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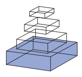

Calcium-activated chloride current expression in axotomized sensory neurons: what for?

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Calcium-activated chloride currents (CaCCs) are activated by an increase in intracellular calcium concentration. Peripheral nerve injury induces the expression of CaCCs in a subset of adult sensory neurons in primary culture including mechano- and proprioceptors, though not nociceptors. Functional screenings of potential candidate genes established that *Best1* is a molecular determinant for CaCC expression among axotomized sensory neurons, while *Tmem16a* is acutely activated by inflammatory mediators in nociceptors. In nociceptors, such CaCCs are preferentially activated under receptor-induced calcium mobilization contributing to cell excitability and pain. In axotomized mechano- and proprioceptors, CaCC activation does not promote electrical activity and prevents firing, a finding consistent with electrical silencing for growth competence of adult sensory neurons. In favor of a role in the process of neurite growth, CaCC expression is temporally correlated to neurons displaying a regenerative mode of growth. This perspective focuses on the molecular identity and role of CaCC in axotomized sensory neurons and the future directions to decipher the cellular mechanisms regulating CaCC during neurite (re)growth.

Keywords: DRG, CaCC, bestrophin, regeneration, axotomy, electrical activity, membrane tension

INTRODUCTION

After peripheral nerve injury, sensory neurons switch from a transmitting mode to a regrowth mode. Axotomy of adult peripheral neurons induces rapid axon regeneration. This has been demonstrated *in vivo* (Tanaka et al., 1992; Jacob and McQuarrie, 1993) and *in vitro* (Smith and Skene, 1997; Lankford et al., 1998; Andre et al., 2003) where a conditioning lesion increases the ability of the associated primary afferent neuron to regenerate successfully. Molecular mechanisms responsible for this increased neuronal growth ability are accompanied by a shift in cellular organization, such as the appearance of growth cones at the proximal tip of the lesioned axons and the swelling of the neuronal cell body associated with a strong increase in cellular metabolism and protein synthesis (Makwana and Raivich, 2005). Transcriptomic analysis of gene expression following peripheral nerve injury has led to the identification of many injury-related and regeneration-associated genes encoding such as regulatory proteins including growth factor receptors and transcription factors (Araki et al., 2001; Costigan et al., 2002; Xiao et al., 2002; Mechaly et al., 2006).

Axotomy upregulates Ca^{2+} activated chloride current (CaCC) expression in sensory, sympathetic, and nodose neurons (Sanchez-Vives and Gallego, 1994; Lancaster et al., 2002; Andre et al., 2003). Using an *in vitro* model of regenerative growth, we have shown that a close relationship exists between the growth competence of sensory neurons and CaCC expression (Andre et al., 2003). In addition, its expression is limited to a subset of neurons including those of medium and large somatic diameter, i.e., the mechano- and proprioceptors. No expression was observed among small somatic diameter neurons, i.e., the nociceptors.

Under physiological conditions, the reversal potential of Cl^- currents is around –40 mV among most sensory neurons due to the expression and activity of an inwardly directed Na^+-K^+-$2Cl^-$ co-transporter, NKCC1 (Sung et al., 2000). This corresponds to an intracellular chloride concentration ($[Cl^-]_i$) of sensory neurons amounting to 20–30 mM (Alvarez-Leefmans et al., 1988) thus conferring a depolarizing effect of Cl^- currents at resting potential. Following nerve injury, Cl^- reversal potential shifts toward depolarized potentials reaching up to –20 mV corresponding to a two to threefold increase in $[Cl^-]_i$ which increases further the Cl^- current driving force relative to the resting potential (Pieraut et al., 2007). Thus, CaCC expressed in axotomized, regenerating sensory neurons is potentially suited to inducing membrane depolarization and triggering electrical activity. Several years ago, Scott et al. in their review on CaCC pointed out the complexity of defining a role for these channels presumed to have a variety of different functions depending on their co-localization with other channels and the type of physiological mechanism involved in raising the $[Ca^{2+}]_i$ (Scott et al., 1995). Recently, the identification of several candidate genes for CaCC has also to be considered to define the complexity in their role (Duran et al., 2010). Indeed, besides a potential role in electrical activity, CaCC has also been shown to be involved in other cellular functions such as secretion, proliferation, and apoptosis (Kunzelmann et al., 2011).

BESTROPHIN-1: A MOLECULAR DETERMINANT FOR CaCC EXPRESSION AND PERIPHERAL NERVE REGENERATION

CaCC AND BESTROPHIN-1 FUNCTIONALLY LINKED IN TRAUMATIZED SENSORY NEURONS

Calcium-activated chloride channels are coded by a great diversity of genes. To date, research has identified four families of CaCC: the CLCA (Pauli et al., 2000), the bestrophin (Sun et al., 2002), the tweety (Suzuki and Mizuno, 2004) and the TMEM16 families (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). Members of all four families have been found expressed in the DRG following nerve injury (Al-Jumaily et al., 2007; Boudes et al., 2009). CLCA proteins have high homology to known cell adhesion proteins and it is suggested that CLCA proteins modulate endogenous Cl^- channels (Hartzell et al., 2005). While some doubt remains regarding the exact channel function of each of the proteins from the three other families, they are all still considered as putative chloride channels based on bioinformatics and functional data. This great diversity renders the exploration of their function difficult, due to the lack of specific pharmacological and molecular tools to inhibit each of the channels specifically. An elegant method to modulate gene expression by RNA interference using single-cell electroporation in cultured adult DRG neurons was developed to decipher the molecular nature of CaCC expressed in injured sensory neurons (Boudes et al., 2008). With a functional screening strategy, we demonstrated that among six candidates, only siRNA targeted against *Best1* down-regulated CaCC expression in axotomized DRG neurons. Moreover, Bestrophin-1 overexpression with an expression plasmid generated CaCC with biophysical properties close to native currents in putative mechano- and proprioceptors (Boudes et al., 2009). However, no definitive conclusions could be drawn since *Best1* knockout mice expressed a CaCC following *in vivo* peripheral nerve axotomy which could result from a functional compensation by *Best3* in the null mice.

BESTROPHIN-1, A KEY PLAYER IN FUNCTIONAL NEURITE OUTGROWTH

The genetic ablation of Bestrophin-1 (using both knockout mice and RNA interference strategies) induces a decrease in neurite outgrowth velocity in cultured injured sensory neurons (Boudes et al., unpublished result). It is noteworthy that Best3 compensation in the knock-out mice could not sustain neurite growth suggesting the importance of a subcellular localization for correct cellular functioning. Unfortunately, the lack of specific antibodies prevented to determine the cellular and subcellular localizations of Bestrophin-1 and 3, both *in vitro* and *in vivo*. Nevertheless, the observation of positive effects of Bestrophin-1 on neurite growth led us to check for any benefits to the functioning of nerve regeneration *in vivo*. The lack of a direct relationship between morphological data and functional studies, led us to use behavioral test to assess nerve fiber regeneration (de Medinaceli, 1995; Baptista et al., 2007). Following nerve injury, measuring the paw withdrawal threshold in response to mechanical stimulus using a series of graded von Frey filaments can assess the time for recovery of sensitivity to mechanoreceptors function. In agreement with studies in mice (Vogelaar et al., 2004), measurement of the withdrawal threshold before and after left sciatic nerve crush injury indicated that basal mechanical sensitivity needed 15 days to recover (12 mice). In a preliminary study, we observed that, following sciatic nerve crush, *Best1* knockout mice display a roughly 5 days delay for recovery of basal sensibility (10 mice). This strongly indicates a slowing down of the functional outgrowth of a subset of afferent fibers.

Altogether, these data show that Bestrophin-1 is a positive player in the regenerative process of the mechanosensitive afferent fibers. The next and crucial challenge is to understand how CaCC is involved in the regeneration of injured sensory neurons.

CaCC AND REGENERATION, HOW DOES IT WORK?

REGENERATION, ELECTRICAL ACTIVITY AND CaCC, A TRIPTYCH?

A recent study reported that loss of electrical activity following peripheral deafferentation is an important signal to trigger axon regrowth and that concordantly electrical activity strongly inhibits axon outgrowth in cultured adult sensory neurons (Enes et al., 2010). It is proposed that electrical silencing is an important cue in eliciting the conditioning effect on growth competence of adult DRG neurons. Consistent with this notion, no somatic sensations are experienced following peripheral nerve transection, except under certain circumstances when neuropathic pain is felt.

To address the role of CaCC in axotomized adult sensory neurons, we first postulated an involvement in the control of their electrical activity. This led us to analyze the calcium sensitivity of CaCC by simultaneously recording intracellular Ca^{2+} variation and electrophysiological measurements under opening of voltage gated Ca^{2+} channels, VGCCs. Surprisingly, CaCC displayed a rather low Ca^{2+} sensitivity such that one action potential was unable to mobilize enough Ca^{2+} to activate CaCC. In the same study, a train of action potentials was necessary to increase Ca^{2+} to levels sufficient to activate CaCC. Even under such conditions, the CaCC-induced membrane depolarization could be observed only provided that K^+ currents were partially inhibited (Hilaire et al., 2005a). Importantly, under high $[Cl^-]_i$ as determined in axotomized neurons (E_{rev} close to –20mV), once K^+ current inhibition had elicited firing activity, CaCC was able to progressively depolarize the resting membrane potential leading to Na^+ current inactivation and the consequent cessation of electrical activity. Afterwards, a progressive repolarization occurred due to the loss of Ca^{2+} entry through the VGCCs (among 13 recorded axotomized sensory neurons displaying an after-depolarization, six generated firing activity with progressively decreasing amplitude until membrane depolarization reached -28 ± 2 mV, a value close to Cl^- reversal potential and sufficient to inactivate Na^+ channels. All neurons expressed CaCC without differences in amplitude). Although we never recorded spontaneous electrical activity in conditioned axotomized mechano- or proprioceptors, they did display a lower excitability threshold and a subset of them could fire action potentials under intracellular Ca^{2+} buffering, which prevented CaCC activation (Hilaire et al., 2005b). This last result confirms that a subset of axotomized neurons is more sensitive to fire; the molecular changes responsible for increased excitability under intracellular Ca^{2+} buffering remain to be determined. From these observations, it appears that activation of CaCC under intense electrical activity and K^+ current inhibition could induce a negative retro control on neuronal firing properties, therefore preventing unfavorable conditions for neurite regrowth.

The source of Ca^{2+} to activate CaCC is not necessarily through the opening of VGCCs. Indeed, receptor-mediated intracellular Ca^{2+} mobilization is also known to activate CaCC (Scott et al., 1995). To demonstrate whether activation of CaCC could trigger electrical activity in axotomized sensory neurons, caffeine was used to mobilize Ca^{2+} stores. Consistent with other studies, caffeine activated CaCC as efficiently as VGCC and induced membrane depolarization. However, it never triggered electrical activity in axotomized sensory neurons (unpublished result). In fact, unless K^+ currents were inhibited, CaCC-induced membrane depolarization never reached a threshold able to trigger electrical activity.

Consistent with the concerted action of CaCC activation together with K^+ current inhibition on neuronal excitability, the bradykinin-elicited electrical activity observed among nociceptors was due to activation of CaCC together with inhibition of (Kv7) M-current. Interestingly, a lack of coupling between CaCC and VGCC in nociceptors observed in this study was suggested to be required in order to avoid a self-maintaining positive feedback loop. Indeed if Ca^{2+} influx through VGCC was to significantly activate CaCC (and inhibit M channels), this would then cause depolarization and further Ca^{2+} influx through VGCC and an amplification of the cycle (Liu et al., 2010). The authors concluded that in most small DRG neurons (in contrast to the medium/large ones), the coupling between VGCC and CaCC is minimal, which may suggest either poor spatial co-localization of CaCC and VGCC and/or a different molecular identity of CaCC in small compared with large DRG neurons. CaCC amplitude is clearly smaller in nociceptors (pA range) than in axotomized mechano-proprioceptors (nA range) which could be related to both cell size and different channel conductances (Hartzell et al., 2005). This difference in current density could also account for the outward rectification for CaCC-voltage relationships in nociceptors versus linear in axotomized medium-large neurons (Yang et al., 2008). Although both CaCCs were sensitive to niflumic acid and NPPB inhibition, DIDS had not effects in mechano- and proprioceptors (Andre et al., 2003). Consistent with a different type of CaCC expressed in nociceptors, the expression of TMEM16A is observed in the majority of small DRG neurons but not in large neurons (Yang et al., 2008). *Tmem16a* is functionally involved in bradykinin-induced CaCC among nociceptors (Liu et al., 2010), while *Best1* accounts for CaCC expression in axotomized sensory neurons (Boudes et al., 2009). Interestingly, in a model of conditioning, the elongating mode of growth *in vitro* was activated only in the population of nociceptors that did not express calcitonin gene-related peptide ($CGRP^-$) or bind isolectin B4 ($IB4^-$). In other words, a lower regenerative capacity was observed in unmyelinated peptidergic and non-peptidergic primary afferent neurons (Leclere et al., 2007; Kalous and Keast, 2010). It would be of interest to verify whether this subset of nociceptors do also express *Tmem16a* under axotomy.

From these studies, a possible role of large amplitude CaCC together with high $[Cl^-]_i$ in axotomized mechano- and proprioceptors would be to prevent the maintenance of intense electrical activity and thereby contribute to the electrical silencing necessary for growth competence.

REGENERATION, MEMBRANE TENSION AND CaCC: THE ELECTRONEUTRAL HYPOTHESIS

Mechanotransduction is the cellular mechanism by which cells, including neurons, sense and respond to their physical surroundings. Given the amphiphilic nature of phospholipids, neuritic expansion during growth is mainly achieved by inserting vesicles into pre-existing membranes at the growth cone (Pfenninger, 2009). This mechanism is highly demanding in energy and neurons need to counteract physical issues to achieve it. Indeed, growth cones deal with external and internal mechanical forces. Under *in vitro* conditions, intracellular positive forces, such as cytoskeleton polymerization and osmotic tension, are probably the main drivers for neurite growth (Geiger et al., 2009). Osmotic tension is induced under changes in the intracellular ionic concentration. An increase in ionic concentration or hypotonic extracellular solution promote rise in osmotic tension due to water entry leading to cell swelling. Conversely, a decrease in ionic concentration promotes a fall in osmotic tension due to water exit and leads to cell shrinkage.

There are no reports linking CaCC activation to cell swelling in neurons. A contribution from CaCC channels is, however, to be expected when volume regulation is associated with an increase in $[Ca^{2+}]_i$. Hypotonic extracellular solution induces $[Ca^{2+}]_i$ increase and triggers electrical activity in a subset of nociceptors and mechanoceptors, but not among most mechano- and proprioceptors (Viana et al., 2001; Alessandri-Haber et al., 2003; Haeberle et al., 2008). Cell specific expression of stretch-sensitive, Ca^{2+} permeable channels, such TRPV4, accounted for these effects suggesting that volume regulatory mechanisms are electrically silent in the remaining neurons. Ionic channels, exchangers and co-transporters contribute to cell volume regulation (Hoffmann et al., 2009). Preferential activation of volume-sensitive K^+ channels could prevent cell excitability. To date, nothing is known concerning the expression of Ca^{2+} permeable stretch channels in axotomized sensory neurons. Interestingly, GAP43 (growth-associated protein 43) a membrane-anchored neuronal protein up-regulated in axotomized dorsal root ganglia and positive regulator of axonal regeneration, is an osmosensory protein that augments $[Ca^{2+}]_i$ in response to hypotonicity (Caprini et al., 2003; Makwana and Raivich, 2005). Besides, it is reported that drosophila bestrophin-1 (dBest1) is dually activated by calcium and cell volume (Chien and Hartzell, 2007). Interestingly, volume-regulated anion current (VRAC) can be rescue with different mutants of dBest1 in drosophila S2 cells although bestrophins are unlikely to be the *classical* VRAC in mammalian cells (Chien and Hartzell, 2008). Altogether, these findings support that variation in cell tension through swelling could contribute to CaCC activation and to cell growth (Raucher and Sheetz, 2000).

Consistent with a role of membrane tension during neurite growth, we found that, *in vitro*, axotomized sensory neurons display more than a twofold increase in $[Cl^-]_i$ due to interleukin-6-induced phosphorylation of NKCC1 (*Slc12a2*), an electroneutral cation-chloride co-transporter (Pieraut et al., 2007, 2011). This plasmalemmal ion transporter not only regulates the basal $[Cl^-]_i$ but also belongs to the set of molecules controlling osmotic force (O'Neill, 1999). Therefore, receptor-mediated chloride

accumulation does indeed create a situation of osmotic tension, something which has been so far overlooked in neuronal physiology and pathophysiology. Osmotic tension needs to be controlled to avoid cell deterioration and the efflux of chloride ions at resting membrane potential through CaCC can be an option. We postulate that under activation of NKCC1, exit of Cl^- through CaCC at resting membrane potential could be balanced with K^+ outflow induced by volume and/or voltage-activated K^+ currents, making CaCC effects electrically silent. Thus *Slc12a2* and *Best1* could belong to the set of genes involved in mechanotransduction through modification of the osmotic force.

Interestingly, we observed that preventing the rise in $[Cl^-]_i$ in axotomized sensory neurons in a low external Cl^- concentration or using NKCC1$^{-/-}$ mice, not only reduced neurite growth velocity (Pieraut et al., 2007) but also prevented CaCC expression: 27% (10/37) under low external Cl^- and 26% (9/35) in NKCC1$^{-/-}$ sensory neurons compared to 66% (25/42). This thus highlighted a link between $[Cl^-]_i$ regulation and CaCC expression during neurite growth. We propose that the osmotic force associated with volume increase due to NKCC1-induced chloride accumulation could be a cellular mechanism contributing to neurite growth velocity. Envisaging neuronal Ca^{2+}-activated chloride channels involved in electrically silent cellular mechanisms to regulate growth is fascinating and needs to be explored.

PERSPECTIVES

A major pitfall in the study of CaCC expressed in regenerating sensory neurons is the actual localization and source of Ca^{2+}. Many studies have addressed the role of Ca^{2+} during developmental neurite growth and synaptic plasticity (Zheng and Poo, 2007). To our knowledge however, the determination of Ca^{2+} transients during the regenerative growth of sensory neurons has never been performed. To address this important issue, time lapse recordings of spontaneous Ca^{2+} with Ca^{2+} sensitive probes will be necessary. It will also be of interest to identify Cl^- variation with Cl^- sensitive fluorescent probes (Chub et al., 2006; Bregestovski et al., 2009) and tentatively correlate, both temporally and spatially, each event.

Despite the identification of *Best1* as a molecular determinant of CaCC in medium/large diameter sensory neurons, the *Best1*$^{-/-}$ mice did express a CaCC which disallowed any definitive conclusions to be drawn concerning the role of CaCC. Moreover, Bestrophin-1 is probably not the only molecular partner involved in CaCC expression as its overexpression in nociceptors did not result in a functional Cl^- current. Identification of the other molecular partners or regulators could lead to the design of new tools enabling a better understanding of CaCC function.

The membrane tension hypothesis could be tested with the use of biophysical techniques such as atomic force microscopy (AFM) (Ricci et al., 2011). AFM has become a well-used tool for high resolution imaging of biological materials. Surface forces and surface properties (hydrophobicity, elasticity) could be measured on a nanoscale providing spatially –resolved maps of the nanomechanical characteristics of growing sensory neurons from NKCC1 or Bestrophin-1 knockout mice.

The lack of growth competence among a subset of nociceptors, in particular the IB4-positive neurons together with their known vulnerability to peripheral nerve injury could be attributed in part to their *Tmem16a* expression and propensity to fire action potentials. While IB4-labeled neurons do fail to synthesize some proteins involved in axonal regeneration (Leclere et al., 2007), the development of intense electrical activity may also participate. Analyzing neurite growth competence in *Tmem16a*$^{-/-}$ neurons should help clarify this issue.

The concomitant appearance of a high $[Cl^-]_i$ in central neurons (Represa and Ben-Ari, 2005) and the expression of CaCC occur during the development of sensory (Bernheim et al., 1989; Currie and Scott, 1992) and spinal neurons (Hussy, 1991, 1992) and in injury models (Nabekura et al., 2002; Andre et al., 2003; Pieraut et al., 2007). Therefore, we can postulate a role of Bestrophin-1 in these physio and/or physiopathological processes. Functional studies focused on developmental and injured models may be able to decipher a universal growth mechanism which would be something worth knowing!

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