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Studies of the antagonist actions of (RS)-2-amino-3-[5-*tert*-butyl-3-(phosphonomethoxy)-4-isoxazolyl]propionic acid (ATPO) on non-NMDA receptors in cultured rat neurones

[1]W.-M. Dai, [2]B. Ebert, [3]U. Madsen & [1,4]J.D.C. Lambert

[1]Department of Physiology, University of Aarhus, DK-8000 Århus C; [2]Department of Pharmacology and [3]Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

1 Whole-cell patch-clamp recordings from single cultured cortical neurones have been used to study the action of (RS)-2-amino-3-[5-*tert*-butyl-3-(phosphonomethoxy)-4-isoxazolyl]propionic acid (ATPO), which has previously been proposed to be a potent selective antagonist of 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors.

2 ATPO competitively reduced peak responses evoked by semi-rapid applications of AMPA ($K_i = 16$ μM) but had variable effects on plateau responses, which were on average unchanged. Following blockade of AMPA receptor desensitization by cyclothiazide (CTZ, 100 μM), the plateau responses were reduced by ATPO to a similar extent as the peak responses, indicating that ATPO reduces desensitization of AMPA receptors.

3 Semi-rapid application of kainic acid (KA) and the KA receptor-selective agonist, (2S,4R)-4-methylglutamic acid (MeGlu) evoked non-desensitizing responses which were competitively antagonized by ATPO (K_i values: 27 and 23 μM, respectively).

4 Responses to MeGlu were unaffected by CTZ (100 μM), but potentiated 3 fold following blockade of KA receptor desensitization by concanavalin A (Con A, 300 μg ml^{-1}). Responses of spinal cord neurones to MeGlu were blocked by ATPO to a similar extent before and after blockade of KA receptor desensitization by Con A.

5 Although selectively potentiated by Con A, plateau responses to MeGlu were reduced by 69.6% by the AMPA selective antagonist, GYKI 53655 (10 μM). The remaining component was further reduced by ATPO with a K_i of 36 μM, which was not significantly different from that in the absence of GYKI 53655, but was greater than that on responses to AMPA.

6 It is concluded that ATPO is a moderate-potency competitive inhibitor of naturally expressed non-NMDA receptors.

Keywords: AMPA; kainate; (2S,4R)-4-methylglutamic acid; (RS)-2-amino-3-[5-*tert*-butyl-3-(phosphonomethoxy)-4-isoxazolyl]-propionic acid (ATPO); cultured neurones; cyclothiazide; concanavalin A; GYKI 53655

Introduction

L-Glutamate (Glu) is a major transmitter in the mammalian CNS, where its effects are mediated by ionotropic receptors, which are involved in excitatory synaptic transmission and synaptic plasticity (Bliss & Collingridge, 1993), and metabotropic receptors, which are coupled to G-proteins. Over-activation of ionotropic Glu receptors can result in excitotoxic processes which are thought to be involved in a wide range of neurodegenerative disorders, including stroke and seizures (Dingledine *et al*., 1990; Meldrum & Garthwaite, 1990; Choi, 1992). A full understanding of the molecular and pharmacological properties of Glu receptors is not only important for our understanding of the normal functioning of the CNS, but is also essential knowledge in the quest to develop rational, effective treatments for neurological disorders (Krogsgaard-Larsen, 1992; Danysz *et al*., 1995).

Ionotropic Glu receptors are divided into two major classes with markedly different structural, mechanistic and pharmacological properties: *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors, respectively (Watkins & Evans, 1981). On the basis of their relative selectivity for agonists, non-NMDA receptors have been further subdivided into 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid (KA) receptors (Monaghan *et al*., 1989). More recently, molecular biological techniques have shown that non-NMDA receptors are pentameric structures which are assembled from a family of subunits (Hollmann & Heinemann, 1994; Bettler & Mulle, 1995). AMPA-gated receptors are assembled from four subunits, GluR1-4, while KA-gated receptors are assembled from five subunits, GluR5-7 and KA1-2. This confers functional diversity on the assembled receptors (Westbrook, 1994; Ruano *et al*., 1995; Jørgensen *et al*., 1995; Audinat *et al*., 1996), which is further increased by splice variants (Sommer *et al*., 1990) and post-transcriptional RNA editing (Verdoorn *et al*., 1991). Although both AMPA and KA receptors can co-exist on the same neurone, immunoprecipitation and electrophysiological experiments would suggest that subunits from the two classes do not aggregate together to form functional channel complexes (Sommer *et al*., 1992; Puchalski *et al*., 1994, but see Wenthold *et al*., 1994).

AMPA has very low affinity for KA receptors (Werner *et al*., 1991), and is therefore a highly selective agonist for AMPA receptors assembled from GluR1-4. On the other hand, binding studies have shown that AMPA receptors have an appreciable affinity for KA (Hollmann & Heinemann, 1994). Indeed, GluR5-7 receptors show only a 10–50 fold selectivity for KA compared to GluR1-4 receptors (Clarke *et al*., 1997).

[4] Author for correspondence.

However, the response evoked by KA on recombinant AMPA receptors is characterized by showing little desensitization, which contrasts to the rapidly desensitizing responses evoked on KA receptors (Egebjerg *et al*., 1991). It should be noted, however, that the action of KA on naturally expressed AMPA receptors is associated with a very rapidly desensitizing response (Patneau *et al*., 1993).

Within the KA receptors, KA1-2 represent high affinity kainate-binding sites (Werner *et al*., 1991), whereas GluR5-7 represent low affinity kainate-binding sites (Bettler *et al*., 1992). As mentioned above, the prototypical agonist, KA, shows relatively poor discrimination between KA and AMPA receptors. However, it has recently been shown that (2S,4R)-4-methylglutamic acid (MeGlu) is a highly selective ligand for KA receptors (Gu *et al*., 1995) which displaces ^{3}[H]-kainic acid binding to wild-type non-NMDA receptors with an 800 fold selectivity compared to AMPA binding (Zhou *et al*., 1997). Initial steps have also been taken to characterize the activity profile of MeGlu in electrophysiological studies on cloned and naturally expressed receptors (Zhou *et al*., 1997; Wilding & Huettner, 1997; Jones *et al*., 1997). Three distinct concentration-dependent features of the action of MeGlu have emerged: (1) Agonist action at KA receptors. A direct agonist action at low μM concentrations consisting of a rapidly and completely desensitizing response. This has been demonstrated for both cloned GluR6 (Zhou *et al*., 1997; Jones *et al*., 1997) and naturally expressed KA receptors on dorsal root ganglion (DRG) neurones (Zhou *et al*., 1997; Jones *et al*., 1997), which are probably composed of GluR5 and KA2 subunits (Herb *et al*., 1992; Sommer *et al*., 1992; Partin *et al*., 1993). (2) Antagonist action at KA receptors. Desensitization of KA receptors to MeGlu renders the ionophores inoperable by other agonists, and therefore constitutes a functional antagonism. Interestingly, this action occurs at low nM concentrations, which can be about 100 times lower than for evoking a direct response. This indicates that the receptor complex might enter a desensitized state before opening (Jones *et al*., 1997). (3) Agonist action at AMPA receptors. At relatively high concentrations, MeGlu evokes a non-desensitizing agonist response on cultured cortical neurones which has been proposed to result from an action on naturally expressed AMPA receptors (Zhou *et al*., 1997).

Non-NMDA receptors are involved in rapid glutamatergic synaptic transmission. The precise combination of subunits determines the kinetics and shaping of the EPSP, along with the ionic permeability of the ionophore (Geiger *et al*., 1995; Lerma *et al*., 1997). Discrimination between receptor sub-types presently relies upon the cumulative evidence provided by a combination of pharmacological, electrophysiological and molecular biological techniques (Fletcher & Lodge, 1996). Blockade of receptor desensitization is often used to distinguish between AMPA and KA receptors. The benzothiadiazine, cyclothiazide (CTZ), selectively blocks desensitization of AMPA receptors and greatly potentiates currents gated by these receptors (Yamada & Tang, 1993; Partin *et al*., 1993; Wong & Mayer, 1993; Paternain *et al*., 1996). The specificity of CTZ is underlined by the fact that it does not potentiate responses to KA of heteromeric combinations of GluR5(R) and KA1 expressed in *Xenopus* oocytes (Partin *et al*., 1993). Within the AMPA receptors themselves, however, the sensitivity to CTZ is determined both by the subunit itself and the splice variant (Fleck *et al*., 1996). The plant lectin, concanavalin A (Con A), shows selectivity towards desensitization mediated by KA receptors (Wong & Mayer, 1993; Yue *et al*., 1995). In addition to blocking desensitization, both CTZ and Con A also antagonize agonist-induced responses to a certain extent (Patneau *et al*., 1993; Paternain *et al*., 1996; Wilding & Huettner, 1997).

By a non-competitive action at an allosteric modulatory site, 2,3-benzodiazepines, such as GYKI 52466 and GYKI 53655 inhibit responses gated by cloned and naturally expressed AMPA receptors much more potently than those gated by KA receptors (Renard *et al*., 1995; Paternain *et al*., 1995; Bleakman *et al*., 1996; Lerma *et al*., 1997). However, not all AMPA receptor subunit combinations are equally sensitive to blockade by GYKI 53655. Furthermore, CTZ affects the affinity of the binding site for 2,3-benzodiazepines. This action of CTZ is probably mediated at a separate, but allosterically linked binding site (Johansen *et al*., 1995; Partin & Mayer, 1996).

We have recently become interested in the physiological and pharmacological properties of non-NMDA ionotropic receptors expressed in neurones cultured from various regions of the CNS. Here we report studies on the antagonist profile of (RS)-2-amino-3-[5-*tert*-butyl-3-(phosphonomethoxy)-4-isoxazolyl] propionic acid (ATPO), which has previously been reported to be a potent and selective antagonist of AMPA responses (Madsen *et al*., 1996). Part of this work has been published as an abstract (Dai *et al*., 1997).

Methods

Tissue culture

Neurones were cultured from the cortex of 15–18 day old and from the spinal cords of 13–15 day old Sprague-Dawley rat embryos using methods based on those described previously (Jensen & Lambert, 1984; Kristiansen *et al*., 1991). The nervous tissue was mechanically dissociated and plated into 35 mm petri dishes (Nunclon) containing three cover slips (1×1 cm) which had been coated with poly-D-lysine (5 mg ml^{-1}). Plating medium was based on Minimal Essential Medium (MEM) with Earle's salts (without glutamine) and with L-alanyl-L-glutamine (Glutamax-1) to which the following were added: 10% horse serum (heat inactivated), 10% foetal calf serum (FCS), 50 i.u. ml^{-1} penicillin, 50 μg ml^{-1} streptomycin. Neurones were cultured in a hypoxic humidified gas mixture (85% N_2, 10% O_2, 5% CO_2; Brewer & Cotman, 1989) in a Heraeus (model BB 6220) incubation oven at 37°C. After one day, the plating medium was completely exchanged with feeding medium (2 ml). This had the same composition as the plating medium, except that the FCS was omitted and horse serum was reduced to 5%. The medium was replenished twice a week by exchanging 1 ml with fresh medium. Mitosis was inhibited with 5′-fluoro-2′-deoxyuridine (FUdR, 15 μg ml^{-1}) plus uridine (35 μg ml^{-1}) at the time when visual inspection showed a confluent background layer of cells (usually 3–4 days). All culturing media and chemicals were purchased from Gibco, with the exception of FUdR, uridine and poly-D-lysine, which were purchased from Sigma.

Electrophysiological recording

Electrophysiological recordings were made from neurones 7–14 days after plating. A glass coverslip containing the culture was transferred to the recording chamber (milled from stainless steel with a quartz glass base) mounted on the stage of an inverted microscope equipped with Nomarski optics (Nikon Diaphot 200), where the individual neurones were viewed at $\times 200$. The recording chamber contained 2–3 ml artificial balanced salt solution (ABSS), which was renewed by

constant perfusion at 0.5–1 ml min^{-1} and from the flushing tube of the drug application micro-manifold. Recordings were made at room temperature (20–24°C). The composition of ABSS was (in mM): NaCl 140, KCl 3.5, Na_2HPO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, glucose 10 and HEPES 10 (osmolarity: 310 mosmol l^{-1}). pH was adjusted to 7.35 at 22°C using NaOH. Tetrodotoxin (0.2 μM) was added to the ABSS to block spontaneous regenerative and synaptic activity. Activation of NMDA-operated ionophores was prevented by inclusion of Mg^{2+} and omission of glycine from the ABSS.

Standard patch-clamp techniques (Hamill *et al*., 1981) were used to record from neurones in the whole-cell configuration using a List EPC-7 amplifier. The patch electrodes were manufactured on the morning of the experiment from 1.2 mm o.d. glass (Clark Electromedical) using a P-87 electrode puller (Sutter Instruments). Electrode tips had internal diameters of around 1 μm and resistances of 2–5 MΩ. The patch electrodes were filled just before use with a solution containing (in mM): CsCl 130, $CaCl_2$ 1, TEA-Cl 10, $MgSO_4$ 1, EGTA 10, Leupeptin 0.1, MgATP 2 and HEPES 10 (290–295 mosmol l^{-1}). The pH was adjusted to 7.35 at 22°C using CsOH.

A holding potential (V_h) of −60 mV was used, from which the agonists, whose equilibrium potentials were around 0 mV, evoked inward whole-cell currents. The whole-cell currents and voltage command signals were filtered at 3 kHz and recorded using Axotape software (Axon Instruments) at a sampling rate of 167 Hz. The currents were also plotted on a low fidelity chart recorder (Servogor, model 124) during the experiment and recorded on a digital audio tape (DAT) recorder (BioLogic model DTR-1200) for off-line analysis.

Compounds and their applications

The compounds to be tested were prepared as stock solutions dissolved in ABSS, except cyclothiazide and AMPA, which were dissolved in dimethyl sulphoxide and NaOH, respectively. The stock solutions were stored at −20°C and diluted to the final concentrations on the morning of experiment. The sources of compounds were: KA and Con A were purchased from Sigma: MeGlu and cyclothiazide were purchased from Tocris Cookson; AMPA and ATPO were synthesized at The Royal Danish School of Pharmacy; NBQX was a gift from Novo Nordisk.

The compounds were applied using a semi-rapid 12 channel application system (DAD-12, Adams & List). The micro-manifold consisted of 12 teflon-coated quartz tubes of 100 μm i.d. fused together with a single 200 μm i.d. tube, which was used for flushing with ABSS. The end of this multi-channel assembly was connected by a short piece of silicone tubing to a final common outlet, consisting of a short (5 mm) piece of 100 μm i.d. quartz tube. The outlet was positioned 100–200 μm from the soma of the recorded neurone. Each of the 12 tubes was connected to a reservoir (a 5 ml syringe) to which positive pressure could be applied. The outlet of each reservoir was led through a solenoid valve to the micro-manifold. Operation of the valves, which was controlled by a 386 computer using DAD-12 software (Adams & List), resulted in flow of the test solution out of the micro-manifold, which engulfed the recorded neurone. By applying solutions of different ionic strength to the open tip of a patch electrode, complete exchange of the solution was shown to be achieved within 50–100 ms, depending on the pressure applied to the reservoir.

Between the applications of compounds, the neurone was continually superfused with normal ABSS by gravity feed from the single 200 μm barrel. This prevented hydrodynamic perfusion artifacts, and also hastened recovery following application of the compounds. Perfusion from this barrel was also controlled by a solenoid valve, which was turned off and on concurrently with the application of test substances.

The agonists were usually applied for 15 s every 55 s. Responses did not usually show appreciable 'run-down' and, after establishment of a constant response level to an agonist, the actions of an antagonist/modulator were studied. To ensure that equilibrium conditions had been reached, the neurone was always pre-treated with the antagonist/modulator before co-application with the agonist. The pre-treatment periods are given in the text. Following an interaction, a series of agonist applications was made until a stable level of response was reached. This could take a few minutes following blockade of receptor desensitization. Results were only used if the response recovered to within ±15% of its original size.

Since the semi-rapid application system allowed up to twelve different combinations of agonist and antagonist, it was used for the quantitative pharmacological studies at the single neurone level. Where extremely rapid (sub ms) application of agonists was required, the 'concentration-jump' technique was used to apply solutions to outside-out membrane patches excised from cell somata. For this purpose, a double-barrelled theta-glass tube (o.d., 2.0 mm; wall thickness, 0.3 mm; septum thickness, 0.12 mm; Hilgenberg, Germany) was used. Normal extracellular medium flowed continuously through one of the barrels. The other barrel was connected *via* a multi-way tap to one of five reservoirs (10 ml syringes), each of which contained a test solution, which was selected by manual operation of the tap. Between applications of the test solutions, the drug-containing channel was thoroughly flushed through with the next solution before it was applied. The theta-glass tube was stepped using a piezo-electric element (Burleigh Instruments). By measuring the change in junction potential between two different buffer solutions, the exchange time was determined to be less than 300 μs.

Quantification of responses and data analysis

Responses were quantified by measuring the peak current during application of agonist and, if the responses faded, the 'plateau' current towards the end of the agonist application. Following blockade of receptor desensitization, the agonist-evoked responses often showed a slow fading, which might result from run-down of the ionic gradient. Therefore, the maximum amplitude of these responses was measured and the ratio was expressed in relation to the control plateau response.

Results were analysed on-line on a Pentium computer using pClamp software (Axon Instruments) and Grafit (Erithacus Software), or off-line by playing back from the DAT recorder. Statistical significance was determined using the two-tailed *t*-test with a P value <0.05 for significant difference.

The concentration-response relationships were fitted and plotted according to the following relationship:

$$I = I_{max} \cdot \left(1/1 + (EC_{50}/[\text{ligand}])\right)^{n}$$

where I is the observed current, I_{max} is the maximum current, [ligand] is the agonist concentration, EC_{50} is the agonist concentration evoking a half-maximal response and n is the Hill coefficient.

Antagonist experiments were carried out using three to five concentrations of agonist chosen such that the concentration-response relationship in the presence of a given concentration of antagonist ([B]) was parallel to that in the absence of antagonist. The dose ratio (r) between these two curves was transformed to a K_i value using the equation:

$$r - 1 = [B]/K_i$$

Values are presented as means ± s.e.mean of at least four individual experiments.

Results

Rapid application of AMPA to cultured neurones evokes an inward current which rises rapidly to a peak and then decays by a process of desensitization to a steady-state plateau level, whereas the currents elicited by KA show little or no desensitization (Patneau & Mayer, 1991). We have obtained similar responses in the present study using a semi-rapid localized application system to apply agonists to cultured cortical neurones.

Action of ATPO on responses of cortical neurones to AMPA

As mentioned in the Introduction, ATPO is a novel non-NMDA receptor antagonist showing marked selectivity towards AMPA receptor-mediated responses (Madsen *et al*., 1996). Firstly, we investigated the effect of 100 μM ATPO on responses of cortical neurones to 100 μM AMPA. As shown in Figure 1, ATPO inhibited AMPA-evoked peak responses. Interestingly, however, AMPA-evoked plateau responses could be either enhanced or decreased: 40% (8/20) of plateau responses to AMPA were enhanced in the presence of 100 μM ATPO (Figure 1A). Figure 1B summarizes the effects of 100 μM ATPO on responses to 100 μM AMPA. Peak responses to AMPA were reduced to 58 ± 5.2% of control ($n = 20$; $P < 0.001$), while plateau responses were reduced from

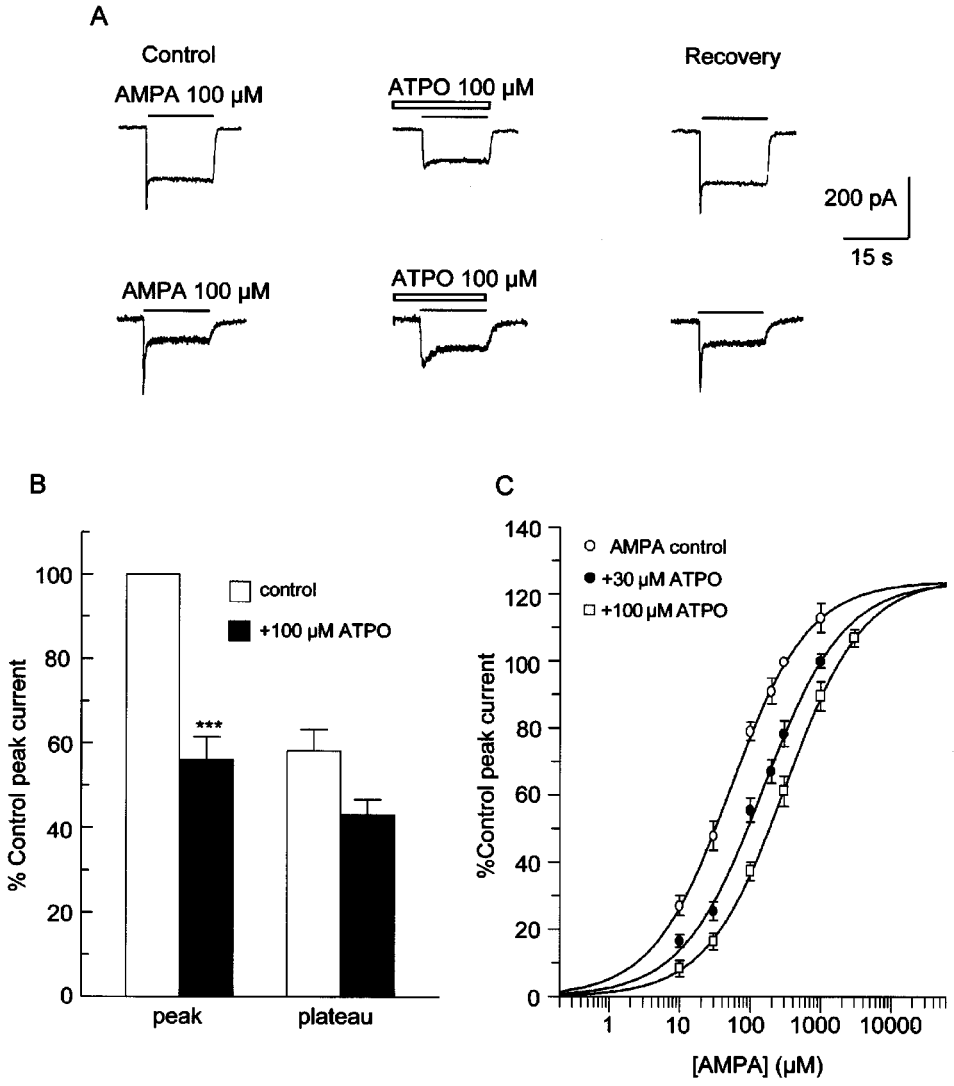

Figure 1 The effect of ATPO on AMPA-induced responses. (A) Representative experiments performed on two cortical neurones. AMPA (100 μM) was applied for 15 s with a 40 s pause between applications. The neurones were pre-treated with 100 μM ATPO for 20 s before concomitant application with AMPA (middle panel). ATPO inhibited peak responses to AMPA, while plateau responses were either depressed (experiment in upper traces) or potentiated (lower traces). The effects of ATPO were reversible on washing (right panel). All data were recorded at a holding potential (V_h) of −60 mV. (B) Summary of the action of 100 μM ATPO on responses to AMPA. Responses for each neurone ($n = 20$) were normalized to the peak control response to 100 μM AMPA and data bars represent means ± s.e.mean. ATPO caused a marked reduction of the peak responses to AMPA ($P < 0.001$) and a small, but not significant, reduction of plateau responses. (C) Concentration-response curves showing ATPO competitively inhibits peak responses of cortical neurones to AMPA. 30 μM ATPO ($n = 7$) and 100 μM ATPO ($n = 5$) produced a parallel dextral shift of the AMPA concentration-response curve, corresponding to a K_i value for ATPO of 16 μM. Data points represent means ± s.e.mean after normalization to the peak responses induced by 300 μM AMPA alone. The four parameter logistic equation was used to fit the curves and calculate the EC_{50} values (see text).

56±5.4% of the control peak response to 43±3.7%. This represents a relative reduction in the plateau response of 23%, which was not, however, significant ($P>0.05$).

We then investigated the effect of ATPO on peak responses of cortical neurones to a range of AMPA concentrations (Figure 1C). At concentrations of 30 and 100 μM, ATPO produced a parallel dextral shift of the concentration-response relationship with EC_{50} values for AMPA of 51±4.5 μM (control), 146±19 μM (+30 μM ATPO) and 308±20 μM (+100 μM ATPO), corresponding to a K_i value for ATPO of 16±2.2 μM. The Hill slope of the relationship was 0.86±0.04.

A possible explanation for the fact that ATPO acts as a competitive antagonist of the peak response to AMPA yet has variable actions on the plateau response is that ATPO reduces receptor desensitization. This would counteract the antagonist action on the later phase of the response to a greater or lesser extent. We therefore investigated the effect of 100 μM ATPO on responses to 100 μM AMPA following blockade of desensitization by 100 μM CTZ (Figure 2). CTZ potentiated the plateau response to AMPA to 744±68% ($n=17$). In the presence of ATPO, the response to AMPA was reduced to 390±55% ($n=7$) of the control, corresponding to a reduction by 47±5.6%. This is similar ($P>0.05$) to the effect of ATPO on peak responses to AMPA, which were reduced by 42±5% (Figure 1).

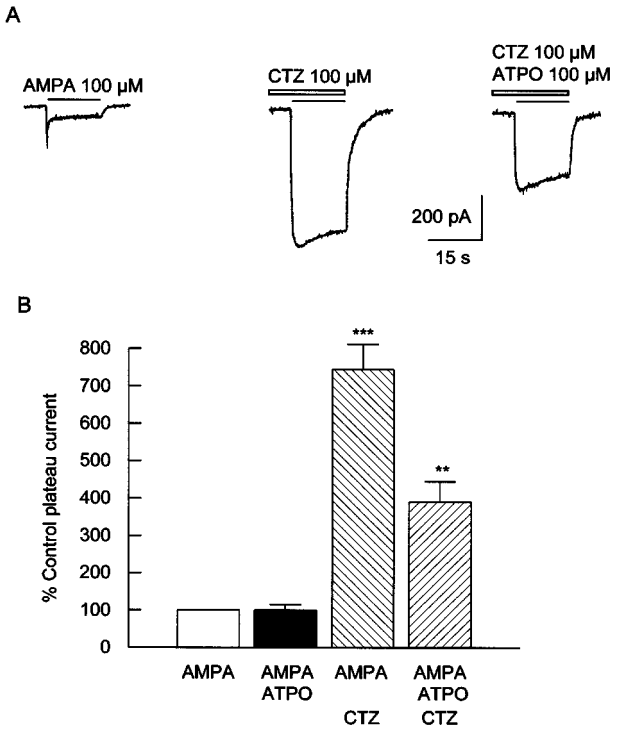

Figure 2 ATPO reduces the plateau phase of responses to AMPA following the blockade of receptor desensitization by cyclothiazide (CTZ). (A) Representative traces showing marked enhancement of the response to AMPA in the presence of 100 μM CTZ. This response was reduced by about half in the presence of 100 μM ATPO. (B) Summary of the action of ATPO before and following blockade of receptor desensitization. Responses for each neurone were normalized to the control plateau response to 100 μM AMPA and the data bars represent means±s.e.mean of these values. ATPO had no significant effect on the plateau response to AMPA ($n=7$). CTZ caused a 7.5 fold enhancement of the plateau response to AMPA ($n=17$), following which ATPO reduced the response by 47.6% ($n=7$). (***$P<0.001$, **$P<0.01$ by two tailed t-test compared with control).

Action of ATPO on responses of cortical neurones to KA and MeGlu

We then investigated the effect of ATPO on responses evoked by KA. Figure 3 illustrates that 30 μM ATPO depressed responses of cortical neurones to KA and caused a parallel dextral shift of the concentration-response relationship. EC_{50} values for KA were 89±7.3 μM (control) and 178±12 μM (+30 μM ATPO), corresponding to a K_i value for ATPO of 27±2.8 μM. The Hill slope of the relationship was 1.18±0.17. Although these results would indicate that ATPO acts as an antagonist on KA receptors, it is known that higher concentrations of KA will activate AMPA receptors. We therefore studied the action of ATPO on responses to MeGlu, which has been proposed to be a selective agonist at KA receptors without significant activity on AMPA receptors (Gu *et al*., 1995). We will be presenting a detailed report of the actions of MeGlu on cultured cortical and spinal cord neurones elsewhere (Dai *et al*., in preparation). Semi-rapid application of MeGlu to cortical neurones evoked a non-desensitizing response (Figure 4A) with an EC_{50} of 303±41 μM. While responses to MeGlu were competitively antagonized by NBQX, the K_i value for this action was some 3.5 times greater than for responses to AMPA and KA, for which the K_i values were similar (Table 1).

30 μM ATPO reversibly depressed the response to 300 μM MeGlu (Figure 4A). At concentrations of 30 and 100 μM, ATPO caused a parallel dextral shift of the concentration-response relationship for MeGlu (Figure 4B), indicating that

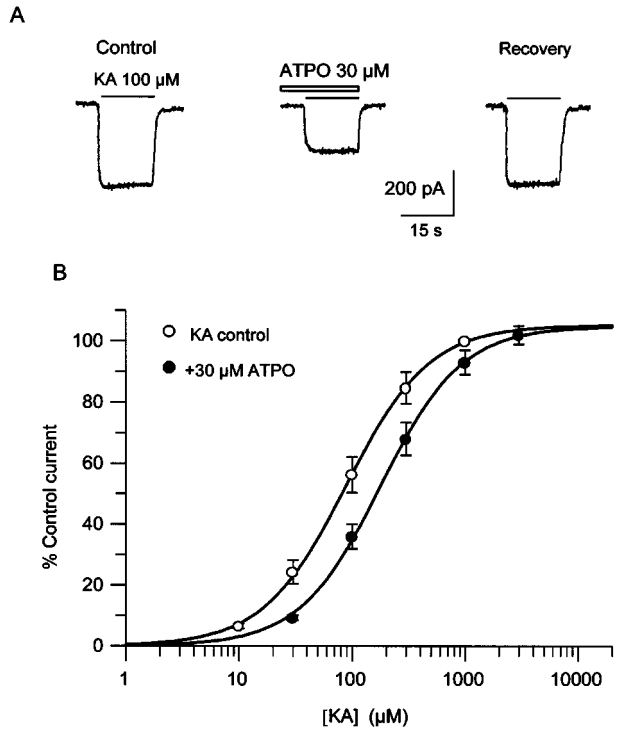

Figure 3 ATPO competitively inhibits responses of cortical neurones to KA. (A) Representative traces from a cortical neurone showing that 100 μM KA induced a non-desensitizing response which was reduced by about half during concomitant application of 30 μM ATPO. The effect was fully reversible. (B) 30 μM ATPO caused a parallel dextral shift in the concentration-response relationships for KA, corresponding to a K_i value of 27 μM. Data points represent means±s.e.mean of results for eight neurones plotted as a percentage of the current evoked by 1000 μM KA in the absence of ATPO. The four parameter logistic equation was used to fit the curves and

ATPO competitively antagonizes the responses to MeGlu. The EC_{50} values for MeGlu were 310 ± 11 μM (control; $n=21$), 790 ± 83 μM ($+30$ μM ATPO; $n=6$) and $1{,}460 \pm 120$ μM ($+100$ μM ATPO; $n=6$), corresponding to a K_i value for ATPO of 23 ± 2.6 μM. The Hill slope of the relationship was 1.26 ± 0.28. Table 1 shows that ATPO has a similar efficacy ($P>0.05$) towards responses to AMPA, KA and MeGlu, which contrasts with the lower potency of NBQX towards responses to MeGlu.

Analysis of the concentration-inhibition curves for ATPO on responses to 500 μM MeGlu (Figure 4C) yielded an IC_{50} value for ATPO of 17 ± 1.9 μM ($n=6$).

Blockade of receptor desensitization

To gain insight into which receptors participate in the responses of the cortical neurones to the three agonists, we blocked desensitization of AMPA and KA receptors using CTZ and Con A, respectively, and compared the effects on responses to MeGlu with those evoked by AMPA and KA. Representative responses are shown in Figure 5. 100 μM CTZ potentiated the plateau responses to AMPA (to 826 ± 77% of control; $n=7$), but had no significant effect on responses to 100 μM MeGlu (increased to 112 ± 5%; $n=9$; $P>0.05$). Con A (300 μg ml^{-1}) caused a 3 fold potentiation of responses to 300 μM MeGlu (to 306 ± 25%; $n=11$; $P<0.001$), which was considerably greater than the potentiation of responses to 100 μM KA (to128 ± 6%; $n=13$; $P<0.01$).

It is possible that the application system is not fast enough to disclose a rapidly desensitizing component of the response to MeGlu. We therefore investigated the effect of ATPO before and after blockade of KA receptor desensitization by Con A. Since, in contrast to cortical neurones, responses of spinal cord neurones to KA are not affected by CTZ (Frerking *et al*., 1997), these were used for the present experiments. The action of 100 μM ATPO on the response to 300 μM MeGlu was tested first. Following complete recovery to control size, Con A (300 μg ml^{-1}) was applied using an application protocol similar to that shown in Figure 5. Finally, the effect of ATPO on responses to MeGlu in the presence of Con A was tested. The results from seven spinal cord neurones in which the whole of this protocol was completed are summarized in Figure 6. ATPO depressed the response to MeGlu to 26.5 ± 3.4%

Table 1 Comparison of K_i values for ATPO and NBQX on responses to AMPA, KA and MeGlu

	K_i (μM)	
Agonist	*ATPO*	*NBQX*
AMPA	16 ± 2.2	0.083 ± 0.012
KA	27 ± 2.8	0.079 ± 0.010
MeGlu	23 ± 2.6	0.28 ± 0.03
MeGlu + GYKI 53655	36 ± 4.3	N.D.

N.D., not determined.

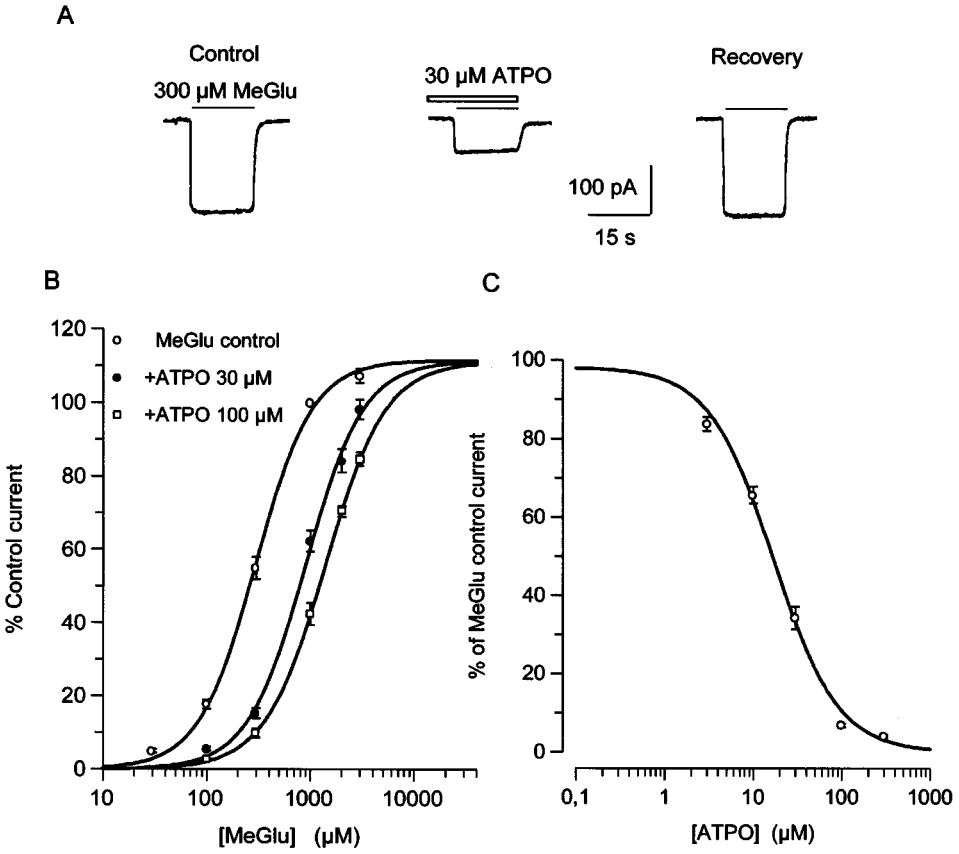

Figure 4 Action of ATPO on responses of cortical neurones to MeGlu. (A) Representative traces showing 30 μM ATPO caused a marked and reversible reduction of about 70% of the response to 300 μM MeGlu. (B) Concentration-response curves for MeGlu showing ATPO at 30 and 100 μM caused a parallel dextral shift of the relationship, indicating that ATPO acts as a competitive inhibitor with a K_i value of 23 μM. Data points represent means$\pm$s.e.mean of results from 6–21 cells plotted as a percentage of the response evoked by 1000 μM MeGlu in the absence of ATPO. (C) Concentration-inhibition curve for the effect of ATPO on responses to 500 μM MeGlu. The IC_{50} value for ATPO was 17 ± 1.9 μM ($n=6$). The four parameter logistic equation was used to fit the curves and calculate EC_{50} (B) and IC_{50} (C) values.

($P<0.001$) corresponding to a 3.8 fold reduction. Following return to control level, the responses to MeGlu were potentiated to $315\pm10\%$ by Con A. ATPO then reduced the Con A-potentiated response to $38.1\pm5.5\%$ ($P<0.001$) of its initial size, corresponding to a 2.6 fold reduction. There was no significant difference ($P>0.05$) in the degree of antagonism by ATPO before and after blockade of receptor desensitization (Figure 6).

Rapid application of MeGlu

In order to disclose whether a rapidly desensitizing component of the response to MeGlu is present on our cortical neurones (cf. Zhou *et al.*, 1997), we also applied MeGlu using a 'concentration-jump' technique to outside-out patches. Because extensive flushing of the theta-tube was required (see Methods) and run-down of agonist-induced responses necessitated recording control responses between each application with antagonist, it was only possible to test a limited number of drug combinations. We therefore chose to test the interaction of 100 μM ATPO with responses to 300 μM MeGlu (these concentrations being approximately five times the K_i and the EC_{50} values, respectively). Figure 7 shows that rapid application of 300 μM MeGlu evoked a transient response: the peak reached a maximum after 9 ms, and then decayed back to a steady-state level (peak : plateau = 4.2). 100 μM ATPO reduced the peak response by 55% and slowed its kinetics markedly (the time-to-peak was prolonged to about 85 ms). Since the patch was not pre-treated with ATPO, this change in kinetics probably reflects the binding of ATPO with the receptors. The histogram in Figure 7B shows that 100 μM ATPO reduced peak responses to 300 μM MeGlu by $54.8\pm3.9\%$ ($n=10$, $P<0.001$).

Effect of GYKI 53655 on responses to MeGlu

Although the response of cortical neurones to semi-rapid application of MeGlu was not affected by CTZ (Figure 5), it is nevertheless possible that part, if not all, of the response is mediated by an action on AMPA receptors (Zhou *et al.*, 1997). We therefore tested the action of GYKI 53655 on responses to MeGlu. We chose to use GYKI 53655 at a concentration of

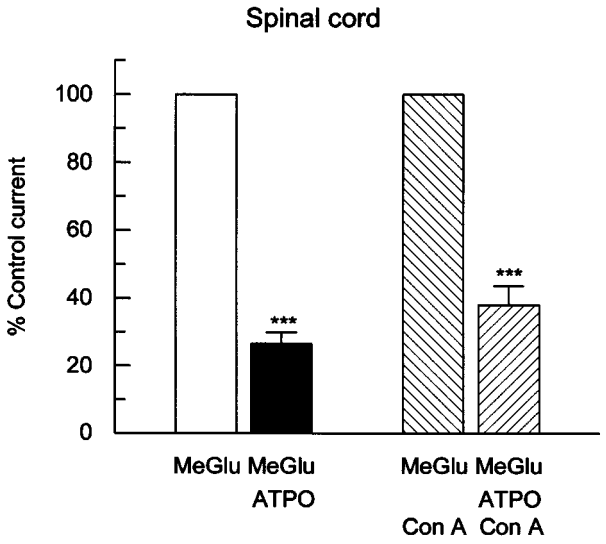

Figure 6 The effect of ATPO on responses of spinal cord neurones to MeGlu in the presence and absence of KA receptor desensitization. The histogram summarizes results from seven neurones in which the effect of ATPO was tested both before and after blockade of receptor desensitization by Con A (300 μg ml^{-1}). ATPO caused a 3.8 fold reduction of the response to MeGlu ($P<0.001$, ***). Con A caused a 3 fold increase in the response to MeGlu (not shown), following which ATPO reduced the response 2.6 fold ($P<0.001$, ***). In both cases, responses are normalized to the respective responses before ATPO was applied. There was no significant difference ($P>0.05$) in the degree of antagonism by ATPO before and after blockade of receptor desensitization.

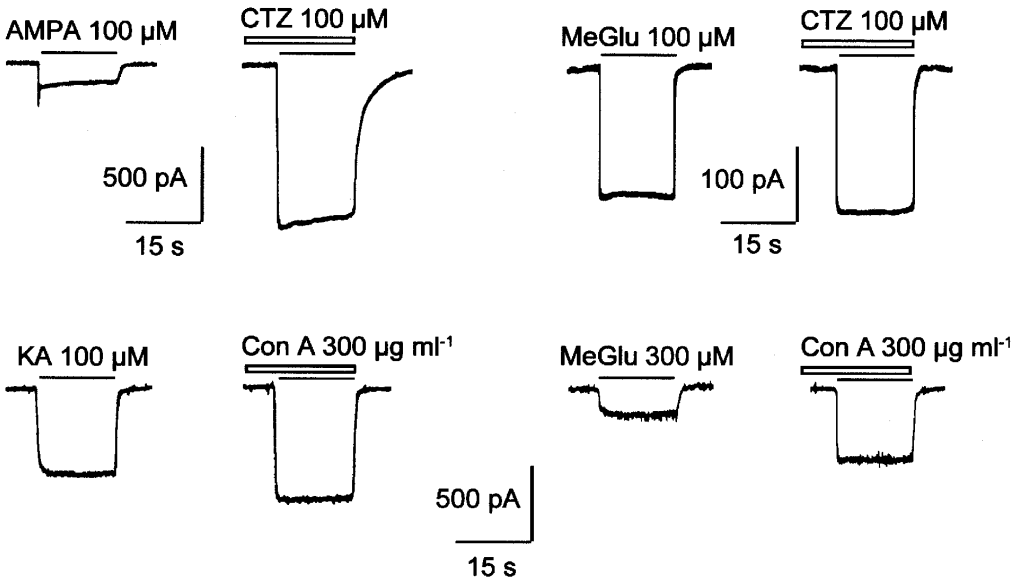

Figure 5 The effect of blockade of receptor desensitization on responses of cortical neurones to AMPA, KA and MeGlu. In the upper traces, 100 μM CTZ caused an 8.7 fold potentiation of the plateau response to 100 μM AMPA, but had little effect on the response to 100 μM MeGlu. The lower traces show that Con A (300 μg ml^{-1}) potentiated the responses to 100 μM KA (by 1.3 fold) and 300 μM MeGlu (by 3 fold). Due to the slow development of blockade of receptor desensitization, the neurones were pre-treated with CTZ for 30 s and with Con A for 3 min before co-application with the respective agonists.

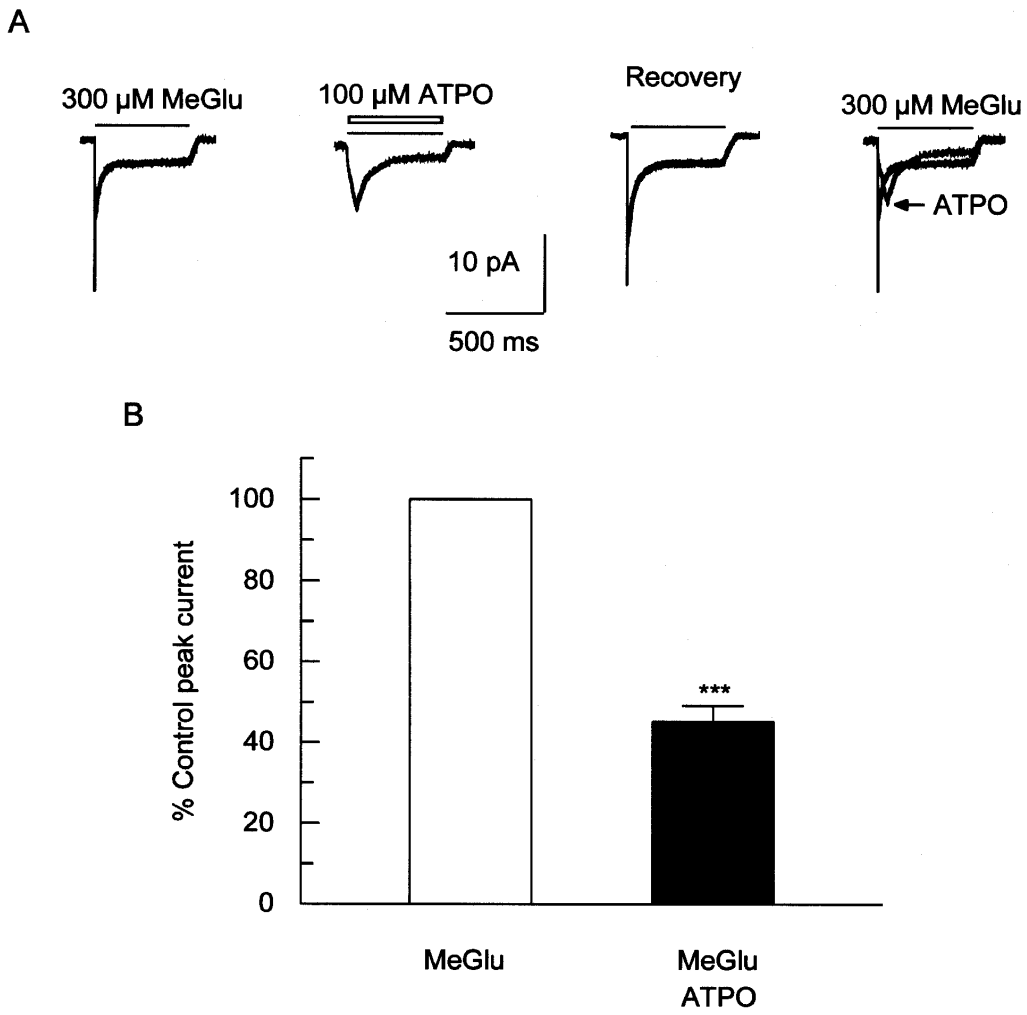

Figure 7 ATPO reduces responses evoked by rapid application of MeGlu. (A) Representative responses evoked by concentration-jump application of 300 μM MeGlu (for 500 ms) to an outside-out patch. The response reached a peak after 9 ms from which it rapidly declined back towards the base-line. The patch was then perfused with a solution containing 300 μM MeGlu + 100 μM ATPO. As also illustrated by the superimposition to the right, the peak response was reduced by about 55% and somewhat delayed with respect to the control. The effect of ATPO was reversible on washing (not shown). (B) Histogram showing that 100 μM ATPO reduced peak responses to 300 μM MeGlu to 45.2 $\pm$ 3.9% ($n = 10$, $P < 0.001$, ***).

10 μM, which is about 10 times the IC_{50} value (Paternain *et al.*, 1995). GYKI 53655 caused a reduction of the response to 300 μM MeGlu by 69.6 $\pm$ 3.6% ($n = 6$; $P < 0.001$). It is reasonable to assume that the GYKI 53655-resistant component of the response to MeGlu is mediated by KA receptors. Interestingly, this response exhibited an initial peak phase, which was about 45% larger than the following plateau phase (Figure 8A). 200 μM ATPO then caused a further reduction of the GYKI 53655-resistant component to about 23% of the initial control size (the peak and plateau phases were reduced proportionately, (Figure 8A)). Since the neurone was pre-treated with GYKI 53655 and ATPO, it should be at equilibrium with respect to the antagonists before application of MeGlu. We then performed quantitative pharmacology on the GYKI 53655-resistant component. 200 μM ATPO caused a parallel dextral shift of the GYKI 53655-resistant component with a K_i value of 36 $\pm$ 4.3 μM ($n = 11$, Figure 8B). This is not significantly different from the K_i for ATPO on responses to MeGlu in the absence of GYKI 53655, though it is significantly different ($P < 0.01$) K_i for ATPO towards AMPA responses.

Discussion

ATPO is an analogue of AMPA itself and was originally described as a potent and selective AMPA receptor antagonist (Madsen *et al.*, 1996). Based on studies in the rat cortical wedge preparation and homogenate binding studies, it was concluded that ATPO is devoid of affinity for KA receptors (Madsen *et al.*, 1996). However, recent data obtained with cloned non-NMDA receptor subunits expressed in HEK293 cells have clearly shown that the actions of ATPO are more complex (Wahl *et al.*, 1998). At recombinant receptors containing homo- and heteromeric combinations of AMPA preferring subunits (GluR1-4), ATPO inhibited responses to KA with a K_i value ranging from 3.9–26 μM. A similar value for ATPO antagonism of responses of GluR1 receptors to AMPA was observed, indicating that the response to KA at AMPA receptors is inhibited *via* an agonist-antagonist interaction similar to that of AMPA and ATPO. On the other hand, ATPO was found to be inactive at GluR6-containing receptors, while it behaved as a weak partial agonist at GluR5 and GluR5 + KA2 receptors (Wahl *et al.*, 1998).

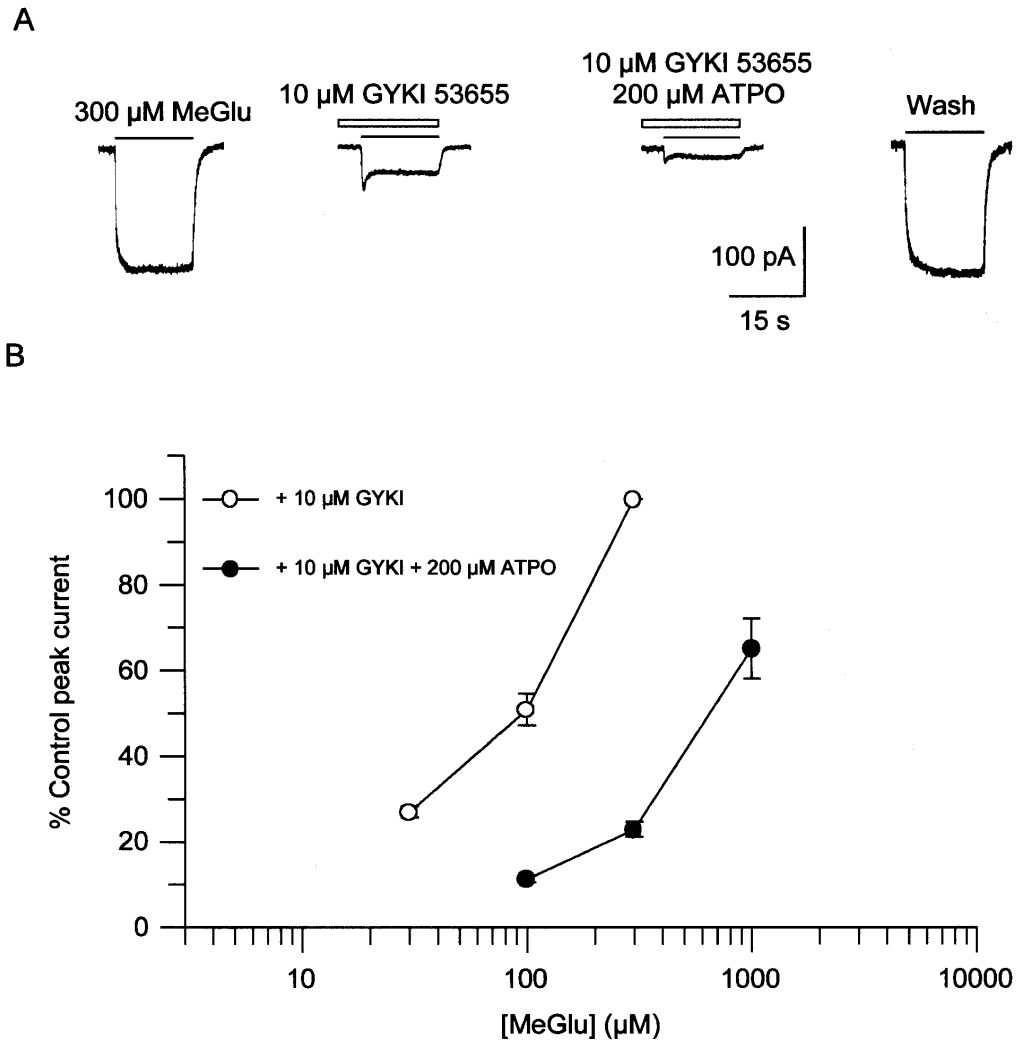

Figure 8 ATPO antagonizes the GYKI 53655-resistant component of the response to MeGlu. (A) Representative traces showing that 10 μM GYKI 53655 reduced the response to 300 μM MeGlu by about 70% and that 200 μM ATPO reduced the residual response by a further 77%. Note the appearance of an early transient peak component of the response in the presence of GYKI 53655. This peak persisted in the presence of ATPO, although it was reduced correspondingly in magnitude. (B) Concentration-response relationships to show the effect of ATPO on the GYKI 53655-resistant component of the response to MeGlu. In order that the full experimental protocol could be completed for each neurone ($n=11$), only three concentrations of MeGlu were used. 200 μM ATPO caused a dextral shift of the relationship corresponding to a K_i value of 36 ± 4.3 μM.

These results of Wahl *et al.* (1998) could provide clues about the identity of the naturally expressed receptors in the neurones studied here. Since ATPO always caused depression of responses to MeGlu and low concentrations of KA (Figures 3 and 4), none of these actions can be mediated by GluR6 receptors, which are insensitive to ATPO. Furthermore, ATPO never evoked a direct response, making it unlikely that GluR5, either alone or in combination with KA2, is expressed in the neurones. It is possible, however, that GluR5 and GluR6 may behave differently when expressed in other heteromeric combinations. This is illustrated by the example that AMPA evokes a relatively persistent inward current when applied to heteromeric combinations of GluR6 and KA-2, despite the fact that neither subunit alone either binds or is activated by AMPA (Herb *et al.*, 1992). This would suggest that the agonist-binding site is probably located between adjacent sub-units and furthermore serves to illustrate that restraint should be exercised when extrapolating pharmacological data derived from recombinant receptors to those expressed naturally.

In the present study, ATPO caused a parallel dextral shift of the concentration-response relationships for AMPA, KA and MeGlu, indicating that it competitively antagonizes responses at non-NMDA receptors. K_i values for the action of ATPO on responses to MeGlu and KA were similar (23 and 27 μM, respectively) and not significantly different from responses to AMPA (16 μM). This indicates either that ATPO discriminates only weakly between AMPA and KA receptors on cultured cortical neurones, or that the responses to the three agonists are mediated by one receptor population. We discuss the evidence for these two possibilities below.

Action of ATPO at AMPA receptors

Because of the variable effects on plateau responses to AMPA, it was necessary to study the actions of ATPO on peak responses, which were antagonized in a competitive manner. Although the agonist-evoked peak current is a convenient response to quantify, it reflects the instant when recruitment of new ionophores to conducting states is exactly balanced by the passage of others into the desensitized state

(Patneau *et al*., 1993) and is thereby very labile. The largest peak response is attained when receptors are activated as rapidly and synchronously as possible. Although our semi-rapid application system is not fast enough to achieve this, the peak response will nevertheless be related to the number of occupied receptors providing the kinetics of applications are constant and reproducible. Justification for using the peak response to measure K_i is also borne out by the fact that ATPO reduced responses AMPA to the same extent following blockade of receptor desensitization with CTZ. Since AMPA is a selective agonist at AMPA receptors (Bettler & Mulle, 1995), it may be concluded that ATPO acts as a medium potency competitive antagonist at AMPA receptors.

ATPO had no significant effect on plateau responses to AMPA, though both increases and decreases were seen in individual cases. During the plateau phase, there is equilibrium between ionophores passing between the open and desensitized (closed) states. Therefore, an increase in the plateau phase during application of the antagonist could result from a reduction in receptor desensitization, which opposes the antagonist effect. Indeed, potent receptor antagonists, such as the quinoxalinediones, have less efficacious actions on the plateau response and reduce desensitization (Parsons *et al*., 1994), while weak antagonists, such as AMOA, cause a net potentiation of the plateau response at high agonist concentrations (Wahl *et al*., 1992). That ATPO indeed causes a moderate block of receptor desensitization is confirmed by the fact that 100 μM ATPO reduced responses to AMPA by 47% following blockade of receptor desensitization with CTZ. If ATPO had no effect on desensitization, it would be expected to reduce the plateau response in untreated neurones by the same amount. Since the plateau phase of responses to AMPA were on average unchanged, this difference reflects the block of desensitization by ATPO. In the absence of ATPO, CTZ increased the response to AMPA by 744%. Thus, the relative effect of ATPO on desensitization in relation to the presence of CTZ is given by $(47/744) \times 100 \approx 6\%$.

Action of ATPO at KA receptors

ATPO antagonized responses to KA and MeGlu with a similar potency. Since KA is a mixed agonist at non-NMDA receptors, interpretation of the responses to MeGlu is crucial in deciding whether ATPO acts at KA receptors. Semi-rapid application of MeGlu evoked non-desensitizing responses (Figure 4). Zhou *et al*. (1997) have previously reported similar responses of cultured cortical neurones to high concentrations of MeGlu with an EC_{50} of 325 μM, with which our value of 303 μM is in good agreement. Zhou *et al*. (1997) furthermore suggested that this response to MeGlu results from activation of AMPA receptors, although this was not tested directly. However, we show here that responses to MeGlu were completely unaffected by CTZ, which caused an 8 fold potentiation of plateau responses to AMPA (Figure 5). Responses to MeGlu were also potentiated to a significantly greater extent by Con A than were responses to KA (Figure 5). These results would strongly suggest that plateau responses to MeGlu are mediated by activation of KA receptors. This is further supported by the fact that NBQX, which has previously been shown to be three times more selective towards AMPA preferring receptors in cortical neurones than KA receptors in dorsal root ganglion cells (Paternain *et al*., 1996), was 3.5 times less potent towards responses to MeGlu than towards plateau responses to AMPA.

Although the above results strongly indicate that MeGlu selectively activates KA receptors, the same responses were depressed by 70% in the presence of a supra-maximal concentration of the AMPA-selective antagonist, GYKI 53655 (Figure 8). This would indicate that the majority of the response is actually mediated by AMPA receptors. The reasons for these apparently discordant results remain to be clarified. Nevertheless, the GYKI 53655-resistant component, which is thought to be mediated by KA receptors (Paternain *et al*., 1995), was competitively antagonized by ATPO (Figure 8) with a K_i value of 36 μM. While this was not significantly different from the K_i value in the absence of GYKI 53655 (23 μM), it was significantly different from that of ATPO towards AMPA responses (16 μM). Using a rapid application system, MeGlu was applied to outside-out membrane patches with the aim of disclosing a rapidly desensitizing component which is known to be mediated by KA receptors (Zhou *et al*., 1997; Jones *et al*., 1997). ATPO reduced this transient peak response by 55% (Figure 7).

In conclusion, patch-clamp recordings from cultured neurones have shown that ATPO is a moderate-potency competitive antagonist of naturally expressed non-NMDA receptors with K_i values of 16 μM, 27 μM and 23 M towards responses evoked by AMPA, KA and MeGlu, respectively.

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