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Effects of heparin on the properties of solubilized and reconstituted rat brain AMPA receptors

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Abstract

Heparin was found to bind to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and to alter their functional properties. AMPA receptors solubilized in 0.4% Triton X-100 bound to a heparin-agarose column and were eluted by 0.4 M NaCl. Soluble heparin inhibited 10 nM [^{3}H]AMPA binding to detergent-solubilized receptors by 75% (IC_{50} = 10 μg/ml), but had little effect on binding to membrane-associated receptors. The inhibition of [^{3}H]AMPA binding to detergent-solubilized receptors was not observed when binding was measured in the presence of 0.4 M NaCl, and no effect of heparin was observed on binding of the AMPA receptor antagonist [^{3}H]6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Scatchard analyses of [^{3}H]AMPA binding to solubilized receptors revealed that the inhibition induced by heparin was caused by a decrease in the apparent affinity of a portion of the total binding sites. Studies on AMPA receptors reconstituted in artificial lipid bilayers indicated that 10 μg/ml heparin enhanced cooperativity between channels and prolonged the lifetime of the open channel, but did not affect the amplitude of single channel currents. Thus, heparin may be added to the list of compounds known to modulate AMPA receptor function. These data also raise the possibility that heparin-containing proteoglycans, which are known to be concentrated at synaptic junctions, might be able to bind AMPA receptors and influence their functional characteristics.

Keywords: Glutamate; 6-Cyano-7-nitroquinoxaline-2,3-dione; Binding; Affinity; Solubilization; Reconstitution; Glycosaminoglycan

Analyses of the properties of neurotransmitter receptors may be greatly enhanced by the use of compounds which modulate receptor function. Experiments utilizing a number of such compounds have provided insight into the properties of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors: these modulatory agents include aniracetam [10], cyclothiazide [16,24], GYKI 52466 [25], argiotoxin [6] and potassium thiocyanate [1,7]. During the course of studies aimed at purifying native AMPA receptors from rat brain [2], it was noted that AMPA receptors bound with high affinity to a heparin-agarose column. Since heparin is known to alter the biological activity of other proteins to which it binds [11], the possibility was raised that the interaction of AMPA receptors with heparin might lead to altered receptor properties; if this were the case, then heparin might prove to be a useful tool in analyses of AMPA receptor properties. The possibility of a modulatory association between heparin and AMPA receptors is furthermore intriguing since various heparin-containing proteoglycans are known to play key roles in the formation and maintenance of synaptic junctions [14]; the potential for interactions of these species with neurotransmitter receptors, however, has not been widely explored. The present experiments describe the association of AMPA receptors with heparin and the effects of this interaction on receptor function.

Preparation of brain membranes, solubilization and

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[^{3}H]AMPA binding assays were as described [2,3]. In brief, membranes were prepared from whole rat brain by differential centrifugation and osmotic lysis and suspended in a buffer of 50 mM Tris acetate, pH 7.2. Membranes were solubilized with 0.4% Triton X-100 and incompletely solubilized material was removed by centrifugation (48 000g for 2 h); endogenous glutamate was removed by extensive dialysis. Samples assayed for [^{3}H]AMPA binding in the presence of heparin (sodium salt, grade I-A from Sigma) were incubated with the appropriate concentration of heparin for 30–60 min before mixture with radiolabeled ligands. All heparin concentrations are expressed in milligram/millilitre, as precise estimates of molarity are not possible since the molecular weight of heparin varies between 6000 and 20 000 g/mol depending on preparation. For the lipid bilayer reconstitution experiments, receptors were purified and reconstituted as previously reported [2,23]. The electrical measurement system, assay procedures and data processing were as described [22].

Fig. 1 shows the interaction of AMPA receptors with heparin immobilized on an agarose support. For this experiment, the Triton X-100 solubilizate obtained from ~3 mg brain membrane protein was loaded onto a 7.0 ml heparin-agarose column. The column was then washed with approximately 6 volumes of solubilization buffer and the eluted fractions were examined for [^{3}H]AMPA binding activity. No binding was evident in the fractions following the addition of the solubilized membranes to the column, suggesting that the AMPA receptors in the solubilizate had been quantitatively adsorbed to the heparin-agarose matrix. Proteins were then eluted from the column with a step gradient of progressively higher concentrations of NaCl; the peak of [^{3}H]AMPA binding was eluted at 0.4 M NaCl.

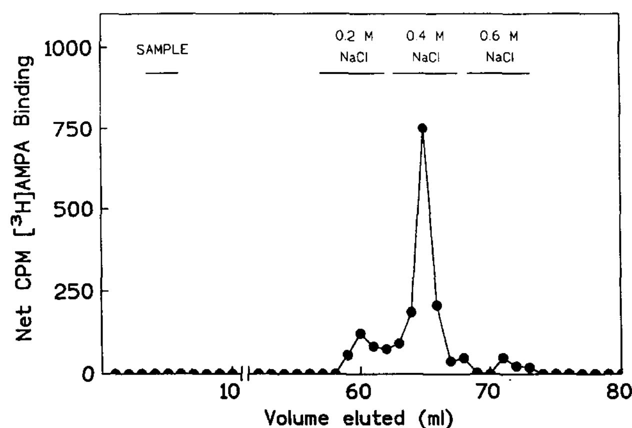

Fig. 1. Adsorption of detergent-solubilized AMPA receptors to heparin-agarose. The bars at the top of the graph indicate the application of the soluble sample onto the column ('sample') and the elution of [^{3}H]AMPA binding by a step gradient of increasing concentrations of NaCl. This experiment was performed twice with very similar results; the points on the graph represent the averages from the two experiments.

The chromatography experiments suggested that heparin associates avidly with AMPA receptors at physiological salt concentrations. The nature of this interaction was explored further in experiments in which detergent-solubilized receptors were treated with soluble heparin and then examined for changes in their binding and electrophysiological properties. Fig. 2A shows the effect of increasing concentrations of heparin on the binding of 10 nM [^{3}H]AMPA. Binding was decreased and reached a minimum plateau at approximately 26% of control binding; the IC_{50} for heparin was determined to be 10 μg/ml. In contrast, binding of the AMPA receptor antagonist [^{3}H]6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was not significantly altered by 1 mg/ml heparin (Fig. 2B). It is also of interest that [^{3}H]AMPA binding to membrane-associated receptors was relatively insensitive to heparin; binding of 10 nM [^{3}H]AMPA to intact brain membranes was 98 ± 4% of control when assayed in the presence of 10 μg/ml heparin (n = 4). At heparin concentrations higher than 1 mg/ml, a slight reduction in [^{3}H]AMPA binding to membrane-associated receptors was observed, but the maximum extent of inhibition was much smaller than that for solubilized receptors (data not shown).

Since high ionic strength disrupted the interaction between receptor and heparin in the chromatography experiments, we next examined whether 0.4 M NaCl would prevent the heparin-induced inhibition of [^{3}H]-AMPA binding to detergent-solubilized receptors. Under control conditions, binding of 10 nM [^{3}H]AMPA to solubilized brain membranes was inhibited by 51% in the presence of 1 mg/ml heparin. Assays carried out in the presence of 0.4 M NaCl showed considerably lower binding in the control sample, consistent with the previously reported effect of ionic strength on apparent [^{3}H]AMPA binding affinity [4]; in this case, however, binding in the presence of heparin was not reduced relative to its control (Fig. 2B). Further binding experiments were conducted to test the specificity of the effects of heparin. It was found that glucosamine-2,3-disulfate, one of the two monomeric constituents of heparin, had no detectable effect on [^{3}H]AMPA binding at 4 mg/ml (n = 3); the polysaccharide dextran at 10 mg/ml also had no significant effect (n = 3; data not shown).

The nature of the heparin effect on binding was characterized in greater detail by Scatchard analysis; Fig. 2C shows averaged data for [^{3}H]AMPA binding at 10 ligand concentrations. In the absence of heparin, the data were adequately fitted by a straight line (r = 0.99) which yielded a K_D estimate of 33 ± 3 nM; this value is consistent with previous determinations for solubilized AMPA receptors [3,9]. Heparin at 1 mg/ml did not change the overall B_{max} but introduced a significant degree of curvature into the Scatchard plot. Non-linear regression analysis of the untransformed binding data indicated the presence of a low-affinity component with a K_D of 344 ± 200 nM and a B_{max} equal to 3.6 ± 0.4 pmol/mg in addition to a

high-affinity component which had an unaltered K_D of 39 ± 7 nM and a reduced B_{max} of 2.5 ± 0.7 pmol/mg; curve fits carried out under the assumption of two sites rather than a single site were superior according to statistical criteria (F-squared test, $P < 0.05$). These data suggest that heparin reduced the affinity of a portion of the total binding sites by a factor of about 10.

The channel properties of partially-purified AMPA receptors reconstituted in artificial lipid bilayers [2,23] were examined in the absence and presence of heparin. The top and bottom panels of Fig. 3 show representative traces of events activated by 283 nM AMPA before and after equilibration in 10 μg/ml heparin, respectively. Under control conditions, the majority of recordings revealed open channel events in the range of 40–50 pS. Some bilayers showed robust channel activity, with average summed amplitudes of approximately 500–900 pS which could be divided into multiple sub-conductance levels of 40–50 pS. Occasionally, large open events occurred without apparently moving through any sub-conductance levels. The 40–50 pS events correspond well in size to AMPA-activated single channel conductances observed in neurons [19,20], but the larger events, observed here and in reconstitution experiments examining *Xenopus* glutamate receptors [13], bear a much more tenuous relation to the behavior of native neuronal glutamate receptors. These larger events may reflect cooperativity between multiple receptors incorporated into the lipid bilayer, or alternatively may represent a completely different mode of channel gating behavior. In the presence of heparin, the average lifetime of the 40–50 pS open channel events was increased by several-fold while their average amplitude was unchanged. Moreover, a higher percentage of total channel activity was in the form of the larger aggregate events, with markedly fewer sub-conductance levels observed. These effects were specific for heparin as another polysaccharide, dextran (1 mg/ml), failed to have any effect on channel behavior. A more detailed analysis of these reconstitution data will be presented separately.

The data presented here demonstrate that heparin associates with AMPA receptors and that this interaction alters receptor properties. The most straightforward interpretation of these data is that heparin associates with AMPA receptors and causes either a change in receptor conformation or a restriction in conformational changes induced by agonists. An alternative interpretation, given that heparin has been shown to facilitate associations between various proteins in vitro [17], is that heparin facilitates interactions either between soluble receptors or

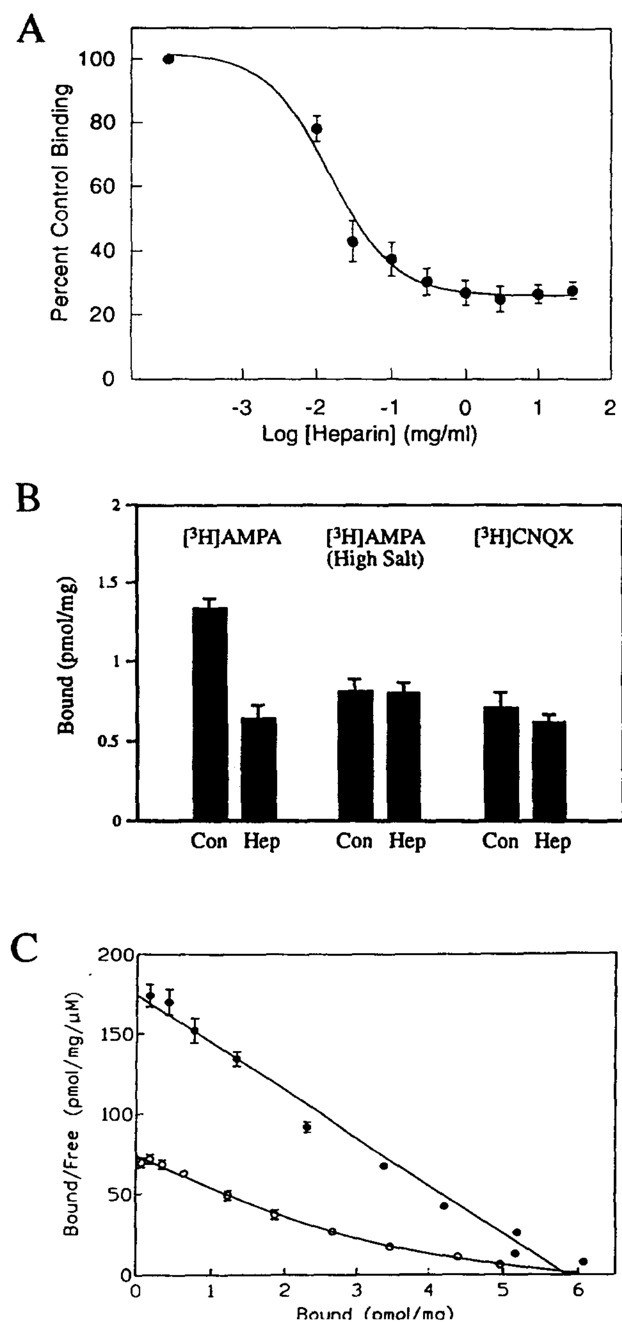

Fig. 2. (A) Inhibition by heparin of [^{3}H]AMPA binding to detergent-solubilized receptors. The percentage of control 10 nM [^{3}H]AMPA binding remaining in the presence of increasing concentrations of heparin is shown. The x-axis represents the log of the concentration of heparin in mg/ml. Points and error bars represent the mean ± SEM for three experiments. (B) Lack of effect of heparin on [^{3}H]CNQX binding and on [^{3}H]AMPA binding in the presence of high salt. The set of bars to the left represent binding of 10 nM [^{3}H]AMPA to detergent-solubilized receptors in the absence and presence of 1 mg/ml heparin. The middle set of bars represent identical experiments, except performed in the presence of 0.4 M NaCl. The set of bars to the right represent the binding of 50 nM [^{3}H]CNQX to the soluble samples in the absence and presence of 1 mg/ml heparin. Bars and error bars indicate the mean ± SEM for four independent determinations. (C) Scatchard plots of [^{3}H]AMPA binding. The binding of 10 concentrations of [^{3}H]AMPA to soluble samples in the absence (filled circles) and presence (open circles) of 1 mg/ml heparin is shown. The values and error bars represent mean ± SEM for three determinations. For the control soluble data, the best linear regression fit is shown; for the heparin samples, the best two-site fit curve is superimposed, as these data were significantly better fit by two-site regression analysis than by a one-site fit (F-squared test, $P < 0.05$).

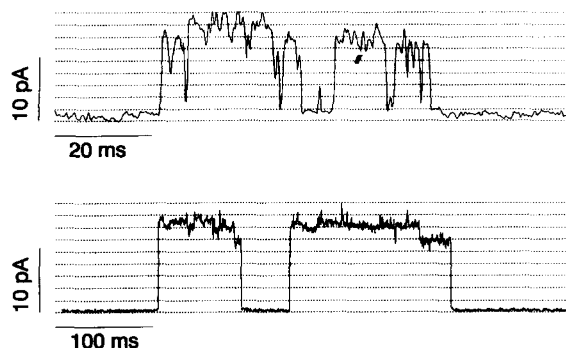

Fig. 3. Effects of heparin on the channel properties of detergent-solubilized AMPA receptors functionally reconstituted in an artificial lipid bilayer. Openings are upwards. Ten microlitres of a suspension of partially-purified AMPA receptor (approximately 1 pmol receptor/ml) was added to the cis compartment (1 ml) with stirring. AMPA (283 nM) was then added to the same compartment and the membrane current was registered with the voltage clamped at 42.2 mV. The traces shown in this figure consist of 512 data points recorded at 0.2 ms intervals. The dotted lines correspond to conductance levels of 100 pS. The top panel represents recordings made under control conditions, while the bottom panel represents recordings made from the same bilayer following equilibration in 10 μg/ml heparin.

between soluble receptors and other proteins, with these protein-protein interactions leading to the observed alteration in properties. The present set of data do not allow for determination of the mechanism of heparin's actions nor for calculation of the stoichiometry of the heparin/AMPA receptor association. Perhaps the only assertion which can presently be made about the nature of the physical interaction between heparin and AMPA receptors is that it must involve the extracellular domain of the receptors since heparin in the reconstitution studies was added on the same side of the membrane as AMPA.

In the present study, heparin was found to induce changes in the properties of soluble AMPA receptors in two different systems: (1) in binding assays, heparin reduced the apparent affinity of receptors, and (2) in reconstitution experiments, heparin increased the mean time of channel opening. These two observations might seem to be unrelated. However, it has previously been shown that most AMPA receptors in conventional binding assays are in a desensitized state [5]. Since there is a difference in agonist affinity between the sensitized and desensitized forms of the receptor [5,15,21], changes in any aspect of receptor kinetics are capable of influencing the apparent affinity of receptors in a binding assay. If the main action of heparin is to stabilize the open channel conformation of the AMPA receptor (as suggested by the reconstitution data), this might be expected to shift the balance in an equilibrium binding assay toward the lower affinity sensitized form of the receptor since receptors held in the open state cannot readily desensitize according to traditional models [12]. This would result in some of the receptors in the binding assay exhibiting a lower apparent affinity for agonist, which is precisely the result observed. Thus, the binding data and the reconstitution data presented here may simply represent different descriptions of a single underlying effect of heparin on AMPA receptor properties.

One result from the present study which might seem anomalous is that heparin had little effect on the binding of the AMPA receptor antagonist [^{3}H]CNQX to soluble AMPA receptors. However, [^{3}H]CNQX binding is known to be insensitive to other treatments which affect affinity for [^{3}H]AMPA, such as incubation with potassium thiocyanate [4,8]. Thus, heparin's lack of effect on [^{3}H]CNQX binding is not inconsistent with the observation that heparin reduces the affinity of [^{3}H]AMPA binding; indeed, the discrepancy between the results obtained with the two radiolabeled ligands highlights the point that heparin does not cause a generalized degradation or occlusion of the binding domain but rather acts by influencing specific aspects of receptor kinetics. A second result reported here which might seem anomalous is that heparin, which has such a profound effect on the binding properties of solubilized AMPA receptors, has little effect on the properties of membrane-associated AMPA receptors. However, this finding could be explained by assuming either (1) that membrane-associated AMPA receptors are in a conformation which is not susceptible to alteration by association with heparin or (2) that the heparin-association domains of membrane-associated AMPA receptors are already occupied by endogenous proteoglycans. With regard to the second possibility, it may be of interest that most membrane-associated AMPA receptors exhibit relatively low affinity for [^{3}H]AMPA [3]; the K_D for the majority of membrane-associated receptors is in the same range (300–600 nM) as that reported here for solubilized receptors treated with heparin. This observation suggests the possibility that the characteristic low-affinity state of membrane-associated AMPA receptors might result from stable interactions between synaptic AMPA receptors and endogenous proteoglycans.

In summary, heparin has been shown to bind to and alter the properties of AMPA receptors. Heparin has effects on a large number of physiological processes [11], and this broad spectrum of action may preclude the use of heparin in electrophysiological studies of AMPA receptors in intact preparations. However, heparin could prove to be a useful tool in studying the properties of purified AMPA receptors in reconstituted systems [2,13,23]. Moreover, the demonstration of an interaction between AMPA receptors and heparin raises the possibility that native AMPA receptors might associate with endogenous proteoglycans, such as agrin [18], which are known to be involved in the formation and regulation of synaptic junctions [14].

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