Adenosine Triphosphate Acts as Both a Competitive Antagonist and a Positive Allosteric Modulator at Recombinant N-Methyl-D-aspartate Receptors

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ABSTRACT

ATP and glutamate are fast excitatory neurotransmitters in the central nervous system acting primarily on ionotropic P2X and glutamate [N-methyl-D-aspartate (NMDA) and non-NMDA] receptors, respectively. Both neurotransmitters regulate synaptic plasticity and long-term potentiation in hippocampal neurons. NMDA receptors are responsible primarily for the modulatory action of glutamate, but the mechanism underlying the modulatory effect of ATP remains uncertain. In the present study, the effect of ATP on recombinant NR1a + 2A, NR1a + 2B, and NR1a + 2C NMDA receptors expressed in Xenopus laevis oocytes was investigated. ATP inhibited NR1a + 2A and NR1a + 2B receptor currents evoked by low concentrations of glutamate but potentiated currents evoked by saturating glutamate concentrations. In contrast, ATP potentiated NR1a + 2C receptor currents evoked by nonsaturating glutamate concentrations. ATP shifted the glutamate concentration-response curve to the right, indicating a competitive interaction at the agonist binding site. ATP inhibition and potentiation of glutamate-evoked currents was voltage-independent, indicating that ATP acts outside the membrane electric field. Other nucleotides, including ADP, GTP, CTP, and UTP, inhibited glutamate-evoked currents with different potencies, revealing that the inhibition is dependent on both the phosphate chain and nucleotide ring structure. At high concentrations, glutamate outcompetes ATP at the agonist binding site, revealing a potentiation of the current. This effect must be caused by ATP binding at a separate site, where it acts as a positive allosteric modulator of channel gating. A simple model of the NMDA receptor, with ATP acting both as a competitive antagonist at the glutamate binding site and as a positive allosteric modulator at a separate site, reproduced the main features of the data.

ATP and glutamate are fast excitatory neurotransmitters in the central nervous system acting through multimeric ligand-gated P2X (Edwards et al., 1992; Mori et al., 2001) and NMDA and non-NMDA glutamate receptors (McBain and Mayer, 1994). NMDA receptors are involved in many physiological and pathological processes, including the modulation of cell responses to painful stimuli, fine-tuning of synaptic activity, and neuronal pattern formation during development (Singer, 1995; Bennett, 2000; Castellano et al., 2001). Persistent activation of NMDA receptors leads to hypoxic-ischemic neuronal cell death (Choi, 1990) and contributes to initiation and propagation of seizure activity associated with epilepsy (Dingledine et al., 1990; Olney, 1990; Chapman, 1998). Corelease of ATP with other neurotransmitters (Zimmermann, 1994), including glutamate (Wieraszko et al., 1989), from nerve terminals raised the possibility of interaction between the glutamatergic and purinergic components of synaptic transmission. This was further supported by the finding that P2X receptor activation stimulates the release of glutamate at sensory synapses, an event associated with physiological and pathological pain sensation (Gu and MacDermott, 1997).

The hippocampal region of the mammalian brain is associated with long-term potentiation (LTP) whereby intense stimulation of presynaptic neurons results in an increase in synaptic efficiency (Bliss and Collingridge, 1993). Synaptic plasticity is believed to underlie memory and learning (Bliss and Collingridge, 1993; Castellano et al., 2001). Although induction of LTP requires the activation of NMDA receptors, this event is not sufficient to fully explain LTP (Kauer et al., 1988), indicating that molecules other than glutamate are also involved in this process. Induction of LTP by electrical stimulation leads to the release of ATP, suggesting a possible

ABBREVIATIONS: NMDA, N-methyl-D-aspartate; LTP, long-term potentiation; ATP-γ-S, adenosine 5'-O-(3-thiotriphosphate); APV, DL-2-amino-5-phosphopentanoic acid; D-CPP, R-3-[2-carboxypiperazin-4-yl]propyl-1-phosphonic acid; TPEN, N',N',N',N'-tetrakis(2-pyridyl-methyl)ethyl-enediamine; α,β-MeATP, α,β-methyleneadenosine 5'-triphosphate.

role of ATP in synaptic potentiation (Wieraszko et al., 1989; but see Hamann and Attwell, 1996). In fact, Wieraszko and Seyfried (1989) demonstrated a biphasic effect of ATP on stimulated hippocampal slices, where low concentrations of ATP increased the potentiation in pyramidal neurons, whereas a high ATP concentration had the opposite effect, leading to the synaptic depression. Furthermore, ATP applied extracellularly amplified the magnitude of the population spikes and induced LTP in hippocampal slices (Wieraszko and Ehrlich, 1994). Although several mechanisms have been proposed (Dunwiddie and Hoffer, 1980; DiCori and Henry, 1984; Wieraszko et al., 1989; Fujii et al., 2002), the precise physiological role of ATP on neuronal excitability remains unclear. One possibility is that ATP modulation of glutamatergic transmission in the hippocampus (Wieraszko and Seyfried, 1989; Motin and Bennett, 1995) influences LTP. More recently, P2X receptor activation has been reported to regulate the threshold for LTP by controlling the level of activity of NMDA receptors (Pancratov et al., 2002). The proposed mechanism involves altering Ca^{2+}-dependent inactivation of NMDA receptors. Another recent study suggests ATP promotes the induction of LTP via a direct action on NMDA receptors, with no involvement of P2X or P2Y receptors (Fujii et al., 2002). Inhibition of NMDA receptors by guanine nucleotides has been reported previously using radioligand binding assays (Monahan et al., 1988; Baron et al., 1989).

To evaluate further the mechanism by which ATP may regulate neurotransmission at glutamatergic synapses, we examined the effects of ATP on recombinant NMDA receptors expressed in *Xenopus laevis* oocytes. Our results demonstrate that ATP has both direct inhibitory and facilitatory effects on glutamate currents through NMDA receptor channels, which may modulate synaptic plasticity and LTP.

Materials and Methods

Preparation of RNA. Clones of rat NMDA receptor subunits were obtained from Dr. J. Boulter (University of California Los Angeles, Los Angeles, CA). Plasmid cDNA of NR1a, NR2A, NR2B, and NR2C were linearized with NheI, EcoRI, NotI, and BamHI, respectively. Linear templates were used for in vitro synthesis of capped cRNA with either T3 (NR1a and NR2A receptors) or T7 (NR2B receptor) polymerase using the mMessage mMachine transcription kit (Ambion, Austin, Texas).

Expression in *X. laevis* Oocytes. Mature *X. laevis* female frogs were anesthetized by immersion in 0.2% of 3-aminobenzoic acid ethyl ester solution for 15 to 30 min. Harvested ovarian lobes were defolliculated by incubation in 2 mg/ml collagenase dissolved in ND96 media containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5, at room temperature for 2 to 4 h. Subsequently, oocytes were rinsed and incubated for 10 to 15 min in Ca^{2+}-free ND96 solution to remove remaining follicular cells. Selected stage-V and -VI oocytes were stored at 18°C in ND96 media supplemented with 1 mM sodium pyruvate and 0.01 mg/ml gentamycin. NR1a and NR2 cRNAs were mixed in a molar ratio of 1:3 to minimize formation of NR1a monomers. Oocytes were microinjected with 50 nl of the final cRNA mixture (15–30 ng total) into oocyte cytoplasm. Oocytes were incubated in ND96 media at 18°C for 2 to 5 days before electrophysiological measurements.

Electrophysiology. Oocytes were placed in the recording chamber (0.1 ml volume) and continuously perfused with Ca^{2+}- and Mg^{2+}-free solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.3. Membrane currents were recorded using a two-electrode virtual ground voltage-clamp circuit with a GeneClamp 500B amplifier (Axon Instruments Inc., Union City, CA), filtered at 200 Hz, and digitized using a Digidata 1200A interface and pClamp software (Axon Instruments Inc.). Electrodes were filled with 3 M KCl and had resistances of 0.2 to 1 MΩ. Current magnitude was determined by the steady-state plateau response elicited by various concentrations of glutamate in the presence of either 10 or 100 µM glycine at a holding potential of −70 mV unless otherwise indicated. Current-voltage curves were obtained by either measuring steady-state currents in voltage-clamped oocytes at different holding potentials or by applying voltage ramps from −120 to +40 mV during steady-state responses. Net currents were obtained by subtracting currents recorded in the absence (control) from those recorded in the presence of agonists.

Data Analysis. Concentration-response curves for ATP and GTP were fit to the equation:

$$I_{ATP/GTP} = I/(1 + (IC_{50}/[antagonist])^{n_H}) \quad (1)$$

where I is the steady-state current evoked by glutamate, $I_{ATP/GTP}$ is the current after steady-state block by either ATP or GTP, IC_{50} is the concentration of ATP or GTP resulting in 50% block, and n_H is the Hill coefficient describing the steepness of the curve.

Data for glutamate concentration-response relations were fit to an equation of a similar form:

$$I/I_{max} = 1/(1 + (EC_{50}/[glutamate])^{n_H}) \quad (2)$$

where I is the measured current, I_{max} is the maximum current, and EC_{50} is the concentration of glutamate that elicits a half-maximal response.

Schild analysis, that is, a linear regression of log (dose ratio − 1) versus log [antagonist] was performed to estimate the slope and the x-axis intercept (log K_B). Sigma Plot (SSPS Inc., Chicago, IL) was used for all curve fitting. Data are expressed as mean ± S.E.M.

Chemicals. ATP (Na_2ATP and MgATP) was purchased from either Roche Diagnostics Australia Pty. Ltd. (Castle Hill, NSW Australia) or Sigma-Aldrich (St. Louis, MO). The following chemicals were purchased from Sigma-Aldrich: HEPES, 3-aminobenzoic acid ethyl ester, collagenase, pyruvic acid, gentamycin, glutamate, glycine, ADP, AMP, α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP), adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S), CTP, GTP, UTP, DL-2-amino-5-phosphopentanoic acid (APV), (R)-3-[2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (D-CPP), EDTA, and N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). All other chemicals were analytical grade.

Results

The application of various concentrations of glutamate and 10 µM glycine elicited inward currents in voltage-clamped *X. laevis* oocytes injected with NR1a + NR2A, NR1a + NR2B, or NR1a + NR2C recombinant NMDA receptors. No glutamate-evoked currents were recorded from control oocytes (noninjected or injected with sterile water). ATP (0.1–10 mM) applied alone or together with glycine (10 µM) did not activate detectable currents in any of the oocytes tested. The amplitude of glutamate-evoked currents varied between 0.2 and 2 µA and was dependent on the batch of oocytes and days after injection as reported previously by other investigators (e.g., Kleckner et al., 1999). Despite the variability in current amplitude, there was no difference in the potency of ATP and other nucleotides or in agonist/antagonist interactions with the receptors.
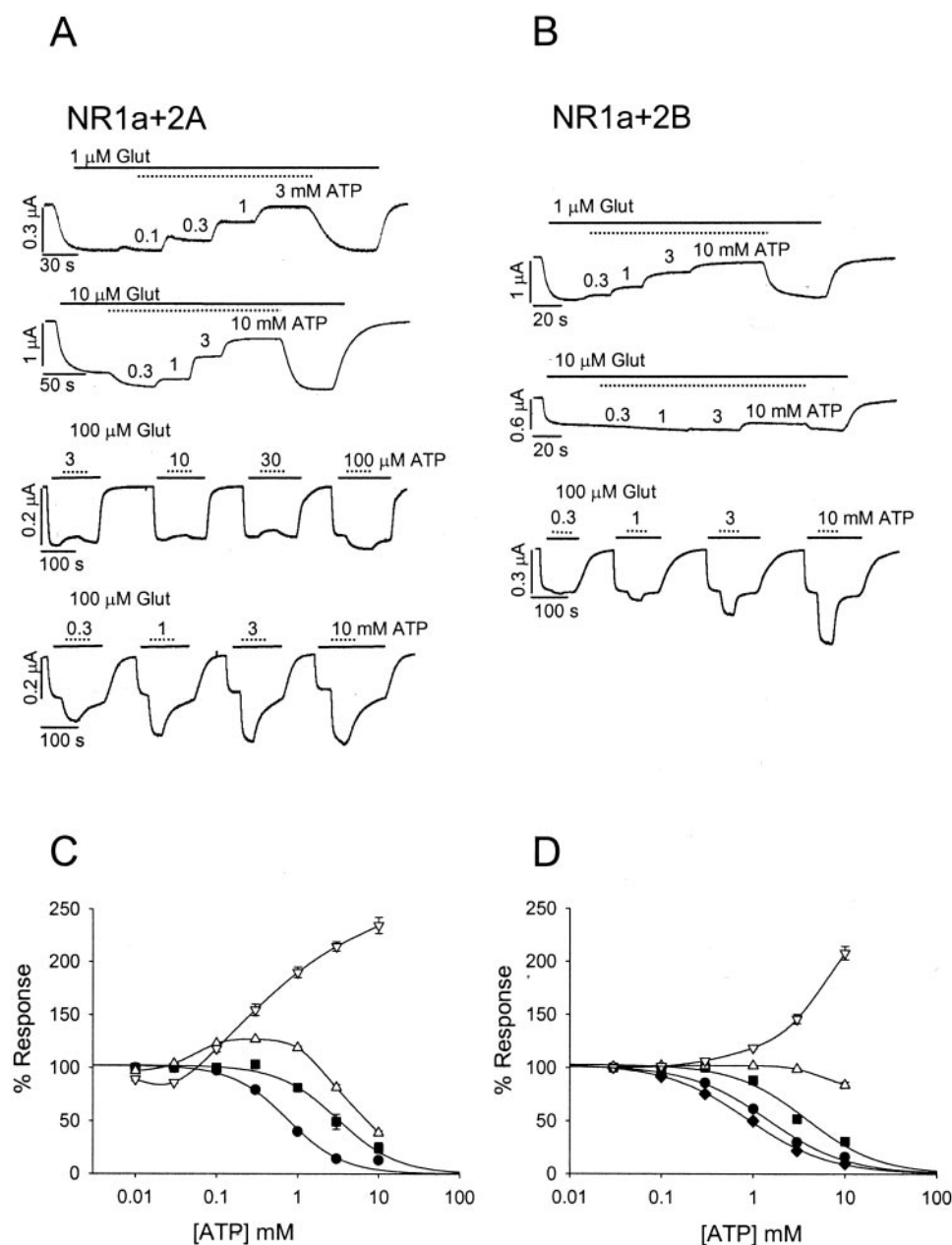
The Effect of ATP on Glutamate-Activated NMDA Receptors Is Biphasic. The effect of extracellular ATP on NR1a + 2A and NR1a + 2B recombinant NMDA receptors

was examined by measuring peak current amplitude in response to fixed concentrations of glutamate (in the presence of 10 μM glycine) in the absence and presence of various concentrations of ATP (Fig. 1). ATP (0.1–10 mM) inhibited glutamate-induced currents in a concentration-dependent manner. ATP suppression of NMDA receptor-mediated currents could be overcome by increasing the concentration of glutamate. For the NR1a + 2A receptor combination, the concentration of ATP that produced half-maximal inhibition (IC_{50}) was 0.6 $\pm$ 0.2 mM and 2.9 $\pm$ 0.6 mM for currents elicited by 1 and 3 μM glutamate, respectively. ATP was less potent at the NR1a + 2B receptor combination, exhibiting IC_{50} values of 0.9 $\pm$ 0.1 mM, 1.5 $\pm$ 0.2 mM, and 3.8 $\pm$ 0.7 mM for ATP inhibition of 0.3, 1, and 3 μM glutamate-evoked currents, respectively.

The effect of ATP on glutamate-evoked currents was biphasic depending on the glutamate concentration (Fig. 1, A–D). For the NR1a + 2A subunit combination, currents induced by 10 μM glutamate (+10 μM glycine) were potentiated by 0.1 to 1 mM of ATP and inhibited by 3 to 10 mM ATP. Increasing concentrations of glycine up to 100 μM did not significantly affect the action of ATP on NR1a + 2A receptors (data not shown). Membrane currents evoked by 100 μM glutamate were potentiated in a concentration-dependent manner over the concentration range 0.1 to 10 mM ATP. For the NR1a + 2B combination, 0.1 to 1 mM ATP did not have a significant effect on currents evoked by 10 μM glutamate, although there was a tendency toward a slight potentiation, whereas 3 to 10 mM ATP produced a small inhibitory effect. In contrast, currents evoked by 100 μM glutamate were potentiated in a concentration-dependent manner by 0.3 to 10 mM ATP (data not shown).

The inhibitory effect of ATP was subunit specific. At low concentrations of glutamate (1 μM), 0.1 to 1 mM ATP failed

Fig. 1. The biphasic effect of ATP on glutamate-evoked currents of recombinant NR1a + 2A and NR1a + 2B NMDA receptors. A and B, the effect of various concentrations of ATP on membrane currents evoked by the application of 1, 10, and 100 μM glutamate recorded from different oocytes expressing NR1a + 2A and NR1a + 2B NMDA receptors, respectively. Glutamate was applied as indicated by the solid horizontal lines. The application of ATP is marked by broken lines. C and D, concentration-response curves obtained for ATP inhibition and potentiation of glutamate-induced currents derived from data shown in A and B. For the NR1a + 2A subunit combination (C), steady-state currents were induced by 1 ($\bullet$), 3 ($\blacksquare$), 10 ($\triangle$), and 100 μM glutamate ($\triangledown$) and measured in the presence of various concentrations of ATP. Similar measurements were performed for the 0.3 ($\blacklozenge$), 1 ($\bullet$), 3 ($\blacksquare$), 10 ($\triangle$), and 100 μM ($\triangledown$) glutamate-evoked currents of NR1a + 2B receptors (D). Lines connecting closed symbols are the best-fit curves of eq. 1 to the data, whereas lines connecting open symbols were drawn by hand. Data are expressed as a percentage of control glutamate currents (n = 5–20 oocytes for NR1a + 2A and 5–13 oocytes for NR1a + 2B at each concentration tested).

to inhibit currents through NR1a + 2C receptors, whereas higher concentrations of ATP (3–10 mM) significantly potentiated the glutamate-evoked currents (Fig. 2A, upper trace). Increasing concentrations of glutamate (3–100 μM) markedly enhanced the concentration-dependent potentiation by ATP, with the maximum obtained with 100 μM glutamate (Fig. 2, A, lower trace and B). The potentiation by ATP of glutamate currents through NR1a + 2C was not dependent on voltage (Fig. 2C). Furthermore, ADP also potentiated currents evoked by 100 μM glutamate in a concentration-dependent manner at NR1a + 2C receptor combination (Fig. 2D). Similar results for ADP potentiation of glutamate-evoked currents were obtained for NR1a + 2A (data not shown). ADP ($\leq$3 mM) did not inhibit NR1a + 2C NMDA receptor-mediated currents evoked by low concentrations ($<$10 μM) of glutamate.

ATP Does Not Bind in the Channel Pore Nor Act as a Heavy Metal Chelator. The mechanism underlying the inhibitory effect of ATP on glutamate-evoked currents at NMDA receptors was further examined by testing the effect of membrane potential on the inhibition of glutamate (1 μM) responses by MgATP and Na_2ATP (Fig. 3). Antagonism of glutamate-activated currents by Na_2ATP was independent of voltage for both NR1a + 2A and NR1a + 2B receptors. In contrast, antagonism by MgATP exhibited a voltage dependence as described previously for Mg^{2+} block of the NMDA receptor channel at hyperpolarized membrane potentials (see review by Mayer et al., 1992). These results indicate that ATP inhibition is not caused by residual Mg^{2+} ions present in

the recording solution and that ATP acts outside of the membrane electric field.

To exclude the possibility that the potentiating effect of ATP could be caused by the chelation of contaminating Zn^{2+} and subsequent removal of tonic Zn^{2+} inhibition (Paoletti et al., 1997), a series of experiments was carried out in the presence of heavy metal chelators. The addition of either TPEN (1 μM) or EDTA (10 μM) failed to abolish the potentiation of glutamate-evoked currents by ATP at NR1a + 2B (Fig. 4, B and D) and zinc-insensitive NR1a + 2C receptors (data not shown). Both heavy metal chelators potentiated glutamate-induced currents at zinc-sensitive NR1a + 2A receptors, consistent with that reported previously (Paoletti et al., 1997), and thus reduced ATP potentiation (Fig. 4, A and C).

Competitive Antagonism by ATP at the Glutamate Binding Site. To evaluate the competitive interaction between ATP and glutamate for the agonist binding site, the concentration dependence of ATP modulation was examined. Figure 5, A–D shows the effect of glutamate concentration on ATP inhibition of glutamate-activated currents through NR1a + 2A, NR1a + 2B, and NMDA receptors. In the absence of ATP, the half-maximal activation (EC_{50}) of NR1a + 2A, NR1a + 2B, and NR1a + 2C receptors by glutamate was 2.8 $\pm$ 0.2 μM, 2.1 $\pm$ 0.3 μM, and 2.2 $\pm$ 0.3 μM, respectively. In the presence of ATP, the glutamate concentration-response curves were shifted to the right without a significant change in slope. The EC_{50} values for glutamate concentration-response curves in the presence of 0.3, 1, 3, and 10 mM
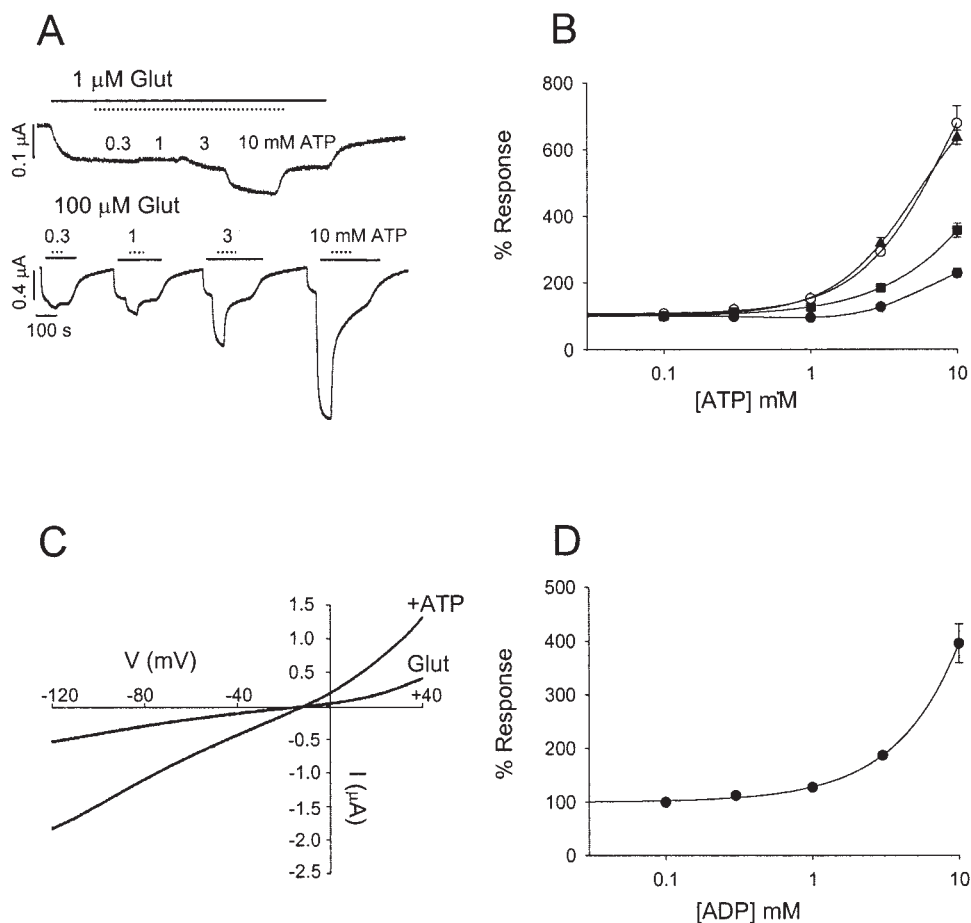

Fig. 2. Potentiation by ATP of glutamate-evoked currents at recombinant NR1a + 2C NMDA receptors. A, representative traces of ATP concentration-dependent potentiation of glutamate-evoked currents recorded from different oocytes expressing NR1a + 2C NMDA receptors. Glutamate and ATP were applied as indicated by the solid and broken horizontal lines, respectively. B, concentration-response curves obtained for ATP potentiation of glutamate-induced currents. Steady-state currents were evoked by 1 (●), 3 (■), 10 (▲), and 100 μM glutamate (○), and amplitude was measured in the presence of various concentrations of ATP ($n \geq 5$ oocytes at each concentration). C, current-voltage curves evoked by voltage ramps applied during steady-state responses induced by 100 μM glutamate in the absence and presence of 3 mM ATP ($n = 9$). D, concentration-response curve obtained for ADP potentiation of 100 μM glutamate-induced currents ($n = 5$).

ATP were: 5.9 ± 0.1 μM, 11.4 ± 0.5 μM, 21 ± 1.3 μM, and 57.4 ± 3.6 μM ($n = 7$–26 oocytes), respectively, for NR1a + 2A; and 2.4 ± 0.02 μM, 3.1 ± 0.2 μM, 5.5 ± 0.3 μM, and 10.6 ± 1.7 μM ($n = 5$–13 oocytes), respectively, for NR1a + 2B receptor subunit combinations. Hill coefficients determined for each of the glutamate concentration-response curves were between $n_H = 1.4$ and 1.5. Schild regression analysis calculated from these data is shown in Fig. 5, E and F. For both NR1a + 2A and NR1a + 2B receptors, the dose ratio increased linearly with ATP concentration. The Schild regression had a slope close to unity for both NR1a + 2B and NR1a + 2A receptors. The potency (pA_2) was 3.6 for NR1a + 2A and 2.7 for NR1a + 2B, which corresponds to K_B values of 0.26 and 2.14 mM, respectively.

Effects of Nonhydrolyzable Analogs and Other Nucleotides on Glutamate-Evoked Currents. The effect of nucleotides other than ATP, such as ADP, CTP, GTP, and UTP, and the nonhydrolyzable analogs, ATP-γ-S and α,β-MeATP, applied at concentrations of 1 mM were investigated on the NR1a + 2A receptor activated by 1 μM glutamate. GTP was the most potent inhibitor, producing $95.6 \pm 1.5\%$ inhibition compared with ATP ($60.1 \pm 1.1\%$). The concentration dependence of GTP in-

hibition of the NR1a + 2A receptor on currents evoked by 1 and 10 μM glutamate is shown in Fig. 6, A and B. Analogous to ATP, GTP (10 μM-1 mM) produced a concentration-dependent inhibition of 1 μM glutamate-evoked currents but with a significantly lower IC_{50} (0.1 ± 0.01 mM) than that observed for ATP. Raising the glutamate concentration to 10 μM shifted the GTP concentration-response curve to the right (IC_{50}, 0.4 ± 0.03 mM). Inhibition of glutamate-evoked currents was obtained with 0.3 to 10 mM GTP; however, potentiation was observed with low (30–100 μM) GTP concentrations. CTP and UTP (1 mM) were less potent than either ATP or GTP, producing only $35.9 \pm 1.9\%$ and $19.4 \pm 1.5\%$ inhibition of glutamate-evoked currents, respectively. The reduced potency of ADP ($15.6 \pm 1.9\%$) and the lack of sustainable inhibition of glutamate-evoked currents by AMP suggest that potency is decreased with the removal of each phosphate (Fig. 6, C and D). Although the nonhydrolyzable analog ATP-γ-S produced an inhibitory response ($65.1 \pm 1.0\%$) similar to that obtained with ATP, α,β-methylene-ATP failed to elicit a sustainable inhibition of glutamate-evoked currents (Fig. 6C). These results indicate that the phosphate chain of nucleotides is essential for the inhibitory
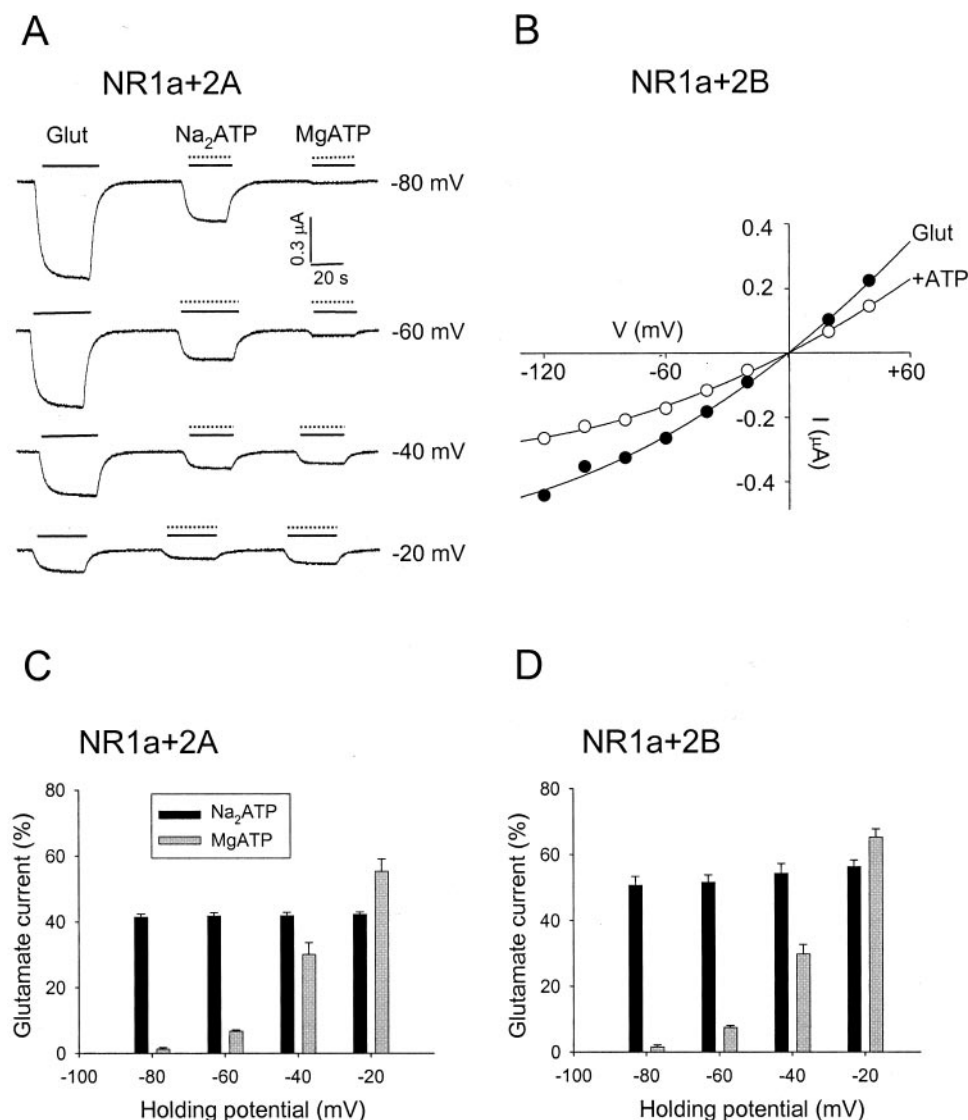
Fig. 3. Effect of membrane potential on ATP inhibition of glutamate-evoked currents of NR1a + 2A and NR1a + 2B receptors. A, representative currents recorded from the same oocyte expressing NR1a + 2A receptors showing 1 μM glutamate-evoked responses (solid lines) before and after steady-state block by 1 mM Na$_2$ATP or MgATP (broken lines) at different holding potentials. B, current-voltage relation for NR1a + 2B receptors activated by 1 μM glutamate in the absence (●) and presence (○) of 1 mM Na$_2$ATP from the same oocyte voltage clamped at -120, -100, -80, -60, -40, -20, $+20$, and $+40$ mV. C and D, bar graphs summarizing the effects of Na$_2$ATP and MgATP on normalized glutamate current amplitude obtained from data similar to and including those shown in A and B. Current amplitudes are expressed as a percentage of the control current evoked by 1 μM glutamate. Bars represent data from four to five oocytes.

response and that the response is stringent with regard to the ring structure or its modification.

NMDA Receptor Antagonists Do Not Alter ATP Potentiation. Coapplication of either of the NMDA receptor antagonists, D-CPP (10–100 nM) or APV (3–10 μM), with 1 mM ATP enhanced the block of 1 μM glutamate-induced currents mediated by NR1a + 2A and NR1a + 2B receptors (Fig. 7, A and B). The inhibitory effects of ATP and CPP were approximately additive. In contrast, ATP potentiation of currents evoked by 100 μM glutamate was not altered when CPP was coapplied (Fig. 7, C–E). This confirms that the potentiation does not result from ATP binding to the agonist/antagonist binding site. Instead, it must bind to a physically distinct site where it acts as a positive allosteric modulator of channel gating.

A Simple Model of the NMDA Receptor. The experimental results were compared with the prediction of a model of the NMDA receptor to test the plausibility of the hypothesis that there are two separate ATP binding sites. For simplicity, it was assumed that the NMDA receptor has a single site where glutamate, CPP, and ATP can all bind. The receptor also has a modulatory site where only ATP can bind. When glutamate is bound to the first site but the modulatory site is vacant, the channel opens with a low probability; but when ATP is bound to the modulatory site, the channel opens with a higher probability (Fig. 8, A and B). Although this model is simplified, it was still able to reproduce all the main features of the data. A series of ATP concentration-response curves was generated in the presence of 0.3, 1, 10, and 100 μM glutamate (Fig. 8C). The simulated curves closely matched the experimental concentration-response curves (Fig. 1C). The model also predicted a systematic shift to the right in the glutamate concentration-response curves with increasing ATP concentrations (Fig. 8D; experimental results in Fig. 5, C and D). It predicted that the inhibition produced by CPP and ATP would be additive at low glutamate concentrations and that the potentiation produced by ATP at high glutamate concentrations would not be affected by CPP (simulation data not shown; experimental results in Fig. 7, B, D, and E).

Discussion

Interactions between neurotransmitters play an important role in the modulation of synaptic transmission. State-depen-
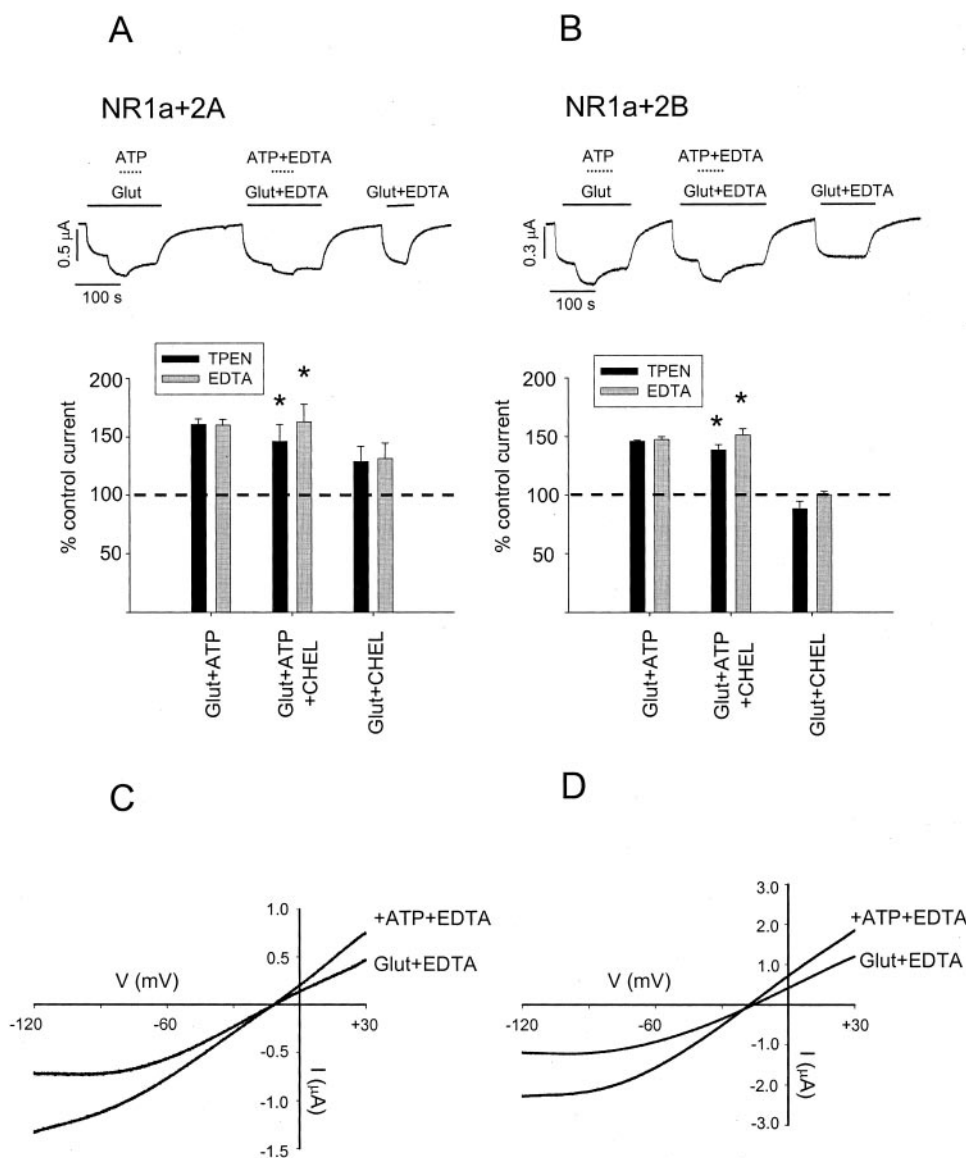

Fig. 4. Heavy metal chelators, TPEN and EDTA, do not prevent ATP potentiation of NMDA receptors. A and B, representative traces and bar graphs of ATP potentiation of glutamate-induced currents mediated by NR1a + 2A and NR1a + 2B NMDA receptors, respectively, in the absence and presence of either 10 μM EDTA or 1 μM TPEN as indicated. Glutamate (100 μM) was applied as indicated by the horizontal solid lines, and ATP application (1 mM for NR1a + 2A and 3 mM for NR1a + 2B) is indicated by the broken lines. $\star$, significantly different from glutamate + chelator controls (n = 5 oocytes for NR1a + 2A and n = 4 oocytes for NR1a + 2B). C and D, current-voltage relations obtained for glutamate-activated NR1a + 2A and NR1a + 2B NMDA receptors in the absence (glutamate + 10 μM EDTA) and presence of ATP (1 mM for NR1a + 2A and 3 mM for NR1a + 2B) and ATP + EDTA. Currents were evoked by 100 μM glutamate, and voltage ramps were applied during steady-state responses before (glutamate + EDTA) and after addition of ATP + EDTA.

dent cross-inhibition between purinergic (P2X) and nicotinic acetylcholine receptors has been demonstrated recently by coexpression of nicotinic and P2X receptor channels in *X. laevis* oocytes (Khakh et al., 2000). In the present study, we examined the interaction between glutamate and ATP at recombinant NMDA receptors expressed in *X. laevis* oocytes. Our results reveal a mechanism distinct from that proposed for nicotinic and P2X receptors. While this work was in progress, a study by Ortinau et al. (2003) reported that ATP inhibited glutamate-evoked currents through NR1a + 2B receptors and rescues hippocampal neurons from NMDA-mediated neurotoxicity. In contrast, our study demonstrates a biphasic effect of ATP at both NR1a + 2A and NR1a + 2B NMDA receptors and potentiation at NR1a + 2C receptors.

Expression of heteromeric NR1a + 2A and NR1a + 2B NMDA receptors in *X. laevis* oocytes yielded functional glutamate-gated channels. Extracellular ATP inhibited gluta-

mate-evoked currents at low glutamate concentrations (≤ 10 μM) but potentiated currents evoked by high concentrations of glutamate (100 μM). Thus, the action of ATP at NMDA receptors is biphasic, acting as an antagonist of NMDA receptors at unsaturated glutamate concentrations and a positive allosteric modulator at glutamate concentrations close to saturation. ATP suppression of NMDA receptor-mediated currents could be surmounted by increasing concentrations of glutamate, shifting the ATP concentration-response curves to the right. In addition, the rightward shift of the glutamate concentration-response curves obtained in the presence of ATP provides further support for a competitive inhibition at the agonist binding site on NR1a + 2A and NR1a + 2B receptors. In contrast, ATP did not compete for the agonist site on NR1a + 2C receptors and potentiated their response even at nonsaturating levels of glutamate.

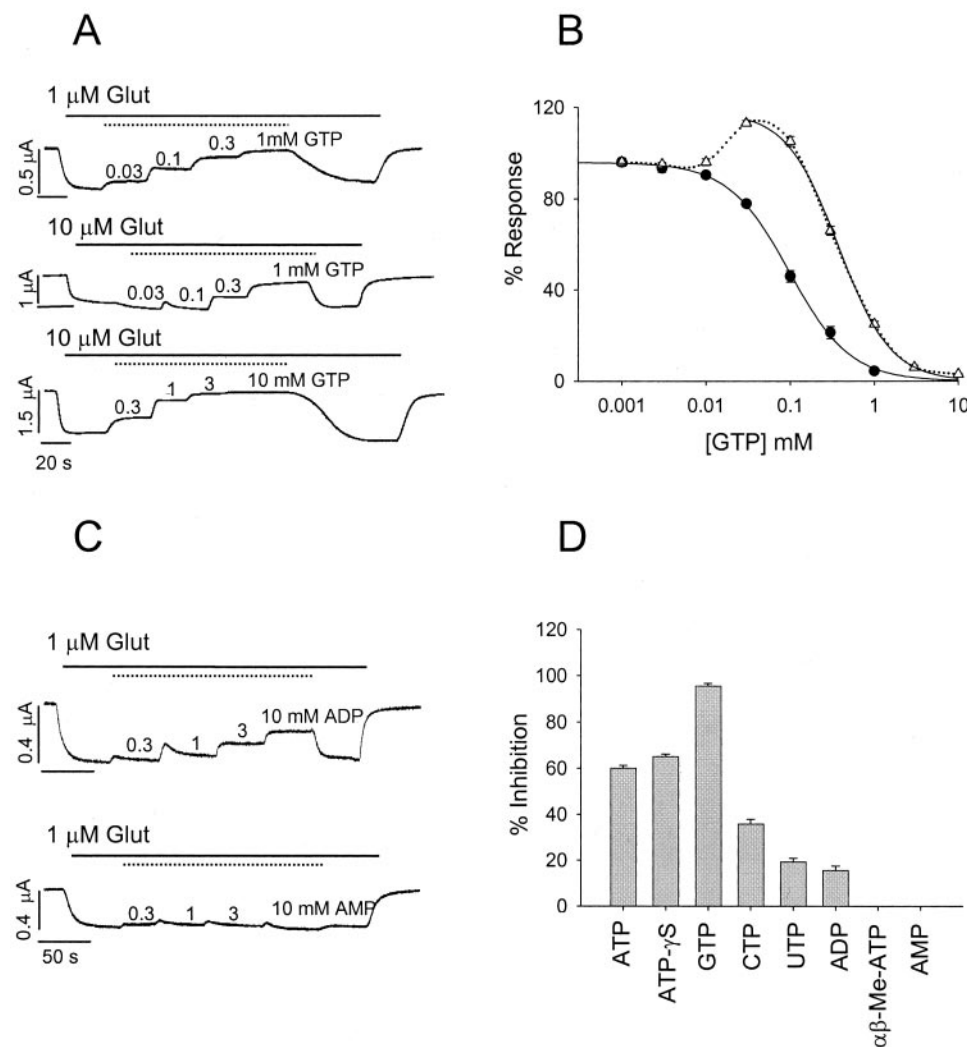ATP inhibition of glutamate-evoked current amplitude re-
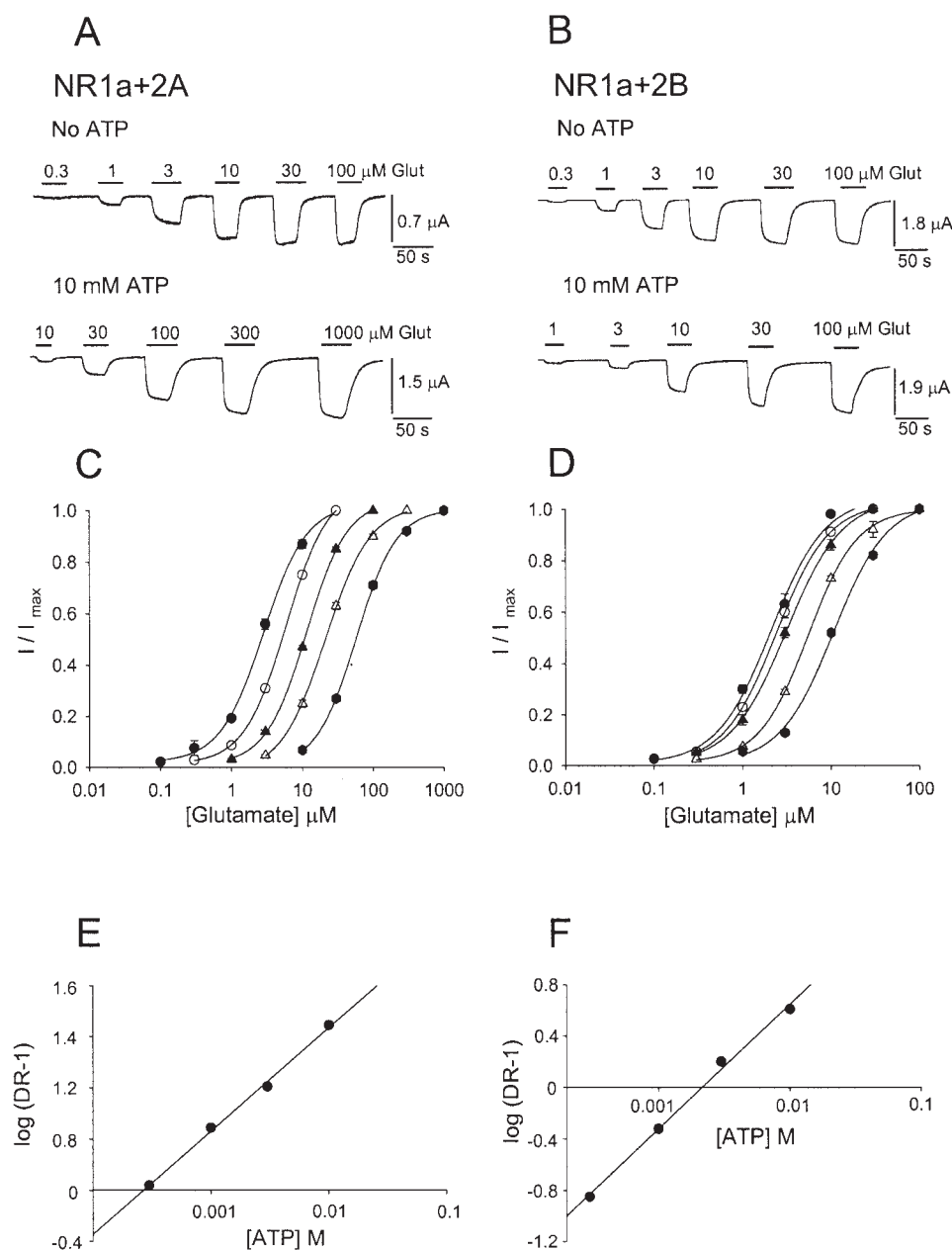
Fig. 5. Competitive interaction of ATP at the glutamate binding site. A and B, representative traces obtained from different oocytes expressing either NR1a + 2A or NR1a + 2B receptor combinations, respectively, showing glutamate responses in the absence (upper traces) and presence (lower traces) of 10 mM ATP. Glutamate application as indicated by the horizontal solid lines. C and D, concentration-response curves for glutamate-evoked currents in oocytes expressing NR1a + 2A and NR1a + 2B receptors, respectively, in the absence ($\bullet$) and presence of 0.3 ($\bigcirc$), 1 ($\blacktriangle$), 3 ($\triangle$), and 10 mM ATP ($\bullet$). Steady-state glutamate current amplitude obtained in the absence and presence of ATP were normalized to the maximum current. The Hill coefficients (n_H) for the glutamate concentration-response curves in the presence of 0.3, 1, 3, and 10 mM ATP were 1.5 $\pm$ 0.04, 1.5 $\pm$ 0.1, 1.4 $\pm$ 0.1, and 1.5 $\pm$ 0.1, respectively, for NR1a + 2A and 1.5 $\pm$ 0.02, 1.4 $\pm$ 0.1, 1.5 $\pm$ 0.1, and 1.4 $\pm$ 0.1, respectively, for NR1a + 2B receptor subunit combinations. n = 7 to 26 oocytes for NR1a + 2A and 5 to 13 oocytes for NR1a + 2B at each concentration. E and F, Schild regressions derived from the data shown in C and D, respectively. The dose ratio was calculated as the EC_{50} for glutamate in the presence of ATP over the EC_{50} for glutamate in the absence of ATP.

mained constant with different holding potentials, indicating that the action of ATP is not influenced by the membrane electric field and thus its site of interaction is unlikely to be in the NMDA receptor channel pore. Other nucleotides, including ADP, GTP, CTP, and UTP, inhibited glutamate-evoked currents with different potencies, revealing that the inhibition is dependent on the phosphate chain as well as the nucleotide ring structure. The rank order of inhibitory potency at the NR1a + 2A receptor (GTP > ATP > CTP > UTP > ADP ≫ AMP) differs from their rank order of potency at any of the cloned P2X receptors (North, 2002). Although guanine nucleotides and their analogs have been reported previously to selectively inhibit agonist (l-[^{3}H]glutamate) and antagonist ([^{3}H]D-CPP) binding to the NMDA receptor (Monahan et al., 1988; Baron et al., 1989), GTP at low concentrations (10–100 μM) could also potentiate glutamate-evoked currents though the NR1a + 2A receptor. Further studies of the mode of nucleotide-receptor interaction may guide the design of novel and selective modulators of the NMDA receptor.

Glutamate-induced (1 μM) currents mediated by NR1a + 2A and NR1a + 2B receptors were inhibited by coapplication of D-CPP (10–100 nM) and APV (3–10 μM) with 1 mM ATP. The inhibitory effects of ATP and CPP were approximately additive. On the other hand, ATP potentiation of currents evoked by high concentrations of glutamate was unchanged by coapplication of either CPP or APV. These results indicate that ATP potentiates the NMDA receptor by binding to a site distinct from that of the competitive antagonists, CPP and APV. We propose that ATP interacts both with the glutamate binding site and with a second, physically separate modulatory site. At nonsaturated concentrations of glutamate, ATP-competitive antagonist actions dominate; whereas at saturating concentrations of glutamate, ATP increases the open probability of the channel via the positive allosteric modulatory site. A semiquantitative model of this effect described below predicts an approximately 5-fold enhancement of the maximal current produced by saturating glutamate (and glycine) concentrations.

The stoichiometry of NMDA receptors is thought to be tetrameric, consisting of two NR1 and two NR2 subunits (Laube et al., 1998). The glutamate binding site has been localized to the NR2 subunit (Laube et al., 1997), whereas the binding site for the coagonist glycine resides on the NR1 subunit (Hirai et al., 1996). Thus, two molecules of glutamate are required for activation of the heteromeric complex (Clements and Westbrook, 1994). The Hill coefficient values of >1 obtained in our experiments for glutamate in the absence and presence of ATP are consistent with these observations and suggest that the NMDA receptor may also harbor one or two

Fig. 6. Nucleotides inhibit glutamate currents of NMDA receptors with different potencies. A, current traces showing the modulatory effect of GTP on 1 μM glutamate-evoked currents. Potentiation (in the presence of 30 and 100 μM GTP) and inhibition (in the presence of 0.3–10 mM GTP) of NR1a + 2A receptor mediated currents evoked by 10 μM glutamate. B, GTP concentration-response curves obtained from data similar to and including those shown in A. Closed and open symbols represent data points for 1 and 10 μM glutamate-induced currents, respectively. Solid lines are the best-fit curves of eq. 2 to the data. The curve drawn to data points indicates the potentiation and inhibition observed for currents evoked by 10 μM glutamate. Data are expressed as a percentage of control glutamate currents (n = 5–18 oocytes at each concentration). C, concentration-dependent inhibition by ADP and lack of effect of AMP on the currents evoked by application of 1 μM glutamate. Solid and broken lines in A and C indicate the application of glutamate and nucleotides, respectively. D, the effect of 1 mM ATP, nonhydrolyzable ATP analogs, and other nucleotides on the 1 μM glutamate-evoked currents in oocytes expressing NR1a + 2A receptors. Bars represent data from 5 to 10 oocytes/treatment group.

ATP binding sites that each overlap the glutamate binding site. We have developed a model of the interaction of ATP with the NMDA receptor that predicts the potentiation of currents by ATP at saturating concentrations of glutamate as well the effects of CPP on NMDA receptor function. It suggests that ATP binds with a 15-fold lower affinity to the modulatory site (K_d, ~3 mM) compared with the agonist site (K_d, ~200 μM). It is possible to predict, using the model, the influence of ATP on synaptic function under a range of conditions. If ATP is coreleased with glutamate into the synaptic cleft, then the high concentration pulse of glutamate (1 mM, 1 ms; Clements, 1996) will prevent ATP from binding to the agonist site. Thus, the predominant effect of ATP will be a potentiation of the synaptic current mediated by the NMDA receptor. If ATP transiently reaches a concentration >3 mM in the cleft, then a large potentiation (~5-fold) is predicted, whereas lower concentrations of ATP would produce less potentiation. Potentiation is also expected to dominate if ATP is not coreleased but instead diffuses into the cleft from a remote release site (volume transmission). Experiments with
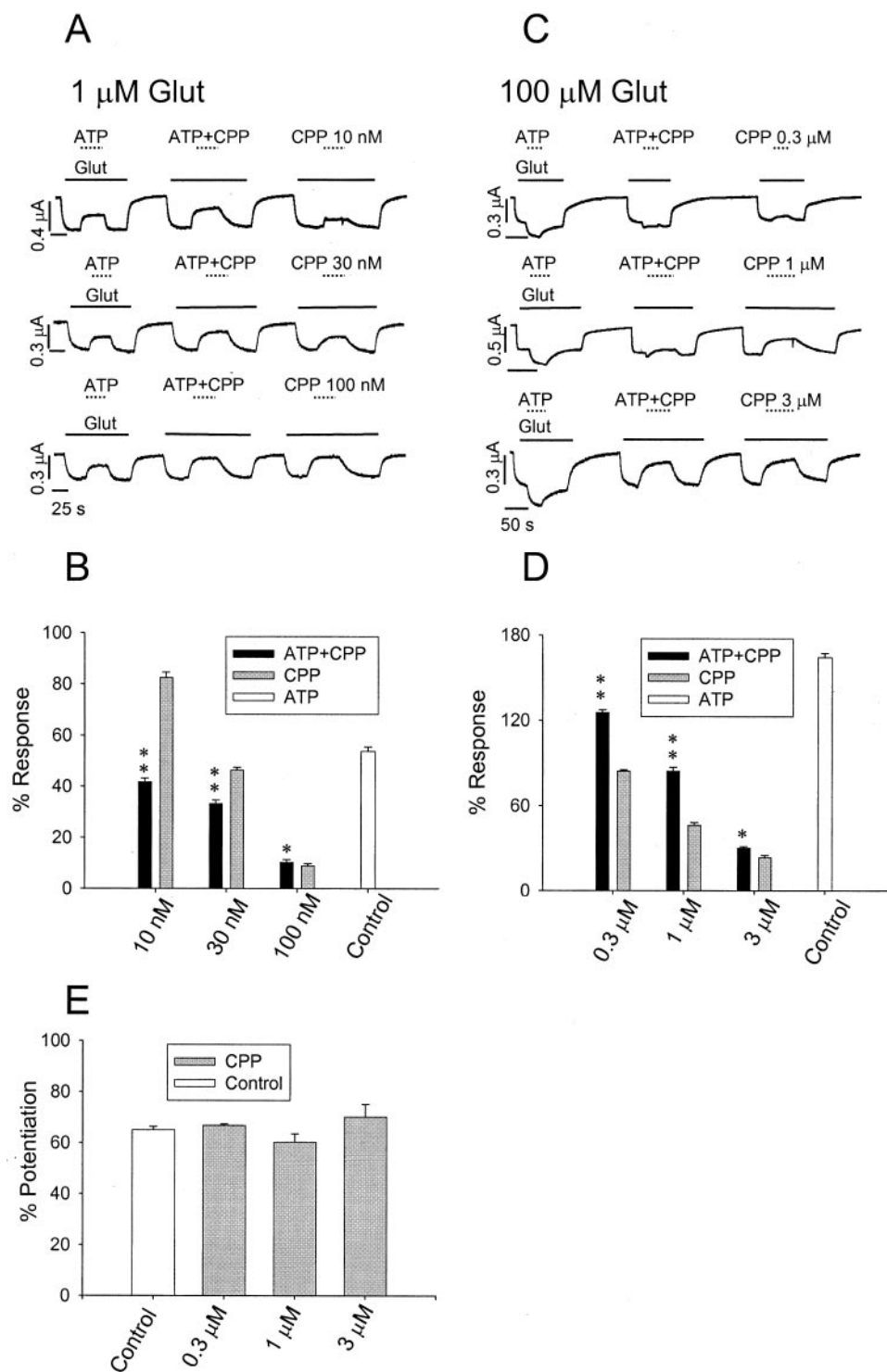
Fig. 7. Effect of NMDA receptor antagonist D-CPP on ATP inhibition and potentiation of glutamate-evoked currents. Representative traces show inhibition of 1 μM glutamate-induced currents by 1 mM ATP in oocytes expressing NR1a + 2A receptors in the presence or absence of 10 to 100 nM CPP (A). B, bar graph of the inhibition of 1 μM glutamate-induced current by 1 mM ATP in the presence and absence of 10 to 100 nM CPP for NR1a + 2A subunit combination (n = 7 oocytes/treatment group). C, representative traces of currents recorded from oocytes expressing NR1a + 2A receptors and the potentiation of 100 μM glutamate-evoked currents by 1 mM ATP in the absence and presence of 0.3 to 3 μM CPP. In A and C, glutamate was applied as indicated by the solid horizontal lines. The application of ATP and CPP is marked by a broken line. D, bar graph summarizing data similar to and including those shown in C (n = 5 oocytes/treatment group). Data in B and D are expressed as a percentage of control glutamate currents. *, significantly different from ATP control; **, significantly different from CPP and ATP control ($P <$ 0.01; paired t test).). E, the percentage potentiation produced by ATP in the presence of various concentrations of CPP. Values not significantly different from control ($P <$ 0.01; paired t test).

the low-affinity competitive antagonist, D-aminoadipate, reveal that synaptically released glutamate can partially displace it from the NMDA receptor on the timescale of synaptic transmission (Clements et al., 1992). The affinity of D-aminoadipate (K_d, 30 μM) is nearly an order of magnitude higher than the affinity of ATP; thus, ATP, even at millimolar concentrations, should be almost completely displaced from NMDA receptors during synaptic transmission, leaving only potentiation. This mechanism may underlie the observation that ATP applied to hippocampal slices amplified the magnitude of the population spikes and induced LTP (Wieraszko and Ehrlich, 1994; Fujii et al., 2002). On the other hand, ATP at moderate to high concentrations could inhibit the activation of NMDA receptors by low concentrations of glutamate produced by spillover from neighboring synapses. Thus, ATP can act to focus and enhance the effects of glutamate at regions near transmitter release sites.

Trace amounts of extracellular Zn^{2+} (<1 μM) tonically inhibit NMDA receptors (Paoletti et al., 1997). At the high concentrations used in this study, ATP chelates Zn^{2+} ions (ZnATP^{2-} log K_1, 4.76; Sillen and Martell, 1971) and potentiates the response of zinc-sensitive NR1a + 2A receptors. However, this mechanism accounts for only part of the potentiation produced by ATP at NR1a + 2A receptors and does not account for any of the potentiation at NR1a + 2B and NR1a + 2C receptors. When tonic zinc inhibition is removed by heavy metal chelators (EDTA and TPEN), the addition of ATP further potentiates the response of NR1a + 2A receptors. Chelators do not alter the response of NR1a + 2B and NR1a + 2C receptors, consistent with their much lower sensitivity to Zn^{2+} inhibition (Paoletti et al., 1997), yet ATP and analogs potentiate the response of these receptors. The potentiation of zinc-sensitive NR1a + 2A receptor is observed not only for ATP but also for ADP and GTP, which have different dissociation constants with zinc (Sillen and Martell, 1971). Taken together, our results demonstrate that ATP acts directly at NMDA receptors. Zn^{2+} can be released into the synaptic cleft at concentrations of nearly 1 μM, so at some synapses, ATP may further potentiate NMDA receptors by chelating Zn^{2+}. The dominant action of ATP during synaptic transmission could be to potentiate the NMDA receptor-mediated responses.

The bidirectional and subunit-specific modulation of the NMDA receptor by ATP may have significant physiological and pathological implications. For example, glutamate-mediated excitotoxicity results in neurodegeneration followed by loss of receptors and deficiency in neurotransmission (Olney, 1990). ATP is released from presynaptic nerve terminals in response to electrical stimulation (Zimmermann, 1994) and therefore, it is possible that ATP can modify glutamate-evoked signals by enhancing transient synaptic responses to high concentrations of glutamate during periods of intense neuronal activity triggered by events such as LTP, seizure, or stroke. On the other hand, ATP may attenuate chronic activation of NMDA receptors by low levels of glutamate, thus preventing excitotoxic neuronal degeneration such as that seen in Alzheimer's disease.

In conclusion, the present study shows that ATP can act directly on NMDA receptors and therefore reveals a potential novel role for ATP as a modulator of synaptic transmission and plasticity. These results indicate that state-dependent cross-interaction may occur not only between distinct receptor types but also between different neurotransmitters for a
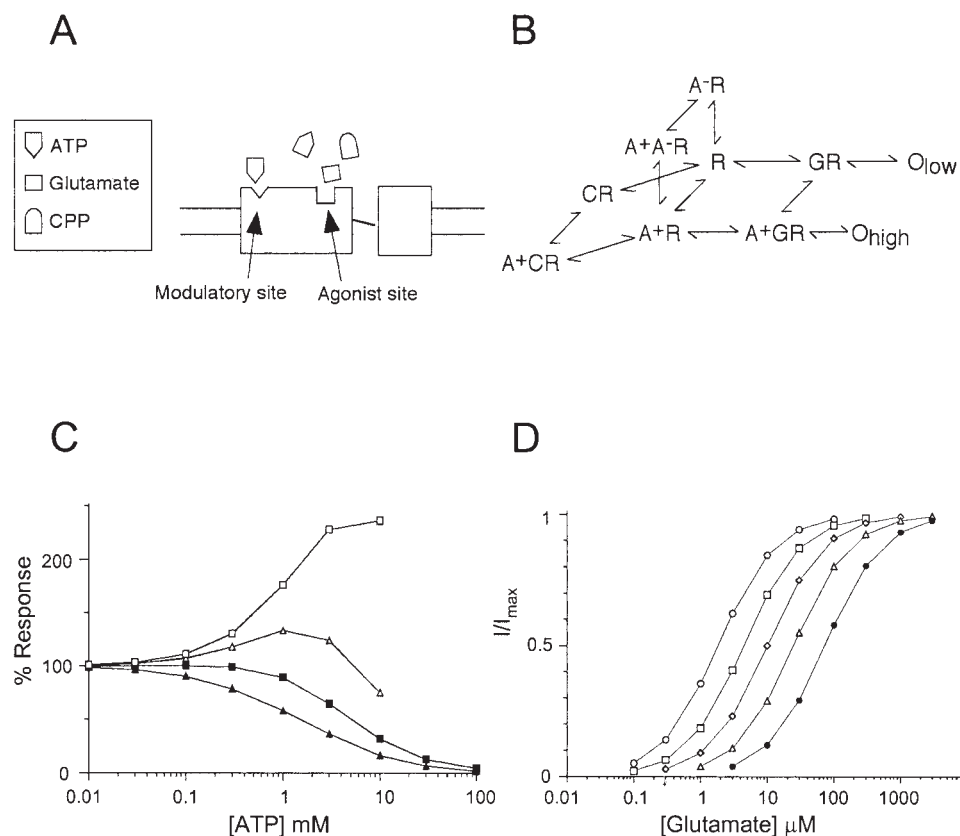
Fig. 8. A model of the modulation of the NMDA receptor by ATP. A, schematic showing a simplified model of the NMDA receptor. The receptor has a single agonist binding site where glutamate, CPP, or ATP can bind and a modulatory site where only ATP can bind. The channel pore opens with low probability when glutamate binds to the agonist site and with higher probability when ATP also binds to the modulatory site. B, reaction scheme corresponding to the model show in A. R is the NMDA receptor, G is glutamate, C is CPP, A$^-$ is ATP bound to the agonist site, and A$^+$ is ATP bound to the modulatory site. Reaction rates for glutamate and CPP binding and unbinding were set to values published previously (see Benveniste and Mayer, 1991; Clements and Westbrook, 1991). ATP binding rate to the agonist site was set to 10 μM^{-1} s^{-1} and unbinding rate to 2000 s^{-1}. ATP binding rate to the modulatory site was set to 10 μM^{-1} s^{-1} and unbinding rate to 30,000 s^{-1}. C, a series of ATP dose-response curves was simulated in the presence of glutamate, 0.3 (▲), 1 (■), 10 (△), and 100 μM (□). D, a series of glutamate dose-response curves was simulated in the absence (○) and presence of 0.3 (□), 1 (◇), 3 (△), and 10 mM ATP (●).

receptor to yield a particular physiological response in conditions of health and disease.

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