Neuroscience Letters 628 (2016) 17–23

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

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

Alexander E. Gaydukov[a,b,*], Polina O. Bogacheva[a], Olga P. Balezina[a]

[a] Department of Human and Animal Physiology, Biological Faculty, Lomonosov Moscow State University, Leninskie Gory 1/12, Moscow 119234, Russia
[b] Department of Physiology, Russian National Research Medical University, Ostrovitjanova Str. 1, Moscow, Russia

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.

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form 1 June 2016
Accepted 6 June 2016
Available online 7 June 2016

Keywords:
Calcitonin gene-related peptide
Quantal size
Neuromuscular junction
Vesamicol
Protein kinase A

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.
© 2016 Elsevier Ireland Ltd. All rights reserved.

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.

* Corresponding author.
E-mail address: gaydukov@mail.bio.msu.ru (A.E. Gaydukov).

http://dx.doi.org/10.1016/j.neulet.2016.06.014
0304-3940/© 2016 Elsevier Ireland Ltd. All rights reserved.

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

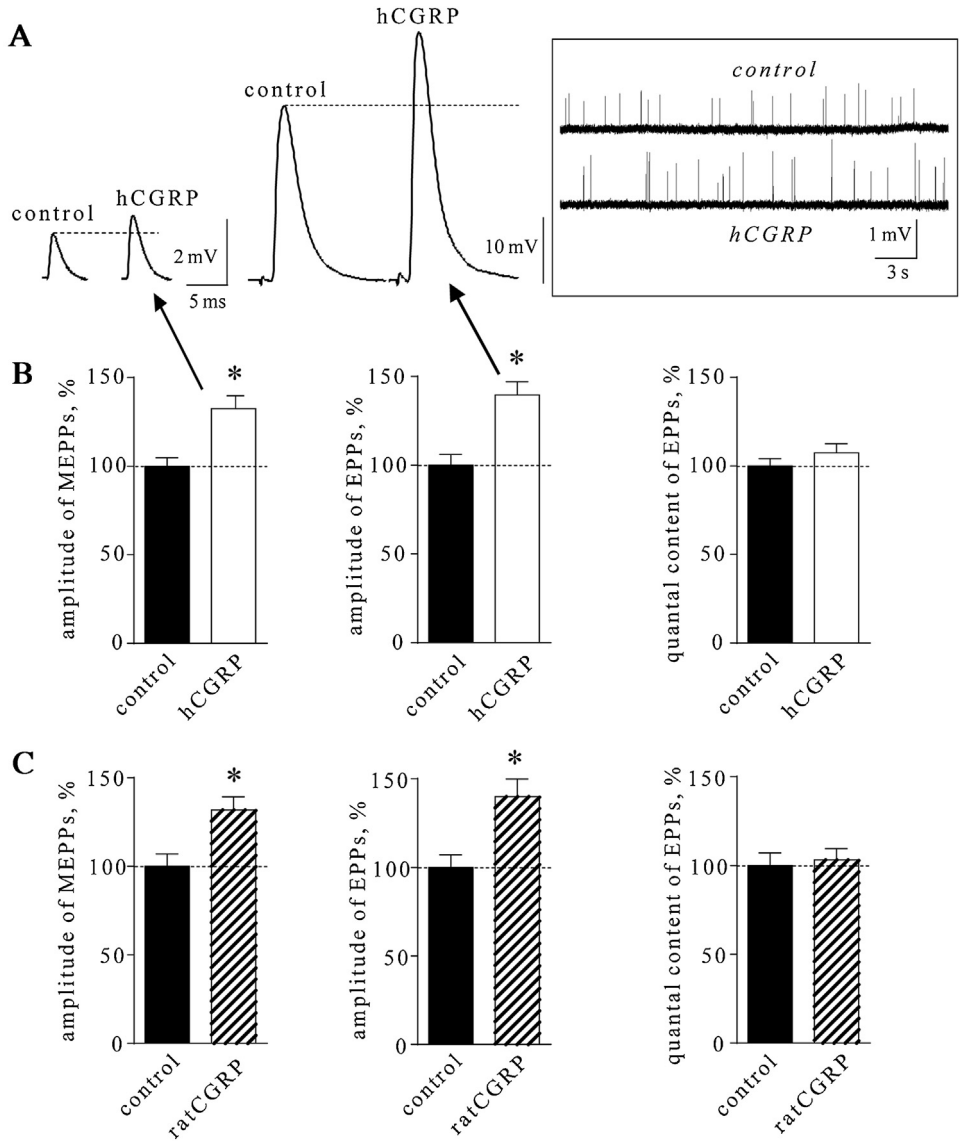

Fig. 1. Both human and rat isoforms of CGRP increase the amplitudes of MEPPs and single evoked EPPs in the mouse NMJ. (A) Representative intracellular recordings of MEPPs (left) and EPPs (right) in control and upon application of 100 nM hCGRP. Insert shows typical timeline of MEPP recordings in control and in the presence of hCGRP. (B) Mean MEPPs amplitude, EPPs amplitude and quantal content of EPPs (left to right) in control (n = 21) and within 1 h of hCGRP application (n = 20). (C) Mean MEPPs amplitude, EPPs amplitude and quantal content in control (n = 15) and within 1 h of ratCGRP application (n = 19). Symbols and error bars represent mean ± SEM. * – $p < 0.05$ compared to control.

EPPs (control—21.06 ± 0.86; hCGRP—22.61 ± 1.09, $p > 0.05$) (Fig. 1A, B).

Next, we studied the action of rat isoform of CGRP (ratCGRP) (100 nM) using recordings of spontaneous and evoked endplate potentials. Application of ratCGRP caused the same effect as the hCGRP. Both MEPP and EPP amplitudes were increased to 131.83 ± 7.38% and 140.04 ± 9.96% (n = 15, $p < 0.05$) of control (n = 19), respectively. No changes were observed in the quantal content of EPPs in the presence of ratCGRP (103.16 ± 6.31% of control, $p > 0.05$) (Fig. 1C).

Thus, we found no differences between effects of hCGRP and ratCGRP: both isoforms enhanced evoked ACh release (augmented the EPP amplitude). This augmentation was due to the increase in the amplitude of individual ACh quantum (MEPP) within multi-quantal EPP rather than of the upregulation of EPP quantal content.

For more detailed analysis of the potentiating effect of CGRP on MEPPs amplitude we used the intact neuromuscular preparations to test the dose-dependence of hCGRP action on the MEPP parameters. We applied 1 nM, 100 nM, and 1 μM of the peptide, considering that in this range of concentrations exogenously applied CGRP potentiated spontaneous neurotransmitter release in mammalian CNS synapses [22] and frog NMJs [19].

Application of hCGRP at all concentrations tested did not affect the RMP of muscle fibers: −62.29 ± 0.74 mV (n = 24) in control and −63.8 ± 0.84 mV (n = 21, $p > 0.05$) in the presence of 1 μM hCGRP. Also, no changes in MEPP time parameters were observed. At the same time, a concentration-dependent increase of MEPP amplitude was revealed. In the presence of 1 nM of the peptide, the amplitude of MEPPs increased by 32%, from 1.21 ± 0.07 mV in control (n = 24) to 1.56 ± 0.08 mV (n = 25, $p < 0.05$). An increase in the peptide concentration to 100 nM induced more pronounced increment of MEPP amplitude—up to 151% of the control (n = 25, $p < 0.05$). This effect seemed to reach maximum because no further increase in MEPPs amplitude was observed in the presence of 1 μM hCGRP (Fig. 2A). Histograms of MEPP amplitude distributions demonstrated a uniform shift towards the larger amplitudes with the increase in concentration of hCGRP from 1 nM to 1 μM (Fig. 2B). Thus, hCGRP evoked a significant increase in MEPP amplitude across the entire

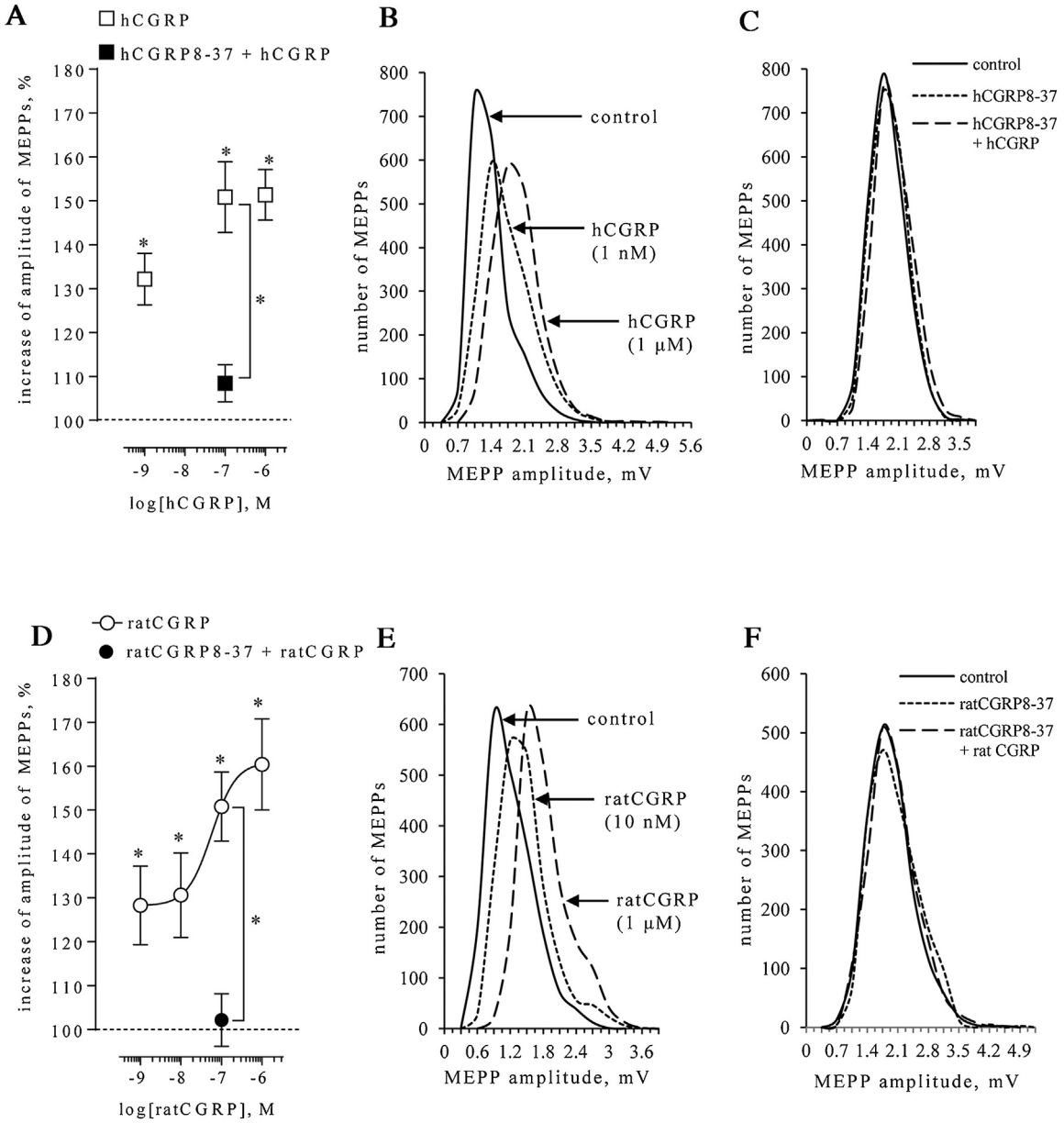

Fig. 2. Concentration-dependent effects of human and rat isoforms of CGRP on the MEPPs amplitude. (A) Increase in mean amplitude of MEPPs during application of hCGRP. Mean MEPPs amplitudes are expressed as percentage of control (set as 100%). (B) MEPP amplitude distributions in control and in the presence of hCGRP (1 nM and 1 μM). (C) Pre-application of hCGRP8-37 (1 μM) prevented the effect of hCGRP (100 nM). (D) MEPPs mean amplitude increase during application of ratCGRP (1 nM–1 μM). (E) MEPP amplitude distributions in control and in the presence of ratCGRP (10 nM and 1 μM). (F) Pre-application of ratCGRP8-37 (1 μM) prevented the effect of ratCGRP (100 nM). Symbols and error bars represent mean ± SEM. * – $p < 0.05$ compared to control.

MEPP population in a concentration-dependent manner without changing other parameters of MEPP.

To compare the effects of human and rat isoforms of CGRP on the amplitude of MEPPs in mouse motor synapses, in the next series of experiments we used ratCGRP at concentrations from 1 nM to 1 μM. We did not find any significant changes in the mean values of RMP, frequency and time parameters of MEPPs during the application of ratCGRP at any of the concentration used. However, amplitude of MEPPs increased by 30% in the presence of 1 or 10 nM of ratCGRP. Application of the peptide at higher concentrations (0.1 and 1 μM) caused a greater increase in MEPP amplitude—up to 150–160% of control (Fig. 2D). As in the case of hCGRP, ratCGRP uniformly shifted MEPP amplitude distribution histograms to the area of the larger MEPP amplitudes in a concentration-dependent manner (Fig. 2E). In summary, we found that in mouse NMJs human and rat isoforms of CGRP had similar effect, a dose-dependent potentiation of MEPPs amplitude.

It was necessary to test the receptor specificity of CGRP effect, because CGRP might activate amylin or adrenomedullin receptors [23]. Truncated molecules of respective peptide isoforms (CGRP8-37) were used due to the ability of these classical concurrent antagonists bind to CGRP-receptors with greater potency compared to receptors for amylin or adrenomedullin [23]. Both human and rat CGRP8-37 had no significant effect on parameters of spontaneous ACh release but completely prevented the potentiating effect of human or rat CGRP on the amplitude of MEPPs, respectively ($p < 0.05$) (Fig. 2A,C,D,F). Thus, the obtained results suggested that the effect of human or rat CGRP isoforms on MEPP amplitude was mediated by peptide action on their specific receptor. Taking into account the similarities in qualitative and quantitative strength of

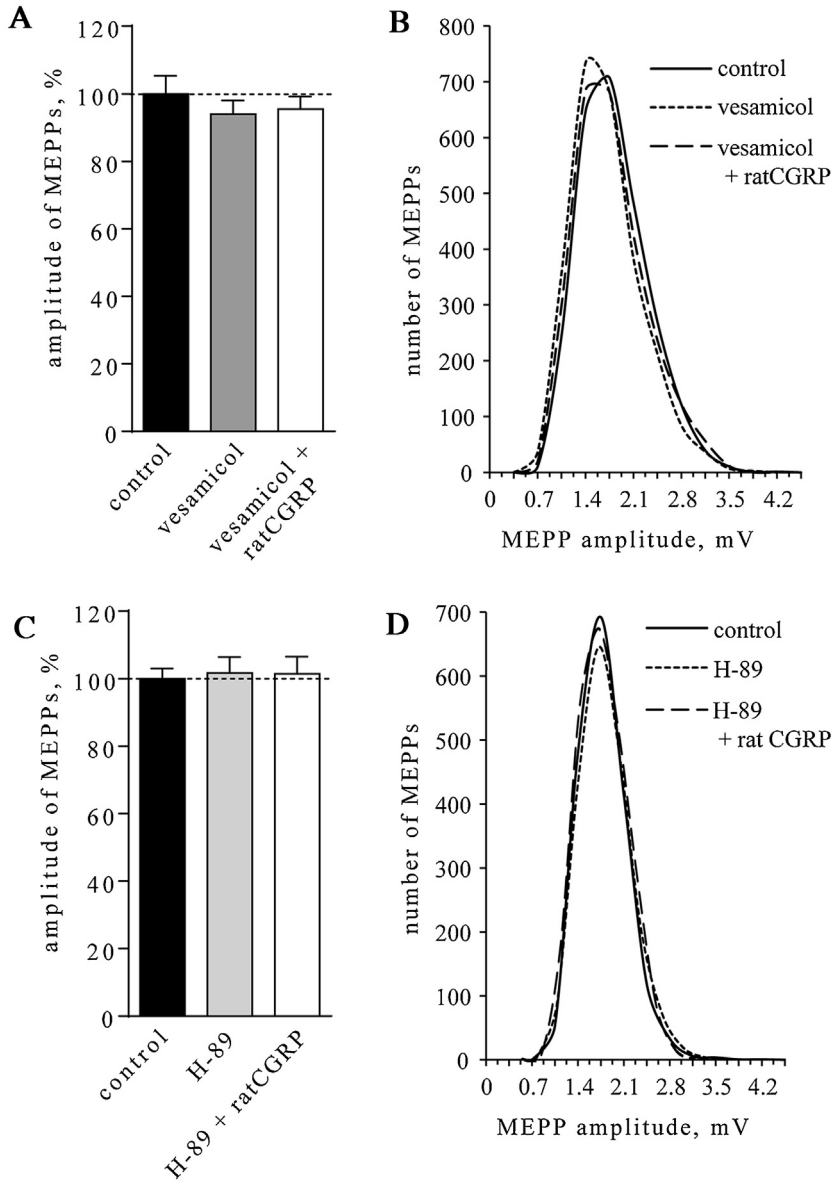

Fig. 3. Intracellular mechanisms of CGRP action at the mouse NMJs. (A) Vesicular ACh transporter blocker vesamicol (1 μM) abolished MEPP amplitude increase induced by ratCGRP (1 μM). (B) MEPP amplitude distribution in control; during application of vesamicol and ratCGRP in the presence of vesamicol. (C) Inhibition of PKA by H-89 (1 μM) prevents the increase of MEPP amplitude caused by ratCGRP (1 μM). (D) MEPP amplitude distributions in control; during application of H-89 and ratCGRP in the presence of H-89. Symbols and error bars represent mean ± SEM.

the effect between hCGRP and ratCGRP further analysis was performed using just one peptide isoform.

We next examined the input resistance of diaphragm muscle fibers using two-electrode recordings. Under control conditions, the mean input resistance of fibers was 0.67 ± 0.06 MΩ (n = 15) and remained at the same level—0.66 ± 0.06 MΩ (n = 15, $p > 0.05$) for 45 min of ratCGRP (1 μM) application. The obtained results together with unchanged RMP and MEPP time parameters argued against any postsynaptic actions of CGRP in the mouse NMJs.

CGRP-induced augmentation of postsynaptic potentials amplitude might be the consequence of the presynaptic increment of quantal size due to increased ACh loading into the synaptic vesicles. To test this hypothesis, we used vesamicol, which directly inhibited vesicular ACh transporter [24,25]. Vesamicol (1 μM) alone had no significant effect on MEPP amplitude: 1.72 ± 0.09 mV (n = 24) in control versus 1.62 ± 0.07 mV (n = 31, $p > 0.05$) under vesamicol. Further application of ratCGRP (1 μM) under these conditions did not change the MEPP amplitude significantly: 1.65 ± 0.06 mV (n = 26, $p > 0.05$) (Fig. 3A,B). Hence, preliminary blocking of the vesicular ACh transport completely prevented the increase in MEPP amplitude induced by ratCGRP. The obtained data provided strong argument in favor of our suggestion that the effect of CGRP on transmitter release in mouse NMJs was presynaptic and resulted from an increase in quantal size due to upregulation of ACh transport into vesicles.

It has been shown in different types of cells that intracellular mechanisms of CGRP signaling may involve activation of cAMP-pathway leading to an increase in PKA activity [4,26]. Therefore, we studied the effect of ratCGRP in the presence of PKA inhibitor, H-89. The preliminary application of H-89 (1 μM) for 50 min by itself provided no significant changes in MEPP characteristics: the mean MEPP amplitude was 1.77 ± 0.05 mV (n = 19) in control and 1.80 ± 0.08 mV (n = 18, $p > 0.05$) in the presence of H-89. However, under these conditions ratCGRP (1 μM) lost its ability to increase

the amplitude of MEPPs: 1.80 ± 0.09 mV ($n = 19$, $p > 0.05$) (Fig. 3C,D). Overall, the obtained data indicated that CGRP acted on its putative presynaptic receptors and triggered an intracellular signaling cascade involving PKA and leading to the increased loading of ACh in synaptic vesicles and upregulation of ACh quantal size in mouse motor nerve endings.

4. Discussion

In the present study we for the first time analyzed CGRP-induced changes in MEPPs and EPPs parameters in mouse motor synapses. We used human and rat isoforms of CGRP, which differed in 4 amino acids in the peptide molecule [4]. We found that both isoforms of CGRP acted presumably via CGRP-receptors and produced a similar concentration-dependent increase in MEPP amplitudes. Such similarity in the manifestation of physiological effects for CGRP isoforms derived from different vertebrate species was described in literature [27]. It is consistent with the view that differences in the amino acid composition in the different isoforms do not lead to fundamental changes in the three-dimensional structure of CGRP that would prevent peptide binding to the receptor complex and its activation [3]. Moreover, the primary structure of the ligand-binding part of CGRP-receptors in different species has a very high degree of homology—91–96%, and species-specific activation only differs slightly among the full-size peptides [27]. It seems that this is the reason for similarity between the concentration-dependent actions of rat and human CGRP in mouse NMJs.

One of the major findings of our study is that both isoforms of CGRP uniformly potentiate the amplitudes of MEPPs and EPPs in mouse NMJs due to the increase of quantal size without changing the quantal content of EPPs, this effect requires PKA activation. CGRP was also found to increase the ACh release by prolonged high-frequency stimulation of synapses, however, co-activation of presynaptic adenosine A_{2A}-receptors is needed [18]. It is possible that during intensive synaptic activity interaction of CGRP- and A_{2A}-receptors becomes necessary since each of them is positively coupled to the cAMP/PKA pathway. PKA can potentiate the ACh release in motor synapses in response to activation of different presynaptic receptors and mechanisms [28,29]. Along with our results, PKA-dependent modulation of quantal size was also described in several other studies at NMJs [19,30,31].

It has been shown that CGRP decreased the AChE expression in motor synapses [16] which may lead to an increase in MEPP and EPP amplitudes during the peptide application. However, we may rule out such cause of increased MEPP and EPP amplitudes in our experiments because in our study CGRP-induced changes in postsynaptic potential amplitudes were not paralleled by the changes in their time course. Furthermore, suppression of the expression and the clearance of the AChE molecules from the synaptic cleft requires more time (hours) in comparison with short-term CGRP potentiating action (within 30–60 min) on the MEPP amplitude which we observed in isolated nerve-muscle preparation.

Further analysis revealed that enlargement of MEPP amplitude was due to presynaptic CGRP action directed to increased loading of ACh into synaptic vesicles in mouse NMJs. This possibility is supported by the effects of vesamicol which has been traditionally used to prove the involvement of the vesicular ACh transport into increase of the quantal size followed by MEPP amplitude augmentation [19,25]. In our experiments, vesamicol by itself did not induce any changes in MEPP amplitude—and it was consistent with the literature data [25,31,32]. This phenomenon may be explained by the fact that single quanta of ACh released spontaneously at rest reflects the exocytosis of synaptic vesicles already prefilled with ACh and do not undergo accelerated ACh pumping which takes place during vesicle recycling after evoked synaptic activity or under special treatment [25,32]. Thus, vesamicol may prevent only some specifically accelerated, additionally enhanced loading of ACh into synaptic vesicles [19,31]. This is consistent with our observation of vesamicol completely preventing the CGRP-induced increase in MEPP amplitude.

Presynaptic CGRP effects revealed in our studies, could be mediated by direct peptide action at presynaptic CGRP-receptors, although their presence on nerve terminals has not been described yet. At the same time activation of muscular CGRP-receptors is also possible, as they are found on a muscle membrane at the region of the endplate [33]. Therefore we cannot rule out the possibility that increase of ACh quantal size in nerve terminal might result from a retrograde signaling triggered by CGRP at postsynaptic site with subsequent action on nerve terminal. Both hypotheses need additional experimental testing.

Along with generally accepted ideas about the long-term trophic and postsynaptic effects of CGRP in mammalian skeletal muscle, we revealed the acute presynaptic activity of the peptide in mouse motor synapses, which leads to an increase in the amplitude of the postsynaptic potentials via presynaptic enlargement of ACh quantal size. Physiological consequences of CGRP upregulation of quantal size may play important role in maintaining the ACh release during prolonged rhythmic synaptic activity when quantal content is reduced significantly, and secretion of endogenous CGRP may occur [8,10]. Finally, the ability of CGRP to potentiate presynaptically the size of uniquantal miniature signals represents a rather novel effective way of enhancing synaptic transmission via modulation of mediator quantal size. This modulation may occur alongside with traditionally accepted mechanisms that lead to changes the number of quanta released.

5. Conclusions

In summary, it was revealed for the first time that in mouse NMJ two exogenous isoforms of CGRP facilitated the neurotransmitter release by increasing presynaptically the quantal size of ACh. This phenomenon leads to the enlargement of both MEPP and EPP amplitudes. Thus, exogenous CGRP has replenished the limited selection of known effective regulators of neurotransmitter quantal size which may potentiate cholinergic transmission in motor synapses of mammals [25,31,34]. Taking into account the presence and release of CGRP in motor nerve terminals, newly obtained data on exogenous CGRP suggest in favor of similar specific presynaptic autoregulatory role for endogenous CGRP in cholinergic synaptic transmission. Therefore, the next step of CGRP study should be directed at identification of the particular conditions that facilitate the endogenous CGRP exocytosis and enable its potentiating effects during synaptic activity in NMJs.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Author contributions

AEG and POB performed the experiments and analyzed the data. AEG and OPB conceived the experiments and wrote the manuscript. All authors reviewed the manuscript.

Acknowledgements

This work was supported by Russian Foundation for Basic Research grant 16-04-00554a.

References

[1] S.G. Amara, V. Jonas, M.G. Rosenfeld, E.S. Ong, R.M. Evans, Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products, Nature 298 (1982) 240–244.
[2] M.G. Rosenfeld, J.J. Mermod, S.G. Amara, L.W. Swanson, P.E. Sawchenko, J. Rivier, W.W. Vale, R.M. Evans, Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing, Nature 304 (1983) 129–135.
[3] H.A. Watkins, D.L. Rathbone, J. Barwell, D.L. Hay, D.R. Poyner, Structure-activity relationships for α-calcitonin gene-related peptide, Br. J. Pharmacol. 170 (2013) 1308–1322.
[4] F.A. Russell, R. King, S.-J. Smillie, X. Kodji, S.D. Brain, Calcitonin gene-related peptide: physiology and pathophysiology, Physiol. Rev. 94 (2014) 1099–1142.
[5] D. van Rossum, U.K. Hanisch, R. Quirion, Neuroanatomical localization pharmacological characterization and functions of CGRP, related peptides and their receptors, Neurosci. Biobehav. Rev. 21 (1997) 649–678.
[6] B. Csillik, L. Tajti, T. Kovács, E. Kukla, P. Rakic, E. Knyihár-Csillik, Distribution of calcitonin gene-related peptide in vertebrate neuromuscular junctions: relationship to the acetylcholine receptor, J. Histochem. Cytochem. 41 (1993) 1547–1555.
[7] M. Matteoli, C. Haimann, F. Torri-Tarelli, J.M. Polak, B. Ceccarelli, P. De Camilli, Differential effect of alpha-latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 7366–7370.
[8] S. Uchida, H. Yamamoto, S. Iio, N. Matsumoto, X.B. Wang, N. Yonehara, Y. Imai, R. Inoki, H. Yoshida, Release of calcitonin gene-related peptide-like immunoreactive substance from neuromuscular junction by nerve excitation and its action on striated muscle, J. Neurochem. 54 (1990) 1000–1003.
[9] M. Sakaguchi, Y. Inaishi, Y. Kashihara, M. Kuno, Release of calcitonin gene-related peptide from nerve terminals in rat skeletal muscle, J. Physiol. 434 (1991) 257–270.
[10] I. Kimura, M. Okazaki, H. Nojima, Mutual dependence of calcitonin-gene related peptide and acetylcholine release in neuromuscular preparations, Eur. J. Pharmacol. 330 (1997) 123–128.
[11] J.-P. Changeux, A. Duclert, S. Sekine, Calcitonin gene-Related peptides and neuromuscular interactions, Ann. N. Y. Acad. Sci. 657 (1992) 361–378.
[12] V.L. da Costa, A.J. Lapa, R.O. Godinho, Short- and long-term influences of calcitonin gene-related peptide on the synthesis of acetylcholinesterase in mammalian myotubes, Br. J. Pharmacol. 133 (2001) 229–236.
[13] S.G. Rossi, I.M. Dickerson, R.L. Rotundo, Localization of the calcitonin gene-related peptide receptor complex at the vertebrate neuromuscular junction and its role in regulating acetylcholinesterase expression, J. Biol. Chem. 278 (2003) 24994–25000.
[14] R. Laufer, J.P. Changeux, Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: possible neurotrophic role for a coexisting neuronal messenger, EMBO J. 6 (1987) 901–906.
[15] I. Kimura, Calcium-dependent desensitizing function of the postsynaptic neuronal-type nicotinic acetylcholine receptors at the neuromuscular junction, Pharmacol. Ther. 77 (1998) 183–202.
[16] H.L. Fernandez, G.S. Ross, I. Nadelhaft, Neurogenic calcitonin gene-related peptide: a neurotrophic factor in the maintenance of acetylcholinesterase molecular forms in adult skeletal muscles, Brain Res. 844 (1999) 83–97.
[17] C.G. Caratsch, F. Eusebi, Effect of calcitonin gene-related peptide on synaptic transmission at the neuromuscular junction of the frog, Neurosci. Lett. 111 (1990) 344–350.
[18] P. Correia-de-Sá, J.A. Ribeiro, Potentiation by tonic A2a-adenosine receptor activation of CGRP-facilitated [3H]-ACh release from rat motor nerve endings, Br. J. Pharmacol. 111 (1994) 582–588.
[19] W. Van der Kloot, W.B. Benjamin, O.P. Balezina, Calcitonin gene-related peptide acts presynaptically to increase quantal size and output at frog neuromuscular junctions, J. Physiol. 507 (1998) 689–695.
[20] J.A. Barstad, G. Lilleheil, Transversaly cut diaphragm preparation from rat. An adjuvant tool in the study of the physiology and pbarmacology of the myoneural junction, Arch. Int. Pharmacodyn. Theír. 175 (1968) 373–390.
[21] E.M. McLachlan, A.R. Martin, Non-linear summation of end-plate potentials in the frog and mouse, J. Physiol. 311 (1981) 307–324.
[22] J.S. Han, H. Adwanikar, Z. Li, G. Ji, V. Neugebauer, Facilitation of synaptic transmission and pain responses by CGRP in the amygdala of normal rats, Mol. Pain 6 (2010) 10.
[23] D.L. Hay, D.R. Poyner, R. Quirion, International Union of Pharmacology. LXIX. Status of the calcitonin gene-related peptide subtype 2 receptor, Pharmacol. Rev. 60 (2008) 143–145.
[24] C. Prior, I.G. Marshall, S.M. Parsons, The pharmacology of vesamicol: an inhibitor of the vesicular acetylcholine transporter, Gen. Pharmacol. 23 (1992) 1017–1022.
[25] S.W. Fong, I.S. McLennan, A. McIntyre, J. Reid, K.I.J. Shennan, G.S. Bewick, TGF-beta2 alters the characteristics of the neuromuscular junction by regulating presynaptic quantal size, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 13515–13519.
[26] C.S. Walker, A.C. Conner, D.R. Poyner, D.L. Hay, Regulation of signal transduction by calcitonin gene-related peptide receptors, Trends Pharmacol. Sci. 31 (2010) 476–483.
[27] D.R. Poyner, Calcitonin gene-related peptide: multiple actions multiple receptors, Pharmacol. Ther. 56 (1992) 23–51.
[28] M.M. Santafé, N. Garcia, M.A. Lanuza, M. Tomàs, J. Tomàs, Interaction between protein kinase C and protein kinase A can modulate transmitter release at the rat neuromuscular synapse, J. Neurosci. Res. 87 (2009) 683–690.
[29] L. Oliveira, A.C. Costa, J.B. Noronha-Matos, I. Silva, W.L.G. Cavalcante, M.A. Timóteo, A.P. Corrado, C.A. Dal Belo, C.R. Ambiel, W. Alves-do-Prado, P. Correia-de-Sa, Amplification of neuromuscular transmission by methylprednisolone involves activation of presynaptic facilitatory adenosine A_{2A} receptors and redistribution of synaptic vesicles, Neuropharmacology 89 (2015) 64–76.
[30] W. Van der Kloot, D.D. Brănişteanu, Effects of activators and inhibitors of protein kinase A on increases in quantal size at the frog neuromuscular junction, Pflügers Arch. Eur. J. Physiol. 420 (1992) 336–341.
[31] A.E. Gaydukov, O.P. Balezina, Potentiating effect of allatostatin on transmitter quantal secretion in the mouse nerve-muscle synapse, J. Evol. Biochem. Physiol. 42 (2006) 699–705.
[32] T. Searl, C. Prior, I.G. Marshall, Acetylcholine recycling and release at rat motor nerve terminals studied using (−)-vesamicol and troxpyrrolium, J. Physiol. 444 (1991) 99–116.
[33] H.L. Fernandez, M. Chen, I. Nadelhaft, J.A. Durr, Calcitonin gene-related peptides: their binding sites and receptor accessory proteins in adult mammalian skeletal muscles, Neuroscience 119 (2003) 335–345.
[34] W. Van der Kloot, Loading and recycling of synaptic vesicles in the Torpedo electric organ and the vertebrate neuromuscular junction, Prog. Neurobiol. 71 (2003) 269–303.