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Effect of *N*-acetylaspartylglutamate (NAAG) on non-quantal and spontaneous quantal release of acetylcholine at the neuromuscular synapse of rat

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Abstract

N-Acetylaspartylglutamate (NAAG), known to be present in rat motor neurons, may participate in neuronal modulation of non-quantal secretion of acetylcholine (ACh) from motor nerve terminals. Non-quantal release of ACh was estimated by the amplitude of the endplate membrane hyperpolarization (H-effect) caused by inhibition of nicotinic receptors by (+)-tubocurarine and acetylcholinesterase by armin (diethoxy-*p*-nitrophenyl phosphate). Application of exogenous NAAG decreased the H-effect in a dose-dependent manner. The reduction of the H-effect by NAAG was completely removed when *N*-acetyl-β-aspartylglutamate (βNAAG) or 2-(phosphonomethyl)-pentanedioic acid (2-PMPA) was used to inhibit glutamate carboxypeptidase II (GCP II), a presynaptic Schwann cell membrane-associated ectoenzyme that hydrolyzes NAAG to glutamate and *N*-acetylaspartate. Bath application of glutamate decreased the H-effect similarly to the action of NAAG but *N*-acetylaspartate was without effect. Inhibition of NMDA receptors by DL-2-amino-5-phosphopentanoic acid, (+)-5-methyl-10,11-dihydro-*5H*-dibenzocyclohepten-5,10-imine (MK801), and 7-chlorokynurenic acid or inhibition of muscle nitric oxide synthase (NO synthase) by N^G-nitro-L-arginine methyl ester and 3-bromo-7-nitroindazole completely prevented the decrease of the H-effect by NAAG. These results suggest that glutamate, produced by enzymatic hydrolysis of bath-applied NAAG, can modulate non-quantal secretion of ACh from the presynaptic terminal of the neuromuscular synapse via activation of postsynaptic NMDA receptors and synthesis of nitric oxide (NO) in muscle fibers. NAAG also increased the frequency of miniature endplate potentials (mEPPs) generated by spontaneous quantal secretion of ACh, whereas the mean amplitude and time constants for rise time and for decay of mEPPs did not change.

Keywords: glutamate, muscle endplate, nitric oxide, *N*-methyl-D-aspartate receptor.

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N-Acetylaspartylglutamate (NAAG) is the most abundant and widely distributed neuropeptide in the mammalian CNS (see Neale *et al*. 2000 for review). NAAG is an excitatory neurotransmitter at central synapses of the optic nerve and cerebellum (Mori-Okamoto *et al*. 1987; Moffett *et al*. 1990; Tsai *et al*. 1990; Molinar-Rode and Pasik 1992), directly activating metabotropic $mGluR_3$ glutamate receptors (Wroblewska *et al*. 1997, 1998). It also has been reported to be a weak partial agonist/antagonist of ionotropic NMDA receptors (Westbrook *et al*. 1986; Sekiguchi *et al*. 1992; Koenig *et al*. 1994; Valivullah *et al*. 1994) but that activity has

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Abbreviations used: ACh, acetylcholine; AP5, DL-2-amino-5-phosphopentanoic acid; GCP II, glutamate carboxypeptidase II; L-NAME, N^G-nitro-L-arginine methyl ester; mEPP, miniature endplate potential; MK801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartylglutamate; βNAAG, *N*-acetyl-β-aspartylglutamate; NO, nitric oxide; 2-PMPA, 2-(phosphonomethyl)-pentanedioic acid.

recently been challenged (Losi *et al*. 2004). The NAAG signal is terminated by its hydrolysis to form glutamate and *N*-acetylaspartate (NAA) by the glial membrane-associated ectoenzyme glutamate carboxypeptidase II (GCP II, EC 3.4.17.21), also known as N-acetylated α-linked acidic dipeptidase (NAALADase; Blakely *et al.* 1988; Cassidy and Neale 1993) or by a second NAAG peptidase (GCP III) recently discovered in knockout mice (Bacich *et al*. 2002; Bzdega *et al*. 2004).

High concentrations of NAAG or NAAG-like immunoreactivity have been found in spinal cord motoneurons, motor components of cranial nerve nuclei (Forloni *et al*. 1987; Ory-Lavollée *et al*. 1987; Fuhrman *et al*. 1994), sciatic nerve (Ory-Lavollée *et al*. 1987; Miyake *et al*. 1981) and phrenic nerve terminals (Berger *et al*. 1995a). GCP II has been immunocytochemically identified in non-myelinating presynaptic Schwann cells surrounding motor nerve terminals (Berger *et al*. 1995a,b) and NMDA receptors are found in the endplate membrane (Berger *et al*. 1995a; Urazaev *et al*. 1995, 1998; Grozdanovic and Gossrau 1998; Lück *et al*. 2000), consistent with the hypothesis that NAAG may participate in the function of the neuromuscular junction.

Our recent studies indicate that the activation of muscle NMDA receptors by bath-applied glutamate increases synthesis of nitric oxide (NO) in muscle fibers and NO acts in a retrograde manner on motor nerve terminals to inhibit non-quantal release of acetylcholine (ACh) (Malomouzh *et al*. 2003). Non-quantal secretion of ACh has been shown to participate in various processes at the neuromuscular synapse, from synapse assembly in development and reinnervation (Sun and Poo 1985; Vyskočil and Vrbová 1993; Nikolsky *et al*. 1996), maintenance (Bray *et al*. 1982; Urazaev *et al*. 1987a, 1997; Nikolsky *et al*. 1996), and modulation (Thesleff 1990; Giniatullin *et al*. 1993; Nikolsky *et al*. 1994) to a desensitizing action on the postsynaptic nicotinic receptors that shortens postsynaptic response to quantal mediator (Giniatullin *et al*. 1993). The loss of non-quantal secretion of ACh appears to be the earliest presynaptic change following nerve section (Stanley and Drachman 1986; Nikolsky *et al*. 1996) and may trigger initiation of the denervation changes seen in the postsynaptic membrane (Urazaev *et al*. 1987a, 1997, 1999, 2000).

We report here that the extracellular hydrolysis of NAAG could provide glutamate for activation of postsynaptic NMDA receptors and synthesis of NO in muscle fibers that modulates non-quantal release of ACh from motor nerve endings.

Methods

Materials and animals

N-Acetylaspartylglutamate (NAAG), *N*-acetyl-β-aspartylglutamate (βNAAG), glutamic acid, *N*-acetylaspartate (NAA), NMDA receptor antagonists DL-2-amino-5-phosphopentanoic acid (AP5) and 7-chlorokynurenic acid, a NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), and nicotinic receptor antagonist (+)tubocurarine were purchased from Sigma, St Louis, MO, USA. [^{3}H]NAAG (50 Ci/mmol) labeled in the glutamate moiety was purchased from PerkinElmer (Boston, MA, USA). The GCP II inhibitor 2-(phosphonomethyl)-pentanedioic acid (2-PMPA) was a gift of Guilford Pharmaceuticals (Baltimore, MD, USA). The NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine (MK801) and the selective inhibitor of neuronal NO synthase 3-bromo-7-nitroindazole were purchased from Tocris Cookson Inc., Ballwin, MO, USA. Armin (diethoxy-*p*-nitrophenyl phosphate), an inhibitor of acetylcholinesterase, was manufactured in the Institute of Organic Chemistry, Moscow, Russia. All other chemicals were purchased from Sigma.

TLC analysis indicates that the commercial NAAG contained < 1% contamination with glutamate. In experiments with 20 μmol/L NAAG, 0.1–0.5% contamination would add too little glutamate to influence the H-effect. In experiments with 500 μmol/L NAAG, 0.5% glutamate contamination could add to the effect of glutamate released from enzymatic hydrolysis of NAAG, but would not be expected to change the H-effect.

Male Wistar rats (150–200 g body weight) were used for all experiments. The animals were kept in sawdust-lined plastic cages in a well-ventilated room. A standard diet and water were available at all times. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the protocol of the experiments was approved by the Animal Care and Use Committee of Kazan State Medical University.

Tissue preparation and bathing solution

Diaphragms with a 10–15 mm nerve stump were removed from animals killed by cervical dislocation and decapitation under ether anesthesia. The diaphragms were cut lengthwise into strips of intact muscle fibers, which were placed in transparent 5-mL plastic dishes with a Sylgard 184 silicone elastomer coating (Dow Corning Co, Midland, MI, USA) and superfused with a standard oxygenated (95% O_2 + 5% CO_2) Ringer–Krebs solution containing, in mmol/L: NaCl 120.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 11.0, NaH_2PO_4 1.0, D-glucose 11.0; pH 7.2–7.4. In some experiments, specially designated in the text, phosphate-free Ringer–Krebs solution was used to avoid the possibility of phosphate inhibition of GCP II activity (Robinson *et al*. 1987; Cassidy and Neale 1993). This solution contained, in mmol/L: NaCl 154.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, HEPES 5.0, D-glucose 11.0; pH 7.2–7.4. Solutions of drugs were prepared just prior to their use. Drugs were directly added to the recording chamber at the time of experimentation. Muscle strips were incubated in these solutions without superfusion. All experiments were performed at 20°C, the temperature at which the H-effect is maximal (Lupa *et al*. 1986).

Electrophysiological recordings

Standard recording techniques for electrophysiological measurement of endplate potentials were used and previously described (Vyskočil and Illes 1977, 1978; Vyskočil *et al*. 1983; Mukhtarov *et al*. 2000; Malomouzh *et al*. 2003). Glass microelectrodes filled with 2.5 mol/L KCl and tip resistances between 15 and 25 MΩ

were used for recording of endplate membrane potential. The bath ground consisted of a 3 M NaCl-filled glass tube bridge in contact with a silver–silver chloride wire-to-system ground. Continuous digital recordings of membrane potentials were collected for subsequent double-blind analysis of the traces. Typically, the criteria used to differentiate acceptable from non-acceptable impalements of muscle fibers include: (i) a rapid (ms) change in recorded potential from the zero reference to a resting membrane potential maintained for several seconds and (ii) upon withdrawal of the electrode the recorded potential rapidly returns to the zero reference level. Using these criteria the ratio of acceptable/total impalements was 15–20/25.

Following 15–20 min of superfusion in physiological solution, muscles were incubated with the irreversible cholinesterase inhibitor Armin (10 μmol/L) for 30 min and then rinsed with several changes of normal saline over a 10-min period. Endplate membrane potential measurements were begun 15 min post anticholinesterase treatment, with various drugs added. The neuromuscular synapses occur along the intramuscular branch of the phrenic nerve and can be localized by the presence of muscle contractures in the endplate region due to the accumulation of ACh in the synaptic cleft following inhibition of cholinesterase. Further evidence that the region of contracture is, indeed, the endplate region is obtained by microelectrode recording of miniature endplate potentials (mEPPs) generated from the spontaneous quantal secretion of ACh. Non-quantal release of ACh, which causes depolarization of muscle fibers at the endplate zone, was quantified statistically by measurement of membrane potentials in 25 superficial fibers in each strip during a 5–10-min period before and another 25 fibers 5–10 min after addition of (+)-tubocurarine (10 μmol/L) to the medium. The difference between the mean resting membrane potentials under these two conditions (i.e. the H-effect) is considered to be due to the non-quantal release of ACh (Katz and Miledi 1977; Vyskočil and Illes 1977, 1978; for details see Vyskočil *et al*. 1983). In each group, four to eight muscles from different animals were used. In these experiments, choline chloride (10 μmol/L) was added to Ringer–Krebs solution to delay postdenervation decrease of non-quantal ACh secretion (Nikolsky *et al*. 1991).

The effect of NAAG on postsynaptic membrane receptor properties was estimated by analysis of the amplitude and time constant of the rise and decay of mEPPs generated from the spontaneous quantal secretion of ACh. Changes in presynaptic terminal properties of spontaneous quantal secretion of ACh were estimated by analysis of mean mEPP frequencies. For these experiments, recordings were made from endplates with resting membrane potentials between −65 mV and −75 mV. For analysis, mEPPs were simultaneously displayed on an oscilloscope and collected by PC software that digitized the signals at 100 kHz. Recordings of 100 mEPPs were made from the same endplate every 3–5 min, first during 10–15 min with control solution, then during a 30 min incubation with NAAG added, and finally during a 20–30 min wash with control solution. The effect of NAAG treatment was studied only on preparations in which the resting membrane potential remained stable. The mean value of mEPP amplitudes, rise times (from 20 to 80% of maximum amplitude), and decay time constant were calculated. The mean values were determined from 300 mEPPS in the initial control period, 400 mEPPs during the 15th through 30th min of NAAG exposure (comparable to the time allowed for measuring the result of drug exposure on the H-effect), and 300 mEPPs following 20 min of wash. Only endplates with mEPP amplitudes that demonstrated a normal distribution, rise times not greater than 400 μs and amplitudes not less than 0.5 mV were used. These values are five to six times higher than the standard deviation of electrical noise.

N-Acetylaspartylglutamate peptidase assay

Diaphragms were dissected from 100–125 g male Wistar rats under deep isoflurane anesthesia. The muscles were cut transversely in the central region, where the neuromuscular junctions are localized, and the phrenic nerve innervation was trimmed as close as possible to the muscle to prepare samples that should be relatively enriched in NAAG peptidase associated with perisynaptic glia (Berger *et al*. 1995a,b). Enzyme activity was assayed in duplicate by a procedure like those of Berger *et al*. (1995a,b) and Fuhrman *et al*. (1994). Briefly, tissue homogenates prepared in 50 mmol/L Tris-Cl buffer, pH 7.5, were centrifuged, and the pellet was washed and resuspended in Tris buffer containing 0.5% Triton X-100. Aliquots (70–170 μg protein, measured with BCA reagent (Pierce, Rockford, IL, USA) were incubated at 37°C for 45–120 min in 40 μL total volume containing 40 mmol/L Tris-Cl, 1 mmol/L $CoCl_2$, 4 μmol/L NAAG, and 0.1 μCi [^{3}H]NAAG. Sodium phosphate buffer, pH 7.4, was included in some samples at 1 or 10 mmol/L final concentration. The reaction was terminated by addition of 40 μL 0.2 mol/L HCl and the samples were applied to 0.6 × 2 cm AG 50W-X4 (200–400 mesh) resin, H^+ form (Bio-Rad, Richmond, CA, USA). After washing NAAG off with 4 mL 0.1 mol/L HCl, glutamate was eluted with 2 mL 1 mol/L HCl and its radioactivity determined with 14 mL Ecolume scintillation fluid in a Beckman Coulter LS 6500 counter.

Statistics

The Tukey–Kramer Multiple Comparisons Test for One-way Analysis of Variance (ANOVA) was performed using GraphPad InStat program (GraphPad Software, Inc., San Diego, CA, USA) for statistical analysis of physiological data. A paired *t*-test was used to analyze biochemical data on the effect of phosphate on NAAG peptidase activity. A *p*-value of ≤ 0.05 was considered to indicate a significant difference between two groups of experimental data. Data are presented as means ± 1 SEM.

Results

The effect of *N*-acetylaspartylglutamate on the H-effect

The resting potential of the endplate membrane of muscle fibers treated with acetylcholinesterase inhibitor was −70.2 ± 0.2 mV ($n = 200$ from eight animals). Superfusion of these muscle fibers with (+)-tubocurarine hyperpolarized the endplate membrane to −75.5 ± 0.2 mV ($n = 200$; $p < 0.001$), representing an H-effect in control animals of 5.3 ± 0.2 mV ($n = 200$). Bath application of NAAG decreased the amplitude of the H-effect in a dose-dependent manner (Fig. 1). The concentration of NAAG required for a

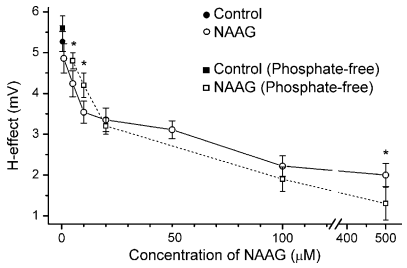

Fig. 1 The dose–response relationship between *N*-acetylaspartylglutamate (NAAG) and the amplitude of the H-effect. The H-effect is the endplate hyperpolarization following inhibition of nicotinic cholinoreceptors by (+)-tubocurarine (10 μmol/L) in muscle fibers treated with armin to inhibit acetylcholinesterase. The H-effect was used to estimate the non-quantal release of acetylcholine from the motor nerve terminals. The amplitude of the H-effect in control experiments was 5.3 mV (filled circle) and 5.6 mV (filled square) in Ringer–Krebs solutions with and without phosphate, respectively. The open circles and squares represent effects of different concentrations of NAAG on the amplitude of the H-effect (open circles – in solution containing 1 mmol/L phosphate; open squares – in phosphate-free solution). NAAG reduced the H-effect in a dose-dependent manner. The concentration of NAAG required for half maximal reduction of the H-effect (EC_{50}) was 9.2 μmol/L. These results suggest that NAAG inhibits the non-quantal release of acetylcholine in the neuromuscular synapse of rat. Data are presented as means ± 1 SEM pooled from four to eight animals (75–200 muscle fibers). Asterisks (*) indicate the small, but statistically significant difference ($p < 0.05$) between the effects of the same concentration of NAAG in solutions with and without phosphate.

half maximal decrease of the H-effect (EC_{50}) was 9.2 μmol/L. In a previous study, we found that the EC_{50} for glutamate action on the H-effect was 25.4 μmol/L and for glutamate potentiated by glycine was 4.8 μmol/L (Malomouzh *et al.* 2003).

The solution used to superfuse muscle strips contained phosphate to supplement buffering by bicarbonate. Since phosphate has been reported to inhibit the activity of GCP II in brain homogenates and in brain *in vivo* (Robinson *et al.* 1987; Stauch *et al.* 1989; Cassidy and Neale 1993), we measured the H-effect in phosphate-free Ringer–Krebs solution by the same procedures to look for any effect of phosphate on the action of NAAG. As shown in Fig. 1, the action of NAAG on the H-effect was nearly identical with and without phosphate.

We also measured the effect of phosphate on NAAG peptidase activity of diaphragm muscle homogenates. NAAG hydrolysis by homogenates of the end-plate region of diaphragm muscle was linear with protein and time in the ranges studied. Product formation was about twice the no tissue background, amounting to about 25 pmol/mg protein/h, and was completely blocked by the GCP II inhibitor 2-PMPA at 50 nmol/L. Contrary to published studies that indicate a competitive inhibition of GCP II activity in brain homogenates (Robinson *et al.* 1987), phosphate stimulated NAAG peptidase activity of diaphragm muscle homogenates by 35.9 + 7.6% above the no phosphate control level at 1 mmol/L ($p < 0.05$; $n = 6$) but had no effect at 10 mmol/L (94.2 ± 3.9% of control; $p > 0.05$; $n = 4$).

Throughout the remainder of this investigation NAAG was used at a concentration of 20 μmol/L, which is approximately 2 × the EC_{50}. At this concentration, the H-effect was 3.4 ± 0.3 mV ($n = 125$; $p < 0.001$; Figs 1–4) and 3.2 ± 0.2 mV ($n = 225$; $p < 0.001$; Fig. 1), or 64% and 57% of control levels in solutions with and without phosphate, respectively. The experiments described below were therefore performed in phosphate-containing saline since the H-effect was altered only a little by phosphate, at only three of seven NAAG concentrations tested but not at the one selected for further experimentation, and did not inhibit NAAG peptidase activity at the phosphate concentration used.

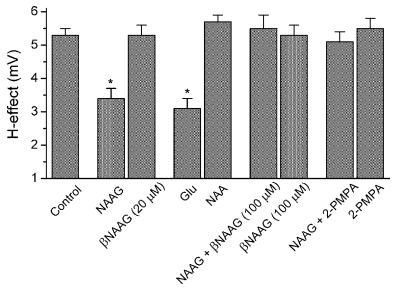

Fig. 2 Effects of *N*-acetylaspartylglutamate (NAAG) and inhibitors of glutamate carboxypeptidase II (GCP II) on the H-effect in phosphate-containing Ringer–Krebs solution. Bath-application of 20 μmol/L NAAG reduced the H-effect by about 36% compared to control conditions. However, when the muscle fibers were superfused with 20 μmol/L of *N*-acetyl-β-aspartylglutamate (βNAAG), an analog of NAAG that cannot be hydrolyzed by GCP II, the amplitude of the H-effect did not change. These results suggest that the products of NAAG hydrolysis, glutamate (Glu) and/or *N*-acetylaspartate (NAA), rather than NAAG itself, may be responsible for the reduction of the H-effect in the experiments with bath application of NAAG. In fact, only glutamate, but not NAA (both 20 μmol/L), could mimic the effect of application of NAAG on the amplitude of the H-effect. These data imply that only one product of NAAG hydrolysis, i.e. glutamate, elicits the decrease of the H-effect. At the neuromuscular junction, GCP II that is associated with the membrane of presynaptic Schwann cells is the only NAAG hydrolyzing enzyme that has been found so far. The inhibition of this enzyme activity by either 100 μmol/L βNAAG, which acts as a competitive inhibitor of the enzyme at this concentration, or by potent non-competitive inhibitor 2-(phosphonomethyl)-pentanedioic acid (2-PMPA, 0.1 μmol/L), completely prevented the reduction of the H-effect by NAAG. The results suggest that NAAG hydrolysis by NAAG peptidase yields glutamate to decrease the non-quantal secretion of acetylcholine. Data are presented as means ± 1 SEM pooled from five to eight animals (125–200 muscle fibers). Asterisks (*) indicate the statistically significant difference ($p < 0.05$) between the H-effects in control condition and following bath-application of drugs.

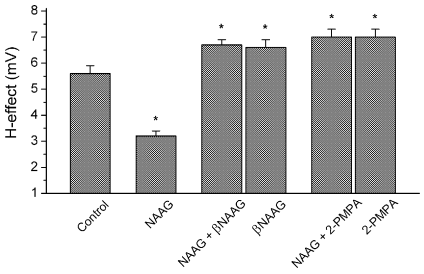

Fig. 3 Effects of *N*-acetylaspartylglutamate (NAAG) and inhibitors of glutamate carboxypeptidase II (GCP II) on the H-effect in phosphate-free Ringer–Krebs solution. Bath application of 20 μmol/L NAAG reduced the H-effect by about 43% compared to control conditions. Similarly to their action in phosphate-containing solution (Fig. 2), GCP II competitive inhibitor *N*-acetyl-β-aspartylglutamate (βNAAG, 100 μmol/L) and non-competitive inhibitor 2-(phosphonomethyl)-pentanedioic acid (2-PMPA, 0.1 μmol/L) completely prevented the reduction of the H-effect by NAAG. These results support our conclusion that the product of NAAG hydrolysis by NAAG peptidase develops the inhibitory action on the H-effect. Bath application of either 100 μmol/L βNAAG or 0.1 μmol/L 2-PMPA, without NAAG, increased the amplitude of the H-effect by 18 and 25%, respectively ($p < 0.05$ relative to the control); that was in contrast to the results in phosphate-containing solution (Fig. 2), in which neither of these GCP II antagonists changed the H-effect. These data may have uncovered the action of endogenously released NAAG, which is hydrolyzed by a NAAG peptidase in intact tissue in phosphate-free saline to release a small amount of glutamate sufficient to reduce the H-effect. Data are presented as means ± 1 SEM pooled from six to seven animals (150–175 muscle fibers). Asterisks (*) indicate the statistically significant difference ($p < 0.05$) between the H-effects in Control condition and following bath application of drugs.

H-effect at endplates treated with *N*-acetylaspartylglutamate and glutamate carboxypeptidase II inhibitors

The following experiments were predicated on the hypothesis that glutamate produced when NAAG is hydrolyzed by NAAG peptidase may affect the non-quantal release of ACh (Malomouzh *et al*. 2003). As illustrated in Fig. 2, βNAAG, a non-hydrolysable structural analog of NAAG, used at the same concentration at which NAAG was effective (20 μmol/L), did not change ($p > 0.05$) the H-effect as compared to the control: 5.3 ± 0.3 mV ($n = 150$) and 5.3 ± 0.2 mV ($n = 200$; Fig. 2), respectively. Neither did bath application of 20 μmol/L NAA (5.7 ± 0.2 mV, $n = 200$; $p > 0.05$), whereas 20 μmol/L glutamate caused a considerable decrease in the amplitude of the H-effect (to 3.1 ± 0.3 mV; $n = 125$; $p < 0.001$), in accord with our previous study (Malomouzh *et al*. 2003). These results suggest that the reduction of the H-effect at NAAG-treated endplates is caused by glutamate, a product of NAAG hydrolysis, rather than by the other by-product, NAA, or by a direct action of NAAG itself.

To test this hypothesis, the muscle fibers were exposed to one of two inhibitors of GCP II, i.e. βNAAG or 2-PMPA, with and without NAAG. βNAAG at high concentration (e.g. 100 μmol/L) is known to act not only as a non-hydrolysable analog of NAAG but also as a competitive inhibitor of GCP II (Serval *et al*. 1990). 2-PMPA is a potent and specific inhibitor of GCP II (Thomas *et al*. 1999) and of GCP III (Bacich *et al*. 2002). Superfusion of muscle fibers with 100 μmol/L βNAAG completely eliminated the reduction in the H-effect by 20 μmol/L NAAG (5.5 ± 0.4 mV; $n = 125$; $p > 0.05$). However, without NAAG addition, 100 μmol/L βNAAG did not change the H-effect as compared to controls (5.3 ± 0.3 mV; $n = 150$; $p > 0.05$; Fig. 2). Similarly, 2-PMPA (0.1 μmol/L) completely prevented the action of 20 μmol/L NAAG on the H-effect (5.1 ± 0.3 mV; $n = 200$; $p > 0.05$). 2-PMPA itself, without NAAG, did not change the H-effect as compared to the control (5.5 ± 0.3 mV; $n = 175$; $p > 0.05$; Fig. 2).

As seen in comparing Figs 2 and 3, βNAAG and 2-PMPA had the same effect on the NAAG-induced decrease in the H-effect in phosphate-containing and phosphate-free saline. On the other hand, superfusion of the muscle strips with either of these inhibitors alone, without NAAG, caused an H-effect increase ($p < 0.05$) in phosphate-free saline in contrast to the results in phosphate-containing saline ($p > 0.05$).

Effect of inhibitors of *N*-methyl-D-aspartate receptors and nitric oxide-synthase on the reduction of the H-effect by *N*-acetylaspartylglutamate

We have recently demonstrated that bath-application of glutamate activates endplate NMDA receptors in a dose-dependent manner, leading to synthesis and release of NO from muscle fibers and inhibition of non-quantal secretion of ACh from the motor nerve terminals (Malomouzh *et al*. 2003). We therefore tested whether bath-applied NAAG, hydrolyzed by GCP II, can be a source of glutamate at the neuromuscular junction.

As expected, the decrease of the H-effect by 20 μmol/L NAAG was prevented by a potent competitive antagonist of NMDA receptors, DL-2-amino-5-phosphopentanoic acid (AP5, 100 μmol/L), by a selective non-competitive blocker of NMDA receptors, MK801 (0.2 μmol/L), and by a potent competitive NMDA receptor inhibitor acting at the glycine site of the receptor, 7-chlorokynurenic acid (20 μmol/L). The amplitude of the H-effect did not change when muscle fibers were superfused with any of these NMDA receptor inhibitors in the absence of NAAG (Fig. 4a).

Non-quantal secretion of ACh at the rat neuromuscular junction has been shown to be regulated in a retrograde fashion by NO released from muscle fibers following activation of postsynaptic NMDA receptors and/or muscle muscarinic M1 cholinoreceptors (Urazaev *et al*. 1997, 2000; Mukhtarov *et al*. 2000; Malomouzh *et al*. 2003).

As illustrated in Fig. 4(b), the competitive inhibitor of NO-synthase L-NAME (100 μmol/L) and the selective neuronal NO-synthase inhibitor, 3-bromo-7-nitroindazole

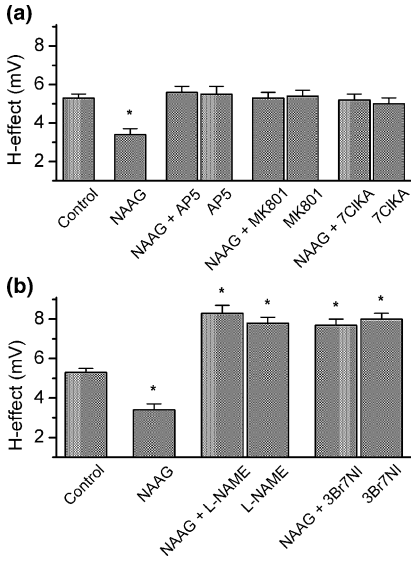

Fig. 4 The effects of NMDA receptor and nitric oxide (NO)-synthase antagonists on the decrease of the H-effect caused by the bath application of *N*-acetylaspartylglutamate (NAAG). As seen in both panels, bath application of 20 μmol/L NAAG decreased the amplitude of the H-effect by about 36% compared to control muscle fibers. (a) The inhibition of postsynaptic NMDA receptors by DL-2-amino-5-phosphopentanoic acid (AP5, 100 μmol/L), (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine (MK801, 0.2 μmol/L), and 7-chlorokynurenic acid (7ClKA, 20 μmol/L) completely removed the reduction of the H-effect by NAAG. The results suggest that glutamate released from NAAG hydrolysis by NAAG peptidase reduces the non-quantal secretion of acetylcholine via activation of NMDA receptors at the endplate membrane. (b) As expected, a preincubation of muscle fibers with NO synthase inhibitors N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L) or 3-bromo-7-nitroindazole (3Br7NI, 10 μmol/L) increased the amplitude of the H-effect compared with control experiments, suggesting that there is a background synthesis of NO in the sarcoplasm of the control muscle fibers that decreases the non-quantal secretion of acetylcholine to some extent. Under these circumstances, NAAG did not reduce the H-effect. These results suggest that glutamate release from NAAG hydrolysis by NAAG peptidase attenuates the non-quantal secretion of acetylcholine through the activation of NO production in the sarcoplasm of muscle fibers. In both (a) and (b), data are presented as means ± 1 SEM pooled from four to eight animals (100–200 muscle fibers). Asterisks (*) indicate the statistically significant difference ($p < 0.05$) between the H-effects in control condition and following bath application of drugs.

(10 μmol/L), in the absence of exogenous NAAG, significantly increased the H-effect, consistent with our previous findings (Mukhtarov *et al*. 2000; Malomouzh *et al*. 2003). NAAG (20 μmol/L) did not change the H-effect in muscle fibers pretreated with either of these NO-synthase inhibitors, suggesting that NO is involved in the reduction of the H-effect caused by the bath application of NAAG.

Taken together, the results suggest that hydrolysis of bath-applied NAAG yields glutamate to activate NMDA receptors in the postsynaptic membrane and NO synthase in the sarcoplasm of muscle fibers.

The effect of *N*-acetylaspartylglutamate on the quantal release of acetylcholine

In a previous study, we found that the concentration of bath-applied glutamate required for a half-maximal decrease of the amplitude of the H-effect is 25.4 μmol/L (Malomouzh *et al*. 2003), i.e. that glutamate is nearly threefold less effective than NAAG (EC_{50} 9.2 μmol/L) in phosphate-containing solution not supplemented with glycine. In phosphate-free solution the EC_{50} for NAAG was increased to 17.4 μmol/L. The difference in potencies between glutamate and NAAG is the reverse of what would be expected and seemingly contradicts our hypothesis that glutamate released upon NAAG hydrolysis, but not NAAG itself, reduces the amplitude of the H-effect. We therefore tested whether NAAG might alter postsynaptic receptor sensitivity or presynaptic transmitter release.

One possible explanation for the apparent difference in the EC_{50} is that NAAG might alter the sensitivity of postsynaptic nicotinic receptors to non-quantal ACh causing an increased endplate depolarization. The sensitivity of endplate nicotinic receptors to ACh can be evaluated by analysis of the amplitude and temporal parameters of mEPPs generated by spontaneous quantal secretion of ACh. In each experiment, the recordings were made from the same neuromuscular junction that was first superfused with physiological saline and then incubated in solution containing 20 μmol/L NAAG. The mean amplitude and time constants for rise time and decay of mEPPs in control conditions were 1.38 ± 0.21 mV, 0.33 ± 0.02 ms and 1.58 ± 0.12 ms, respectively ($n = 1500$ mEPPs from five animals, for each measurement). Following a 25–30 min incubation of endplate membrane with NAAG, these values were 1.45 ± 0.31 mV, 0.33 ± 0.03 ms and 1.60 ± 0.14 ms, respectively, i.e. not significantly different from the control values ($p > 0.05$; $n = 2000$). These results indicate that NAAG did not change the sensitivity of postsynaptic nicotinic receptors to ACh. Similarly, bath-applied glutamate had no effect on the sensitivity of postsynaptic nicotinic receptors and kinetics of their ionic channels (Malomouzh *et al*. 2003).

Whereas NAAG reduces evoked, vesicular release of transmitter in brain via presynaptic metabotropic receptors (Zhao *et al*. 2001; Sanabria *et al*. 2004), it elicited a presynaptic stimulatory effect on spontaneous quantal secretion at the neuromuscular junction. NAAG increased the frequency of mEPPs by about 60%, from 0.992 ± 0.162 s^{-1} in control experiments to 1.527 ± 0.279 s^{-1} ($p < 0.05$; in each group, $n = 1500$ mEPPs from five animals). MEPP frequency returned to 89% of the control value (0.970 ± 0.269 s^{-1}; $p > 0.05$) following a 30 min washout period in a control solution. By contrast, bath-applied

glutamate did not change the frequency of spontaneous quantal secretion of ACh (Malomouzh *et al*. 2003).

Discussion

Progress in the understanding of synaptic development and plasticity has been advanced, to a great extent, by the use of the neuromuscular junction as a model system. The modulation and auto-regulation of synaptic transmission at that site is believed to involve cholinergic, adrenergic, and purinergic mechanisms (for reviews see Lu and Fu 1995; Ribeiro *et al*. 1996; Wood and Slater 2001). In addition to these well-characterized mechanisms, recent studies revealed that glutamate (Waerhaug and Ottersen 1993; Berger *et al*. 1995a; Grozdanovic and Gossrau 1998; Malomouzh *et al*. 2003; Pinard *et al*. 2003) and neuropeptides (see for reviews Lu and Fu 1995; Sanes and Lichtman 1999) also modulate neuromuscular transmission.

In the peripheral nervous system, relatively high concentrations of NAAG have been found in both spinal motoneurons and their nerve terminals (Miyake *et al*. 1981; Forloni *et al*. 1987; Ory-Lavollée *et al*. 1987; Berger *et al*. 1995a). Since GCP II is found on the surface of presynaptic Schwann cells and inactivates NAAG, NAAG may be a direct participant in the regulation of transmission at the neuromuscular synapse (Berger *et al*. 1995a,b). Alternatively, it is possible that extracellular NAAG hydrolysis by NAAG peptidase provides glutamate and/or NAA for modulation of neuromuscular transmission. A recent study from our laboratory presented strong evidence that glutamate can activate postsynaptic NMDA receptors and synthesis of NO in sarcoplasm and thereby modulate non-quantal release of ACh into the neuromuscular synaptic space by a cGMP-dependent mechanism (Malomouzh *et al*. 2003). In the investigation reported here, we found that in the absence but not presence of GCP II inhibitors NAAG reduced the non-quantal secretion of ACh from the motor nerve endings. This confirms our prediction that NAAG peptidase activity at the synapse is sufficient to produce enough bioactive glutamate from NAAG to block the H-effect.

The lack of inhibition of NAAG peptidase activity by 1 mmol/L phosphate in our physiological experiments and the stimulation measured in our biochemical assays were at first unexpected and contrary to published literature on GCP II of brain homogenates. Robinson *et al*. (1987) reported that phosphate competitively inhibits activity of the brain enzyme with a half-maximal activity at 100 μmol/L. However, those assays, and similar GCP II assays conducted by other investigators (e.g. Tiffany *et al*. 2001), were performed with a total NAAG concentration of only 30 nmol/L whereas our reaction mixture contained about 100 × as much NAAG, i.e. 4 μmol/L. Apparently 1 mmol/L phosphate not only is insufficient to inhibit NAAG peptidase activity of neuromuscular homogenates at that higher NAAG concentration, but actually stimulates the enzyme in some way. The fact that 10 mmol/L phosphate did not increase the enzyme activity further suggests that phosphate may have a dual effect on the enzyme, dependent on concentration. These observations warrant a further study to compare the properties of the brain and neuromuscular enzymes. As far as we are aware, ours is the first published report of the biochemical activity of the enzyme at the neuromuscular junction.

Our finding that NAAG is nearly 3 × as effective as glutamate in reducing the H-effect in a phosphate-containing solution and 46% more effective in the absence of phosphate was surprising, because we predicted that NAAG as a source of glutamate would be equally or less potent than glutamate itself. We do not currently have a comprehensive explanation for the disparity in efficacy of the same concentration of NAAG and glutamate but suggest that this difference could result, at least in part, from NAAG's presynaptic action that increases the spontaneous mEPP frequency. Endogenous glutamate is released with quantal ACh from motor nerve terminals (Vyas and Bradford 1987; Israel *et al*. 1993; Meister *et al*. 1993; Pinard *et al*. 2003; Kraus *et al*. 2004; Landry *et al*. 2004), so a NAAG-stimulated increase in released glutamate could supplement exogenous glutamate derived from hydrolysis of NAAG to reduce the H-effect. In a recent study, Li *et al*. (2004) demonstrated corelease of glutamate and ACh from the same synaptic vesicle during its spontaneous secretion in single identified premotoneurons and motoneurons during development of the hatching frog tadpole.

Another possible explanation is that bath-applied NAAG increased the response to glutamate that was formed by hydrolysis of some of the NAAG or to glutamate released presynaptically in response to exogenous NAAG. Hess *et al*. (1999) reported that NAAG potentiates the effect of glutamate on human recombinant glutamate receptors (NMDAR1/2D) expressed in oocytes. Losi *et al*. (2004), on the other hand, recently reported that NAAG does not potentiate the response of NMDA receptors to NMDA at rat cerebellar granule neurons in primary culture. We do not know whether NAAG affects the response to glutamate at the neuromuscular junction.

It is well-documented that NMDA receptors are present in the endplate membrane along with nicotinic ACh receptors (Berger *et al*. 1995a; Urazaev *et al*. 1995, 1998) and colocalized with NO synthase in the junctional sarcolemma (Grozdanovic and Gossrau 1998; Lück *et al*. 2000). Since bath-application of glutamate activates postsynaptic NMDA receptors and triggers Ca^{2+}-dependent synthesis of NO in sarcoplasm (Urazaev *et al*. 1995; Malomouzh *et al*. 2003), we predicted that glutamate released by enzymatic hydrolysis of bath-applied NAAG would activate postsynaptic NMDA receptors to initiate synthesis of NO in muscle fibers. This hypothesis was confirmed in the present investigation using

selective NMDA receptor antagonists and NO synthase inhibitors.

Evidence for a role of neuronal NO synthase in skeletal muscle fibers is well established (Kobzik *et al*. 1994; Brenman *et al*. 1995; Grozdanovic *et al*. 1995a,b, 1996; Chang *et al*. 1996; Frandsen *et al*. 1996; Gossrau *et al*. 1996; Kusner and Kaminski 1996; Oliver *et al*. 1996; Urazaev *et al*. 1996; Stamler and Meissner 2001) and more recently, a role in control of non-quantal release of the mediator from the motor nerve terminals has been described (Mukhtarov *et al*. 2000; Malomouzh *et al*. 2003).

The results of this and previous investigations suggest the model shown in Fig. 5 of glutamatergic regulation of function at the neuromuscular synapse. This model illustrates how glutamate formed from released NAAG could suppress non-quantal secretion of ACh from motor nerve terminals. These studies with bath-applied glutamate and NAAG provide a framework for considering whether and how endogenous glutamate and NAAG might contribute to regulation of non-quantal transmitter release at the neuromuscular junction. This issue will require further investigation. On the one hand, in the present study inhibitors of GCP II (in phosphate-free saline) and of NO synthase augmented the size of the H-effect in the absence of exogenous NAAG. This is consistent with the hypothesis that endogenously released NAAG is hydrolyzed to yield small amounts of glutamate sufficient to provoke muscle fibers to generate NO and decrease the H-effect. However, on the other hand, inhibitors of GCP II (in phosphate-containing saline) and of NMDA receptors did not change the H-effect in the absence of exogenous NAAG. These results are not consistent with the hypothesis. We are currently designing experiments to test whether endogenous NAAG and glutamate influence the H-effect when the nerve fibers are at rest and when they are generating action potentials at high frequency, conditions that would favor release of NAAG.

Our experiments revealed an increase in mEPP frequency induced by NAAG. A working hypothesis at this point is that NAAG activates a presynaptic second messenger cascade via mGLUR3, with the end result being an increase in the spontaneous release of ACh. That would be the opposite of the known effect of NAAG on presynaptic mGLUR3 to decrease glutamate release.

Regulation of NO production in muscle by NAAG and/or glutamate could contribute to regulation of muscle cell volume (Khairova *et al*. 2002, 2003) and modulation of metabolism and contraction, including muscle glucose homeostasis, creatine kinase activity, autoregulation of blood flow, mitochondrial oxygen consumption, differentiation of myocytes, excitation–contraction coupling, and activation of guanylate cyclase and nitrosylation of proteins (see reviews by Reid 1998; Kaminski and Andrade 2001; Stamler and Meissner 2001).

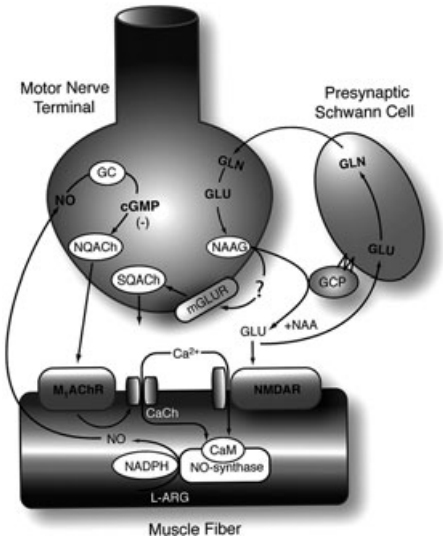

Fig. 5 Working model of the physiological interrelationships between *N*-acetylaspartylglutamate (NAAG), NAAG peptidase (abbreviated as GCP for simplicity) and non-quantal acetylcholine (ACh) at the neuromuscular synapse. NAAG was found in motor nerve terminals (Berger *et al*. 1995a) and, presumably, can be secreted into the synaptic cleft by physiological stimuli, as observed at synapses of CNS (for review see Neale *et al*. 2000). NAAG, upon its secretion from the nerve terminal, could modulate the non-quantal secretion of ACh (NQACh) from the same terminal by NAAG peptidase-mediated hydrolysis of NAAG to produce glutamate (GLU). The release of glutamate leads to activation of postsynaptic NMDA receptors (NMDAR; Berger *et al*. 1995a; Urazaev *et al*. 1995) and Ca^{2+}-dependent stimulation of nitric oxide (NO) synthesis in sarcoplasm (Urazaev *et al*. 1995, 1996, 1997, 1998). NO diffuses from muscle fibers to the cytoplasm of the nerve ending to attenuate the non-quantal secretion of ACh through a cGMP-dependent mechanism (Urazaev *et al*. 1996, 1997; Mukhtarov *et al*. 2000). Non-quantal ACh maintains high resting membrane potential in the muscle fibers (Bray *et al*. 1982) through muscarinic M1 receptor (M_1AChR)-mediated (Urazaev *et al*. 2000) suppression of the activity of the furosemide-sensitive Na^+,K^+,$2Cl^-$-cotransport in sarcolemma (Urazaev *et al*. 1987b, 1999). In addition, non-quantal ACh modulates its own release through a negative feedback mechanism that involves activation of voltage-dependent Ca^{2+} channels (CaCh) in muscle membrane and synthesis of NO in the sarcoplasm (Urazaev *et al*. 1997; Mukhtarov *et al*. 2000). Both muscle denervation, which impairs non-quantal ACh release (Stanley and Drachman 1986; Nikolsky *et al*. 1996), and NO synthase inhibition *in vivo* (Khairova *et al*. 2002, 2003) eliminate the negative control of active Cl^- cotransport, leading to an elevation of sarcoplasm Cl^- and a decrease in the Cl^- equilibrium potential (Urazaev *et al*. 1987b). This causes early denervation depolarization of muscle fibers (Albuquerque *et al*. 1971; Urazaev *et al*. 1999). The metabolic recycling of NAAG is believed to require the uptake of glutamate by presynaptic Schwann cells and its delivery to the nerve terminal through a glutamate (GLU)–glutamine (GLN) shuttle. Future experiments will investigate whether the effect of NAAG on the spontaneous quantal secretion of ACh (SQACh) involves presynaptic metabotropic glutamate receptors (mGLUR).

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