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Glutamate-stimulated phosphatidylinositol metabolism in the avian cochlear nucleus

Lance Zirpel[a,c], Neil M. Nathanson[b], Edwin W Rubel[a,c,*], Richard L. Hyson[c,**]

[a]*Department of Physiology and Biophysics, SJ-40, School of Medicine, University of Washington, Seattle, WA 98195, USA*
[b]*Department of Pharmacology, SJ-30, School of Medicine, University of Washington, Seattle, WA 98195, USA*
[c]*Virginia Merrill Bloedel Hearing Research Center, RL-30, University of Washington, Seattle, WA 98195, USA*

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Abstract

This study examined the ability of the excitatory amino acid glutamate and its analogs to stimulate phosphatidylinositol metabolism in isolated cochlear nucleus tissue from young chicks. In the presence of lithium chloride, glutamate and (±)-1-aminocyclopentyl-*trans*-1,3-dicarboxylate (ACPD) stimulated the formation of inositol phosphates to levels significantly above unstimulated control levels. Unexpectedly, quisqualate did not stimulate inositol phosphates formation. The *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-5-phosphonovalerate (APV), the ionotropic kainate/quisqualate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the putative metabotropic glutamate receptor antagonist 2-amino-3-phosphonopropionate (AP3) had no effect on the glutamate stimulated formation of inositol phosphates. We conclude that a metabotropic glutamate receptor is present on cochlear nucleus neurons of posthatch chicks and is able to stimulate formation of inositol phosphates.

Key words: Metabotropic; IP_3; Deafferentation; Auditory system; ACPD; Nucleus magnocellularis

Neurons of the chick cochlear nucleus, nucleus magnocellularis (NM), are critically dependent upon eighth nerve activity for maintenance of their metabolic activity and for their survival [24]. Eliminating this input, either by cochlea ablation or perilymphatic injection of TTX, results in the death of approximately 30% of the NM neurons [3]. Within minutes to hours after the elimination of afferent activity, NM neurons display a variety of changes in their metabolic activity including decreased protein synthesis [25]. Using an *in vitro* brain stem slice preparation, Hyson and Rubel [11] showed that transneuronal regulation of protein synthesis appears to require calcium-dependent release of a 'trophic substance' from the eighth nerve terminals.

*Corresponding author. Department of Physiology and Biophysics, SJ-40, School of Medicine, University of Washington, Seattle, WA 98195, USA. Fax: (1) (206) 543-5152.

***Present address:* Department of Psychology, R-54, Florida State University, Tallahassee, FL 32306, USA.

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One obvious candidate 'trophic substance' in this system is the neurotransmitter at the eighth nerve–NM synapse, the excitatory amino acid glutamate [12,16,19]. It is possible that glutamate regulates the metabolism of postsynaptic NM neurons through activation of a metabotropic glutamate receptor and the resulting phosphoinositide second messenger cascade [28]. In this report, we demonstrate that at least one subtype of metabotropic glutamate receptor is present in NM. These results have been previously reported in abstract form [37].

Isolation of NM. NM samples were isolated from brain stem slices prepared from hatchling chicks 8–16 days old (P8–P16) as described by Hyson and Rubel [11]. Slices containing NM were then placed in a small, Sylgard-lined Petri dish and 'punches' of NM were obtained using an electrolytically-beveled 27 gauge hypodermic needle. The NM punches were placed in Eppendorf tubes (2 punches per tube) containing 60–100 μl oxygenated ACSF.

Tissue labeling. The tissue was incubated for 45–60

min in 250 μl ACSF containing 0.3 μM *myo*-2-[^{3}H]inositol (American Radiolabeled Chemicals Inc., St. Louis, MO; 30 Ci/mmol) and 5 mM LiCl to inhibit the conversion of [^{3}H]inositol-1-phosphate into inositol by the enzyme inositol-1-phosphatase. Transmitter agonists were then added directly to the tubes. When used, antagonists were added 10 min prior to agonist addition. An equal volume of ACSF vehicle was added to control tissue in conjunction with the addition of agonists and antagonists. After 15 min of stimulation, the interaction was terminated by addition of 940 μl of ice-cold chloroform/methanol (1:2). Another 0.3 ml each of chloroform and deionized, distilled water (dH_2O) were added 20 min later and the tissue was sonicated for 20 min. An additional 0.15 ml of each chloroform and dH_2O were added and the mixture was vortexed and centrifuged at 1000 × *g* for 15 min.

Separation of inositol phosphates. Inositol phosphates were quantified using a modification of the standard anion exchange column method for cultured cells described by Nathanson et al. [18]. Total inositol phosphates (IP_x) were eluted into a scintillation vial with 3 ml 1.0 M ammonium formate in 0.1 N formic acid. Scintillation fluid was added and the samples counted. IP_3 is more rapidly metabolized than the other inositol phosphates, but since they are regulated in parallel by agonist stimulation [2], changes in the level of total inositol phosphates is a relatively accurate indication of changes in IP_3 levels [6,9].

Data analysis. IP_x counts were normalized by assaying the total [^{3}H]phosphatidylinositol pool size of the tissue sample using a modification of the method described by Scherer and Nathanson [18] and dividing total IP_x counts by total phosphatidylinositol pool size counts for each data point. Basal levels were obtained from unstimulated tissue (controls). All other groups were expressed as a percentage difference from these control levels: control = 0; positive numbers indicate an increase in PI metabolism; negative numbers indicate an inhibition of PI metabolism. Statistical analyses (*t*-test or analysis of variance) were performed on these difference scores. For group analyses, scores were pooled across doses since no reliable dose effects were observed for the range of doses used in this study. Numbers reported are means followed parenthetically by the standard error. All statistical procedures were performed using Statview II software (Abacus Concepts).

Drugs, chemicals and media. (±)-ACPD was acquired from Tocris Neuramin, Essex, UK. Quisqualate, AMPA, CNQX and APV were from Research Biochemicals Inc., Natick, MA. Glutamate, AP3 and ammonium formate were from Sigma Chemical Company, St. Louis, MO. All other chemicals were reagent grade unless otherwise noted. All agonists and antagonists were dissolved in ACSF with the exception of CNQX which was dissolved in DMSO and then diluted to working concentration in ACSF. ACSF consisted of (in mM): NaCl 130, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, glucose 10. .

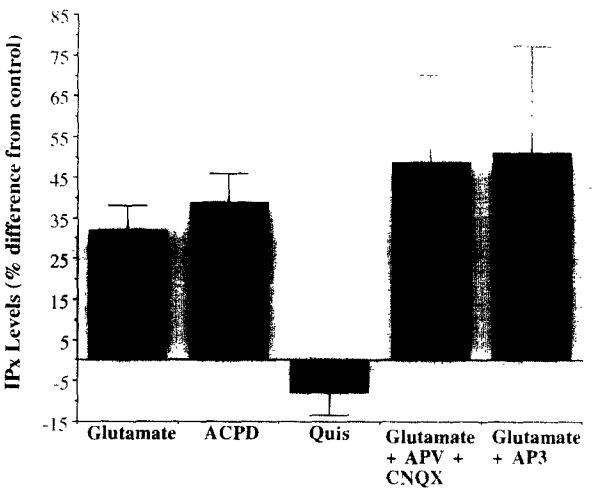

Fig. 1. Pharmacology of IP_x formation in isolated NM tissue. Glutamate (2.5–1000 μM) and ACPD (2.5–100 μM) stimulated increased IP_x formation, whereas quisqualate (2.5–100 μM) showed no reliable effect. CNQX (30 μM) and APV (200 μM) or AP3 (1mM) had no effect on the response to glutamate.

The data for each agonist group and agonist plus antagonist group are shown in Fig. 1.

Agonists. Glutamate stimulated a 31.9% (6.1) increase in IP_x formation (n = 103, P = 0.0001) in isolated NM tissue. Responses to doses from 2.5 to 1000 μM glutamate ranged from 24.79% (7.05) to 39.19% (18.45) above control levels. These levels of stimulation of IP_x formation are not large, but they are within the normal range for this type of assay performed on intact tissue [24].

ACPD, a specific, but not necessarily potent, agonist to the metabotropic glutamate receptor [15,21,29] also stimulated a 38.6% (7.3) increase in IP_x formation (n = 49, P = 0.0001) in isolated NM. The responses to doses from 2.5 to 100 μM ACPD, ranged from 30.09% (20.11) to 50.35% (16.47) above control levels.

Quisqualate is reported to be a potent, but non-selective agonist of the metabotropic receptor in several systems [20,28,32]. Our results show that quisqualate had no reliable effect on IP_x formation in isolated NM tissue within the concentration range tested. Doses from 2.5 to 100 μM quisqualate produced responses ranging from −2.98% (15.28) to −18.28% (8.88) difference from control levels with a mean of −8.05% (5.6) (n = 41, P = 0.1565).

Antagonists. IP_x formation was measured in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist for the kainate and AMPA ionotropic receptors [17], 2-amino-5-phosphonovalerate (APV), an antagonist for the NMDA receptor [17], and 2-amino-3-phosphonopropionate (AP3), a putative antagonist for the ACPD metabotropic receptor [27]. Blockade of the

ionotropic receptors with a combination of 30 μM CNQX and 200 μM APV had no reliable effect on baseline IP_x formation ($n = 6$; $P = 0.2052$, data not shown) and also had no effect on the level of IP_x formation stimulated by glutamate. Concentrations of glutamate from 12.5 to 500 μM still produced a 48.7% (21.5) increase in IP_x formation ($n = 16$, $P = 0.0391$). These concentrations of CNQX and APV completely eliminate postsynaptic field potentials in NM evoked by eighth nerve stimulation [36]. Incubation in 1 mM AP3, also had no reliable effect on IP_x formation in either baseline ($n = 12$; $P = 0.6148$, data not shown) or glutamate-stimulated conditions ($F_{3,65} = 0.3246$, $P = 0.8076$).

The results show that a form of 'metabotropic' glutamate receptor is present in NM and its pharmacological properties are similar to metabotropic receptors described in some other systems. PI metabolism in NM tissue was stimulated by glutamate and ACPD. Glutamate stimulation of PI metabolism is consistent with the very definition of a metabotropic glutamate receptor. Similarly, ACPD is a specific agonist of the metabotropic receptor [15,21,28,29] and our observation of ACPD-stimulated PI metabolism is further evidence in support of the presence of functional metabotropic receptors on NM neurons.

Unexpectedly, quisqualate did not stimulate PI metabolism in NM tissue and even tended to show an inhibitory effect. Quisqualate is a very potent agonist of the metabotropic receptor in rat hippocampus, cerebral cortex, cerebellum, striatum and various cultured cells [1,28,32]. However, quisqualate is ineffective in stimulating PI metabolism in primary cultures of cerebellar granule cells [4]. In addition, a family of metabotropic receptors has been isolated and cloned from rat brain that show differential sensitivity to quisqualate [34]. Thus, although quisqualate is frequently a potent agonist for metabotropic receptors, it is not a universal feature for this family of receptors. It appears that chick NM neurons may express a subtype of the metabotropic receptor which has little if any sensitivity to quisqualate.

The effects of receptor antagonists in NM are also similar to the effects reported in some, but not all, other systems. Neither the kainate/AMPA receptor antagonist, CNQX, nor the NMDA receptor antagonist, APV, has any antagonistic effect on activation of the metabotropic receptor in any tissue type studied to date [28]. CNQX and APV also have no effect on glutamate-stimulated PI metabolism in tissue from NM. The same concentration of CNQX does, however, block postsynaptic field potentials in NM evoked by electrical stimulation of the eighth nerve [36].

Our results also indicate that AP3 has no effect on glutamate-stimulated PI metabolism in NM neurons. This is in contrast to the reports that AP3 is a specific antagonist of the metabotropic receptor in rat hippocampal slices [27] and *Xenopus* oocytes injected with rat brain cDNA [10]. However, AP3 is without effect on receptor-mediated PI metabolism in transfected Chinese hamster ovary cells [1] and inconsistent effects of AP3 have been reported for cultured rat hippocampal neurons [23] and isolated pyramidal neurons from rat cortex [25]. AP3 has even been shown to *stimulate* PI metabolism in rat hippocampal slices [14].

As noted earlier, the impetus for this research was that the presence of the metabotropic receptor on NM neurons of chicks may be important for transneuronal regulation of these neurons by eighth nerve activity. NM neurons of young chicks undergo dramatic changes in metabolism and morphology following removal of eighth nerve activity [24]. Electrical activity alone is insufficient to prevent these effects; a Ca^{2+}-dependent release of some presynaptic 'trophic substance' appears to be required [11]. One possible trophic interaction is that activity-dependent release of glutamate from the eighth nerve regulates the metabolic activity of the NM neuron via a metabotropic receptor. There is a large body of evidence demonstrating the diversity and power of the effects of metabotropic glutamate receptor stimulation on cellular functioning and regulation [5,7,8,13,30, 31,33]. Thus, the functional presence of this receptor on NM neurons of young chicks may allow eighth nerve activity to regulate multiple enzyme systems, intracellular calcium stores, ionic fluxes across the plasma membrane and protein phosphorylation, thereby influencing virtually every cellular function involved in maintenance and survival of the neuron.

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