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Modified autonomic regulation in mice with a P/Q-type calcium channel mutation

Takayoshi Ohba [a], Eiki Takahashi [b], Manabu Murakami [a,*]

[a] Department of Physiology, Akita University School of Medicine, 1-1-1 Hondoh, Akita 010-8543, Japan
[b] Riken Brain Science Institute, 2-1 Hirosawa, Wako, Saitama, Japan

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ABSTRACT

Recent genetic analyses revealed an important association between P/Q-type channels and hereditary neurological disorders. The α1 subunit of P/Q-type channels is coded by a single CaV2.1 gene. Since calcium entry via neuronal calcium channels is essential for neurotransmission, P/Q-type channels may play an important role in cardiac autonomic neurotransmission. To elucidate the physiological importance of P/Q-type channels in autonomic nerve control, we used *rolling Nagoya* (tg^{rol}) mice, which have a mutation in the CaV2.1 gene and decreased P/Q-type channel currents with reduced voltage sensitivity.

The tg^{rol} mice demonstrated unmodified expression of other calcium channel subunits. Electrocardiogram and echocardiographic analyses revealed decreased heart rate. Furthermore, ω-agatoxin IVA, a P/Q-type channel inhibitor, decreased heart rate and ejection fraction only in wild-type mice, thus suggesting a significant involvement of P/Q-type channels in chronotropic regulation. Atrium contraction analyses revealed a minor but significant role for P/Q-type channels in sympathetic and parasympathetic nerve regulation.

Introduction

The autonomic nervous system, which includes sympathetic and parasympathetic pathways, is an essential regulator of the circulatory system. Calcium-influx across plasma membranes augments cytosolic free calcium concentrations, which facilitate neurotransmitter release at synaptic termini [1]. The existence of five different high-threshold types (L, N, P, Q, and R) of channels has been identified, in addition to a low-threshold T-type voltage-dependent calcium channels in neurons [2,3]. Several types of these calcium channels are colocalized in a single neuron and contribute to the regulation of neuronal activity. In particular, the P- and N-types are involved in transmitter release at the synaptic terminal [4–6]; however, the role of calcium channels in autonomic nerve signal transduction has not been clearly identified.

Voltage-gated calcium channels are composed of a main pore-forming α1 subunit, which is encoded by a family of 10 different genes (CaV1.1-4, CaV2.1-3, and CaV3.1-3), and the accessory α2/δ, β, and γ subunits [7]. The CaV2.1 (α1A) subunit was originally characterized via pharmacological methods using the N-type-selective inhibitor ω-conotoxin GVIA and the L-type inhibitor dihydropyridines [8]. The CaV2.1 gene codes for two pharmacologically different high-voltage-activated calcium channels, (i.e., P- and Q-type channels).

In the human Ca2.1 gene, missense and nonsense mutations and CAG expansion have been shown to underlie neurological disorders such as familial hemiplegic migraine, episodic ataxia type-2 [9], and autosomal dominant spinocerebellar ataxia (SCA6) [10]. For example, α1A-null mutant mice show rapidly progressive ataxic syndrome a few weeks after birth, thus indicating the importance of α1A in the central nervous system [11].

To identify treatment options for these human channelopathies, spontaneous α1A subunit gene-mutant mice can be used as models. A missense mutation was found in *tottering* (*tg*) mice, which display a delayed-onset, recessive disorder consisting of ataxia and absence seizure-like petit mal epilepsy [12]. In mice, the allelic tottering mutation *leaner* (tg^{la}) causes truncation of the open reading frame.

The mouse mutation *rolling Nagoya* (tg^{rol}) shows an allelic mutation of CaV2.1 in a voltage-sensing S4 transmembrane region of the third transmembrane domain [13,14]. Homozygous tg^{rol} mutant mice show poor motor coordination and stiffness of the hindlimbs; however, seizures are not observed [13,15]. The mouse mutation *rolling Nagoya* is characterized by reduced voltage sensitivity and diminished activity of P/Q-type calcium channels. Action potential generation in cerebellar Purkinje cells is impaired, and currents in P/Q-type channel currents are decreased (~40% reduction). These characteristics underlie the ataxic phenotype of tg^{rol} mice.

* Corresponding author. Fax: +81 18 834 8930.
E-mail address: mmura0123@hotmail.co.jp (M. Murakami).

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In the present study, we used biochemical and physiological analyses in the *rolling Nagoya* (tg^{rol}) mouse to elucidate the physiological role of P/Q-VCCs in regulation of the autonomic nerve system.

Materials and methods

Animals. The mouse mutation *rolling Nagoya* (tg^{rol}) shows an allelic mutation in CaV2.1 [13,14]. The mutant gene was introduced into a C3H background by cross–intercross matings. As a consequence, a C3H-tg^{rol} congenic was established [15]. All experiments were carried out in accordance with the Guidelines for the Use of Laboratory Animals of the Akita University School of Medicine.

RNA isolation and RT-PCR analyses. Total RNA was isolated from the mouse heart, and comparative RT-PCR reactions were performed under the same conditions for 30 PCR cycles, as previously described [16]. The CaV2.1 (α1A), CaV2.2 (α1B), CaV2.3 (α1E), CaV1.2 (α1C), and CaV1.3 (α1D) subunit-specific sequences were amplified by PCR (Supplementary Information). The mutational sequence of CaV2.1 was amplified with NagoyaA1 and AntiA1, which correspond to the L_{1255}RPLKTIKG$_{1262}$ and V_{1275}NSLKNVF$_{1282}$ sequences, respectively, of the murine CaV2.1 (α1A) subunit.

As a control, murine β-actin cDNA was amplified for 25 PCR cycles using the primers BA1 (5′-CAACTGGGACGAATGGAGAA-3′) and BA2 (5′-CAGCCTGGATGGCTACG TACA-3′), which amplify a 185-bp fragment.

Western-blot analyses. Partially purified superior cervical ganglion (SCG) and adrenal gland membranes from wild-type (W) and tg^{rol} (P) mice were prepared and suspended in 50 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors [17]. Aliquots of homogenate (100 μg) from each mouse were resolved by 6% SDS–polyacrylamide gel electrophoresis. Commercially available polyclonal antibodies specific for CaV2.1 (α1A, Alomone, Jerusalem, Israel), β4 (Abcam plc, Cambridge, UK) were used for immunodetection.

As a control, an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology, CA, USA) was used to confirm comparable sample loading.

Tissue preparation. Tissues of WT and tg^{rol} mice were excised under ether anesthesia. The tissues were fixed immediately in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4 °C and then embedded in paraffin wax.

Immunohistology. After deparaffinization, slides were treated with 0.1% Triton X-100. To detect CaV2.1 subunit, the permeabilized slides were labeled with rabbit polyclonal anti-CaV2.1 antibody (Alomone) at 4 °C. Slides were then labeled with Alexa Fluor 594-labeled goat anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). The slides were mounted using VECTA Shield with DAPI (Vecter Laboratories, Burlingame, CA, USA), which stained the nuclei, and were analyzed under an inverted Zeiss LSM510META (Carl Zeiss, Oberkochen, Germany) confocal laser-scanning microscope [18].

ECG telemetry. ECG telemetry was performed as previously described [17]. During the recording period (60 min), animals were free in their home cage. ω-Agatoxin IV (3 μg/kg) was administered intraperitoneally to block P/Q-type channels. Analog ECG signals were transferred to a receiver device and directly digitized by an analog-to-digital converter system (Mini-Digi-1A, Axon Instruments, Foster City, CA, USA).

Echocardiography. Mice were anesthetized with 2% inhaled sevoflurane. All mice were permitted to breathe spontaneously during the echocardiographic studies [16]. A commercially available echocardiography machine equipped with a 7.5-MHz transducer (SSD6500; ALOKA, Tokyo, Japan) was used to record the B-mode echocardiogram of the left ventricle. M-mode echocardiographic recordings were analyzed to determine the left-ventricular inner diameter at end-diastole and -systole and the left-ventricular ejection fraction using software supplied with the system.

Contraction of the atrium. Left and right atria were isolated (Hartleystrain, 0.35–1.1 kg). Preparations were bathed in Tyrode solution (composition in mM: 136.9 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 10.0 glucose, and 5.0 HEPES) maintained at 37 °C and pH 7.4. Contractile forces were recorded isometrically by means of a strain gauge force transducer (CD200; NihonKoden). Excitation of autonomic nerves was measured using a field stimulation assembly consisting of one pair of punctate electrodes for pacing, which were placed in the cardiac muscle and juxtapositioned to the center in the right atria. The other pair of bar electrodes for field stimulation of the autonomic nerves was positioned to stimulate the entire cardiac ventriculum.

Atropine (5.5 μM) or metoprorol (1 μM) was added to evaluate sympathetic or parasympathetic nerve activities, respectively. Sympathetic positive inotropic responses (increases in contractile force) were evaluated as an increase from baseline force in response to electrical field stimulation (EFS) after atropine pretreatment. Parasympathetic negative inotropic responses (inhibitory effects on contractile force) were evaluated as decreases in baseline force in response to EFS after metoprorol treatment. The heart was paced electrically at 400 beats/min (bpm). The EFS was applied in conjunction with the pacing stimulation (delay, 4 ms; duration, 1 ms for 5 s). The baseline contraction was evaluated as 1.0. The contractile responses were calculated as the corresponding changes of the baseline contraction.

Statistical analysis. The data are presented as means ± SEM. Differences were evaluated using unpaired Student's *t*-tests and were considered statistically significant at $p < 0.05$.

Results and discussion

Detection of the mutant allele in the rolling Nagoya (tg^{rol})

Three weeks after birth, homozygous mice began to show the typical ataxic phenotype (Supplementary information, Video 1). Mice were genotyped using PCR and the mutation was confirmed (Fig. 1A, right panel).

To investigate the influence of CaV2.1 mutant expression on Ca^{2+} channel assemblies, we analyzed the expression of the α1 and β subunits in the SCG and the adrenal glands of tg^{rol} and wild-type (wt) mice. For these experiments, RT-PCR and immunoblotting (Fig. 1) were used. The wild (Wild) and Mutant (Mut) alleles of CaV2.1 were detected with specific primers able to distinguish between the two genotypes (Fig. 1A).

Specific expression of the wild-type and mutant CaV2.1 allele was confirmed in the SCG and/or adrenal gland of wt and tg^{rol} mice. No apparent differences in the expression of the other calcium channel-forming subunits were detected between wt and tg^{rol} mice (Fig. 1B). No significant changes were detected in the expression of N-type channel-forming CaV2.2 and L-type channel-forming CaV1.2 subunits, thus suggesting that there were no compensatory increases. Another L-type channel-forming CaV1.3 subunit was also detected in the adrenal gland; however, no apparent differences between wild and tg^{rol} mice were detected.

Additionally, expression levels of the β1, β2, β3, and β4 subunits in the SCG and adrenal glands were not significantly different, as shown in Fig. 1B (β1, β2, β3, and β4, respectively). These results suggest that a compensatory increase in these accessory subunits

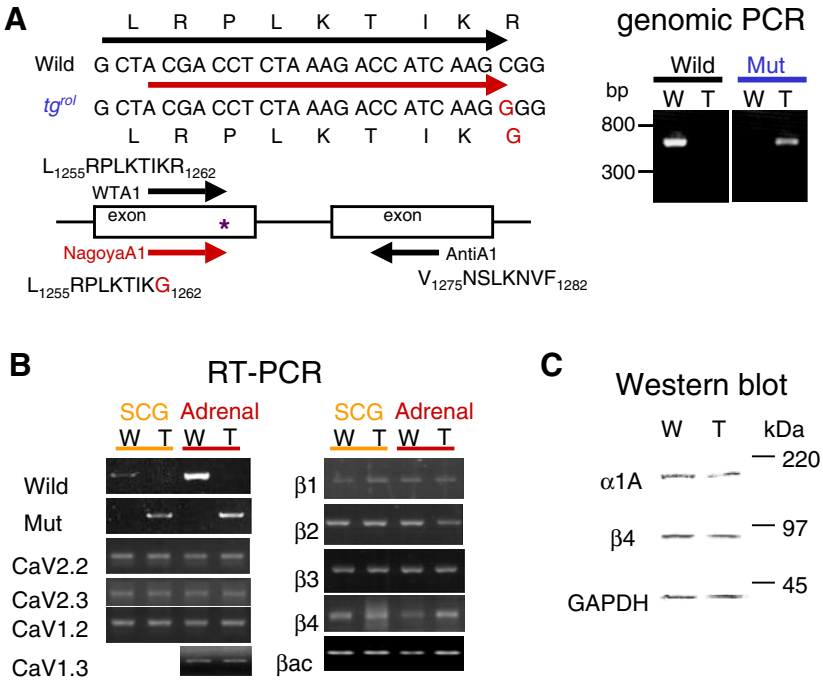

Fig. 1. Biochemical analysis from wild-type (wt, +/+) and P/Q-mutant *rolling Nagoya* (tg^{rol}) mice. (A) Sequence alterations in the tg^{rol} mutant. tg^{rol} contains a cytosine (*C*) to guanine (*G*) change at nucleotide position 3784 (asterisk), which results in an arginine (*R*) to glycine (*G*) alteration at amino acid position 1262. Exons are boxed. Lines indicate introns. PCR primers are indicated as arrows. Genotyping of *rolling Nagoya* (tg^{rol}) mice. Site specific primers (WTA1 or NagoyaA1 with AntiA1) enabled to us to distinguish wild-type and mutant alleles (577 bp in both alleles). (B) Expression profiles of various calcium channel subunits (RT-PCR) in the SCG and adrenal gland from wild-type (W) and tg^{rol} (*rolling Nagoya*, T) mice. (βac: β-actin). (C) Western-blot analysis (100 μg/lane) of adrenal glands from wild-type (W) and tg^{rol} (*rolling Nagoya*, T) mice. CaV2.1 (α1A), β4 or GAPDH as indicated.

did not occur. RT-PCR analysis with a β-actin specific primer pair was used as control (βac).

Western analysis revealed a CaV2.1-specific band in the adrenal gland (Fig. 1C). Protein levels of P/Q-type channel-forming CaV2.1 were not altered in the tg^{rol} mice. In addition, there were no apparent changes in β4 expression in the tg^{rol} mice. Furthermore, anti-GAPDH analyses confirmed that comparable amounts of protein were loaded in each lane of the gel.

Immunohistological examination of α1A subunits in the SCG and adrenal gland

Immunohistochemical analysis using anti-CaV2.1 antibodies revealed that CaV2.1 is homogeneously distributed in the SCG and adrenal medulla of wt and tg^{rol} mice (Supplementary Figs. 1ii and 2ii). No clear differences in the patterns of immunohistological labeling between the wt and tg^{rol} mice were detected.

ECG telemetry

The tg^{rol} ECG revealed a regular pattern indicative of physiological pacemaking and excitation propagation (Fig. 2A). Calculating heart rate (HR) over 1 h revealed that the HR of tg^{rol} mice was decreased as compared with wt mice (476 ± 13 and 388 ± 46 bpm, respectively; $p < 0.05$, Fig. 2B). The P/Q-VCC blocker ω-agatoxin IVA significantly decreased HR in the wt mice (Δ, 69 ± 12 bpm); however, ω-agatoxin IVA only marginally affected HR in tg^{rol} mice (Δ, 8 ± 3 bpm, Fig. 2C). Since ω-agatoxin IVA injection decreased HR, we speculated that P/Q-type channels mainly regulate sympathetic tone.

Echocardiography

Representative records of M-mode echocardiograms are shown in Fig. 3A. When heart rate, left-ventricular end-diastolic (LVED) and -systolic (LVES) dimensions, and ejection fraction were evaluated, it was discovered that the HR of tg^{rol} mice was slightly decreased (Fig. 3B). These results are consistent with our ECG-telemetry analysis. In addition, the ejection fraction was slightly reduced (Fig. 3C). The P/Q-VCC blocker ω-agatoxin IVA caused a significant decrease in HR and ejection fraction in wt mice; however, only marginal changes were observed in tg^{rol} mice (Fig. 3B and C). These data suggest that decreased HR and ejection fraction are mainly dependent upon the inhibitory effects of the toxin on the P/Q-type channels.

Atrium contraction

To further analyze the importance of P/Q-type VDCCs in autonomic nerve regulation, we examined contraction patterns of isolated heart atriums. Fig. 4A shows typical contractile force responses to field stimulation in the mouse atrium. The heart was electrically paced at 400 beats/min (bpm). Electrical field stimulation (EFS) was applied in conjunction with pacing stimulation (delay, 4 ms; duration, 1 ms for 5 s). To determine which component (adrenergic or cholinergic) was affected in tg^{rol} mice, subjects were pretreated with either atropine or metoprorol. Sympathetic nerve activity was evaluated after treatment with atropine (5.5 μM) for 60 min (Fig. 4A, upper panel). Parasympathetic nerve activity was evaluated after treatment with metoprorol (1 μM) for 60 min (Fig. 4A, lower panel).

Supplementary Fig. 3 shows representative contractile traces induced by EFS (15 V) in wt (left panel) and tg^{rol} mice (right panel) at baseline following atropine treatment. The contractile forces of tg^{rol} mice were significantly smaller than those of wt mice (Fig. 4B, Supplementary Fig. 3). However, variance within each preparation exists.

With regard to sympathetic neural (adrenergic, positive inotropic) responses, ω-agatoxin IVA pretreatment (1 μM for 60 min) showed slight, but significant effects in the wt mice. However,

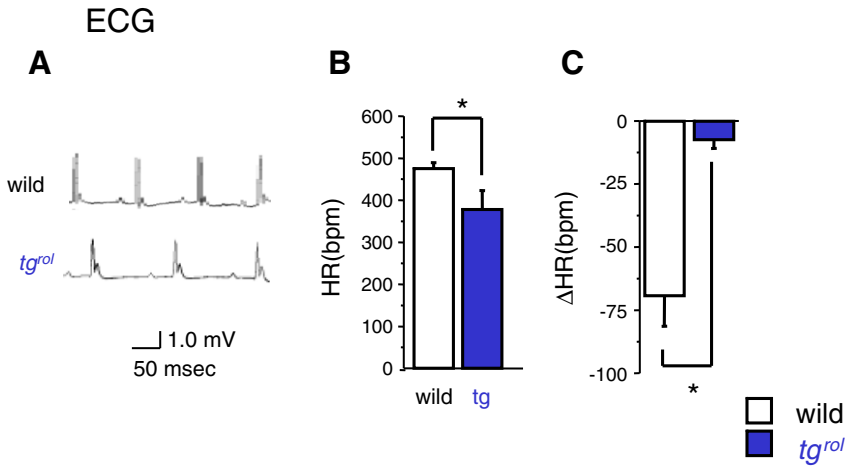

Fig. 2. ECG telemetry. (A) Representative ECGs from wild-type (upper panel) and (ii) tg^{rol} (lower panel). (B) Statistical results of ECG from wild-type (open bar) and tg^{rol} (blue bar) at baseline. tg^{rol} mice displayed a significantly decreased HR. (C) Pharmacological responses to 3 μg/kg ω-agatoxin IVA in the ECG-telemetry response. Data are expressed as means ± SE of the change in HR of at least seven animals. $^{*}p < 0.05$. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

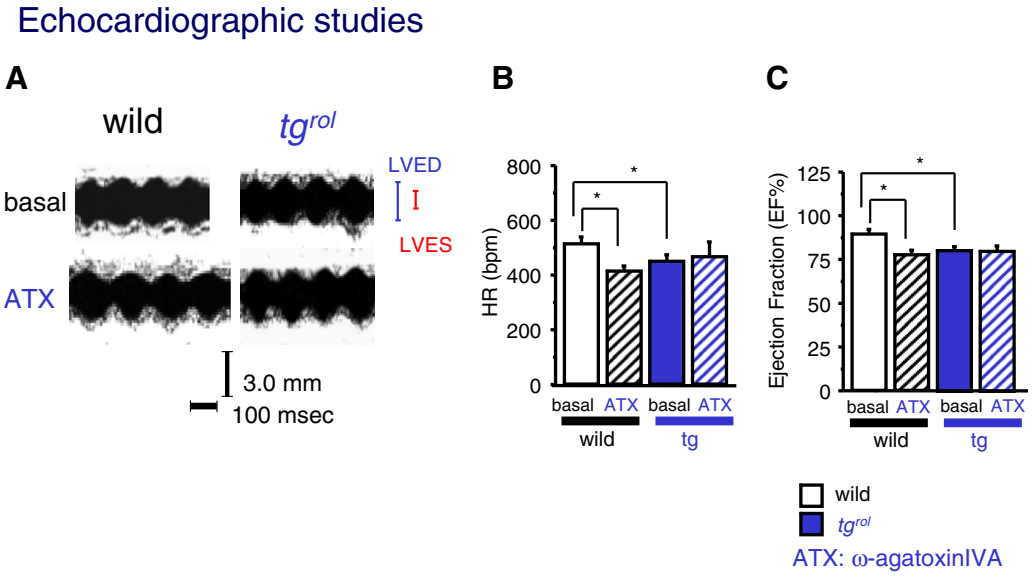

Fig. 3. Echocardiographic studies. (A) Representative B-mode results in the wild-type (left panels) and tg^{rol} mice (right panels). Bars indicate the left-ventricular diameters of diastole (LVED, blue bar) and systole (LVES, red bar). Effects of ω-agatoxin IVA (lower panels). Effect of intraperitoneal administration of ω-agatoxin (3 μg/kg) on heart rate (HR, B) and ejection fraction (EF, C) in wild-type (open bars) and tg^{rol} (blue bars) mice. Data are expressed as means ± SE of eight animals. $^{*}p < 0.05$. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

the tg^{rol} mice were unaffected (Fig. 4C, asterisk). From previous work it is clear that N-type channels play a major role in sympathetic neural responses [17,19,20]. The results of the present study suggest that P/Q-type channels are also involved in sympathetic nerve control; however, their contribution may be minor. Hong and Chang also reported a major contribution of N-type channels and a minor contribution of P/Q-type channels in sympathetic neural responses. They speculated that these results were due to the non-specific effects of ω-agatoxin IVA [21]. ω-agatoxin IVA shows a lower affinity to Q-type channels (IC_{50}; ~150 nM [6]) as compared with P-type channels (IC_{50}; 2–10 nM, [6]). In combination with Hong's report, our results suggest that Q-type channels play a more significant role in autonomic nerve regulation than do P-type channels. Nevertheless, the present results strongly suggest the involvement of P/Q-type channels in sympathetic nerve regulation.

In the experiments examining vagal (cholinergic, negative inotropic) stimulation, atria pretreated with β-blockers [metoprorol (1 μM) or ω-conotoxin GVIA (1 μM for 60 min] showed significant inhibitory effects in both wt and tg^{rol} mice. These results suggest a major contribution of N-type channels in vagal nerve regulation (Fig. 4D). No difference between wt and tg^{rol} mice was detected in the sensitivity to ω-conotoxin GVIA, thus suggesting that N-type channels were not affected in tg^{rol} mice. Our results, i.e., a major contribution of N-type channels in negative inotropic responses, correspond to Serone's report but not Hong's, in which P/Q-subtype channels play a major role [20,21].

We also analyzed the effects of ω-agatoxin IVA (1 μM for 60 min) on negative inotropic responses. A slight but significant effect of agatoxin IVA on parasympathetic relaxation was found in the wt mice. This effect was only marginal in the tg^{rol} mice, suggesting a minor but significant role of P/Q-type channels in para-

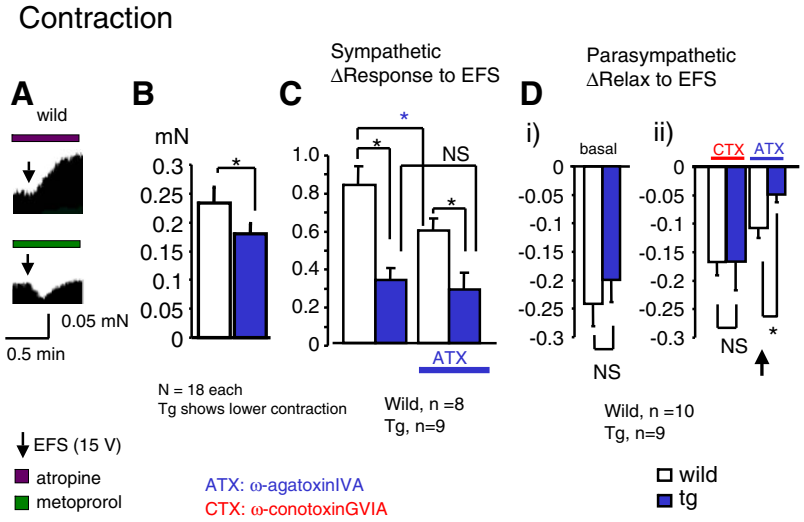

Fig. 4. Modified cardiac contractility in tg^{rol} mice. (A) Representative contractile traces induced by EFS (15 V) in wild-type mice after pretreatment with atropine (upper panel) or metoprorol (lower panel). (B) Summarized results of atrium contraction at baseline of wild-type (open bar) and tg^{rol} (blue bar) mice. (C) Sympathetic responses to EFS after atropine treatment in wild-type (open bars) and tg^{rol} (blue bars) mice. tg^{rol} mice showed significantly lower responses to EFS (black asterisk). ω-Agatoxin IVA induced significant effects only in wild-type mice (blue asterisk). (D) Parasympathetic responses to EFS after metoprorol treatment in wild-type (open bars) and tg^{rol} (blue bars) mice. At the baseline, wild-type and tg^{rol} mice showed similar negative inotropic effects in response to EFS (i). Inhibitory effects of ω-conotoxin GVIA and ω-agatoxin IVA on the parasympathetic relaxation were examined (ii). In both mice types, ω-conotoxin GVIA (CTX) induced significant inhibitory effects on relaxation. The application of ω-agatoxin IVA induced limited effects only in the wild-type mice (arrow); only marginal effects were observed in tg^{rol} mice. The data represent means ± SEM of 10 (wild-type) and nine (tg^{rol}) animals. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

sympathetic nerve regulation. Taken together, the present results suggest that N-type channels play a major role and P/Q-type channels play a minor role in vagal nerve regulation within the heart.

This report describes the cardiac phenotype of mice with a mutation in CaV2.1P/Q-type Ca^{2+} channels. The spontaneous subtle mutation in CaV2.1 did not affect the expression of other calcium channel subunits. ECG and echocardiographic analyses revealed significant involvement of P/Q-type channels in chronotropic regulation. Atrium contraction analyses revealed minor, but significant involvement of P/Q-type channels in sympathetic and parasympathetic nerve regulation.

A reduction in P-type calcium channel currents in tg^{la} Purkinje cells (~60%) as compared with those in *tg* and tg^{rol} cells (~40% reduction) has been reported [14]. Additionally, P-type currents in tg^{rol} and tg^{la} mutants display a shift in voltage dependence inactivation. In this study we found that tg^{rol} mutants displayed decreased responsiveness in ECG and atrium contraction to ω-agatoxin IVA, a specific P/Q-type channel blocker. These results strongly suggest the involvement of P/Q-type channels in the autonomic nerve system. Recent transgenic approaches have enabled scientists to construct a CAG repeat mouse model of the CaV2.1 gene, which resembles spinocerebellar ataxia type 6 (SCA6) of CAG repeat expansions [23]. It is therefore possible to make various types of CaV2.1 mutant mouse models that correspond to human CaV2.1 gene mutations.

Previously, we examined mice deficient in β3 and CaV2.2 [17,22], two major subunits of N-VCCs, and β3-overexpressing mice [16]. In our previous study, we identified a major role for N-type calcium channels in sympathetic nerve regulation. In the present study, we discovered a major role for N-type channels in parasympathetic nerve control. Furthermore, we found that P/Q-type channels play a significant role in sympathetic and parasympathetic nerve control. Nevertheless, our present results suggest the involvement of P/Q-type channels in autonomic nerve regulation. Future studies should examine the involvement of different calcium channels in autonomic nerve system.

In conclusion, our present results using mice and ω-agatoxin IVA strongly suggest that P/Q-type calcium channels play an important role in sympathetic and parasympathetic nerve regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.184.

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