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Cyclothiazide decreases [^{3}H]AMPA binding to rat brain membranes: evidence that AMPA receptor desensitization increases agonist affinity

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The effects of cyclothiazide, a drug which blocks AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor desensitization, were tested on binding of [^{3}H]AMPA to rat brain membranes. Cyclothiazide reduced [^{3}H]AMPA binding by lowering the apparent affinity of the AMPA receptor. The magnitude of the decrease was temperature dependent and greater for membrane-bound than for solubilized receptors. These data provide evidence that desensitization increases the affinity of the AMPA receptor for agonists and indicate that a significant percentage of AMPA receptors in conventional equilibrium binding assays are in a desensitized state.

Physiological experiments indicate that glutamate receptors of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subclass rapidly desensitize following agonist binding; i.e. the receptor channel fails to open despite the continued presence of appropriate ligand[6,9]. The diuretic drug cyclothiazide greatly reduces desensitization and prolongs the duration of excitatory postsynaptic currents in cultured hippocampal neurons[7,12]. Physiological experiments utilizing rapid agonist application have suggested that desensitization might increase the affinity of the AMPA receptor for agonists[6,9] as in the case for the nicotinic cholinergic receptor[5]. Estimates of AMPA receptor affinity derived from equilibrium binding studies might thus be distorted by the presence of receptors which have shifted into the desensitized state during incubation of brain membranes with agonists. If this were so, and if desensitization was linked to a change in affinity, then cyclothiazide would be expected to alter the K_d for [^{3}H]AMPA binding without affecting the number of binding sites. The present experiments tested this prediction. Tests of the drug's effects were also carried out following solubilization of membrane receptors, a manipulation which converts low-affinity membrane receptors into a higher affinity state[1]. The factors controlling this conversion are poorly understood, but the two affinity states could potentially reflect sensitized versus desensitized states of the receptor. If this were the case, cyclothiazide would be expected to have greater effects on solubilized receptors than on membrane-bound receptors.

Membranes and fractions solubilized with 0.4% Triton X-100 were prepared as previously described[2]. The final buffer composition of the samples was 100 mM Tris acetate, pH 7.2, with 50 μM EGTA (with 0.4% Triton X-100 in the case of the soluble samples). [^{3}H]AMPA binding in the presence of 50 mM potassium thiocyanate (KSCN) was measured using the centrifugation technique for the membrane samples and the filtration technique for the soluble samples, as previously described[1,2]. Briefly, 80–120 μg of protein in a final volume of 100 μl were incubated with [^{3}H]AMPA for 40–60 min in either an ice water bath (0°C), a room temperature rack (23°C) or a heated water bath (35°C). Membrane samples were centrifuged at 48,000 $\times g$ (at appropriate temperature) for 20 min. The supernatants were then aspirated and the pellets superficially rinsed with ice-cold 0.4 ml 100 mM Tris acetate. The pellets were then dissolved in 10 μl Beckman Tissue Solubilizer and counted with an effi-

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ciency of 0.40. Soluble samples were filtered through Whatman GF/B filters (soaked in 0.03% polyethylenimine for at least 30 min) with 3 washes of 4.0 ml buffer (50 mM Tris-HCl, pH 7.2, 100 mM KSCN). Results were expressed as specifically bound [^{3}H]AMPA, which equals the difference between the total bound and non-specifically bound ligand. For the saturation studies, AMPA concentrations were increased between 2 and 3000 nM. Concentrations between 2 and 50 nM were achieved by increasing the concentration of radiolabeled AMPA, while those above 50 nM were produced by adding unlabeled AMPA to a fixed [^{3}H]AMPA concentration of 50 nM. Non-specific binding was defined as the amount of [^{3}H]AMPA bound in the presence of 2.5 mM l-glutamate. For both the membrane and soluble fractions, non-specific binding increased from approximately 10% to 70% of the total binding as AMPA concentration increased from 2 nM to 3000 nM. Cyclothiazide dilutions were made from a stock of cyclothiazide in pure dimethyl sulfoxide (DMSO). All samples assayed (both control and cyclothiazide) contained 1% DMSO; this was shown in preliminary experiments to have no effect on [^{3}H]AMPA binding. Two-site analyses of binding data were performed by non-linear regression using the Inplot program by GraphPAD Software Inc. Protein concentrations were determined with the protein assay reagent obtained from Bio-Rad with bovine serum albumin as the standard. [^{3}H]AMPA was purchased from NEN/Dupont and AMPA was obtained from Tocris.

Fig. 1 shows a dose–response curve for the effects of cyclothiazide on 50 nM [^{3}H]AMPA binding at 0°C in the presence of 50 mM KSCN for membranes and soluble fractions. The K_i for cyclothiazide in the membrane samples was 31 μM; in the soluble fractions, the

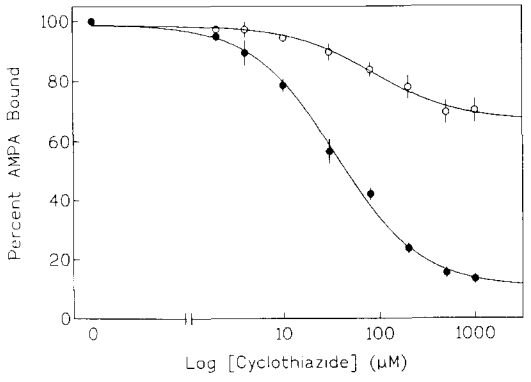

Fig. 1. Dose–response curves for the effects of cyclothiazide on [^{3}H]AMPA binding in membranes (●) and soluble fractions (○). Points and error bars indicate the means and S.E.M. for 3 determinations. Two-site fits were not significantly better than one-site fits for either curve (F-squared test, $P < 0.05$).

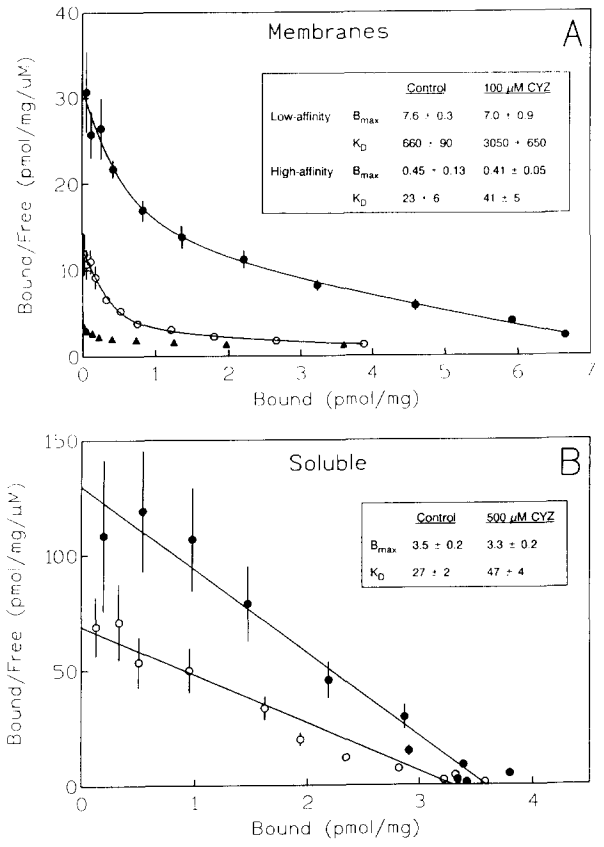

Fig. 2. Top: Scatchard transformations of [^{3}H]AMPA binding to membranes in the absence of cyclothiazide (●) and the presence of 100 μM (○) and 500 μM (▲) cyclothiazide (CYZ). The curves show the best two-site fits for the control and 100 μM cyclothiazide samples. Bottom: Scatchard transformations of [^{3}H]AMPA binding to soluble fractions in the absence (●) and presence (○) of 500 μM cyclothiazide. Points and error bars indicate the means and S.E.M. for 3 determinations. The inset for the membranes provides B_{max} and K_d values for two-site regression analyses, while the inset for the soluble fractions provides values from linear regression analyses. B_{max} values are in pmol/mg, while K_d values are in nM.

K_i was 32 μM. The binding appeared to reach a minimum plateau at cyclothiazide concentrations of 500 μM to 1 mM; however, since 1 mM was approximately the limit of drug solubility, higher concentrations could not be tested.

The effects of cyclothiazide across a wide range of [^{3}H]AMPA concentrations were measured. These data are shown in Fig. 2 for binding to both the membranes (top) and soluble fractions (bottom) at 0°C. For the membrane-binding data, a two-site fit was significantly better (F-squared test, $P < 0.05$) than a one-site fit both in the presence and absence of 100 μM cyclothiazide. In the presence of 500 μM cyclothiazide, specific binding was so low that resolution of two sites was not possible. Cyclothiazide (100 μM) did not significantly affect B_{max} estimates, but there was an approximately 5-fold decrease in the apparent affinity of the low-affinity binding site and a 2-fold decrease in the apparent

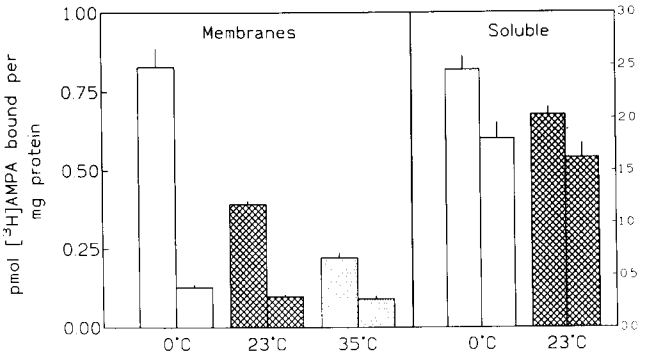

Fig. 3. Binding of 50 nM [^{3}H]AMPA to membranes and soluble fractions at different temperatures. The left bar of each pair represents binding under control conditions, while the right bar represents binding in the presence of 500 μM cyclothiazide. The bars and error bars represent means and S.E.M. for 3–4 determinations.

affinity of the high-affinity binding site. B_{max} and K_d values are provided in the insets to Fig. 2, and, as shown, the effects of cyclothiazide were much greater for the membranes (especially the low-affinity site) than for the soluble fractions.

[^{3}H]AMPA binding at various temperatures in the presence and absence of cyclothiazide (500 μM) is shown in Fig. 3. Binding to membrane samples was much less at 23°C than at 0°C, in accord with previous studies[3]. Cyclothiazide reduced the binding still further although its percentage effects were substantially smaller than at 0°C. Binding in membrane samples incubated at 35°C was less than that obtained at lower temperatures and this was accompanied by a further decrease in the proportional effects of cyclothiazide. The affinity of soluble receptors appeared to be less affected by the change from 0°C to 23°C than was that of membrane samples. Cyclothiazide was still effective in reducing the high-affinity binding at 23°C but to a lesser degree than was found at the lower temperature. High-affinity AMPA receptors appear to degrade during incubation at 35°C (data not shown) and this precluded tests of binding to soluble fractions at this temperature.

As mentioned, it has been suggested on the basis of rapid agonist application experiments that the desensitized state of the AMPA receptor is of higher affinity than the sensitized state[6,9]. Assuming that in an equilibrium binding assay a significant percentage of bound receptors are in a desensitized state (i.e. one that does not lead directly to an open state) and that there is an increase in agonist affinity upon entering this state, then a drug which reduces the time spent in the desensitized state (e.g. by slowing the rate of desensitization or by increasing the rate of resensitization) should reduce binding, since a higher proportion of receptors in the binding assay would remain in the sensitized state. The present results satisfy this prediction and thus constitute evidence that AMPA receptor desensitization is accompanied by an increase in the affinity of the receptor.

Increases in temperature caused a marked reduction in [^{3}H]AMPA binding, as well as a decrease in the relative effects of cyclothiazide. Higher temperatures might increase the off-rate constant of the receptor and thus the percentage of bound receptors present at any given moment. An increase in the off-rate constant would also allow the receptors less time to desensitize and thereby would shift the equilibrium of the binding assay toward the sensitized state. Assuming that the sensitized state is of lower affinity than the desensitized state, this would serve to further decrease the apparent affinity of the receptor, and would also cause drugs which affect desensitization kinetics (like cyclothiazide) to have a smaller effect on binding. Other potential explanations for the decreased effects of cyclothiazide at higher temperature might be that temperature alters the desensitization kinetics of the receptor, or that the binding of cyclothiazide to the receptor is temperature sensitive.

It does not seem likely that the previously described high- and low-affinity states of the AMPA receptor[1,3] reflect desensitized vs. sensitized forms of the receptor. Since desensitization causes an increase in affinity, the low-affinity state of the AMPA receptor might conceivably represent the sensitized form of the receptor while the high-affinity state might correlate with the desensitized form; from this it would be predicted that cyclothiazide would have no effect on the affinity of low-affinity AMPA receptors. Therefore, the observation that cyclothiazide reduces the apparent affinity of both states provides evidence contrary to the notion that the low-affinity state simply represents the sensitized version of the receptor.

Finally, it is of interest to compare the present results with those obtained previously with the nootropic drug aniracetam which, like cyclothiazide, prolongs the open time of AMPA receptor channels and the duration of EPSCs at glutamatergic synapses[4,8,10]. Millimolar concentrations of aniracetam induce only a marginal reduction of [^{3}H]AMPA binding to membranes[11]. It is possible that aniracetam at higher concentrations might reduce [^{3}H]AMPA binding as much as cyclothiazide does, but this cannot be tested due to the limited solubility of aniracetam in aqueous solutions. Alternatively, the differences between the effects of aniracetam and cyclothiazide in physiological studies and binding analyses may be due to distinct molecular actions of the two drugs.

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