Subcellular localization and molecular pharmacology of distinct populations of [³H]-AMPA binding sites in rat hippocampus

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- 1 The subcellular distributions of [³H]- α -amino-3-hydroxy-5-methylisoxazolepropionate ([³H]-AMPA) and [³H]-kainate binding sites in rat hippocampus were investigated by cell fractionation techniques.
- 2 Two major populations of [³H]-AMPA sites were detected with the majority of binding located intracellularly in the microsomal (P₃) fraction. Most of the remaining sites were in the synaptosomal membrane fraction but some were also present in the nuclear fraction. In contrast, essentially all of the [³H]-kainate binding sites were in the synaptosomal membrane fraction.
- 3 Saturation binding analysis yielded K_D and B_{max} values for [³H]-AMPA of 147 nM and 2.6 pmol mg⁻¹ protein respectively for the synaptosomal membrane-associated sites and 129 nM and 5.3 pmol mg⁻¹ protein respectively for the microsomal sites.
- 4 Both main populations of [³H]-AMPA sites displayed the same rank order of inhibition by competitive ligands, the apparent Mr values of GluR1 subunits were equivalent, suggesting the same degree of post-translational modification and the hydrodynamic properties of the receptor complexes were identical.
- 5 These data are consistent with the hypothesis that the movement of AMPA receptors between cellular compartments in the postsynaptic neurone could constitute one mechanism underlying long-term potentiation in the hippocampus.

Keywords: Glutamate receptors; excitatory amino acid receptors; AMPA; synaptic plasticity; long-term potentiation; kainate; subcellular fractionation; receptor localization

Introduction

Glutamate receptors (GluRs) are the major class of excitatory receptors in the vertebrate central nervous system. They mediate fast excitatory neurotransmission, they are involved in synaptic formation and stabilization and their prolonged activation is potentially neurotoxic (Choi, 1992; Bliss & Collingridge, 1993). Ionotropic GluRs are usually classified into N-methyl-D-aspartate (NMDA) and non-NMDA (kainate and α -amino-3-hydroxy-5-methylisoxazolepropionate, AMPA) subtypes. The cDNAs encoding many of the ionotropic receptor subunits have been cloned and characterized. The sequences which code for the AMPA receptor subunits GluR1–GluR4 show levels of amino acid identity of greater than 70% and they share multiple putative sites for regulation at both the nucleic acid and protein levels (Sommer & Seeburg, 1992; Wenthold *et al.*, 1992; Hollmann & Heinemann, 1994).

The molecular pharmacology and cellular and subcellular distributions of AMPA receptors are of widespread interest because of their underlying roles in normal physiological and pathological processes (Choi, 1992; Bliss & Collingridge, 1993). It is becoming increasingly apparent that AMPA receptors are subject to tight cellular controls and a variety of processes which regulate AMPA receptor expression at the nucleic acid level and which modulate their function at the protein level have been described. To date, these mechanisms include alternative RNA splicing and RNA editing at the nucleic acid level, post-translational modification events such as phosphorylation and glycosylation at the protein level and regulation by phospholipase A₂ and receptor-associated modulatory proteins at the cellular level (for recent reviews see Barnes & Henley, 1992; Swope *et al.*, 1992; Seeburg, 1993).

Another way by which the neurone can control receptor activity is by subcellular compartmentalization. Indeed, several groups have observed significant levels of GluR1–GluR4 immunoreactivity inside neurones although quantitative analyses

of the plasma membrane and intracellular localisations have not been performed (Eshhar *et al.*, 1993; Molnar *et al.*, 1993; Baude *et al.*, 1994). However, recent quantitative autoradiographic experiments carried out on fractionated homogenates from *Xenopus* brain have suggested that in that tissue up to 80% of the [³H]-AMPA and [³H]-6-cyano-7-nitroquinoxaline-2,3-dione ([³H]-CNQX) (but not [³H]-kainate) binding is missing from synaptosomal membrane preparation compared to whole cell homogenates (Barnes & Henley, 1993).

The aim of this study, therefore, was to determine quantitatively the subcellular compartmentalization of [³H]-AMPA binding in rat hippocampus. The pharmacological and biochemical properties of the sites were compared to gain insight into possible mechanisms by which AMPA receptor processing and compartmentalization may effect the molecular processes underlying synaptic plasticity.

Methods

Wistar rats (250 g) were killed by decapitation and the brains rapidly removed and dissected. Hippocampi were pooled from several rats and either homogenized immediately or frozen rapidly and stored at –80°C.

Subcellular fractionation by differential centrifugation

Fractionation procedures were based on previously described protocols (Maeno *et al.*, 1971; Rodríguez de Lores Arnaiz & Pellegrino de Iraldi, 1984). Rat hippocampi were homogenized with a glass-Teflon homogenizer in 10 volumes of ice-cold 320 mM sucrose and then spun for 10 min at 1000 g in a JA 20 rotor. All centrifugation steps were carried out at 4°C and all homogenizations were in ice-cold buffer. The supernatant was saved and the pellet was rehomogenized and recentrifuged twice more. The final pellet (P₁) consists of nuclei and cell debris. The pooled supernatant was centrifuged at 11,500 g for 20 min. The supernatant was saved and the pellet was re-

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suspended and respun. The pooled supernatants were spun at 100,000 *g* for 90 min, the resultant pellet from this step comprises microsomes and the supernatant is defined as cell sap. The combined pellets from the 11,500 *g* spins were resuspended in 0.32 M sucrose and layered in 4 ml aliquots onto discontinuous sucrose gradients (4 ml 1.2 M sucrose, 4 ml 0.8 M sucrose) in 12 ml Beckman SW40 centrifuge tubes. The gradients were centrifuged at 58,000 *g* for 110 min. Cell components migrated to the interfaces of the gradient; the 0.32/0.8 M sucrose interface (P₂A) is myelin; the 0.8/1.2 M sucrose interface (P₂B) comprises synaptosomal membranes and the pellet (P₂C) consists of mitochondria. Each of the fractions was frozen at -80°C, thawed and resuspended to 35 ml in ice-cold Tris-citrate buffer (Tris 50 mM, EDTA 1 mM, EGTA 1 mM, pH 7.4 at 0°C with citric acid) and centrifuged at 48,000 *g* for 20 min to pellet the membranes. This was repeated a total of three times to ensure complete elimination of any remaining endogenous L-glutamate. The final pellet from each fraction was resuspended in Tris-citrate buffer to a final protein concentration of 0.1–0.5 mg ml⁻¹ and stored in aliquots at -80°C. A schematic of the subcellular fractionation procedure is shown in Figure 1.

Radioligand binding

Hippocampal cell fractions (usually 50 µl) in Tris-citrate buffer were added to test-tubes containing 10 µl of competing compound or vehicle and 10 µl [³H]-AMPA (58 Ci mmol⁻¹) at a final concentration of 25 nM. Potassium thiocyanate was included in all [³H]-AMPA binding assays to a final concentration of 100 mM (Honoré & Drejer, 1988). The final assay volume was adjusted to 100 µl with Tris buffer. The reaction mixture was incubated at 0°C for 90 min and then terminated by rapid filtration through GF/B filters pre-soaked in 0.05% polyethylenimine. The filters were washed rapidly with three × 3 ml aliquots of ice-cold Tris buffer and the total wash time 2–3 s. Non-specific binding was defined by inclusion of 1 mM L-glutamate. Radioactivity remaining on the filters was assayed by liquid scintillation spectroscopy at an efficiency of approximately 48%. Binding assays for [³H]-kainate (58 Ci mmol⁻¹; 25 nM final concentration) were exactly the same except KSCN was not included and non-specific binding was defined by 100 µM kainate. The binding affinities (*K_D*) and total number of sites (*B_{max}*) for [³H]-AMPA were determined by direct fitting to saturation binding curves. The data were

Figure 1 Schematic diagram of the differential centrifugation protocol used to isolate subcellular fractions. Details of the procedure are given in the Methods section.

analysed with the KaleidaGraph programme using the logistic equation:

$$b = B_{\max} \cdot [L]^n / [L]^n + K^n$$

where $[L]$ is the free ligand concentration, K is the dissociation constant and n is the Hill number. A two-site model did not fit the data significantly better than the one-site model used.

SDS-PAGE and Western blotting

The anti-peptide antibody Ab9T was a generous gift from Dr Robert Wenthold, NIH, Bethesda, U.S.A. Hippocampal subcellular fractions were subjected to discontinuous SDS-PAGE on 7.5% acrylamide gels. The electrophoresed protein was blotted on to nitro-cellulose for 30–40 min at ~50 V (~170 mA–100 mA) in CAPS (3-[cyclohexylamino]-1-propanesulphonic acid) transfer buffer (10 mM CAPS pH 11, 10% methanol). Non-specific binding to the blots was blocked by incubation overnight at 4°C in TN buffer (50 mM Tris-HCl (pH 7.4), 0.5 M NaCl) containing 5% (w/v) dry milk powder.

For immunolabelling, blots were incubated for 1 h at 28°C in a Hybaid hybridisation oven with 1.0 $\mu\text{g ml}^{-1}$ Ab9T and 0.15 $\mu\text{g ml}^{-1}$. The blots were extensively washed (3 × 15 min) in TNT buffer (TN buffer plus 0.1% Triton X-100) and then incubated with the secondary antibody, horse-radish peroxidase (HRP) conjugated swine anti-rabbit IgG for 1 h. Fol-

lowing further extensive washes (3 × 15 min in TNT buffer) the blots were soaked in 3,3-diaminobenzidine-hydrogen peroxide (DAB-H₂O₂) solution to develop the HRP stain. The immunostained blots were washed in distilled water to stop the HRP reaction.

Protein solubilisation

[³H]-AMPA sites were solubilized essentially as described elsewhere for *Xenopus* CNS (Henley & Barnard, 1989). Well-washed freeze-thawed synaptosomal membrane or microsomal fractions were resuspended in Tris-citrate buffer containing 1% n-octyl- β -glucopyranoside (octylglucoside). Following incubation with gentle agitation for 1 h at 4°C, the mixture was centrifuged for 1 h at 100,000 g and the supernatant containing solubilized protein was collected and either used immediately or stored at –80°C until required.

Sucrose density gradient centrifugation

Gradients were constructed and sampled as described previously (Henley *et al.*, 1992). Briefly, octylglucoside-solubilized synaptosomal membrane or microsomal extracts (0.5 ml) were layered on to 15 ml 5%–20% sucrose gradients. The gradients were made in Tris-citrate buffer containing 0.8% octylglucoside and were poured using the gradient maker facility on the BioRad Econosystem. Centrifugation was at 4°C for 17 h at $g_{\max}$ 260,000 in a Beckman SW-40 rotor. Thirty drop fractions were collected from the bottom of the gradient and assayed for [³H]-AMPA binding as described above.

Electron microscopy

The micrographs show representative examples of freshly prepared subcellular fractionations that were not subjected to any freeze-thaw-wash steps. All of the electron microscopy was performed by use of standard protocols on a commercial contract basis by Birmingham University Electron Microscopy Service.

Protein assays

Protein concentrations were determined by use of the BioRad assay kit with BSA as standard.

Results

The subcellular localizations of [³H]-AMPA and [³H]-kainate binding are shown in Figure 2. Using 25 nM [³H]-AMPA, binding in rat hippocampus was localized predominantly in the microsomal fraction and to a lesser extent in the synaptosomal membrane fraction. In contrast, [³H]-kainate sites were located almost exclusively in the synaptosomal membrane fraction.

Electron micrographs prepared from subcellular fractions which were not subjected to freeze-thaw-wash protocols are shown in Figure 3. Structures and membrane fragments characteristic of each of the sucrose gradient fractions are apparent in each of the pictures. The P₁ nuclear and the P₂A myelin fractions (b, c) comprise mainly filamentous myelin membranes with an assortment of smaller vesicular structures also present. The P₃ microsomal fraction (d) contains mostly small vesicular structures consistent with ribosomes and intact microsomes. In addition some small filamentous membranes are also present in the microsomal fraction. The P₂B synaptosomal membrane fraction (e) shows a very different profile comprising mainly much more electron-dense structures, a characteristic of postsynaptic membranes, and a few mitochondria. The P₂C fraction (f), consists almost entirely of mitochondria with some smaller electron dense structures also present.

Saturation binding curves for [³H]-AMPA binding to both populations of sites are shown in Figure 4. Under the experi-

Figure 2 Distributions of (a) [³H]-AMPA and (b) [³H]-kainate binding to rat hippocampal subcellular fractions prepared by differential centrifugation. The data are expressed as pmol of radioligand bound per mg of protein at a single concentration of 25 nM for both radioligands. The results are the means $\pm$ s.e. mean of 4 separate experiments for [³H]-AMPA and 3 separate experiments for [³H]-kainate.

mental conditions used in this study, a single class of binding sites was detected in each of the subcellular fractions. The K_D and B_{max} values were 147.5 ± 25.6 nM and 2.6 ± 0.3 pmol mg^{-1} protein respectively for synaptosomal membranes and the Hill number (n_H) was 0.97. For microsomal membranes the K_D and B_{max} values were 129.3 ± 22.7 nM and 5.3 ± 0.6 pmol mg^{-1} protein respectively and the n_H was 0.94. The data are the means and s.e.mean values from 4 experiments each using hippocampal tissue pooled from two rats. The K_D values obtained for [^3H]-AMPA binding to both subcellular fractions are well within the range of previously reported values fitted to

both one and two site models (Honoré & Drejer, 1988; Smith & McIlhinney, 1992). Furthermore, both the K_D and B_{max} values are in good agreement with saturation binding results from different membrane preparation protocols and utilising much higher concentrations of [^3H]-AMPA (up to 1 μM) (K.K. Dev, T. Honoré & J.M. Henley, unpublished observation).

Displacement experiments for [^3H]-AMPA binding to the two main populations of sites are shown in Figure 5. The inhibition curves were similar for both the microsomal and synaptosomal sites. For microsomal membranes the IC_{50} values were: CNQX, 0.20 ± 0.04 μM ; kainate, 6.5 ± 1.5 μM ; quisqua-

Figure 3 Representative electron micrographs showing the structures present in the subcellular fractions. The panels are: (a) crude homogenate; (b) P_1 , nuclear fraction; (c) P_2A , myelin fraction; (d) P_3 , microsomal fraction; (e) P_2B , synaptosomal fraction; (f) P_2C , mitochondrial fraction. All of the micrographs are at the same magnification and were prepared by the specialist e.m. facility at Birmingham University. The scale bar in panel (f) corresponds to 1 μm .

Figure 4 Saturation binding curves for [3 H]-AMPA to extensively freeze-thaw-washed synaptosomal membrane fragments (●) and microsomes (○). The data are representative of 4 separate experiments each of which used different synaptosomal and microsomal membrane preparations. Values for the binding affinities and total number of sites are given in the text.

Figure 5 Competition binding curves for [3 H]-AMPA to (a) extensively freeze-thaw-washed synaptosomal membrane (P₂B) and (b) microsome fragments by (○) CNQX, (■) L-glutamate, (●) kainate and (△) quisqualate. The data are normalized to 100% in the absence of displacing drug and are the mean $\pm$ s.e. mean of at least 4 separate experiments for each displacer. The IC₅₀ values are given in the text.

late, $0.05 \pm 0.01 \mu\text{M}$; L-glutamate, $6.3 \pm 2.0 \mu\text{M}$. The IC₅₀ values for synaptosomal membranes were: CNQX, $0.15 \pm 0.01 \mu\text{M}$; kainate, $14.4 \pm 2.5 \mu\text{M}$; quisqualate, $0.09 \pm 0.05 \mu\text{M}$; L-glutamate, $11.9 \pm 3.6 \mu\text{M}$. Data for the displacement curves are the means and s.e. mean values of 3–4 experiments each using hippocampal tissue pooled from two rats.

Figure 6 Sedimentation profiles of octylglucoside-solubilized microsomal (○) and synaptosomal (●) [3 H]-AMPA binding sites in sucrose density gradients. Approximately 30,000 specific d.p.m., determined with 25 nM [3 H]-AMPA, were added to the gradients and the amount of protein was adjusted accordingly. The data show the specific [3 H]-AMPA binding activity, expressed in d.p.m., in each of the gradient aliquots. The arrow indicates the position of haemoglobin in fraction 17 in all of the gradients (sedimentation coefficient = 4.5 S).

The hydrodynamic properties of the microsomal and synaptosomal [3 H]-AMPA binding sites were analysed by sucrose density centrifugation (Figure 6). Within the resolution of the technique, both sets of sites yielded identical sedimentation profiles indicating equivalent sizes, therefore implying that the microsomal sites comprise fully assembled multimeric receptor complexes.

Western blot analysis of the microsomal and synaptosomal fractions was performed using an anti-peptide antibody specific for GluR1 subunit of AMPA receptors. Immunoblots were taken from SDS-PAGE gels run using identical amounts of protein from each fraction. Under exactly the same labelling conditions, darker labelling was observed in the microsomal fraction and qualitatively, the intensity of the HRP-labelled blots were consistent with the [3 H]-AMPA binding data in that the microsomal fraction stained more intensely than the synaptosomal fraction. Furthermore, the GluR1 labelled band was the same Mr value in both fractions indicating that the GluR1 polypeptide in the microsomal fraction was glycosylated to the same extent as the synaptosomal membrane subunits. These data suggest that for GluR1 subunits at least, the intracellular and plasma membrane sites have been subjected to the same extent of post-translational processing.

Discussion

The data presented here indicate that a high proportion of [3 H]-AMPA binding in the hippocampus is located intracellularly, predominantly in the microsomal fraction. The distribution of [3 H]-kainate sites, however, shows a markedly different profile with binding confined mainly to the synaptosomal fraction with little intracellular binding.

Active postsynaptic regions comprise mainly dendritic spines which are approximately $1 \mu\text{m}$ long protrusions from the body of the dendrite. A possible complicating factor that could influence the interpretation of the results obtained in this study is the contamination of microsomal fraction with postsynaptic synaptosomal membranes originating from these spine structures. During tissue homogenization it is possible that some spines sheared from their parent dendrites could separate from the presynaptic terminal and reseal to form vesicles including the postsynaptic membrane. Vesicles of this size might then migrate to the microsomal rather than the synaptosomal fraction and give an inaccurate distribution of [3 H]-AMPA binding.

Two main criteria were used to exclude any artefactual re-

sults of this kind. Firstly, in contrast to the structures observed in the synaptosomal membrane fraction, the micrographs showed no evidence for characteristic postsynaptic densities in the microsomal fraction. Secondly, the subcellular distribution of [3 H]-kainate binding was markedly different from that for [3 H]-AMPA. High affinity [3 H]-kainate binding sites, comprising the KA-1 and KA-2 and/or GluR 5, 6 and 7 subunits, are extremely abundant in areas of the hippocampus, particularly in the CA3 pyramidal cells. However, for [3 H]-kainate binding to provide a good control it is crucial that at least a proportion of kainate receptors must be located at the postsynaptic membrane.

Evidence for presynaptic kainate receptors in the hippocampus has come from the observations that high affinity [3 H]-kainate binding appears in the CA3 region of the hippocampus between post-natal days 7 and 21, exactly matching the development of the mossy fibres which are axonal projections from the dentate granule cells (Miller *et al.*, 1990). Similarly, a dramatic decrease in high affinity [3 H]-kainate binding in the CA3 region occurs if dentate granule cells are lesioned with the resultant degeneration of the mossy fibres, which terminate on the dendritic trees of the CA3 pyramidal cells (Repressa *et al.*, 1987). On the other hand, strong evidence for postsynaptic kainate receptors in the hippocampus comes from: (1) high levels of the kainate receptor subunit cDNA KA-1 in CA3 pyramidal cells (Wisden & Seeburg, 1993); (2) the retention of high affinity [3 H]-kainate binding in denervated dentate gyrus molecular layer (Ulas *et al.*, 1990); and (3) direct immunocytochemical evidence for exclusively dendritic localizations of GluR 5, 6 and 7 subunits in primate hippocampus with no immunoreactivity in the soma (Good *et al.*, 1992). Thus high affinity [3 H]-kainate binding represents a valid marker for synaptic membranes and the lack of [3 H]-kainate activity in the microsomal fraction provides good evidence that cross-contamination of the subcellular fractions is not a major factor in the unusual distribution of [3 H]-AMPA binding.

The immunohistochemical localizations of the AMPA receptor subunits GluR1–GluR4 have been studied by several different groups. In rat brain sections the regional distribution of antibodies raised against GluR1–GluR4 AMPA receptor subunits were differentially localized within neuronal cell bodies and processes in cerebral cortex, basal ganglia, limbic system, thalamus, cerebellum and brainstem and the precise patterns and cellular localizations appear to be unique for each antibody (Martin *et al.*, 1993). Ultrastructurally, the subunit immunoreactivities were present within cell bodies, dendrites and dendritic spines of specific subsets of neurones and, in the case of GluR1 and GluR4, in some populations of astrocytes (Martin *et al.*, 1993). In rat hippocampal cultures the same group showed that the GluR1 antibody stained all neurones, with the somata and dendrites, but not axons, showing high levels of immunoreactivity. Variable degrees of staining observed in different individual neurones between the dendritic spines and the dendritic shafts with highly punctate hot spots visible, many of which corresponded to synapses. Essentially identical distributions were also observed with a GluR2/3 antipeptide antibody (Craig *et al.*, 1993). Other equally elegant immunohistochemical studies have also shown that the AMPA receptor subunits GluR1–GluR4 are located in postsynaptic densities, the associated dendritoplasm and in the cytoplasm of the soma (Petralia & Wenthold, 1992; Eshhar *et al.*, 1993; Molnar *et al.*, 1993).

Nonetheless, a major constraint on these immunohistochemical investigations is the lack of pharmacological characterization and quantitative data about the actual numbers of receptors present in each of the cellular and subcellular locations. These latter questions have been addressed by the alternative and complementary techniques employed in the present study. My results indicate that in excess of 70% of AMPA receptors are located inside the neurone, predominantly in the microsomal fraction with less than 30% of receptors located in the plasma membrane at any given time.

The pharmacological and biochemical characteristics of the synaptosomal membrane and microsomal AMPA receptors appear to be very similar with no marked differences in their [3 H]-AMPA binding affinities or displacement profiles.

Although the precise subunit composition of the population of intracellular receptors was not addressed in this study, the abundance of the GluR1 subunit shown in Figure 7 is consistent with the distribution of [3 H]-AMPA binding. Furthermore, the similarity of the hydrodynamic profiles of the receptor complexes together with the identical M_r values of the GluR1 subunits from synaptosomal membranes and microsomes suggest that polypeptide glycosylation is complete by the time the protein is inserted into the microsome and that no significant additional glycosylation of GluR1 occurs upon insertion into the synaptosomal membrane.

Long-term potentiation (LTP) in the rat hippocampus is an established model system for the study of the mechanisms of learning and memory (Bliss & Collingridge, 1993). AMPA receptors are integral to LTP since they mediate the synaptic responses that are enhanced. The pivotal role of AMPA receptors in LTP is well documented (Bliss & Collingridge, 1993). While the decrease in the efficacy of synaptic transmission mediated by AMPA receptors could be achieved by a variety of mechanisms at transcriptional, translational and post-translational levels, one attractive mechanism by which rapid onset alterations in the efficacy of synaptic responses could be brought about is the recruitment of additional AMPA receptors from an intracellular pool. These recruited receptors may be identical to those already in the synaptosomal membrane and act solely by increasing the physical number of AMPA receptors available at the postsynaptic membrane. Alternatively, receptors recruited to the synaptosomal membrane could have different subunit compositions and/or different phosphorylation states from those already present. Such differences could lead to a higher affinity for L-glutamate thereby increasing the sensitivity of the postsynaptic membrane. The results from the present study suggest the former of these two possibilities.

The presence of intracellular AMPA receptor subunits in immunohistochemical studies has been explained in terms of normal protein synthesis, transport and degradation. While the results presented in this study are compatible with this hypothesis, the quantitative comparison of the synaptosomal and intracellular AMPA receptor populations, together with the markedly different results obtained for [3 H]-kainate binding sites suggests that either AMPA receptor turnover is very rapid or that they are stored in the microsomes, possibly for rapid insertion into the synaptic membrane. With the currently

Figure 7 Western blots of (a) synaptosomal and (b) microsomal fractions with the anti-peptide antibody Ab9T directed against GluR1. The same amount of protein (10 μ g) was loaded into each lane and the SDS-PAGE gel was run and blotted as described in the Methods. The arrows indicate the positions of BioRad high molecular weight standards.

available data it is not possible to distinguish between these two possibilities and definitive analysis of these dynamic processes will require time course experiments.

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