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Guanine nucleotides protect against kainate toxicity in an ex vivo chick retinal preparation

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Abstract Ex vivo preparations of chick neural retina have been successfully used in the assessment of excitotoxicity and in the evaluation of the protective effects of glutamate antagonists. Using a variation of this approach, and measuring the acute and delayed toxic effects of kainate (KA) in terms of lactate dehydrogenase release, we have shown that guanine nucleotides behave as effective neuroprotecting agents. The anti-excitotoxic potency of guanine nucleotides (in the case of GMP and GDPβS it is about 100 times lower than that of DNQX, a powerful kainate antagonist) correlates well with their ability to displace KA from retinal KA receptors.

Key words: Kainate; Guanosine monophosphate; Guanine nucleotide; Excitotoxicity; Neuroprotection; Chick retina

1. Introduction

The interaction of guanine nucleotides (GNs) with excitatory amino acid receptors, at the extracellular level, is already a well-established fact: both the common native nucleotides, GTP, GDP and GMP, and the non-hydrolyzable, synthetic analogs have been shown to displace agonists (and, in some cases, antagonists) from both ionotropic receptors, including NMDA [1–3] and non-NMDA [4–6] types, and metabotropic receptors [7,8]. The structural basis for this phenomenon has been at least partially elucidated [5,7,9], although some contradictions concerning the mechanisms of competition between amino acid agonists and GNs remain to be explained [10].

Unequivocal neuroprotective effects have furthermore been demonstrated for GMP in characteristic neurotoxicity experimental paradigms, both in vivo [11] and in tissue slices [12]. The need for a simple system to test the validity and the relative potency of different GN analogs and derivatives as neuroprotectors has prompted us to explore the suitability of the chick retina as a model to analyze the ability of GNs to antagonize the toxic actions of excitatory amino acids. Although early descriptions of the deleterious effects of glutamate on the mouse retina [13] were based on the parenteral administration of the amino acid (see [14] for a discussion), the use of the ex vivo chick (or mouse) retinal preparation for excitotoxicity analysis was later successfully adopted in several laboratories [15–21]. We have taken advantage of a further adaptation of this retinal model to set up a simple and sensitive system to evaluate the neuroprotective effects of GNs and other EAA antagonists on the acute and delayed effects of a single dose of kainate. Our results confirm once again the striking neuroprotective effect of some guanine nucleotides, especially 5′-GMP, and may contribute to finding practical applications of this interaction between GNs and EAA receptors.

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Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; AP7, 2-amino-7-phosphonoheptanoate; AppHNp, 5′-adenylyl-imidodiphosphate; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EAA, excitatory amino acids; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GN, guanine nucleotide; GppNHp, 5′-guanylyl-imidodiphosphate; KA, kainic acid; KBSS, Krebs balanced salt solution; LDH, lactate dehydrogenase; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclohepten-5,10-imine maleate; NMDA, *N*-methyl-D-aspartate

2. Materials and methods

2.1. Animals

White Leghorn chick (*Gallus domesticus*) embryos of the desired developmental stage were produced by incubation of fertile eggs, in our own facilities, using a forced-draft incubator, with controlled temperature and humidity, and automatic turning.

2.2. Chemicals

[^{3}H]Kainic acid (58 Ci/mmol) was obtained from New England Nuclear, Germany, and purine nucleotides and DNQX from Sigma. All other chemicals were from standard commercial suppliers.

2.3. Dissection of neural retinas and experimental setup

All operations were carried out at room temperature. Thirteen-day chick embryos (13 ± 1) were used for easy separation of the neural retina from the pigment/vascular layer. After decapitation and eye removal the eyecups were placed in a Krebs-like balanced salt solution (KBSS) containing 119 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 24.9 mM $NaHCO_3$, adjusted to pH 7.4 with 95% O_2/5% CO_2 [21]. The eyecups were kept in the dark until dissection. After excision of the cornea and the lens, the vitreous humor was gently pulled out and then the retina was removed as a continuous sheet and placed, inside a small plastic container fitted with a nylon mesh at the bottom, in one of the wells of a 24-well tissue culture plate filled with KBSS. This arrangement facilitates the sequential exposure of the retina to different solutions with a minimum of manipulation.

2.4. Characterization of binding sites for [^{3}H]KA in chick neural retina membranes

E13 embryonic neural retinas, dissected as above, but working always at 4°C, were homogenized, in 20 volumes of distilled water, in a Polytron homogenizer (setting 5; 15 s), and then rehomogenized in a Dounce glass-glass homogenizer (Kontes). The suspension was centrifuged at 100 000 × *g* for 30 min and the pellet was resuspended in water and subjected to two additional cycles of Polytron/Dounce homogenization. The final pellet was resuspended in 1 mM Tris-HCl, pH 7.4, and used for binding experiments.

Binding was carried out as previously described [4,10], using [^{3}H]KA of high specific radioactivity. KA concentrations for saturation experiments ranged from 2 to 1000 nM, whereas 40 nM was used

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in displacement experiments. Non-specific binding was estimated in the presence of 0.4 mM non-labeled KA. Displacing antagonists, at concentrations between 4 nM and 4 mM were added 10 min before [^{3}H]KA.

2.5. Exposure of chick embryo retinas to different substances

Within 15 min after dissection, the containers with the neural retinas were transferred to wells containing, in 1 ml of KBSS at room temperature, kainate, kainate-receptor antagonists and/or nucleotides, in different combinations and at different concentrations (see Section 3). In neuroprotection assays, pre-incubation with antagonists and nucleotides was routinely carried out for 10 min before addition of kainate. After 1 h of exposure to kainate the retinas were thoroughly washed by sequential immersion in wells with KBSS, and then incubated for an additional hour (or longer) in KBSS without kainate. All retinas were frozen at the end of the experiment.

2.6. LDH assays

The acute and delayed toxicity of kainate was assessed by measuring the liberation of LDH into the incubation medium. In routine experiments LDH was measured independently in the two 1-ml experimental medium aliquots (first and second hours) and in the first of the three intermediate washes. LDH was estimated spectrophotometrically by measuring the rate of disappearance of NADH (at 340 nm) in a 1-ml solution containing 0.2 ml of the sample in 50 mM Tris-HCl, pH 7.2, 1.2 mM Na^+-pyruvate, and 0.15 mM NADH (final concentrations) [22]. The frozen retinas were thawed the next day and assayed also for LDH. LDH released by kainate was expressed as percent of the total LDH in the retina. Control retinas, exposed only to KBSS, were carried along in parallel to assess the degradative effects of the dissection and incubation procedures. These control values were always subtracted from experimental values in the same batch of retinas.

3. Results

3.1. Binding sites for [^{3}H]kainate in chick retinal membranes

To compare the efficiency of prospective glutamate antagonists (including GNs), both as displacers in conventional binding experiments and as neuroprotective agents, we carried out a partial characterization of KA-binding sites in a preparation of embryonic chick neural retinal membranes (E13). As seen in Fig. 1, binding data can be adjusted to a two-site model with K_ds of 4 and about 200 nM. This distribution of apparent affinities agrees well with the original paper of Biziere and Coyle [23] except for some quantitative differences in the B_{max} probably related to their using a different membrane preparation and a radioactive KA of much lower specific radioactivity. Routine displacement experiments for screening purposes were carried out at a 40 nM concentration of [^{3}H]KA.

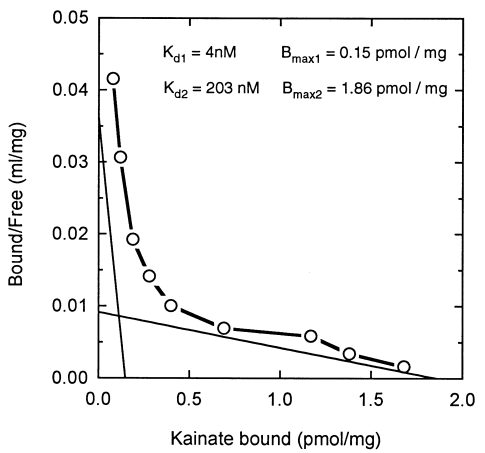

Fig. 1. Saturation analysis of [^{3}H]KA specific binding sites in chick embryonic neural retina (E13): Eadie-Scatchard plot of binding data and adjustment to two discrete populations of binding sites (SigmaPlot).

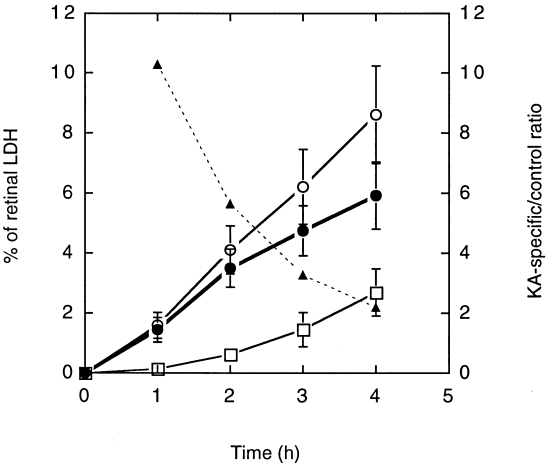

Fig. 2. Cumulative release of LDH after exposure of chick neural retina explants to 0.1 mM KA for 1 h. E13 embryonic chick neural retinas were dissected, and incubated in the presence of 0.1 mM KA, as described in Section 2. They were then transferred to fresh medium without KA and incubated for three additional 1-h periods, with hourly changes of medium. LDH was measured in the four consecutive medium aliquots and expressed as percent of the total retinal LDH (see text). Control retinas, not exposed to KA, were carried along to determine the effect of spontaneous cell damage. Values in the graph are mean ± S.D. for six determinations; ○, accumulated total LDH measured in retinas exposed to KA; □, control retinas; ●, specific KA-induced release of LDH (difference between total and control); ▲, KA-specific/control LDH ratio.

3.2. Optimization of the experimental procedure to assess excitotoxicity

Two hundred and eighty-three chick embryonic retinas were independently processed for the neurotoxicity/neuroprotection experiments. The average LDH activity per retina was 25.2 ± 2.2 OD_{340} units/min. After 1 h of exposure to 0.1 mM kainate, LDH activity in the medium was over 10 times that of the control without kainate (Fig. 2). Washing the treated retinas with KBSS did not stop the leakage of LDH upon incubation in fresh aliquots of KBSS. However, longer incubations also resulted in progressively higher readings in the control retinas. Incidentally, this continued (delayed) release of LDH after removal of the toxin was not inhibited by inclusion of either non-NMDA or NMDA receptor antagonists (1 mM DNQX, MK-801 or AP7), suggesting that it was not due to traces of kainate, or to depolarization-induced release of glutamate, but to irreversible damage of the cell upon the initial exposure to KA. For routine experiments we therefore settled for 1 h of exposure to the excitotoxin plus one additional hour to collect LDH in the absence of KA. In Fig. 2 this accounts for 62% of the total LDH released in the 4-h period, and the KA-specific/control ratio is still higher than 5 (corresponding to a highly significant difference between the KA-specific and control LDH values: $P < 0.0001$ in Student's paired *t*-test). Using this acute/delayed 2-h protocol, exposure to 0.1 mM kainate produced a KA-specific LDH release of 3.6 ± 0.7% (mean ± S.D. of 47 determinations, after deduction of control (blank) values) of the total retinal LDH whereas spontaneous release in controls was 0.7 ± 0.3%

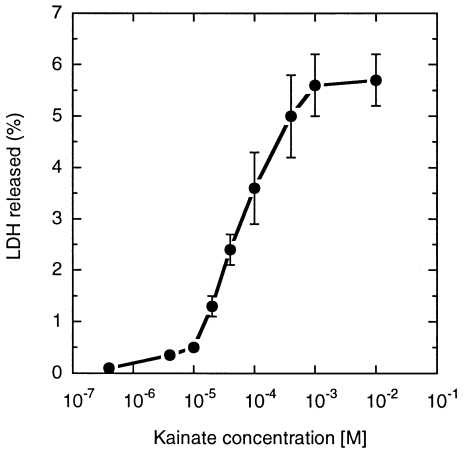

Fig. 3. Percent of retinal LDH released after exposure to different concentrations of KA. Chick embryonic retinas were exposed for 1 h to KA and further incubated in KBSS without KA for one additional hour. LDH released during this 2-h period, expressed as percent of total retinal LDH (see text), has been plotted against KA concentration. Values are mean ± S.D. for six independent experiments. The 50% release concentration was estimated as 0.056 mM.

(32 determinations). The rates of release of LDH during the first and second hours were nearly identical, and so were the protective effects of antagonists. Then, although LDH was systematically measured after each 1-h period, both readings were routinely added and considered the total toxic effect.

The dependence of the retinal damage on the concentration of KA, using this standard protocol, is illustrated in Fig. 3. The sigmoidal profile of the curve is compatible with the notion of a receptor-dependent, saturable phenomenon, typical of EAA-mediated excitotoxicity [24]. The EC_{50} in Fig. 3, 0.056 mM, is of the same order of magnitude as the KA concentration used routinely (0.1 mM), which produces a sufficiently high level of toxicity but can still be overcome by reasonable concentrations of the antagonists.

The high-slope region of the curve occurs between 10^{-5} and 10^{-3} M KA, which would suggest the preferential involvement of the low-affinity receptors in the excitotoxic action of KA (see Fig. 1), although due allowance has to be made for the difference in accessibility of the KA receptors in the washed, fragmented membrane preparation and in the retinal explant.

Table 1
Protection by DNQX and different nucleotides against the toxicity of a 0.1 mM dose of kainate in 13-day embryonic chick retina

Compound	PC_{50} (M)	IC_{50} (M)
DNQX	3.2×10^{-6}	8.4×10^{-7}
5′-GppNHp	2.1×10^{-3}	4.9×10^{-4}
5′-GDPβS	4.0×10^{-4}	1.3×10^{-4}
5′-GMP	4.0×10^{-4}	1.2×10^{-4}
5′-ITP	6.2×10^{-3}	8.9×10^{-3}
5′-IDP	4.0×10^{-3}	$>10^{-2}$
5′-IMP	2.8×10^{-3}	7.6×10^{-3}
5′-AppNHp	$>10^{-2}$	$>10^{-2}$
5′-ADPβS	$>10^{-2}$	$>10^{-2}$
5′-AMP	$>10^{-2}$	$>10^{-2}$

Protection curves (see Fig. 4) were carried out for DNQX and the different series of purine nucleotides featured in the table, and PC_{50}s (PC_{50}: concentration of neuroprotective drug that reduces LDH release by half) were calculated from the curves. Standard IC_{50}s were obtained from displacement experiments, in retinal membranes of the same age, using 40 nM [^{3}H]KA against different concentrations of DNQX or nucleotides.

3.3. Neuroprotection experiments

The presumptive neuroprotective drugs were first tested at a single 10 mM concentration (100 times the KA concentration) and a dose-dependence protection curve was further carried out when the preliminary experiments were positive. The protection curves for GppNHp, GDPβS and GMP are shown together in Fig. 4. Besides the evident neuroprotective effect of all three GNs, the GppNHp profile suggests that this GTP analog may be acting as a partial agonist or co-agonist, adding to the toxicity of KA at low concentrations and displacing it at higher concentrations (GppNHp is not toxic when used alone). PC_{50}s for different nucleotides (defined as the concentration that affords a 50% protection level) are given in Table 1 and compared with the protective efficiency of DNQX, a strong and specific antagonist of the AMPA/KA receptors. The ability of other types of purine nucleotides to counteract the toxicity of KA has also been explored: whereas the inosine series has about 10% of the protective activity of GNs, adenine nucleotides appear to have no measurable effect up to 0.01 M.

Since the neuroprotective effect of GNs must be related to their ability to displace KA from the specific retinal receptors, we also carried out a series of displacement experiments and calculated the IC_{50}s for the same nucleotides used in the protection experiments. As can be seen in Table 1, there is a fair degree of correspondence between the relative efficiency of most nucleotides as displacers and neuroprotectors. The most active in both tests are GMP and GDPβS, which are still 100–150 times less potent than the reference antagonist DNQX.

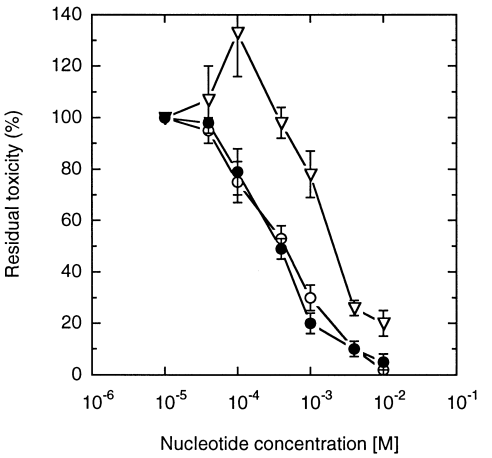

Fig. 4. Protection by GNs against KA toxicity in embryonic chick neural retina. Retinas were exposed for 1 h to 0.1 mM KA, with or without GNs, at the concentrations given, and further incubated in KBSS without KA for one additional hour (GNs were routinely added 10 min before KA). LDH released at different concentrations of GNs, expressed as percent of the enzyme released by KA in the absence of GNs, was taken as residual toxicity and plotted against GN concentration. Exposure to the GNs, without KA, gave LDH readings indistinguishable from control values using KBSS alone. ▽, GppNHp; ●, GDPβS; ○, GMP. Values are mean ± S.D. for four independent experiments.

4. Discussion

As described long ago by Biziere and Coyle [23], and confirmed by us in the present work, two populations of kainate receptors can be distinguished in chick retinal membranes by conventional binding techniques (Fig. 1). Thus retina differs from other chick brain regions, such as tectum and cerebellum, where only the low-affinity site has been found [10,25]. These receptors would be, in principle, the molecular substrate for the excitotoxic action of KA in the chick retina, already a firmly established fact [14–21,26,27]. The characteristic sigmoidal shape of the dose-dependence curve for KA (Fig. 3) and the protection afforded by DNQX (Table 1) are equally suggestive of the involvement of typical KA receptors in the observed excitotoxic damage.

Exposure of embryonic chick retinal explants to adequate concentrations of KA, even for a short time, induces a long-lasting toxic effect leading to cell damage and membrane disruption, with release of cytoplasmic contents into the medium, including the intracellular enzyme LDH which is often used as a marker of cell damage in such excitotoxic experiments [19,21]. LDH can be detected in the medium early during exposure to KA (acute phase), and increases for a long time after terminating the exposure to the excitotoxin (delayed toxicity). It is important to mention that both the acute and delayed effects of KA in this specific model are exclusively due to the activation of AMPA/KA receptors, being only blocked by typical AMPA/KA antagonists – and not by the NMDA antagonists AP7 and/or MK801 – when applied together with KA. Furthermore, the delayed toxic effects, once the exposure of the retina to the toxin is terminated, are exclusively due to this initial period of exposure: addition of different glutamate antagonists, with different receptor specificities, during the post-KA incubation does not stop further release of LDH.

The exclusive involvement of AMPA/KA receptors in this neurotoxicity paradigm is compatible with a role for either Cl^-/Na^+ or Ca^{2+} in the causation of cell damage. Whereas the involvement of Cl^- and Na^+ in the acute phase seems to be established [16,18], the hypothetical participation of Ca^{2+} ions in the acute and/or the delayed phase can be equally accepted without the need of implicating NMDA receptors since AMPA/KA receptors associated with Ca^{2+} channels are expressed in chick retina [20,28].

Our present work describes yet another system where the toxicity of EAA agonists can be overcome by the simultaneous addition of GNs [11,12]. We have, as in previous cases, used non-hydrolyzable analogs of GTP and GDP to avoid ambiguous results due to the hydrolysis that takes place upon incubation with living tissue. The biphasic effect of GppNHp (Fig. 4) contrasts with the consistent protective behavior of GDPβS and GMP but agrees with our previous experience in other systems, such as the rat striatum where GTP analogs afford no protection ([11]; our unpublished results), and rat hippocampal slices where GppNHp, and even GTP, are downright toxic in a way that can be blocked by GMP itself [12]. Since GNs appear to bind to part of the agonist site, different GNs may have different effects upon the subsequent status of the channel itself [5,9,10].

Examination of Table 1 shows that the efficiency of the antagonist DNQX and of nucleotides as neuroprotectors parallels their ability to displace KA, as would be expected for a receptor-mediated phenomenon. The displacer/agonist ratio at the IC_{50}, however, is much higher than the protecting agent/KA ratio at the PC_{50}, suggesting that only partial displacement of KA may be necessary to block toxicity or that a critical number of receptors must be activated to cause a substantial release of LDH.

Table 1 also shows that the antagonistic potency of the GNs is low when compared with the reference antagonist DNQX. However, the specificity of the phenomenon (relative to other purine nucleotides) and the negligible toxicity of compounds such as 5′-GMP [11] provide a promising starting point for the design of derivatives of higher efficiency but still acceptable toxicity.

In conclusion, we have presented another fitting example of the potential of some guanine nucleotides (preferentially GMP, altogether) to act as neuroprotecting agents in a number of animal models in vivo, ex vivo and in tissue slices. The simplicity and reliability of our chick retinal preparation and neurotoxicity/protection protocol makes it especially appropriate for the screening of glutamate antagonists (the chick retina is sensitive to NMDA agonists, too), including more powerful synthetic analogs of the GNs.

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