Human retina contains polyamine sensitive [^{3}H]-ifenprodil binding sites: implications for neuroprotection?

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Abstract

***Aims*—This study characterised the pharmacology of [^{3}H]-ifenprodil binding to the polyamine binding sites (PBS) on the N-methyl-D-aspartate (NMDA) receptor channel complex on human retinas. These data were correlated with the known neuroprotective effects of ifenprodil and eliprodil.**

***Methods*—Specific binding of [^{3}H]-ifenprodil (under sigma site blockade) was investigated using human retinal homogenates and radioligand binding techniques. Scatchard and competition analyses were utilised to define the pharmacology of the [^{3}H]-ifenprodil binding sites.**

***Results*—Specific binding of [^{3}H]-ifenprodil comprised 73% (SEM 3%) of total and reflected interaction with two affinity sites (K_ds = 0.39 and 4.3 μM) of different densities (B_{max} = 14.4 and 105 pmol/mg protein) (n = 5). The rank order of affinity of compounds competing for [^{3}H]-ifenprodil binding to the high affinity PBS was: ifenprodil > eliprodil > arcaine > spermine > diaminodecane > spermidine > putrescine >> MK-801 (n = 3–7). However, [^{3}H]-ifenprodil binding was minimally inhibited by glutamate, NMDA, and kainate.**

***Conclusion*—These studies have shown, for the first time, the presence of specific [^{3}H]-ifenprodil binding sites in the human retina with pharmacological characteristics of PBS associated with the NMDA receptor ionophore complex. The neuroprotective effects of eliprodil and ifenprodil may, in part, be mediated via these [^{3}H]-ifenprodil labelled sites.**

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The excitatory amino acids, glutamate and aspartate, are important neurotransmitters in the retina and the rest of the central nervous system (CNS).[1–3] However, excessive accumulation of these amino acids in the extracellular space can lead to neuronal death via excitotoxicity mediated calcium overloading, production of free radicals, and activation of intracellular proteases.[4 5] Ischaemic insults in the retina can also excerbate the situation by enhancing glutamate release and thus increasing its neurotoxic potential.[6] The toxic effects of glutamate in the retina have been known for a long time.[7 8] Although several subtypes of the glutamate receptor can mediate neurotoxicity,[4 5] the N-methyl-D-aspartate (NMDA) subtype appears to be primarily involved in causing retinal ganglion cell death.[9–12]

Optic neuropathies and ischaemic borne retinopathies, including glaucoma, are multifaceted diseases of the eye which can cause blindness.[13] Elevated intraocular pressure (IOP) is a major risk factor in the aetiology of glaucoma.[13] However, increased intravitreal glutamate levels have been recently observed in glaucoma patients and in experimentally induced glaucoma in monkeys.[14] Hence, elevated vitreal glutamate levels coupled with retinal ischaemia[5 11 14 15] and ocular hypertension may be responsible for retinal ganglion cell loss and mechanical damage to the optic nerve head, eventually leading to visual field deficits and blindness.[13] Therapeutic intervention to preserve vision in the long term may therefore not soley rely on IOP lowering drugs[13] but also on drugs which specifically block the neurotoxic effects of excessive amounts of glutamate at the retinal ganglion cell level.[9 15 16]

The NMDA receptor is composed of a number of different subunits forming the ion channel, with additional modulatory sites on the receptor ionophore complex including the polyamine binding sites and strychnine insensitive glycine sites.[2 17] Eliprodil (SL82.0175) and ifenprodil are potent neuroprotectants[18–20] believed to elicit their protective actions by blocking the polyamine binding site[18 19] and/or the NR2B subunit on the NMDA receptor channel complex[17] and thus preventing the sequelae of neurotoxic events mentioned above. Since eliprodil has recently been shown to protect rat retinal ganglion cells from NMDA induced toxicity[12] in vitro and to preserve retinal architecture and function in rats and rabbits subjected to excitotoxic and ischaemic insults in vivo,[21] we decided to investigate whether specific polyamine binding sites existed in human retinas which may help explain the neuroprotective effects of eliprodil and ifenprodil mentioned above. Therefore, the aims of the present studies were to investigate the presence of specific [^{3}H]-ifenprodil binding sites on washed homogenates of human retinas and to characterise these sites pharmacologically, relating this information to the NMDA receptor and its modulatory sites to functions in the retina. To the best of our knowledge, this represents the first such study to address these topics.

Materials and methods

PREPARATION OF RETINAL HOMOGENATES

Human retinas were obtained from human donor cadaver eyes within 8–18 hours of death and immediately frozen in liquid nitrogen until

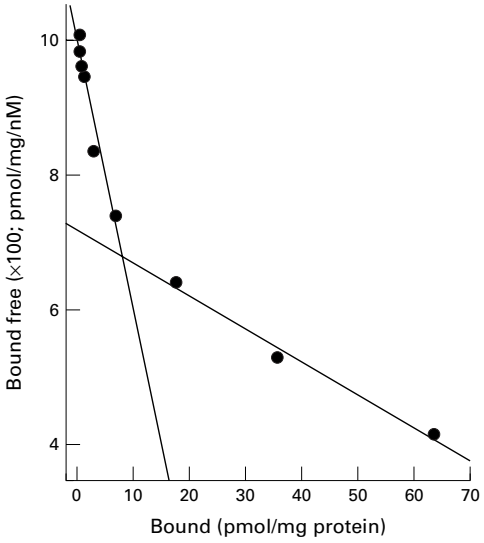

Figure 1 Scatchard analysis of specific [³H]-ifenprodil binding to human retinal homogenates. Experiments were conducted in the presence of 3 μM DTG and 10 μM GBR12909 to block the radioligand from binding to sigma binding sites. Plots shown are from one sample experiment (average of triplicates per point) for illustrative purposes, but composite data from five independent experiments are shown in the Results section.

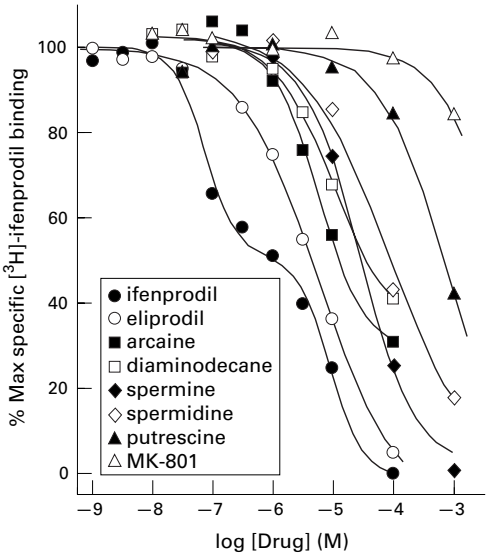

Figure 2 Competition curves for various compounds displacing [³H]-ifenprodil binding from polyamine binding sites on homogenates of human retinas. The assays were conducted in the presence of 3 μM DTG and 10 μM GBR12909 to block the radioligand (2 nM final) from binding to sigma binding sites. The dpm bound in the presence of each competing ligand concentration were converted to an overall percentage of maximum specific [³H]-ifenprodil binding. Data shown are from one experiment (average of triplicates per point) for illustrative purposes, but composite data, in terms of affinity values (K_is), from 3–7 experiments are shown in the Results section.

used for the radioligand binding experiments. None of the donors had any documented ocular diseases and the mean ages of the donors were 80 (SEM 2) years (32 donors). The pooled frozen thawed retinal tissue (usually from six pairs of eyes at a time) was gently homogenised in 50 volumes of ice cold 5 mM TRIS. HCl (pH 7.4) using a Polytron tissue disrupter (setting 5–7 seconds) in a laminar flow hood and centrifuged at 4°C at 30 000 *g* for 30 minutes. The resultant pellets were resuspended by homogenisation in 20 volumes of fresh 5 mM TRIS HCl buffer, recentrifuged as above and then resuspended in more fresh buffer for the binding assays.

RECEPTOR BINDING ASSAYS

The ligand binding assays were performed as previously described.[22] The retinal homogenates (0.2 mg protein final), containing 3 μM DTG (1,3-di(2-tolyl)guanidine, HCl) and 10 μM GBR12909 (final assay concentrations) to block [³H]-ifenprodil binding to sigma binding sites[22–24] were incubated with 2–2.5 nM [³H]-ifenprodil in the absence or presence of 1 mM spermine in a total volume of 500 μl for 30 minutes at 20°C to define total and non-specific binding, respectively. Since DTG and GBR12909 have affinities of 40–60 nM for the sigma sites,[23] the concentrations employed in the current studies were expected to block all the sigma binding sites in the retinal homogenates based on the fractional receptor occupancy principle. The incubations were terminated by rapid vacuum filtration over Wallac glass fibre GF/B filters previously soaked in 0.3% polyethyleneimine using 12 ml of ice cold 50 mM TRIS.HCl (pH 7.4 at 4°C). Filter bound radioactivity was determined on a β scintillation counter and the data analysed using non-linear, iterative curve fitting computer programs (see below).

MATERIALS

Human cadaver donor eyes were obtained from local eye banks within 8–18 hours after death and transported in ice cold Dexol or Optisol corneal preservation medium. [³H]-ifenprodil (68.1 Ci/mmol) was purchased from Amersham Corp (Arlington Heights, IL, USA), while unlabelled drugs including ifenprodil ((±))-(R*,S*)-a-(4-hydroxyphenyl)-α-methyl-4-(phenylmethyl)-1-piperidine-ethanol-(R,R)-2,3-dihydroxy-butanedioate(2:2) (salt), hemihydrate), were purchased from Research Biochemical, Int (Natick, MA, USA). However, eliprodil HCl ((±)-a-(4-chlorophenyl)-4[(4-fluorophenyl)methyl]-1-piperidine-ethanol) was a generous gift from Synthelabo Recherche (Bagneux, France). All other standard chemicals and reagents were purchased from Sigma Chemical Company (St Louis, MO, USA).

DATA ANALYSIS

The original data (dpm bound) were analysed using a non-linear, iterative curve fitting computer program[25 26] incorporating a logistic function. The competition data for ifenprodil *v* [³H]-ifenprodil were processed using the "EBDA" suite of computer programs[27] to perform Scatchard analysis and thus derive the apparent receptor affinity (K_d) and apparent density (B_{max}) parameters. Data are presented as mean (SEM) from several experiments.

Results

[³H]-Ifenprodil (2.0–2.5 nM final) (in the presence of 3 μM DTG and 10 μM GBR12909) binding to human retinal homogenates (0.2 mg protein/assay tube)

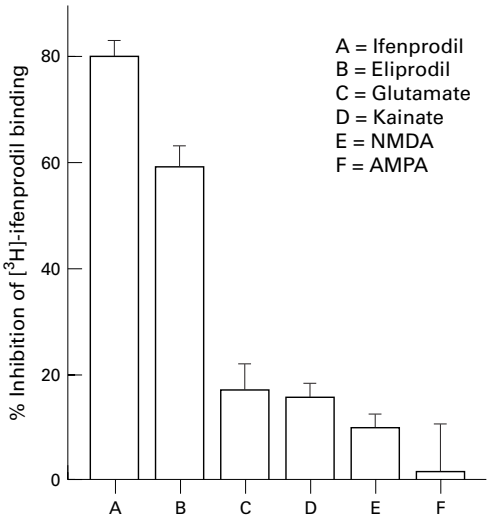

Figure 3 Effect of various glutamate receptor binding site ligands and polyamine antagonists, ifenprodil and eliprodil, on [³H]-ifenprodil binding to human retinal homogenates. Compounds were tested at 10 μM and the data shown are mean from ⩾ 3 experiments; vertical lines are SEM.

Table 1 Affinities of polyamines and polyamine antagonists for [³H]ifenprodil binding to human retinal homogenates

Compound	*Apparent affinity in human retina (K_i; μM)*	*Pseudo-Hill coefficient (nH)*
Ifenprodil	0.7 (0.1)	0.6 (0.1)*
Eliprodil	6.8 (0.7)	1.0 (0.1)
Arcaine	20.2 (4.7)	0.8 (0.1)
Spermine	27.6 (2.6)	1.0 (0.1)
Diaminodecane	51.2 (15.3)	0.8 (0.2)
Spermidine	81.2 (15.9)	0.8 (0.1)
Putrescine	>560	—

Data are mean (SEM) from 3–7 independent experiments. [³H]ifenprodil binding was conducted in the presence of 3 μM DTG and 10 μM GBR12909 to block sigma binding sites.
*nH significantly <1, and dissection of the biphasic competition curves yielded the data presented in the Results section. Preliminary studies with rat and rabbit retinas yielded the following estimated affinities: ifenprodil = 0.5–0.9 μM, eliprodil = 0.9–1.1 μM, spermine = 47–67 μM, spermidine = >100 μM and putrescine = >650 μM.

comprised 2870 (SEM 139) dpm total binding and 790 (99) dpm non-specific binding (yielding 73% (3%) specific binding; 2080 dpm specific), respectively. Scatchard analyses of competition data identified two apparent classes of different affinity sites of the specific [³H]-ifenprodil binding to polyamine binding sites in the human retina (Fig 1). Estimated equilibrium variables (dissociation constant [K_d] and apparent density of binding sites [B_{max}]) for [³H]-ifenprodil binding to the retinal homogenates were: dissociation constant (K_{d1}) = 390 (80) nM and apparent density of sites (B_{max1}) = 14.4 (2.9) pmol/mg protein; (K_{d2}) = 4300 (900) nM and B_{max2} = 105 (19) pmol/mg protein (n = 5 experiments). Preliminary studies on rat and rabbit retinas yielded K_{d1} = 300–500 nM and B_{max1} = 2–4 pmol/mg protein; K_{d2} = 2–4 μM and B_{max2} = 2–11 pmol/ mg protein (data not shown).

Eliprodil, ifenprodil, arcaine, diaminodecane, and polyamines such as spermine, spermidine and putrescine, and other compounds of interest, concentration dependently inhibited [³H]-ifenprodil binding in the human retinal preparations with different affinities (Fig 2; Table 1). The rank order of compound affinities was: ifenprodil > eliprodil > arcaine > spermine > diaminodecane > spermidine > putrescine >> MK-801 (n = 3–7). Again, preliminary studies on rat and rabbit retinas yielded similar data (for example, see Table 1). In view of the apparent biphasic nature of the competition curves for ifenprodil competing for [³H]-ifenprodil binding in the retinal preparations, the data were fitted to the two site model and were reasonably well resolved into two components. The following apparent affinity data for the two sites were obtained: IC_{50high} = 0.1 (0.02) μM (45% (5%) of sites) and IC_{50low} = 12.1 (4.4) μM (54% (5%) of sites). Ligands known to be potent and selective agonists at the glutamate receptor subtype binding sites, such as kainate, NMDA, AMPA ((±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid hydrobromide) and glutamate itself, exhibited low affinity (<20% inhibition at 10 μM final concentration; n = 3) for [³H]-ifenprodil binding to human retinal homogenates under sigma site blockade conditions (Fig 3).

Discussion

Polyamine binding sites represent novel modulatory sites on the heteromeric NMDA receptor channel complex which regulate ionic fluxes across neuronal cell membranes.[17 19] [³H] and [¹²⁵I]-ifenprodil have been used to selectively label these sites in various CNS tissues.[22 28 29] In the current studies we duplicated these assay conditions and have successfully demonstrated, for the first time, the presence of specific polyamine binding sites in the human retinal tissues. Using Scatchard and competition analyses, [³H]-ifenprodil binding in the retinal preparations could be resolved into two apparent binding sites of submicromolar and micromolar affinities. The affinity variables and the pharmacological properties of the high affinity sites in the human retina closely matched those previously reported for rat cerebral cortical synaptic membranes,[22] rat neonate cerebellar membranes,[29] and in rat brain sections studied by autoradiography.[28]

The neuroprotective and anti-ischaemic effects of the polyamine antagonists, eliprodil and ifenprodil, in a variety of cells/tissues in vitro and in many animal models in vivo are now well documented (see Scatton *et al* [19] for review). Additionally, these drugs have been shown to potently and efficaciously antagonise a variety of NMDA induced responses in vitro such as preventing NMDA induced neurotoxicity and ionic fluxes in retinal ganglion cells[12] and brain cells[30–32] and blocking calcium entry into neurons,[33 34] blocking transmitter release from striatum[35] and blocking NMDA induced electrophysiological responses in rat retinal ganglion cells[12] and other neurons.[33] In vivo, eliprodil has been shown to prevent NMDA induced retinal ischaemia and excitotoxicity in the rabbit leading to preservation of the electroretinogram (ERG).[21] Ifenprodil and eliprodil antagonised the majority of the above mentioned functional physiological and

pathological responses with potencies in the submicromolar to micromolar range, thus matching the affinity values (K_d and K_i values) obtained for [³H]-ifenprodil binding in the human retinal preparations in our current studies. Furthermore, the affinity values for the polyamine antagonists/NR2B antagonists (eliprodil, ifenprodil, arcaine, and diaminodecane) and polyamine agonists (spermine, spermidine, and putrescine) obtained here in the human retina (Table 1) also closely resembled those previously reported for rat brain, cerebral cortex, and cerebellum.[22 28 29] Likewise, recent preliminary studies on rat and rabbit retinas yielded similar drug affinities for displacing [³H]-ifenprodil binding from human retinas. The relatively low affinity of MK 801 in competing for [³H]-ifenprodil binding to retinal homogenates is consistent with the fact that MK-801 binds to the NMDA receptor channel[2 25] as opposed to interacting with the modulatory polyamine sites on the receptor channel complex as do the polyamine agonists and antagonists.[19 36] The reason for putrescine showing a lower affinity than spermine or spermidine is the fact that it is a precursor of the latter compounds.[36 37] As expected, glutamate receptor site selective compounds (glutamate, NMDA, AMPA, kainate) exhibited minimal affinities for the specific [³H]-ifenprodil binding sites in human retinal homogenates (Fig 3), thus confirming similar observations for rat CNS tissues[29] and providing further evidence for the polyamine sites being different from the receptor binding sites and/or the receptor ionophore sites associated with the NMDA receptor channel complex in the retina.

The NMDA receptor is an heteromeric complex composed of several subunits forming the ion conducting channel and expressing several modulatory sites including the polyamine and strychnine insensitive glycine sites.[2] Ifenprodil and eliprodil have been shown to selectively interact with the NR1A/NR2B subunits of the NMDA receptor with an affinity of 0.3–1.0 μM,[17 38] in addition to blocking the polyamine binding sites. Our current affinity data for these drugs in the retinal homogenates suggest that [³H]-ifenprodil may also be labelling the NR1A/NR2B subunits of the NMDA receptor in the retinal tissue in addition to labelling the polyamine binding sites. We currently do not have any evidence for this but once the new NR2B subunit specific antagonists such as CP-101,606[39] and Ro-25,6981[40] become commercially available, especially as radioligands, this hypothesis could be readily tested.

Our data for retinal homogenates do not indicate which retinal cells possess the polyamine binding sites/NR2B subunits of the NMDA receptor. However, immunohistochemical localisation of endogenous polyamines in the tiger salamander revealed that ganglion cells and amacrine cells are highly enriched in spermine and spermidine.[41] Furthermore, in situ hybridisation studies have shown that the NMDA receptors carrying the polyamine modulatory sites are also primarily present on retinal ganglion and amacrine cells.[42]

It is concluded that human retinas possess specific [³H]-ifenprodil binding sites, at relatively high concentrations, which exhibit pharmacological properties akin to the polyamine binding sites previously defined in numerous CNS tissues and cells. These specific polyamine sensitive [³H]-ifenprodil binding sites may be involved in mediating some or all of the neuroprotective effects of ifenprodil and eliprodil discussed above. The data presented in this communication suggest that since [³H]-ifenprodil binding sites are present in the human retinas that polyamine antagonists like eliprodil and ifenprodil may also exhibit neuroprotective properties in the human retina in vitro and perhaps in vivo. Studies to elucidate such activities of these compounds are therefore warranted and remain to be conducted.

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