Endogenous Activation of Adenosine A1 Receptors, but Not P2X Receptors, During High-Frequency Synaptic Transmission at the Calyx of Held

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First published February 15, 2006; doi:10.1152/jn.00694.2005. Activation of presynaptic receptors plays an important role in modulation of transmission at many synapses, particularly during high-frequency trains of stimulation. Adenosine-triphosphate (ATP) is coreleased with several neurotransmitters and acts at presynaptic sites to reduce transmitter release; such presynaptic P2X receptors occur at inhibitory and excitatory terminals in the medial nucleus of the trapezoid body (MNTB). We have investigated the mechanism of purinergic modulation during high-frequency repetitive stimulation at the calyx of Held synapse. Suppression of calyceal excitatory postsynaptic currents (EPSCs) by ATP and ATPγS (100 μM) was mimicked by adenosine application and was blocked by DPCPX (10 μM), indicating mediation by adenosine A1 receptors. DPCPX enhanced EPSC amplitudes during high-frequency synaptic stimulation, suggesting that adenosine has a physiological role in modulating transmission at the calyx. The Luciferin-Luciferase method was used to probe for endogenous ATP release (at 37°C), but no release was detected. Blockers of ectonucleotidases also had no effect on endogenous synaptic depression, suggesting that it is adenosine acting on A1 receptors, rather than degradation of released ATP, which accounts for presynaptic purinergic suppression of synaptic transmission during physiological stimulus trains at this glutamatergic synapse.

INTRODUCTION

Synaptic transmission mediated by glutamate acting at postsynaptic AMPA and NMDA receptors (Forsythe and Westbrook 1988) occurs at the majority of central excitatory synapses. In some cases, release and/or corelease of adenosine-5′-triphosphate (ATP) also contributes, with ATP as a neurotransmitter in its own right (Bardoni et al. 1997; Edmonds et al. 1995 et al.) or as acting as a neuromodulator (Li et al. 1998; Nakatsuka and Gu 2001). Following ATP release, ectonucleotidases rapidly convert it to adenosine in the synaptic cleft (Dunwiddie et al. 1997), which can subsequently activate G protein-coupled adenosine receptors, which are widely expressed at both pre- and postsynaptic sites in the CNS. Adenosine application inhibits synaptic transmission (Ginsborg and Hirst 1972) by activating presynaptic A1 receptors in the periaqueductal gray (Bagley et al. 1999) and laterodorsal tegmentum (Arrigoni et al. 2001). Release of endogenous adenosine can also inhibit synaptic transmission (Arrigoni et al. 2001) and contribute to depression through a presynaptic mechanism (Oliet and Poulain 1999). A1 receptors are negatively coupled to adenylate cyclase (van Calker et al. 1979), leading to inhibition of calcium channels (Okada et al. 2001; Wu and Saggau 1994). Adenosine can activate potassium channels (Greene and Haas 1985; Trussell and Jackson 1985), which could contribute to presynaptic inhibition at some sites, but this mechanism is unlikely to be universal, as potassium channel antagonists did not block the suppression of γ-aminobutyric acid (GABA) release by A1 receptor agonists in the hippocampus (Jeong et al. 2003).

The calyx of Held synapse is an excitatory synapse located in the medial nucleus of the trapezoid body (MNTB), which forms one relay in a binaural pathway involved in sound-source localization (Oertel 1999). The MNTB can follow high-frequency trains of action potentials in vivo (Spirou et al. 1990) and up to 800 Hz trains in vitro (Taschenberger and von Gersdorff 2000; Wu and Kelly 1993). During synaptic stimulation at frequencies above 10 Hz, there is a large frequency-dependent reduction in the excitatory postsynaptic current (EPSC) amplitude (short-term depression) (Iwasaki and Takahashi 2001; Taschenberger and von Gersdorff 2000; Wang and Kaczmarek 1998; Wong et al. 2003), which is mediated by presynaptic vesicle depletion (Hjelmstad et al. 1999; von Gersdorff et al. 1997), decreased presynaptic calcium current (Xu and Wu 2005), and postsynaptic AMPA receptor desensitization (Raman and Trussell 1995; Scheuss and Neher 2001; Wong et al. 2003). However, calyceal transmitter release is depressed by a number of G protein-coupled presynaptic receptors, including GABA_A receptors (Isaacson 1998; Takahashi et al. 1998), metabotropic glutamate receptors (Barnes-Davies and Forsythe 1995; von Gersdorff et al. 1997), and presynaptic adenosine receptors (Barnes-Davies and Forsythe 1995; Kimura et al. 2003). Since purines can be coreleased with glutamate at central synapses and previous reports have suggested the presence of P2X receptors in the MNTB (Watanabe et al. 2004), we have tested whether adenosine or P2X receptor activation could mediate short-term depression under physiological conditions. Our results show that P2X receptors are absent from the calyx of Held but that purinergic modulation is mediated by presynaptic A1 receptors, in agreement with a previous report (Kimura et al. 2003). We demonstrate that adenosine receptor activation is not due to metabolism of released ATP, suggesting that adenosine itself is the signaling molecule.

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METHODS

PREPARATION OF BRAIN SLICES. Lister-Hooded rats (age 10–12 days) were killed by decapitation, and brain stem slices containing the superior olivary complex were prepared as described previously (Barnes-Davies and Forsythe 1995). Briefly, transverse slices (250 μm thick) of superior olivary complex containing the MNTB were prepared in a low-sodium artificial CSF (aCSF) solution at a temperature of about 0°C. After 1 h of incubation at 37°C in normal aCSF, slices were maintained at room temperature until required. The normal aCSF composition was (in mM) NaCl, 125; KCl, 2.5; NaHCO3, 26; glucose, 10; NaH2PO4, 1.25; sodium pyruvate, 2; myo-inositol, 3; CaCl2, 2; MgCl2, 1; ascorbic acid, 0.5. The pH was 7.4 when bubbled with 95% O2-5% CO2. For the low-sodium aCSF, 250 mM sucrose was substituted for NaCl, and CaCl2 and MgCl2 concentrations were 0.1 and 4 mM, respectively.

ELECTROPHYSIOLOGY AND IMAGING. Whole cell patch-clamp recordings were made from postsynaptic MNTB neurons using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled in a two-stage vertical pipette puller (PV-83, Narishige, Japan) from thick-walled borosilicate glass capillaries containing an internal filament (GC150F-7.5, outer diameter 1.5 mm; inner diameter 0.86 mm, Clark Electromedical, Reading, UK). Pipettes had a resistance of approximately 8 MΩ when filled with an internal solution, containing (in mM) CsCl, 110; HEPES, 40; EGTA, 0.5; phosphocreatine, 12; QX-314, 5 (pH adjusted to 7.3 with CsOH).

Cells and terminals were visualized in situ using a Zeiss Axioskop fitted with differential interference contrast (DIC) optics and an Olympus LUMPlanFL x 60 0.9NA objective. Presynaptic terminals were identified by including sulforhodamine-101 in the recording pipette. Widespread loss of synaptic connections due to severed axons occurred during the slicing procedure; consequently, intact synaptic connections were detected using an imaging technique as described previously (Billups et al. 2002). Briefly, MNTB neurons were loaded with 7 μM Fura-2 AM for 5 min. Excess Fura-2 AM was washed off for 15 min, before a single image at 380 nm was taken using a Photometrics CoolSnap fx camera. A “region of interest” was then drawn around loaded cells using MetaFluor imaging software (version 4.01, Universal Imaging Corporation, West Chester, PA). Connected cells were identified by imaging for 500 ms at a frequency of 0.7 Hz, and at a wavelength of 380 nm using a xenon arc lamp controlled by means of a Cairn Optoscan (Cairn Instruments, Faversham, UK). Cells were stimulated using an extracellular bipolar platinum electrode placed on the midline, and those which produced a decrease in the 380-nm signal following a 1 s train of 200 Hz at a stimulus intensity of 8 V were then subjected to a second 200 Hz train at 4 V to differentiate between antidromic and orthodromic stimulation. A cell with an intact synaptic connection responded to both an 8 V and a 4 V train. Stimulus trains were delivered through a DS2 isolated stimulator (Digitimer, Welwyn Garden City, UK) and were generated using pClamp 8.2 (Axon Instruments, Foster City, CA). A brightfield photograph was then taken using MetaFluor, and the connected cell was located and then patched under the microscope.

All MNTB neurons and calyx of Held terminals were voltage-clamped at a holding potential of –60 mV. Cells with intact synaptic connections were stimulated with trains of stimuli at 100 Hz and, occasionally, 200 Hz using stimulus intensities of 4–6 V. Non-calyceal synapses require higher stimulus intensities (>10 V) than calyceal inputs (Hamann et al. 2003). Trains were of 1 s duration and were repeated at 30 s intervals. All experiments were performed in the presence of 2 mM calcium, and drugs were applied by bath perfusion in the aCSF. All recordings were made in the presence of 10 μM bicuculline, 1 μM strychnine, 40 μM d-AP5, and 10 μM MK-801 to block GABA, glycine, and NMDA receptors, respectively. In addition, 2 mM kynurenic acid (KYN) was present in all recordings to minimize the effects of desensitization (Wong et al. 2003). The bath volume was 0.8 ml with a perfusion rate of 1 ml per minute. Receptor antagonists were purchased from Tocris Cookson, (Bristol, UK). Other chemicals and drugs were obtained from Sigma (Poole, UK). EPSCs were filtered at 5 kHz and sampled at 20 kHz; series resistances (>16 MΩ) were compensated by 80%. Experiments were conducted at a temperature of 37°C.

All data were acquired using Clampex 8.2 (Axon Instruments, CA). To ensure complete solution exchange and maximal effects of the drugs, we began recordings 3 min after drug application, and only included them for analysis once EPSC amplitudes reached a steady-state level. Analysis of EPSC amplitudes and blanking of stimulus artifacts were performed using Excel (Microsoft, Seattle, WA). Data are expressed as means ± SE from ≥3 separate cells.

IMAGING ATP RELEASE. ATP release from calyceal synapses was imaged using the Luciferin-Luciferase bioluminescence assay described by Newman (2001). Briefly, the bath volume was set to approximately 400 μl, and slices were loaded with 25 μl Luciferin stock solution (Sigma; 10 mg/ml in water) and 25 μl Luciferase stock solution (Sigma; 10 mg/ml in water) to give a final Luciferin-Luciferase concentration of 0.6 mg/ml. In addition, slices were loaded with 7 μM Fura-2 AM as previously described (Billups et al. 2002). Intact calyceal synapses were stimulated with a 200-Hz train for 200 ms, and ATP bioluminescence was detected using a Pentamax cooled-CCD camera via a GenIV image intensifier and analyzed with MetaFluor using a 140 x 120 pixel sub-array and 2 x 2 binning. Data were collected in streams of 500 ms duration, with samples taken every 10 ms during the stream.

STATISTICS. Data were analyzed using a two-tailed, paired Students t-test, and a P value of <0.05 was considered statistically significant. Analysis of steady-state data were done by averaging the last 20 sweeps of the each train during a control period and following drug application.

RESULTS

Repetitive stimulation of the calyceal input was used to study endogenous purinergic modulation. Trapezioid body inputs to MNTB neurons were stimulated at either 100 or 200 Hz in aCSF containing 2 mM extracellular Ca2+. Postsynaptic AMPA receptor desensitization was minimized by conducting the experiments in the presence of 2 mM kynurenate (KYN), which also reduced EPSC magnitude by between 80 and 90% (Wong et al. 2003). Stimulating the calyx of Held with a 1 s stimulus train generated EPSCs that facilitated and then declined in magnitude until near steady-state amplitudes were achieved (Fig. 1A). Facilitation generally occurred between the first and fifth EPSC of the train, and this was subsequently followed by depression. A steady-state level of depression was reached by the 20th EPSC of each train at all stimulus frequencies tested. Repetitions of 1 s trains were at half-minute intervals, and ≥15 trains were obtained per data set per cell.

ATP REDUCES THE AMPLITUDE OF THE INITIAL EPSC OF A TRAIN. Figure 1A shows raw data from the first 100 ms of a 200Hz train. Paired-pulse facilitation occurred at the start of the train, with EPSC amplitude increasing from 794 ± 72 pA to 1,171 ± 113 pA (n = 5 cells). Steady-state EPSC amplitude, expressed as a percentage of the amplitude of the first EPSC, was 26.8 ± 3.7% (Fig. 1D) of initial EPSC amplitude (158 ± 14.6 pA). As ATP and the non-hydrolysable analogue of ATP, ATPγS, have similar effects on spontaneous transmission at this synapse (Watanо et al. 2004), we bath applied ATPγS (100 μM) to minimize any potential complicating effects due to ATP breakdown in the slice (Wieraszko et al. 1989). This significantly
responses in the presence (gray squares) and absence (black diamonds) of 100 control conditions. Between data sets, data are expressed as percent initial EPSC amplitude in plotted for the first 100 stimuli during a 200-Hz EPSC train. For comparison presence of ATP \textsubscript{S} resulted in a significant attenuation in the presence (gray squares) no longer had any effect on the depression kinetics of the train.

Data are expressed as means (gray squares) for clarity. A: facilitation and short-term depression generated by the first 20 EPSCs (100 ms) of a 1-s train at 200 Hz. B: a similar train in the same cell after perfusion of 100 \textmu M ATP\textsubscript{S}. Note the greater inhibition of the initial EPSCs of the train compared with the steady-state EPSCs. Stimulus artifacts have been blanked for clarity. C: ATP\textsubscript{S} has no direct effect on the calyx of Held. The solid black bar shows bath application of 100 \textmu M ATP\textsubscript{S} in the presence of 10 \textmu M DPCPX to a presynaptic terminal. D and E: average EPSC amplitudes are plotted for the first 100 stimuli during a 200-Hz EPSC train. For comparison between data sets, data are expressed as percent initial EPSC amplitude in control conditions. D: average EPSC amplitudes are plotted from 3 different responses in the presence (gray squares) and absence (black diamonds) of 100 \textmu M ATP\textsubscript{S}. E: in the presence of DPCPX (10 \textmu M, black diamonds) ATP\textsubscript{S} (gray squares) no longer had any effect on the depression kinetics of the train. Data are expressed as means \pm SE of 3 cells.

Reduced the initial EPSC amplitude (Fig. 1B), from 794 \pm 72.4 pA to 274 \pm 19.5 pA (Fig. 1D; \( n = 3 \); \( P < 0.05 \)). In addition, application of ATP\textsubscript{S} resulted in a significant attenuation in depression during the train, with the steady-state EPSC being 71.2 \pm 9.0% of the amplitude of the initial EPSC in the presence of ATP\textsubscript{S}, compared with 26.8 \pm 3.7% in control conditions (\( n = 3 \); \( P < 0.05 \)). This reduced depression of evoked EPSC amplitude during the train could be explained by a presynaptic action related to the potentiation of mEPSC frequency observed at this and other central sites (Nakatsu and Gu 2001; Watano et al. 2004). To investigate any presynaptic receptor activation by ATP\textsubscript{S}, we made direct recordings from the presynaptic terminal (calyx of Held) during bath application of 100 \textmu M ATP\textsubscript{S}, in the presence of 10 \textmu M DPCPX, an A1 receptor antagonist (Kimura et al. 2003). However, no discernable current was observed in any of the terminals investigated (Fig. 1C; \( n = 4 \)).

ATP\textsubscript{S} had no effect on the evoked EPSC amplitude or on the magnitude of depression observed during a 100-Hz train applied to in the presence of DPCPX. (Fig. 1E; \( n = 3 \)). These data show that the action of ATP\textsubscript{S}, when applied alone (Fig. 1E), is not mediated by P2X receptors but may be explained by either secondary metabolism of extracellular ATP or release of adenosine.

**ACTIVATION OF ADENOSINE RECEPTORS MIMICS THE EFFECT OF ATP.** In four cells stimulated with 100 Hz trains (Fig. 2A) bath application of adenosine (10 \textmu M) led to a significant decrease in initial EPSC amplitude from control, 608 \pm 47.7 pA to 404 \pm 19.3 pA (Fig. 2B; \( n = 4 \); \( P < 0.05 \)). There was also a concomitant decrease in the amplitude of the last EPSC of the train, from 217 \pm 20.1 pA to 168 \pm 8.8 pA (\( P < 0.05 \)) in the presence of adenosine. Depression curves were best fitted with the sum of two exponentials from the third EPSC of the train, with fast time constants of 39.6 ms (77 \pm 2.4%) and 44.4 \pm 3.3 ms (75 \pm 3.7%) in the presence of adenosine (\( P > 0.05 \); \( n = 4 \)). Paired-pulse facilitation at the start of the train changed from 116 \pm 10% to 130 \pm 20% (Fig. 2B). The effect of adenosine was completely blocked in the presence of 10 \textmu M DPCPX (Fig. 2B, dark gray trace), suggesting that adenosine was acting via presynaptic A1 receptors. Cumulative EPSC amplitude plots have been employed to give an estimate of the readily releasable pool (RRP) size and rates of vesicle recycling (Schneggenburger et al. 1999). The y-axis intercept of a cumulative amplitude plot (Fig. 2C) gives an indication of the RRP size and was obtained by extrapolation of a linear regression line fitted through the steady-state portion of the train.
(EPSCs 50–100). Following adenosine application, this was reduced from 5.0 ± 1.3 nA in control to 3.4 ± 0.9 nA in adenosine (P < 0.05; n = 4).

We have estimated the amplitude of miniature calyceal EPSCs at physiological temperature as being −46 pA at a membrane potential of −60 mV (Postlethwaite and Forsythe, unpublished observations). Taking into account the effect of KYN (85% block, i.e., 15% of control amplitude, −6.9 pA), we estimated the RRP to be 730 ± 223 vesicles (n = 4 cells) in control conditions. The size of the RRP was reduced by 30% to 499 ± 146 vesicles (n = 4) in the presence of 10 μM adenosine, and was reversed by bath application of 10 μM DPCPX (RRP size 631 ± 186 vesicles). The estimates of the RRP size in this study are in general agreement with previous estimates (Meyer et al. 2001; Schuess and Neher 2001; Schneggenburger et al. 1999; Satzler et al. 2002; Wong et al. 2003) of RRP size at this synapse. However, the replenishment kinetics of the RRP remains unaffected, as the RRP recovered with a similar time course in the presence and absence of adenosine (τ 2.5 s; data not shown).

**Endogenous Adenosine Activates A1 Receptors.** An important physiological issue is to test whether endogenous adenosine released during synaptic transmission can result in presynaptic inhibition during a high-frequency train of stimuli. One second trains of EPSCs (100 Hz) were evoked in the presence (black trace) and absence (gray trace) of the A1 receptor antagonist DPCPX (10 μM; Fig. 3A). Application of DPCPX had no effect on the amplitude of the initial EPSC of the train (451 ± 35 pA in control to 464 ± 30 pA in DPCPX), showing that DPCPX had no effect on glutamate receptors, but there was a significant increase in the EPSC amplitude at the end of the train (Fig. 3B; n = 5; P < 0.05). DPCPX reduced the amount of depression observed during the train, from 32 ± 9.8% to 44 ± 10%, but had no effect on the depression time course, with τquin being 40.5 ± 7.0 ms (79 ± 10%) in the absence of DPCPX and 39.5 ± 5.0 ms (90 ± 4.7%) in the presence of DPCPX. In addition, DPCPX had no effect on the size of the RRP (Fig. 3C), suggesting that adenosine receptors are activated progressively during the train. Taken together, these data suggest that endogenously released adenosine (or a precursor such as ATP) can activate A1 receptors during the 1 s duration of the train, as previously reported in hypothalamic synapses (Oliet and Poulain 1999).

**Ectonucleotidase Inhibitors Have No Effect on Synaptic Depression.** An important question is whether the activation of A1 receptors occurs through direct release of adenosine or whether endogenous ATP is released and then subsequently metabolized to adenosine. To test this, we blocked extracellular purine metabolism by bath applying the ectonucleotidase inhibitor 6-N,N-diethyl-b,y-dibromomethylene-o-ATP (ARL 67156, 50 μM) to five MNTB neurons. ARL 67156 prevents breakdown of ATP to ADP (Westfall et al. 1996) and should have an effect on EPSC amplitude or depression during the train if ATP is released and is metabolized to adenosine. However, if adenosine itself is released, then no change should be observed in EPSC amplitude or depression kinetics in the presence of ARL 67156. Figure 4A shows that ARL 67156 results in a small, nonsignificant change in the initial EPSC amplitude (750 ± 52 pA in control to 805 ± 27 pA in ARL). There was also no change in the steady-state EPSC amplitude of a 100-Hz train, or in the depression observed during the train (73 ± 5.9%). ARL 67156 had no significant effect on the RRP size (Fig. 4B) or on the depression kinetics of a 100-Hz train when applied to a different set of four cells in the presence of 10 μM DPCPX (Fig. 4C). Similar results were also obtained when another ectoATPase inhibitor, AMP-CP (250 μM), was tested on three different MNTB neurons. This suggests that adenosine itself is released and that it is not generated as a secondary metabolite from ATP release.

**ATP Is Not Released During a High-Frequency Train.** To test whether ATP is released, we employed the Luciferin-Luciferase assay for detection of ATP release (Newman 2001), combined with an intensified PentaMax CCD camera and electrophysiological recording to confirm activation of the calyceal input. Figure 5A shows a DIC image of an MNTB neuron surrounded by an intact calyx of Held (arrow), with a recording patch pipette in place on the MNTB neuron. Stimulating the presynaptic axon at the midline via a bipolar platinum electrode resulted in a calyceal EPSC of 10 nA (Fig. 5B). It had a fast rise time and decayed back to baseline within 2 ms.

As a positive control, we confirmed that our Luciferin-Luciferase imaging assay was capable of detecting local changes in ATP concentration by using pressure ejection of ATP (100 μM) directly into the slice from a patch pipette. The change in luminescence elicited by local application of ATP is

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**FIG. 3.** Endogenous release of an A1 receptor agonist increases synaptic depression during a stimulus train. A: the A1 receptor antagonist DPCPX leaves the amplitude of the initial EPSC unaffected, but the amplitude of subsequent EPSCs in the train is increased. The first 100 ms of a 1 s train at 100 Hz are shown in the presence (gray trace) and absence (black trace) of 10 μM DPCPX. B: average EPSC amplitude (+ SE; n = 5 cells) in the presence (gray squares) and absence (black diamonds) of 10 μM DPCPX. DPCPX has no effect on the initial EPSC amplitude but significantly increases steady-state EPSC amplitude (P < 0.05; n = 5). C: DPCPX has no effect on the size of the RRP. Cumulative amplitude plots give an RRP size of 507 vesicles in the presence (gray squares) and absence (black diamonds) of DPCPX (n = 5).
To test for synaptic release of ATP from the giant synapse, we monitored the change in luminescence during high-frequency tetanic stimulation of a calyx of Held/MNTB synaptic response (200 ms train delivered at 200 Hz), as shown in Fig. 5D. The leftmost trace is the average light emission recorded from a region of interest that included the synaptic terminal (arrow Fig. 5A). The average of 20 evoked trains was used to reduce noise, but there was no detectable change during or immediately after the train; suggesting that no ATP was released from the calyx. No ATP release could be detected from any of the three functional terminals tested. As a second positive control, we bath applied 10 μM ATP for the duration of the bar in Fig. 5D. The rightmost break represents a 5-min gap in recording to ensure that the ATP had washed into the bath, and there is a 2% increase in luminescence in the presence of 10 μM ATP. The trace in between the two breaks shows baseline luminescence before ATP application (n = 4). These imaging data suggest that even during high-frequency stimulation, ATP release is undetectable and lend support to the pharmacological evidence demonstrating that adenosine is the principal purinergic agonist at the calyx.

**DISCUSSION**

We have established that suppression of transmitter release during high-frequency trains at the calyx of Held/MNTB synapse can be mediated by activation of presynaptic adenosine A1 receptors. The action of endogenous agonist and applied adenosine or ATP were blocked by A1 receptor antagonists. Our results show that ectonucleotidase inhibitors have no effect on EPSC depression, implying that adenosine itself is released rather than being generated by metabolism of released ATP.
Evoked ATP release during high-frequency synaptic stimulation was also undetectable by a sensitive Luciferin-Luciferase assay. Direct recording from the presynaptic terminals showed no inward current in response to ATPγS, so although functional P2X receptors are present at noncalyceal synapses in the MNTB (Watano et al. 2004), we find no evidence for ATP release from, or P2X receptors on, the calyx of Held itself. Our results suggest that release of adenosine from the excitatory terminal serves an autoreceptor function, suppressing transmission in a frequency-dependent manner.

**ATP IS NOT RELEASED AT THE CALYX OF HELD.** There is substantial evidence for release of ATP as a cotransmitter with noradrenaline (Burnstock 1999), acetylcholine (Redman and Silinsky 1994), and GABA (Jo and Schlichter 1999). Direct release of ATP contributes to calcium waves in retinal astrocytes (Newman 2001) and can be detected using the Luciferin-Luciferase bioluminescence assay. It is well established that adenine nucleotide release followed by rapid metabolism by ectonucleotidases leads to adenosine A1 receptor activation in the hippocampus (Dunwiddie et al. 1997), and in the frog spinal cord, adenosine modulation contributes to rhythmic activity and modulation of swimming (Dale 1998). Our data suggests that ATP either is not released from the calyx or is very rapidly broken down (Fig. 5), thereby having no functional effect on transmission at this synapse.

Although there is good evidence for stimulus-dependent ATP release from brain slice preparations (Wieraszko et al. 1989), with a role in fast synaptic transmission (Edwards and Gibb 1993), there is also evidence to suggest that ATP release can be induced by the electrical stimulation itself (electropro- ration) rather than via synaptic transmission (Hamann and Attwell 1996). At the calyx of Held, it was unclear whether physiological activation of A1 receptors was due to direct release of adenosine or secondary metabolic degradation of released ATP. In addressing this question at the calyx of Held, we have the advantage of inducing massive exocytosis at a single giant synapse in which functional synaptic transmission is confirmed using the postsynaptic response (EPSC amplitude of approximately 10 nA; Fig. 5B). In addition, we have used a sensitive intensified CCD camera and remote axonal stimulation to further ensure that transmitter release was through orthodromic invasion of the presynaptic terminal rather than local electropro- ration. Although we show that we could detect extracellular ATP using this system, we were unable to detect activity-dependent ATP release, even during long duration, high-frequency stimulation of the calyx (Fig. 5C). We cannot exclude the possibility that the bioluminescence substrates were excluded from the synaptic cleft; however, it seems unlikely that we would not have detected any spillover during trains of stimuli. Our bioluminescence results are compatible with release of an adenine nucleotide other than ATP and/or adenosine itself.

**P2X RECEPTORS ARE NOT EXPRESSED AT THE CALYX OF HELD.** Presynaptic P2X receptors have been observed at the chick ciliary ganglia (Sun and Stanley 1996) and at synaptic terminals in the spinal cord (Nakatsuka and Gu 2001), and hippocampus (Okada et al. 1999). Recent work from our laboratory has demonstrated that presynaptic P2X receptors are located on inhibitory and excitatory nerve terminals that terminate in the MNTB (Watano et al. 2004), and activation of presynaptic P2X receptors results in an increase in the frequency of noncalyceal mEPSCs (Nakatsuka and Gu 2001; Watano et al. 2004). Although this may be interpreted as an increase in release probability, ATPγS actually depresses evoked calyceal synaptic currents through a mechanism independent of P2X receptor activation (Figs. 1B and 1C). Our experiments show that release of endogenous adenosine (or generation by metabolic degradation) causes activation of presynaptic A1 receptors rather than P2X receptors. Since EPSC depression mediated by ATPγS is blocked by both A1 receptor antagonists and ectonucleotidase inhibitors (Watano et al. in preparation), this strongly implies that ATPγS is not as stable as often claimed in the literature. The absence of presynaptic and postsynaptic P2X receptors at the calyx of Held is consistent with the fact that ATP is not released at this synapse in an activity-dependent fashion.

**PHYSIOLOGICAL IMPLICATIONS OF A1 RECEPTOR ACTIVATION.** The calyx of Held expresses a range of presynaptic metabotropic receptors, including metabotropic glutamate receptors (mGluRs) (Barnes-Davies and Forsythe 1995; Billups et al. 2005; von Gersdorff et al. 1997), α2- adrenergic receptors (Leao and von Gersdorff 2002), and GABA B receptors (Isaacson 1998; Takahashi et al. 1998). While GABA B receptors are present on the calyx of Held, there is no evidence for collateral innervation of the MNTB, as evoked inhibitory responses in the MNTB show higher thresholds than those for stimulation of the calyx input. It is therefore less likely that GABA B receptors are activated during low-frequency stimulation (von Gersdorff et al. 1997) but are able to modulate synaptic state during high-frequency stimulation (Billups et al. 2005).

Adenosine has a presynaptic locus of action at the calyx of Held (Barnes-Davies and Forsythe 1995), with A1 receptors mediating inhibition via a decrease in presynaptic calcium current (Kimura et al. 2003), similar to that previously observed for mGluRs (Takahashi et al. 1996). Activation of mGluRs or A1 receptors has only modest effects on the magnitude of synaptic depression (Kimura et al. 2003; von Gersdorff et al. 1997). In this study, we employed low-affinity glutamate receptor antagonists to minimize AMPA receptor desensitization and conducted the experiments at physiologically relevant frequencies and temperature.

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