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Research article

Calcitonin gene-related peptide increases acetylcholine quantal size in neuromuscular junctions of mice

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HIGHLIGHTS

- Human and rat isoforms of CGRP equally increase ACh release in motor synapses of mice.
- Both isoforms of CGRP concentration-dependently increase MEPPs amplitude.
- Blockage of CGRP receptors with CGRP8-37 prevents the effect of CGRP.
- CGRP upregulates the MEPPs amplitude via the presynaptic increase of quantal size.

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ABSTRACT

We used an intracellular microelectrode technique to study the mechanisms of action of two isoforms (human and rat) of calcitonin gene-related peptide (CGRP) on the evoked and spontaneous quantal secretion of acetylcholine (ACh) in mouse diaphragm motor synapses. Recordings of miniature endplate potentials (MEPPs) and evoked multiquantal endplate potentials (EPPs) in a cut neuromuscular preparation showed that CGRP increased the amplitude of EPPs without influencing their quantal content. Both isoforms of CGRP in a wide range of concentrations (1 nM–1 μM) provoked a similar considerable increase in MEPPs amplitude in a dose-dependent manner (up to 150–160% compared to control) without changing their frequency, rise-time, and decay. Inhibition of CGRP-receptors by truncated CGRP (CGRP8-37) completely prevented the potentiating effect of CGRP on the MEPPs amplitude. The effect of CGRP was not accompanied by changes in input resistance of muscle fiber membrane but was fully prevented by inhibition of vesicular ACh transport by vesamicol. Inhibition of protein kinase A (PKA) by H-89 also prevented CGRP action on the MEPPs amplitude. It is concluded that, in mammalian neuromuscular junctions, different isoforms of exogenously applied CGRP uniformly potentiate amplitudes of evoked and spontaneous postsynaptic potentials acting presynaptically via an increase in ACh quantal size.

1. Introduction

Calcitonin gene-related peptide is a 37-amino acid peptide that is formed as a result of alternative processing of calcitonin gene [1,2]. In spite of difference in 4 amino acids in the peptide molecules of human and rodent CGRP, both isoforms have been used extensively in studies of CGRP physiological activities. Mouse and rat species possess CGRP with the same amino acid content [3,4]. The peptide is widely distributed both in CNS [5] and peripheral synapses [6]. In motor nerve terminals, endogenous CGRP is localized in large dense-core vesicles [6,7] and may be released into synaptic cleft as a co-transmitter [8–10].

In myotube cultures and developing neuromuscular junctions, CGRP acts as a neurotrophic agent: it stimulates nicotinic acetylcholine receptors (nAChRs) synthesis [11], expression and activity of acetylcholinesterase (AChE) [12,13], and muscular cAMP/PKA activity [14]. In adult skeletal muscle, exogenous CGRP induces calcium mobilization, potentiates ACh-induced desensitization of nAChRs [15] and maintains the activity of synaptic AChE isoformes [16]. The acute presynaptic CGRP effects on evoked ACh release were also found [10,17,18]. Moreover, in frog neuromuscular junction (NMJ), CGRP increases the quantal size [19]. It is unclear if the

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CGRP, calcitonin gene-related peptide; EPP, endplate potential; hCGRP, human isoform of calcitonin gene-related peptide; MEPP, miniature endplate potential; NMJ, neuromuscular junction; PKA, protein kinase A; ratCGRP, rat isoform of calcitonin gene-related peptide; RMP, resting membrane potential.

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peptide may have similar potentiating effects on quantal parameters of ACh secretion in mammalian neuromuscular synapses. In the present work, we compared the action of human and rat isoforms of CGRP on evoked and spontaneous quantal ACh release in mouse motor synapses in order to determine possible targets and mechanisms of the peptides effects.

2. Materials and methods

2.1. Animals

Experiments were performed on isolated neuromuscular preparations of the diaphragm-phrenic nerve from adult mice (strain BALB/c) of both sexes (25–30 g body weight). The animals were obtained from the Laboratory of experimental animals, Department of Biology, Moscow State University (Moscow, Russia) and were kept according with the European Communities Council Directive from November 24, 1986 (86/609/EEC). All experimental procedures were approved by the Bioethics Committee of The Department of Biology at Moscow State University. The mice were euthanized by quick decapitation.

2.2. Electrophysiology

All experiments were performed at 20–22 °C. Left hemidiaphragm with the phrenic nerve was excised, mounted to a 3-ml recording chamber, and perfused with oxygenated (95% O_2, 5% CO_2) Liley solution (pH 7.2–7.4) containing (in mM): NaCl—135, KCl—4, NaH_2PO_4—0.9, $CaCl_2$—2, $MgCl_2$—1, $NaHCO_3$—16.3, and glucose—11. Intracellular recordings of the spontaneous (miniature) and multiquantal evoked endplate potentials (MEPPs and EPPs) were performed using glass microelectrodes filled with 2.5 M KCl (microelectrode tip resistance was 20–25 MΩ). Studies on ACh secretion evoked by nerve stimulation were performed with cut neuromuscular preparations to prevent contraction as well as to record both MEPPs and EPPs from the same synapse [20]. After the dissection of the fibers preparation was washed thoroughly in a large volume of the Liley solution (>150 ml) for at least 1 h. Owing to this procedure, a possible blockage of the action potential conduction is prevented and resting membrane potential (RMP) is stabilized at a level somewhat lower (<−50 mV) than in intact fibers. Cutting of the fibers was not performed in preparations when only spontaneous ACh release was recorded. To study evoked synaptic activity, we stimulated the phrenic nerve with suprathreshold stimuli at a frequency of 0.3 Hz and with duration of 0.08–0.1 ms using two silver electrodes connected with an STG4002 stimulator (Multichannel Systems GmbH, Germany). At least 30 EPPs were recorded in each synapse studied, and MEPPs were recorded for 60 s immediately before the nerve stimulation. Mean value of the MEPP amplitudes recorded within this period was used for the calculation of the quantal content of the subsequently recorded EPPs. When only spontaneous activity was studied, we recorded MEPPs for 180 s. Impalement of muscle fiber near endplate was determined by the appearance of MEPPs with rise time (10–90%) less than 1 ms. Bridge balance and microelectrode capacitance neutralization was performed throughout the entire experiment.

We used two microelectrodes that impaled the same fiber near endplate during determination of muscle fiber input resistance. Hyperpolarizing pulses of 100 ms duration and 30 nA amplitude were injected into the fiber via the current-passing microelectrode and the potential at the steady-state hyperpolarization level was recorded with the voltage microelectrode.

Synaptic responses were acquired using amplifiers Axoclamp 2B or Axoclamp 900A (Molecular Devices, USA) and digitized using an E-154 interface with PowerGraph 3.3 software (L-Card, Russia) or a Digidata 1440A interface with pCLAMP 10 software (Molecular Devices, USA), then stored and analyzed using MiniAnalysis software (Synaptosoft, USA).

As a control, MEPPs only or EPPs with MEPPs from 5 or more different synapses were recorded; after that, drugs were added into the perfusion solution in the desired order and the activity of various synapses was recorded for 0.5–1.5 h. In each experimental series, at least three preparations were used. RMP was continuously monitored throughout the signal recordings in each synapse. If the value of RMP dropped by more than 5 mV, the recording was stopped and the data acquired from this synapse were not included into the sample population for further analysis.

2.3. Data analysis and statistics

We estimated RMP of muscle fibers, MEPP and EPP amplitude and time course, and the MEPP frequency. The MEPP and EPP amplitudes were standardized to the membrane potential of −50 mV (to correct the changes in the driving force of the voltage shifts upon RMP changes from one cut muscle fiber to another) using the formula: $A_{st} = A \cdot (-50/RMP)$, where A is the recorded amplitude of postsynaptic potential (MEPP or EPP) and A_{st} is the standardized amplitude of MEPP or EPP. Quantal content of EPP was calculated as a ratio between the mean standardized EPP amplitude corrected to nonlinear summation [21] and the mean standardized MEPP amplitude. The MEPP amplitudes were standardized to the membrane potential of −70 mV when only spontaneous ACh release was studied (on intact fibers). Input resistance was calculated using Ohm's law.

Data are presented as the mean ± SEM; n reflects number of the synapses studied. Statistical significance between sample means was assessed using the Student's *t*-test (in the case of normal distribution) or one-way analysis of variance ANOVA. The difference was considered significant at $p < 0.05$.

2.4. Chemicals

All drugs were applied to preparations via bath perfusion system (0.5 ml/min). The following pharmacological agents were used: human and rat isoforms of CGRP, inhibitors of CGRP-receptors—human and rat CGRP8-37 (Bachem, USA), inhibitor of vesicular ACh transporter—(±)-2-(4-Phenylpiperidino)cyclohexanol hydrochloride ((±) vesamicol hydrochloride), and inhibitor of protein kinase A N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl (H-89) (Sigma-Aldrich, USA). Stock solutions of all drugs except H-89 were prepared in deionized water, H-89 was dissolved in dimethylsulfoxide (DMSO) (Helicon, Russia). The final DMSO concentration did not exceed 0.01% (v/v), at this concentration DMSO did not affect the parameters of spontaneous and evoked ACh release in mouse motor synapses.

3. Results

In the first series of experiments, we tested the action of human CGRP (hCGRP) (100 nM) on parameters of EPPs and MEPPs at mouse hemidiaphragm. Within application of hCGRP for 1 h, the RMP of muscle fibers was −40.19 ± 0.78 mV (n = 21) and did not change significantly from control level −37.95 ± 0.85 mV (n = 20, $p > 0.05$). In the presence of hCGRP we did not reveal any changes in time parameters and frequency of MEPPs. However, the amplitude of MEPPs increased significantly by 32% from 1.32 ± 0.06 mV in the control to 1.74 ± 0.09 mV ($p < 0.05$). The amplitude of single evoked EPPs also increased from 27.71 ± 1.67 mV under control conditions to 38.67 ± 2.06 mV in the presence of CGRP ($p < 0.05$). This effect was not accompanied by a relative increase in quantal content of