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Agmatine acts as an antagonist of neuronal nicotinic receptors

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1 Tritiated agmatine has been used by others in ion flux methods to measure nicotinic receptor function in neurones. However, as shown here, agmatine blocks nicotinic receptor function in both the chick retina and the rat superior cervical ganglion at high concentrations.

2 In intact chick retina, agmatine 1 mM decreases dimethylphenylpiperazinium (DMPP)-induced depolarizations measured in the optic nerve by approximately 70%, while having little effect on responses induced by glutamate analogues. DMPP dose-response curves are reduced in a manner consistent with a non-competitive effect of agmatine, and agmatine at 1 mM does not prevent binding of ^{125}I-labelled neuronal bungarotoxin, a snake venom neurotoxin that competitively binds and blocks functional nicotinic receptors in chick retinal homogenates.

3 Agmatine (10 mM) substantially blocks both DMPP-induced depolarizations of rat superior cervical ganglion and synaptic transmission through the ganglion. Others have established that [^{3}H]-agmatine will pass through nicotinic receptor channels in the rat ganglion. These data suggest that agmatine acts both as a cation and as a weak channel blocker at neuronal nicotinic receptors.

Introduction

Nicotinic receptors are fairly non-selective ligand-activated cationic channels (e.g., Huang *et al.* 1978), Various radiolabelled cations have been used in ion flux methods to take advantage of the non-selectivity of the nicotinic channel and to assay functional receptors (e.g. Creese & England, 1970; Takeyasu *et al.*, 1983; Messing *et al.*, 1984). However, one agent, [^{3}H]-agmatine, has the reported advantage of not only serving as a tracer for ion flux through the receptor channel (Quik, 1985) but also, as being easily visualized by autoradiography once internalized and fixed in cells with functional receptors (Yoshikami, 1981). In this paper, I present evidence that, at high concentrations, agmatine (1-amino-4-guanidobutane) acts as an antagonist of neuronal nicotinic receptors. This pharmacological activity may limit the usefulness of [^{3}H]-agmatine as a probe for functional nicotinic receptor channels.

Methods

Electrophysiological measurements were made of intact chick retina from the ganglion cell population by use of a modification of the method of Loring *et al.* (1989). Briefly, d.c. potentials were measured between a suction electrode placed over the cut optic nerve (which consists of the cut axons of the retinal ganglion cells) and a second electrode placed in the eyecup perfusion medium. The eyecup was superfused at room temperature with Tyrode solution (composition mM: NaCl 130, $NaHCO_3$ 20.5, KCl 3, dextrose 17 and 0.01% phenol red, gassed with 95% O_2:5% CO_2) containing $MgCl_2$ 7mM and $CaCl_2$ 0.1 mM to inhibit synaptic inputs onto the retinal ganglion cells. The major modification was in how the agonist was applied: instead of applying the nicotinic agonist dimethylphenylpiperazinium (DMPP) directly to the eyecup as previously, DMPP was added by a 'sampling loop' consisting of three solenoid valves (Figure 1) located in the perfusion line. The 'sampling loop' was placed within 10 cm of the eyecup to reduce dilution of the applied DMPP *en route* to the preparation. Operation of the solenoid valves during the application of agonist triggered data acquisition (R.C. Electronics Computerscope A/D converter) and storage in a P.C. computer. Agonists were applied at intervals of no less than 5 min to reduce receptor desensitization. For dose-response curves, agonists were applied in a pseudo-random order of concentrations. Antagonists were perfused for at least 5 min

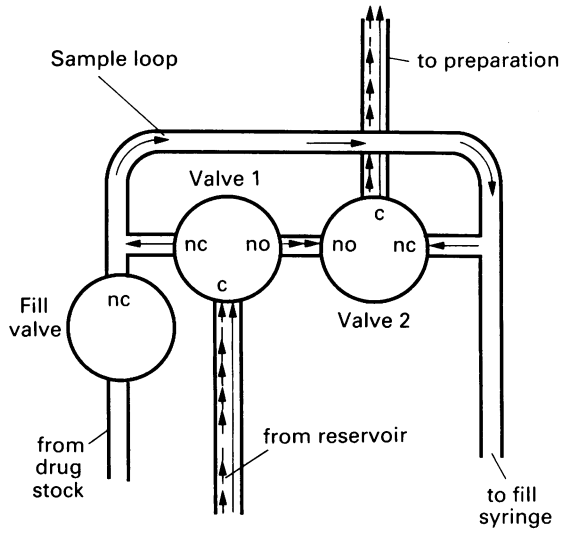

Figure 1 Valve and sample loop configuration: three teflon-coated solenoid valves obtained from General Valve Corp. (Fairfield, N.J., U.S.A.) are mounted as shown above on a 'third hand' manipulator by bolts connected to the valve bodies. To fill the sample loop, the fill valve is opened and solution containing the drug is sucked into the loop by pulling on the plunger of the fill syringe. The fill valve (2 way) is closed before drug applications. The path of perfusion medium through the valve assembly during normal operation is shown as short arrows going from the superfusate reservoir to the preparation. To apply the drug, valves 1 and 2 (3-way valves) are opened by a valve controller (similar to that described by Loring, 1985) and the superfusate travels through the sample loop as indicated by the long arrows.

The volume of the sample loop (about 200 μl in these experiments) and the flow rate of the superfusate (about 8 ml min^{-1} in these experiments) determine the approximate duration that the prepartion is exposed to the maximum concentration of the drug (about 1.5 s in these experiments). To minimize dilution of the drug, the length of line from valve 2 to the preparation is kept as short as possible (about 10 cm). Experiments with dyes suggest that drugs are diluted by no more than 3 fold when the drug reaches the retina. The sample loop can be rinsed between drug applications. All tubing is Intramedic PE 190 polyethylene. The valve ports are labelled as follows: nc, normally closed; no, normally open; c, common (always open).

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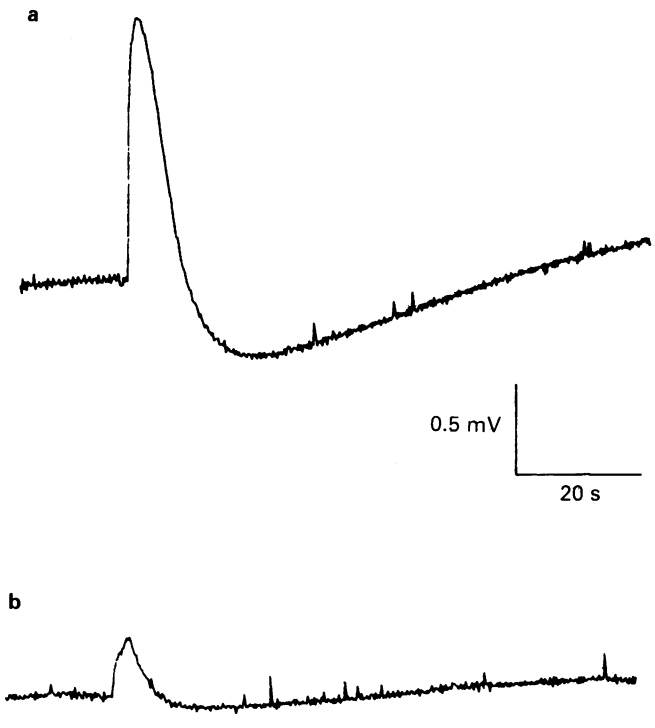

Figure 2 Blockade of nicotinically-mediated depolarizations in chick retina by agmatine. Recordings were made from intact retina in an eyecup preparation as outlined in the text. (a) Application of dimethylphenylpiperazinium (DMPP 100 μM) gives rise to a 1.6 mV depolarization. (b) In the presence of agmatine 1 mM, the same dose of DMPP gives rise to a depolarization of only 0.35 mV. Note that the concentration of DMPP refers to the concentration of agonist loaded into the sample loop and does not account for dilution *en route* to the preparation.

before agonist application. Recordings from rat superior cervical ganglia were made by the method of Loring (1985) similarly modified to use the 'sampling loop'. The rat superior cervical ganglia was maintained at 30°C in Krebs solution (composition mM: NaCl 136, KCl 5.6, $CaCl_2$ 3, $MgCl_2$ 1.5, $NaHCO_3$ 16, NaH_2PO_4 1.2, glucose 11 and 0.01% phenol red, oxygenated with 95% O_2:5% CO_2). The centrifugation

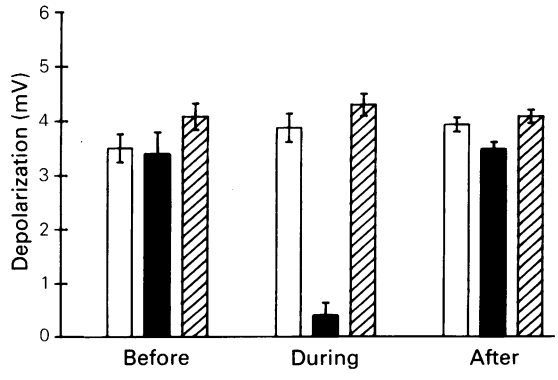

Figure 3 Selectivity of the agmatine effect on nicotinic receptors. An eyecup from a two week old chick was perfused with Tyrode solution and stimulated three times each with kainic acid 100 μM (open column), dimethylphenylpiperazinium (DMPP) 100 μM (solid column) and quisqualic acid (hatched column) (Before). The process was repeated both in the presence of 5 mM agmatine (During) and after washing out the agmatine for 10 min (After). The columns indicate the mean amplitude of the depolarizations obtained with the different agonists $\pm$s.d. (vertical bars) (n = 3 applications of each agonist). The depolarizations induced by DMPP were markedly antagonized (86% decrease) by perfusion with agmatine, while those induced by the glutamate analogues kainic acid and quisqualic acid were unaffected.

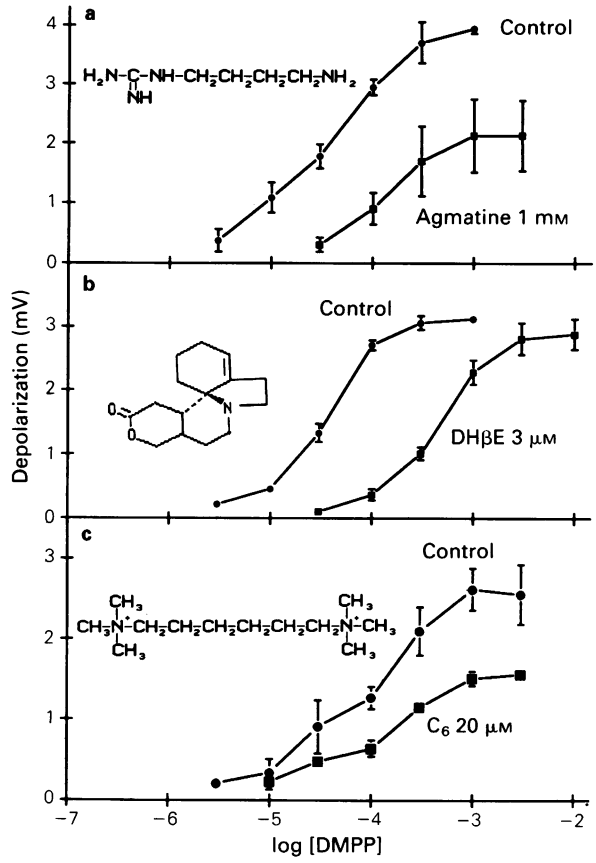

Figure 4 Effects of nicotinic antagonists on dimethylphenylpiperazinium (DMPP) dose-response curves in chick retina. (a) Control curves (●) represents the mean response for three applications of DMPP at each of the indicated concentrations; vertical bars show s.d. The filled squares (■) represent the responses in the same retina in the presence of 1 mM agmatine. Note that the concentrations refer to those loaded into the sample loop and do not take into account any dilution *en route* to the preparation. Due to variability in responses between individual eyecup preparations, a control dose-response curve was included in each experiment. (b) Control responses to the indicated concentrations of DMPP (●) and responses in the same retina in the presence of dihydro-β-erythroidine (DHβE) 3 μM (■). (c) Control responses to the indicated concentrations of DMPP (●) and responses in the same retina to the presence of hexamethonium (C_6) 20 μM (■).

assay for ^{125}I-labelled neuronal bungarotoxin binding in chick retina was performed as previously described (Loring *et al.*, 1989). Similar results were found with agmatine obtained from either Aldrich or Sigma.

Results

Application of DMPP 100 μM to intact chick retina gave rise to depolarization on the order of 1–4 mV (e.g. Figure 2a). Previous work established that these responses were mediated through nicotinic receptors, since the depolarizations were blocked by incubation of nicotinic antagonists such as hexamethonium, dihydro-β-erythroidine, or (+)-tubocurarine (100 μM each, Loring *et al.*, 1989). Repeated application of 100 μM DMPP by the 'sample loop' gave rise to depolarizations with a variability in amplitude generally less than 10% of the total depolarization (n = 10 experiments). As long as intervals of 5 min were used between agonist applications, little evidence of attenuation in the response was observed. However, perfusion of chick retina with 1 mM agmatine sub-

stantially blocked the DMPP response (e.g., Figure 2b: 67 ± 11% blockade in 4 experiments). In contrast, 5 mM agmatine had little or no effect on depolarizations induced by the glutamate analogues kainic acid or quisqualate (Figure 3). In other experiments, agmatine had no effect on depolarizations induced by the glutamate analogue N-methyl-D-aspartate (not shown). These data suggest that agmatine selectively acts at the level of the neuronal nicotinic receptor in the retinal preparation.

In order to determine whether blockade by agmatine was competitive or non-competitive, the effects of agmatine on the DMPP dose-response curve were assessed (Figure 4a). Partial blockade by 1 mM agmatine depressed the maximal response to DMPP suggesting a non-competitive action of agmatine ($n = 4$ experiments). In two of the four experiments, agmatine clearly decreased the slope of the response to DMPP, also suggesting a non-competitive effect of agmatine. However, in the other two experiments (such as shown in Figure 4a), the slope of the dose-response curve was not dramatically affected, but shifted to the right, although the maximal response was always depressed. These data suggest that agmatine may act as a mixed competitive and non-competitive antagonist at nicotinic receptors in the chick retina. For comparison, the effects of a competitive antagonist, dihydro-β-erythroidine (3 μM) are shown in Figure 4b, in which the competitive antagonist clearly shifts the dose-response curve to the right without significantly affecting the slope or maximal response. Also, as shown in Figure 4c, the non-competitive antagonist hexamethonium (20 μM), reduced both the maximum response and the slope of the response to DMPP.

Additional evidence for a non-competitive nature of the blockade by agmatine comes from the displacement of a snake toxin that competitively blocks neuronal nicotinic receptors (Figure 5). Variously referred to as Bungarotoxin 3.1 (Ravdin & Berg, 1979), toxin F (Loring *et al.* 1989), κ-bungarotoxin (Chiappinelli, 1983), and neuronal bungarotoxin (Higgins & Berg, 1988; Loring *et al.* 1989), neuronal bungarotoxin (NBT) blocks nicotinic receptor function in a variety of neuronal preparations including chick ciliary ganglion (e.g., Ravdin & Berg, 1979), rat retina (Lipton *et al.*, 1987), chick retina (Loring *et al.*, 1989), and bovine chromaffin cells (e.g., Higgins & Berg, 1988). In both the chick ciliary ganglion (Halvorsen & Berg, 1986) and the chick retina (Loring *et al.*, 1989), ^{125}I-labelled NBT binding is displaced by competitive but not by non-competitive, antagonists for neuronal nicotinic receptors.

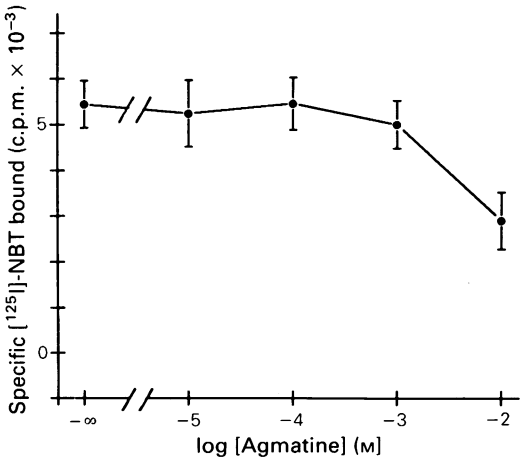

Figure 5 Displacement of [^{125}I]-neuronal bungarotoxin (NBT) by agmatine in homogenates of chick retina. Quadruplicate samples of retinal homogenates from 13 day-old white leghorn cockerels (approximately 1/3 retina per sample) were incubated in the presence of α-bungarotoxin 1 μM, [^{125}I]-NBT 2 nM and the indicated concentrations of agmatine for 2 h at room temperature. Bound label was separated from unbound by a centrifugation assay (Loring *et al.*, 1989). Specific binding was determined by subtracting the counts bound to quadruplicate samples incubated as above but including 1 μM unlabelled NBT.

[^{125}I]-NBT binds to two sites in homogenates of chick retina, one site representing a non-functional binding site shared with the neuromuscular blocking toxin, α-bungarotoxin, and a second site that corresponds to functional nicotinic receptors on the chick retinal ganglion neurones (Loring *et al.*, 1989). To study [^{125}I]-NBT binding to functional nicotinic receptors in chick retina, binding of [^{125}I]-NBT to the non-functional site shared with α-bungarotoxin had first to be blocked by incubation in the presence of 1 μM unlabelled α-bungarotoxin. Under these conditions, 1 mM agmatine did not significantly displace [^{125}I]-NBT binding to chick retinal homogenates (Figure 5) a concentration at which agmatine significantly depressed nicotinic receptor function in the chick retina (Figure 4a). Agmatine did start blocking [^{125}I]-NBT binding at 10 mM, however the displacement was less than 50%. At 5 mM agmatine, nicotinic receptor function was virtually abolished (86% block) in the intact chick retina (Figure 3). Thus, the effects of agmatine on the dose-response curves of DMPP and the lack of displacement of [^{125}I]-NBT binding by agmatine at concentrations that blocked the functional receptors both suggest thet the major action of agmatine was as a non-competitive blocker, while leaving open the possibility of some mixed activity.

Since [^{3}H]-agmatine was reported to be a useful probe for functional nicotinic receptors in intact rat superior cervical ganglia (Quik, 1985), the effect of agmatine on nicotinic receptor function in intact rat ganglia was also determined. DMPP (100 μM) produced depolarizations in rat superior cervical ganglion of greater than 1 mV (Figure 6a). Application of 10 mM agmatine for 5–20 min (Figure 6b) substantially blocked this depolarization (80 ± 15%, $n = 3$ experiments). The effect of agmatine on synaptic transmission through the ganglion was also examined. Figure 6c shows transmission elicited by stimulation of the preganglionic nerve under control conditions. Figure 6d shows that 10 mM agmatine completely blocked nicotinic transmission in the rat ganglion. Agmatine (1 mM) also antagonized both synaptic transmission through the chick ciliary ganglion and depolarizations due to applied agonists (data not shown). Since tetrodotoxin blocks only transmission but not agonist-induced depolarizations in the chick ciliary ganglion preparation (Loring, 1985), the effects of agmatine on the agonist-induced depolarizations in chick ciliary ganglion again strongly suggest that the effect of agmatine was at the level of the postsynaptic receptor and was not due to blockade of axonal conduction.

Discussion

Many isotopes and radiocompounds have been used in isotopic flux studies of nicotinic receptor function. In some of the earliest studies, labelled decamethonium was found to accumulate intracellularly near endplate regions in muscle (Creese & England, 1970; Creese & Maclagan, 1970). In the latter study, pretreatment with (+)-tubocurarine substantially decreased the intracellular accumulation of labelled agonist, suggesting that the decamethonium transport into muscle was mediated through the nicotinic receptor itself. The accumulation of label was demonstrated by light-level autoradiography which also demonstrated a major difficulty found for most radiocompounds that pass through the nicotinic receptor channel: in most cases, the radiocompounds, including decamethonium, cannot be fixed by traditional fixation techniques. This necessitated that the studies of Creese & Maclagan (1970) were done by the technically difficult method of cutting and applying autoradiographic emulsion to frozen tissue sections to prevent diffusion of the labelled compound.

In 1981, Yoshikami used [^{3}H]-agmatine (1 amino-4 guanido butane) to demonstrate nicotinic receptor-mediated

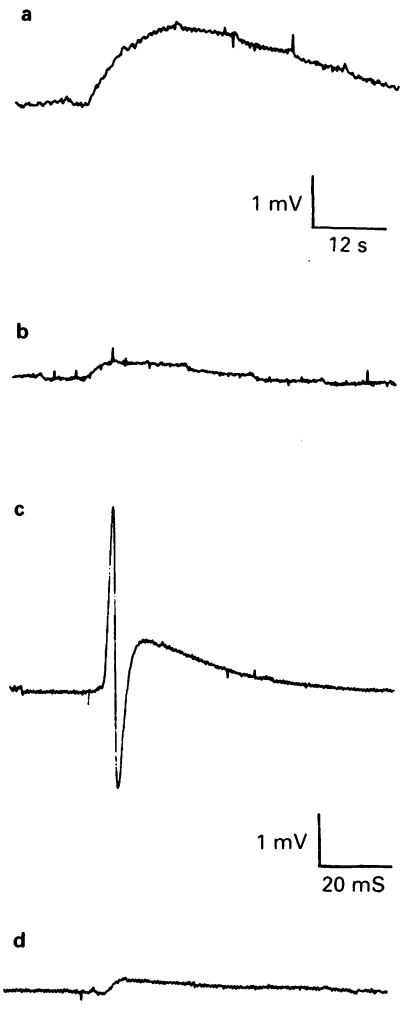

Figure 6 Effects of agmatine in dimethylphenylpiperazinium (DMPP)-induced depolarizations and on synaptic transmission through the rat superior cervical ganglion. (a) A depolarization induced in the rat superior cervical ganglion by application of DMPP 1 mM. The regular smaller inflections are an artifact caused by the cycling of the heater. (b) A 10 min application of agmatine 10 mM substantially blocks the DMPP-induced response. (c) Compound action potentials elicited by stimulating the preganglionic nerve while the ganglion is bathed in normal Krebs solution. (d) Compound action potentials are substantially blocked when the ganglion has been exposed to agmatine 10 mM for 12 min.

flux in the frog sympathetic ganglion. As a polyamine, agmatine is a cation at physiological pH, and is readily fixed by aldehydes. [^{3}H]-agmatine can be obtained at high specific activities by enzymatic decarboxylation of [^{3}H]-arginine. Finally, unlike many other polyamines, agmatine has no known specific uptake mechanism into eucaryotic cells. Subsequently, Quik (1985) used [^{3}H]-agmatine to demonstrate the nicotinic receptor blocking properties of a snake venom neurotoxin in the rat superior cervical ganglion.

This present study was based on earlier attempts to use [^{3}H]-agmatine as a probe to demonstrate the localization of functional nicotinic receptors in the chick retina. Previous work (Loring, *et al.*, 1989) suggests (1) that many retinal ganglion cells in the chick retina possess functional nicotinic receptors, (2) that these receptors are sensitive to blockade by the snake venom neurotoxin referred to as neuronal bungarotoxin, and (3) that [^{125}I]-neuronal bungarotoxin binding is localized to two bands in the inner plexiform layer of the chick retina that correspond to two bands that stain for the enzyme, choline-acetyltransferase. The object was to see if [^{3}H]-agmatine selective uptake could be demonstrated in these same parts of the chick retina following stimulation by nicotinic agonists. However, preliminary experiments failed to demonstrate specific uptake of [^{3}H]-agmatine in chick retina (data not shown), when the labelled compound was applied in either the presence or absence of specific nicotinic agonists. The present study was undertaken to determine whether the pharmacology of the nicotinic receptor on chick retinal ganglion cells differed in some substantial way from that of the nicotinic receptor found in the frog or rat sympathetic ganglion. Instead, the chick retinal receptor was found to differ from the rat superior cervical ganglion receptor only in degree. Nicotinic receptors in both preparations are sensitive to blockade by agmatine and hexamethonium and in both cases, complete blockade required about 10–50 fold more agmatine than hexamethonium. The major difference between the two preparations was that the rat sympathetic ganglion is about 10 fold less sensitive to either of these agents than is the chick retina.

It should be pointed out that agmatine and hexamethonium bear at least superficial similarities in chemical structure. At a physiological pH, the majority of agmatine molecules are positively charged both on the free amino group and on the guanidinium group. Furthermore, the molecular distance between these charges is similar to the distance between the two quaternary amines of hexamethonium. From the work of Quik (1985) it is clear that [^{3}H]-agmatine does penetrate a sufficient number of nicotinic receptor channels in rat ganglion to be a useful probe for ion flux through the receptor. However, those experiments were performed at a concentration of 1–3 μM [^{3}H]-agmatine, concentrations some 1000 fold lower than that needed to block a significant fraction of nicotinic receptors in the rat superior cervical ganglion. One hypothesis to explain these data would be that agmatine has a small but finite probability of blocking the receptor as the compound travels through the open receptor channel. Thus, at high concentrations of agmatine, sufficient agmatine would pass through the open channel that the receptor would most probably be blocked at some point. Other possibilities include a low affinity binding site outside the channel.

Independent of the mechanism by which agmatine blocks neuronal nicotinic receptors, it is reasonable to ask whether hexamethonium, like agmatine, will also pass through nicotinic receptors at concentrations below those causing blockade of the receptor. This possibility seems more plausible since the chemically similar compound, decamethonium, appears to travel through the muscle nicotinic receptor channel (Creese & Maclagan 1970). In addition, decamethonium has been shown to block the muscle nicotinic receptor channel at high concentrations (Adams & Sakmann, 1978). Hexamethonium is believed to act by blocking neuronal nicotinic receptor channels at effective doses (Ascher *et al.*, 1979). Interestingly, the question has also been raised whether acetylcholine itself penetrates the muscle nicotinic receptor channel (Sine & Steinbach, 1984), since it has been demonstrated that, at high concentrations, both acetylcholine and suberyldicholine block nicotinic receptors in vesicles from electric organs of fish (Takeyasu *et al.*, 1983), at the frog neuromuscular junction (Ogden & Colquhoun, 1985), and in a muscle cell line (Sine & Steinbach, 1984).

A major question then, is why [^{3}H]-agmatine is a useful probe for ion flux assays of receptor function in rat and frog sympathetic ganglia and not in the chick retina? One answer may be more anatomical than pharmacological. In the frog and rat ganglia, synaptic profiles and presumably the nicotinic receptors are often observed on or near the postsynaptic neuronal cell body (e.g. Marshall, 1981). In contrast, the localization of [^{125}I]-NBT in the chick retina suggest that the functional nicotinic receptors are located on fine dendritic processes in the inner plexiform layer (Loring *et al.*, 1989). The internal volume of the dendritic processes may simply not be large enough to trap sufficient [^{3}H]-agmatine relative to background. Alternatively, agmatine may discriminate between the ionic channels of the different subtypes of neuro-

nal nicotinic receptors known to be present in various parts of both the rat and chick nervous systems (reviewed by Steinbach & Ifune, 1989).

Regardless of the ultimate differences between nicotinic receptors in the chick retina versus those in the rat superior cervical ganglia, agmatine clearly has a pharmacological effect on both of these preparations. Non-competitive blockade of nicotinic receptor channels may limit the useful concentration of [^{3}H]-agmatine available for studying ion flux through nicotinic channels. However, a clear understanding of how agmatine and similar drugs, such as hexamethonium block receptor channel function may ultimately lead to better understanding of how the receptor channel operates.

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