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KCa1.1 β4-subunits is not responsible for iberiotoxin-resistance in baroreceptor neurons in adult male rats

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A large conductance of Ca^{2+}-activated K^+ channel (BK-KCa, KCa1.1, maxiK,) is one of important ion channel mechanisms in controlling neuroexcitability and neurotransmitter release. In sensory nerve system, KCa1.1 plays important roles in mediating afferent reflex, neuropathic pain, and other sensory functions. KCa1.1 widely expresses in either somatic or visceral afferent neurons including baroreceptor neurons (BRNs), which is composed of a pore-forming α-subunit and modulatory β-subunits. Among β-subunits, β4 expressed abundantly in CNS and plays key roles in Ca^{2+} sensing, modifying voltage-dependent property, current density, and channel cell membrane expression. Even though KCa1.1 β4-subunit is neuronal-specific, but co-expression of KCa1.1 α-/β4-subunits has recently been observed in smooth muscles. However, the information regarding to the expression pattern of KCa1.1 β4-subunits in each category of baroreceptor neurons is still not available and that would be a critical for fully understanding the neuroexcitability and the baroreflex function. Our previous studies indicated that the trajectory of action potential (AP) and repetitive discharge of myelinated A-type BRNs from either intact slice or isolated from male rats have not affected in the presence of N-type Ca^{2+} channel blocker ω-Conotoxin GIVA (ω-CTX) or by simply removing the extracellular Ca^{2+} (1), suggesting that this cell-types may not functionally express the N-type Ca^{2+} channels (2, 3). However, this hypothesis was not supported by the recent observation showing that myelinated A-type BRNs functionally expressed higher density of N-type Ca^{2+} currents compared with unmyelinated C-type BRNs (1). Since it is so, a negative expression of large conductance Ca^{2+}-activated K^+ channel (BK-KCa, KCa1.1) is well expected and several lines of evidence showing AP waveform of A-type BRNs was never changed in the presence of BK-KCa channel blockers charybdotoxin (ChTX) and iberiotoxin (IbTX) (4), and an activity- and frequency-dependent AP widening was not confirmed in identified A-type BRNs as well (5). Even though the electrophysiological observation implies the negative functional expression

of BK-KCa but this hypothesis was not consistent with our Immunohistochemical analysis (1) indicating the positive immunofluorescence of KCa1.1 α-subunits from both A- and C-type BRNs. Interestingly, recent observations have suggested that KCa1.1 β4-subunits (KCNMB4) render a resistance to IbTX but would be sensitive to paxilline (6, 7). To verify this, the experiments were designed to explore whether or not A-type BRNs functionally express KCa1.1 β4-subunits using single-cell RT-PCR and immunostaining conjugated with pharmacological approach and electrophysiological investigation in identified A-type BRNs isolated from adult male Sprague-Dawley (SD) rats.

Due mainly to multi-type afferent neurons housed in nodose ganglia, so, the PCR and western blot analysis in the tissue level would not be useful for the current purpose. In order to understand the expression patterns, we firstly performed single-cell RT-PCR using electrophysiologically and fluorescently (8) identified (9) myelinated A- and unmyelinated C-type BRNs (Fig. 1A) to evaluate mRNA expression for KCa1.1 α- or β4-subunits (Fig. 1B), respectively. These data showed that an identical expression levels of KCa1.1 α-subunits were detected in both A- and C-type BRNs and the expression level of KCa1.1 β4-subunits was more than two folds higher ($P < 0.05$) in C-type than that in A-type BRNs, suggesting that all BRNs co-expressed KCa1.1 α- and β4-subunits mRNA.

⇒ **Figure 1**

In order to further confirm the protein expression, the immunohistochemical staining (Fig. 2) was selected instead of western blot because of very limited quantity of nodose ganglia. In this set of experiments, the nodose slice was incubated with 4',6-diamidino-2-phenylindole (DAPI, blue) for staining the nucleus, isolectin B4 (IB4, green) for staining the unmyelinated neurons, combination with specific

antibody (red) for KCa1.1 α- and β4-subunits, respectively. These data (Fig.2) showed that not only IB4-positive (presumably unmyelinated neurons) but also IB4-negative (presumably myelinated neurons, as indicated by white arrows) neurons that all expressed KCa1.1 α- (Fig. 2, left) and β4-subunits of male rats (Fig. 2, right). These observations were also consistent well with the notion that the mRNAs for KCa1.1 α- and β4-subunits (Fig. 1) could be detected in all cell types in male rats by single-cell RT-PCR.

⇒ **Figure 2**

Based upon the single-cell RT-PCR and immunostaining data, we have a good suggestion that the mRNA for KCa1.1 α- and β4-subunits express in either myelinated A- or unmyelinated C-type BRNs with their targeted proteins. However, whether or not KCa1.1 α- and β4-subunits functionally express in each category BRNs, the functional study with AP recordings using whole-cell patch technique would be necessary. Also according to the literature (6, 7), if neurons positively co-express KCa1.1 β4-subunits, it will render a resistance to IbTX but sensitive to paxilline. In this regards, single AP and repetitive discharge were elicited by a brief pulse and step depolarization before and after 100 nM paxilline and/or 100 nM IbTX through a microperfusion directly onto tested neurons for less toxin used with less contamination to around neurons. In myelinated A-type BRNs, neither the trajectory of AP nor the discharge pattern were altered (Fig. 3A & B, Fig. 3F & G) and the similar results were also not confirmed even switch the application order of two toxins (data not shown), suggesting that KCa1.1 β4-subunits expression indeed rendered a toxin resistance to IbTX, whereas, the paxilline-sensitivity was not observed as supposed in the literature under this experimental condition in A-type BRNs. In the stark contrast in C-type BRNs, the AP duration (APD_{50}) was significantly prolonged and the firing

frequency was dramatically increased in the presence of 100 nM paxilline (Fig. 3C & D, Fig. 3F & G). These finding is consistent with the notion with IbTX (4) and IbTX equivalently mediated APD_{50} prolongation compared with paxilline alone and was not further prolongated by application of paxilline on top of IbTX (Fig. 3E), suggesting that, in C-type BRNs, although KCa1.1 β4-subunits is co-expressed, it does not really render a toxin-resistance to IbTX but sensitive to both toxins through the similar ion channel mechanism in C-type BRNs.

⇒ **Figure 3**

Taken all data together, we have demonstrated for the first time that myelinated A-type and unmyelinated C-type BRNs of male rats co-express mRNAs and targeted proteins for KCa1.1 α- and β4-subunits but they respond very differently to IbTX and paxilline. Collectively, the property of iberiotoxin-resistance rendered by positive expression of KCa1.1 β4-subunits is not confirmed in both afferent type neurons and the lacking of coupling/co-localization (10-13) between N-type Ca^{2+} and BK-KCa channels is the most likely mechanism in myelinated A-type BRNs.

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Conflict of interests

The authors have declared that no competing interest exists.

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Figure legends

Fig. 1. Relative expression levels of mRNAs for KCa1.1 α-subunits and β4-subunits in single electrophysiological and fluorescently identified myelinated A- and unmyelinated C-type baroreceptor neurons (BRNs) isolated from adult male rats. (**A**): Representative action potential (AP) recorded from A- (left) and C-type (right) BRNs form neuronal classification based upon the characteristic trajectory of AP (dark) and derivative current change (blue) over the time course of membrane potential; (**B**): Relative expression level of mRNAs for KCa1.1 α-subunits and β4-subunits in either A-type or C-type BRNs. Averaged data were presented as mean $\pm$ SD, n = 11 for A-type and n = 14 for C-type, *$P < 0.05$ vs A-type.

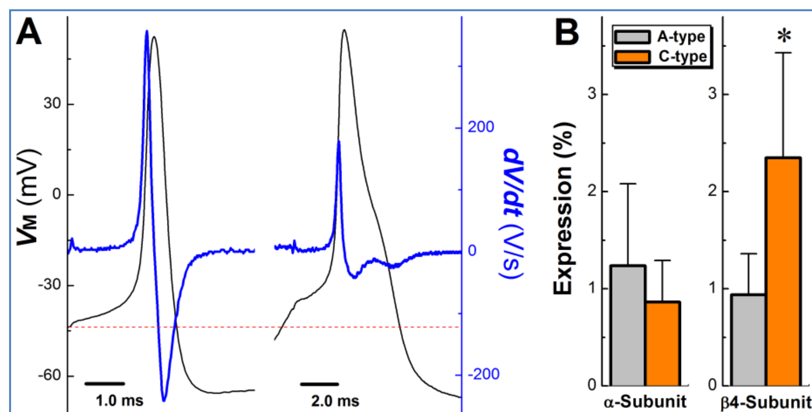

Fig. 2. Immunohistochemical analysis of KCa1.1 α-subunits and β4-subunits. The tissue slices from nodose ganglion of adult male rats were incubated with DAPI (blue) for staining the nucleus, IB4 (Green) for staining unmyelinated neurons, and antibody for either KCa1.1 α-subunits (left panel) or for KCa1.1 β4-subunits (right panel), respectively. White arrows pointed out the IB4-negative neurons presumably myelinated afferent neurons.

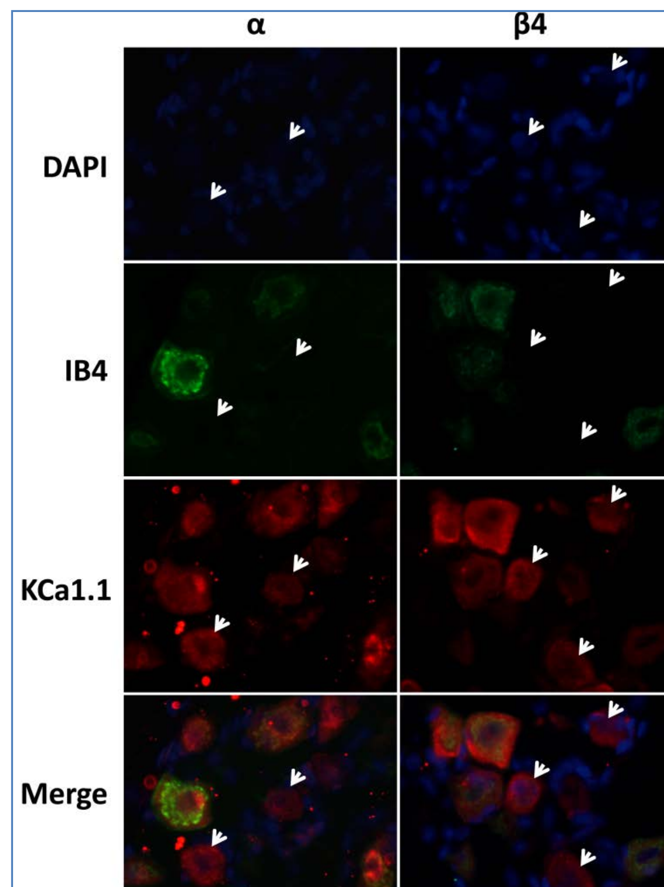

Fig. 3. Effects of paxilline or iberiotoxin (IbTX) on action potential (AP) and discharge profiles in electrophysiologically and fluorescently identified baroreceptor neurons (BRNs) isolated from adult male rats. Single AP and repetitive discharge were elicited by a brief pulse and step depolarization for neuronal classification and then for the following pharmacological tests. 100 nM paxilline or 100 nM IbTX was applied through microperfusion system directly onto tested neurons in order to reduce toxin

used and less contamination to around neurons. (**A**)-(**B**): APs and discharge profiles of myelinated A-type BRNs in the presence of paxilline and paxilline plus IbTX; (**C**)-(**D**): APs and discharge profiles of unmyelinated C-type BRNs before and after application of paxilline; (**E**): APs of C-type BRNs before and after IbTX or IbTX plus paxilline; (F)-(G): The summarized data for AP duration (APD50) and AP firing frequency (APFF) before and after 100 nM paxilline in A- and C-type BRNs. Averaged data were expressed as mean ± SD, n = 9 for A-type and n = 17 for C-type. **$P < 0.01$ vs A-type.