosine itself.

Adenosine (10 μm) attenuated the calcium elevation induced by glutamate. This concentration of adenosine is high enough to stimulate all four adenosine receptors, although the actual concentration reaching the membrane may have been reduced by adenosine deaminase or transporters (Fredholm *et al.*, 2001b). To evaluate the contribution of the A₃ receptor to the block by adenosine, the A₃ antagonist MRS 1191 was added. The Ca²⁺ response to glutamate was substantially inhibited by adenosine. However 1 μM MRS 1191 significantly reduced the block produced by adenosine (Fig. 3A and B). This demonstrates that the A₃ receptor contributes to the attenuation by adenosine.

To determine whether the residual block remaining in the presence of MRS 1191 and adenosine was due to adenosine acting at the A₁ receptor, the effect of A₁ antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was examined (Fig 4A). The mean Ca²⁺ rise induced by NMDA in the presence of both adenosine and MRS 1191 was 15% less than that produced by NMDA alone (Fig. 4B), not significantly different than the reduction of the glutamate rise (p=0.36). DPCPX eliminated this residual, consistent with the theory that both A₁ and A₃ receptors contribute to the inhibitory effects of adenosine on these neurons.

3.4. MRS 3558 and MRS 3630 block response to NMDA

The ability of the A₃ receptor to reduce the NMDA signal was further examined with the A₃ agonist MRS 3558. MRS 3558 is 100-fold more selective for the A₃ receptor over other adenosine receptors and is effective in the low nanomolar range (Tchilibon *et al.*, 2005). While the compound has been shown to prevent ischemic damage in lung tissue (Matot *et al.*, 2006) the effect of MRS 3558 in neurons has not been determined. At 1 nM, MRS 3558 produced a substantial inhibition of the response to NMDA (Fig. 5A-B). MRS 3558 also reduced the response to glutamate (not shown). Given this low effective level, the concentration-dependence of block by MRS 3558 was examined. Exposure of cells to 1 nM, 10 nM or 100 nM MRS 3558 produced a significant reduction in Ca²⁺ response. Even 100 pM MRS 3558 produced a small, albeit insignificant, reduction. The response to NMDA was blocked by MRS 3558 with an IC₅₀ of 400 pM (Fig. 5C), although only 30% of the Ca²⁺ elevation was inhibited by saturating levels.

As the results above, and previous findings by others (Hartwick *et al.*, 2004), suggest the A₁ adenosine receptor can also limit the Ca²⁺ rise from NMDA in these cells, initial experiments were performed with the joint A₁/A₃ agonist MRS 3630. MRS 3630 has a radioligand binding K_i of 17.4 nM against rat A₁ and 5.8 nM against rat A₃ receptors respectively (Jacobson *et al.*, 2005). MRS 3630 significantly inhibited the Ca²⁺ rise (Fig. 5D-E). Of note, MRS 3630 blocked only half the Ca²⁺ elevation even at 1 μM, suggesting the Ca²⁺ rise had both adenosine sensitive and adenosine insensitive components.

4. Discussion

This study has demonstrated that stimulation of the A₃ adenosine receptor limits the rise in Ca²⁺ that accompanies stimulation of the NMDA receptor in rat retinal ganglion cells. Several points support the identification of the A₃ receptor in this role. The calcium elevation triggered by NMDA was inhibited by the agonist CI-IB-MECA. CI-IB-MECA is relatively specific for the A₃ receptor, with a potency of 115/2,100/10,000/11 nM at A₁/A_{2A}/A_{2B} and A₃ receptors respectively (Freedholm et al., 2001a). While the block produced by 1 μM CI-IB-MECA could have theoretically involved the A₁ receptor, antagonism of the adenosine effect with MRS 1191 confirmed action of the A₃ receptor, as MRS 1191 blocks the human A₁/A_{2A} and A₃ receptors with a potency of 40,000/100,000 and 31 nM respectively (Jacobson *et al.*, 1997). Although the compound is somewhat less effective at rat than at human A₃ receptors, 1 μM still acts selectively at rat A₃ receptors (Dunwiddie *et al.*, 1997). As such, a block by 1 μM MRS 1191 strongly implicates the A₃ receptor. Finally the specific A₃ agonist MRS 3558 blocked the Ca²⁺ rise with an IC₅₀ of 400 nM. MRS 3558 has a binding affinity for A₁/A_{2A}/A_{2B} and A₃ receptors of 260/2300/>10,000/0.29 nM respectively (Tchilibon *et al.*, 2005), so an inhibition by 1, 10 and 100 nM confirms actions at the A₃ over the A₁ receptor. The similarity between our effective IC₅₀ of 400 pM and the binding affinity support the correlation. Together, these findings imply that stimulation of the A₃ adenosine receptor can reduce the Ca²⁺ influx accompanying NMDA receptor activation.

While the mechanisms underlying the reduction in Ca²⁺ have not been defined, some inferences can be made. Stimulation of the A₃ receptor never blocked the Ca²⁺ rise completely. Typical inhibition was 30-50%, even with saturating concentrations of MRS 3558 and MRS 3630. Although there are numerous explanations for the partial block, precedent suggests the A₃ receptor does not act directly on the NMDA channels. The depolarization that accompanies NMDA receptor activation can subsequently open voltage-dependent Ca²⁺ channels (Sucher *et al.*, 1991; Alagarsamy & Johnson, 1995), enabling Ca²⁺ to enter through both NMDA channels and Ca²⁺ channels. Stimulation of the adenosine A₁ receptor blocks voltage-gated Ca²⁺ channels by direct inhibition by the G_{βγ} subunit (Schulte & Fredholm, 2002; Dolphin, 2003), and stimulation of the A₁ receptor in salamander retinal ganglion cells inhibited the Ca²⁺ currents (Sun *et al.*, 2002). Stimulation of A₃ receptors can also activate G_{βγ} subunit released from G_{i/o} proteins (Schulte and Fredholm, 2002), suggesting a block of voltage dependent Ca²⁺ channels is possible. However, A₃ receptors were recently shown to stabilize GABA_A receptors in the membrane (Roseti *et al.*, 2008), and as ganglion cells express these receptors (Akopian *et al.*, 1998), this alternative explanation cannot be ruled out.

The demonstration that A₃ receptors can limit excitatory rises in Ca²⁺ does not negate the contribution of the A₁ adenosine receptor in these or other neurons. A₁ receptors have clearly been shown to block Ca²⁺ influx in retinal ganglion cells (Sun *et al.*, 2002; Hartwick *et al.*, 2004), and stimulation of A₁ receptors by endogenously-produced adenosine hyperpolarizes ganglion cells (Newman, 2001, 2003). The ability of specific A₃ antagonist MRS 1191 to limit the effect of adenosine, along with the additive limiting effect of A₁ antagonist DPCPX in the present study, imply that both receptors contribute to the block by adenosine in retinal ganglion cells. Although the A₃ was originally thought to be less sensitive to adenosine, more recent analysis suggests both A₁ and A₃ receptors are highly responsive, with EC₅₀ values of 310 nM and 290 nM respectively (Fredholm *et al.*, 2001b). The overall contribution of each receptor to ganglion cell function will of course depend upon their relative expression in the membrane and the location of this expression, with the latter impossible to estimate using this isolated cell preparation.

Physiological Implications

The primary conclusion of this study is that A₃ adenosine receptors limit the Ca²⁺ rise accompanying NMDA receptor stimulation. As such, it provides a potential explanation for the growing list of modulatory effects of A₃ receptors in the nervous system mentioned above. This block may be restricted to stimulation of NMDA receptors, as A₃ receptor stimulation did not protect against kainate triggered death of cortical neurons (Rebola *et al.*, 2005). The effects will also be regionally and temporally dependent, as suggested by the varied response to A₃ agonists in different neuronal regions. Given the evidence for the neuroprotective actions of the A₃ receptor (Von Lubitz *et al.*, 1999) it is tempting to predict that A₃ agonists may be of some benefit under conditions where neurons succumb to damage from extended stimulation of the NMDA receptor. Any advantage may depend of the ability to restrict these agonists to the appropriate targets. Whether stimulation of the A₃ receptor can protect retinal ganglion cells from ischemic damage as flavonoids and nicotinamide do remains to be determined (Ji *et al.*, 2008; Jung *et al.*, 2008)

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Figure 1. A₃ adenosine receptor agonist CI-IB-MECA reduced the Ca²⁺ elevation triggered by NMDA

(A) Cells were exposed to recurring 15-second applications of 100 μ M NMDA + 10 μ M glycine (referred to as NMDA) followed by an 8-minute wash. Repeated application of these agonists produced multiple elevations in Ca²⁺. (B) CI-IB-MECA (CI-IB) reduced the rise in Ca²⁺ triggered by 100 μ M NMDA. Cells were exposed to 1 μ M CI-IB-MECA for 3 minutes before and 2 minutes after application of NMDA to ensure coverage. The block was reversible and repeatable. (C) Quantification of the block by CI-IB-MECA. The mean peak Ca²⁺ elevation triggered by 10 μ M NMDA was significantly reduced when compared with that in the presence of CI-IB-MECA (n=15, * p < 0.001). Here and throughout the legends, traces indicate representative responses, while the bars represent the mean $\pm$ SEM of the rise in Ca²⁺. Data were normalized to the mean Ca²⁺ increase to NMDA.

Figure 2. The A_3 adenosine receptor reduces Ca^{2+} elevation triggered by glutamate

(A) Recurrent 15 sec application of glutamate (10 μ M glutamate + 10 μ M glycine) induced consecutive increases in Ca^{2+} of similar magnitude. (B) The A_3 adenosine receptor agonist Cl-IB-MECA “Cl-IB” reduced the rise in Ca^{2+} triggered by glutamate. Cells were exposed to 1 μ M Cl-IB-MECA for 3 minutes before and 2 minutes after application of glutamate. (C) Quantification of the block by Cl-IB-MECA. The mean Ca^{2+} elevation triggered by 10 μ M glutamate was significantly reduced by 1 μ M Cl-IB-MECA ($n=5$, * $p = 0.009$).

Figure 3. The block by adenosine involves the A_3 adenosine receptor

(A) The protective effect of adenosine (Ado; 10 μ M) on responses to 10 μ M glutamate (+ 10 μ M glycine; Glut) was largely prevented by 1 μ M of the A_3 adenosine receptor antagonist MRS 1191. Adenosine was present in the perfusate where indicated by the middle bars, while MRS 1191 was present where indicated by the bottom bar. (B) Mean responses from cells exposed to 10 μ M glutamate, 10 μ M adenosine and 1 μ M MRS 1191 using the protocol illustrated in (A). * $p < 0.05$ vs. Glut alone; ** $p < 0.05$ vs Glut + Ado; $n = 10$, Student-Newman-Keuls post-hoc test. Data were normalized to the Ca^{2+} increase induced by glutamate.

Figure 4. The A₁ receptor also contributes to the response

(A) Blocking the A₃ receptor with 1 μ M MRS 1191 reduced the ability of 10 μ M adenosine to prevent the Ca²⁺ rise induced by 100 μ M NMDA (+ 10 μ M glycine). However, the residual protection was eliminated by addition of 1 μ M A₁ adenosine receptor antagonist DPCPX. DPCPX sometimes triggered a rise in baseline Ca²⁺, suggesting adenosine was reducing baseline levels. Adenosine and MRS191 were present in the perfusate where indicated by the middle bars “A+M”, while adenosine, MRS 1191 and DPCPX were present together where indicated by the bottom bar “D”. NMDA was added to relevant solutions as indicated by the top bar. Data are expressed as a ratio of light excited at 340/380 as the need for so many solutions precluded calibration. (B) Mean responses from cells exposed to NMDA in the presence of adenosine and MRS 1191 $\pm$ DPCPX (0.1-1 μ M) using the protocol illustrated in (A). * $p < 0.05$ vs. NMDA alone; ** $p < 0.05$ vs NMDA + Ado + MRS 1191; $n = 13$, Student-Newman-Keuls post-hoc test. Data were normalized to the ratiometric Ca²⁺ increase induced by NMDA. Although addition of DPCPX sometimes led to an increased response as in (A), this was not always the case, and the overall Ca²⁺ rise was not significantly different than NMDA alone (NS = no significant difference).

Figure 5. Novel A₃ receptor agonists effective at blocking Ca²⁺ rise

(A) A₃ receptor agonist MRS 3558 (1 nM) prevented the rise in Ca²⁺ triggered by 100 μM NMDA. (B) The mean rise was significantly reduced by 1 nM MRS 3558 (n=6, * p< 0.001). (C) The concentration-dependence of the inhibition by MRS 3558. The line shows the fit of the data to a single exponential decay, giving an IC₅₀ of 400 pM, and maximum inhibition of 31% (control vs. 100 pM MRS 3558 n=19, p>0.05; control vs. 1 nM MRS 3558 n=30, p<0.001; control vs. 10 nM MRS 3558 n=21, p<0.001; control vs. 100 nM MRS 3558 n=28, * p< 0.001). (D) The joint A₁/A₃ receptor agonist MRS 3630 (1 μM) also inhibited the rise in Ca²⁺ triggered by 100 μM NMDA (+ 10 μM glycine). (E) The mean Ca²⁺ response was significantly reduced by 1 μM MRS 3630 (n=4, * p<0.002).