CHAPTER 5

Mechanosensitive Channels in Neurite Outgrowth

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I. OVERVIEW

The past few years have seen the convergence of two areas of investigation: the first is the study of the molecular basis for Ca^{2+}-dependent axon pathfinding and the second is the molecular and functional characterization of mechanosensitive Ca^{2+}-permeant cation channels (MscCa). The convergence of these two fields has reached a pivotal point when some ion channels belonging to the transient receptor potential (TRP) superfamily of proteins, denoted as TRPCs, were reported to play essential roles in the growth cone guidance and, independently, some of these channels were found to form MscCa of vertebrate cells. Various lines of evidence taken together make likely the idea that MscCa can substantially contribute to the spatial and temporal shaping of Ca^{2+} responses in growing neurites. These findings will be described and the possible contribution of MscCa to the neurite outgrowth will be discussed.

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II. INTRODUCTION

During development, growth cones at the tips of extending neurites guide axons to appropriate target regions, migrating through complex environments. Regeneration and development share basic mechanisms (Letourneau *et al.*, 1991). Although the majority of studies concerning growth cone motility have been performed *in vitro*, the main findings have been validated on neurons developing *in vivo* (Gomez and Spitzer, 1999). The guidance of nerve fibers to their final destination can be considered as a series of short-range projections under the influence of local cues. A variety of simultaneous environmental stimuli is likely to confront the growth cone that must therefore integrate inputs and choose an appropriate final response, consisting in a reorganization of cytoskeleton and adhesion complexes (Lin *et al.*, 1994; Gomez and Zheng, 2006; Wen and Zheng, 2006). Both *in vitro* (Shaw and Bray, 1977) and *in vivo* (Harris *et al.*, 1987) experiments demonstrate that individual growth cones are largely independent of cell body in their responses to environmental cues; accordingly, the essential components for extension and guidance are locally regulated (Ming *et al.*, 2002).

In the next section, a basic summary of current knowledge regarding the mechanisms of calcium-dependent axon pathfindings will be provided. Since many excellent reviews are available in the field (Letourneau *et al.*, 1991; Henley and Poo, 2004; Bolsover, 2005; Chilton, 2006; Gomez and Zheng, 2006; Wen and Zheng, 2006), attention will be focused on a few emerging concepts, which are relevant to answer the question whether MscCa may have a role in the control of growth cone dynamics.

III. ENCODING OF GUIDANCE CUES IN AXON PATHFINDING

A large variety of guidance cues have been found to be encoded into a limited number of intracellular signals. Among others, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and cyclic nucleotides have been extensively studied (Song and Poo, 1999; Ming *et al.*, 2001; Xiang *et al.*, 2002; Nishiyama *et al.*, 2003; Gomez and Zheng, 2006; Wen and Zheng, 2006). Different cues, such as neurotransmitters, growth factors, components of the extracellular matrix (ECM), and cell adhesion molecules (CAMs), through distinct receptor complexes, contribute to change the $[Ca^{2+}]_i$. Elevations of $[Ca^{2+}]_i$ are recognized as a cellular signaling event affecting a variety of cellular processes from cytoskeletal remodeling to transcriptional regulation (Berridge *et al.*, 2003). In many neuronal types, there is an optimal range of $[Ca^{2+}]_i$ that supports maximal neurite outgrowth, while large $[Ca^{2+}]_i$ elevations induce growth cones to slow down or retract, and reductions in $[Ca^{2+}]_i$ promote neurite

growth (Bixby and Spitzer, 1984; Kater *et al*., 1988; Gomez and Spitzer, 2000; Henley and Poo, 2004).

Since the discovery that different classes of growth cones, both *in vitro* and *in vivo*, share the property of generating periodic elevations of $[Ca^{2+}]_i$ as they migrate (Gomez *et al*., 1995), growth-associated calcium transients have been extensively investigated. These appear as a natural signaling mechanism regulating the axon extension because the rate of axon outgrowth has been found inversely proportional to the frequency of calcium transients (Goldberg and Grabham, 1999; Gomez and Spitzer, 1999). Both Ca^{2+}-induced Ca^{2+} release and influx through plasma membrane channels are contributors to the transients. Besides transmitter- and voltage-gated channels, nontraditional calcium channels had been suspected to sustain calcium transients in growth cones. Calcium transients were only partly affected by organic blockers of voltage-dependent Ca^{2+} channels (VDCCs), while they were inhibited by nonspecific inorganic cations (Gu *et al*., 1994; Gomez *et al*., 1995, 2001; Williams and Cohan, 1995).

Analysis at higher resolution shows that adhesion generates in neurons short-lived and highly localized calcium rises (Dunican and Doherty, 2000), and studies elegantly demonstrate that these transients in the growth cone are sufficient to modulate its motility (Zheng, 2000). Furthermore, frequency of filopodial local calcium transients has been found to depend on substrate type and concentration (Gomez *et al*., 2001). Thus, the spatiotemporal shaping of the $[Ca^{2+}]_i$ responses represents a key determinant of signal specificity. One expects that such a shaping of the calcium signal requires mechanisms which are capable of producing large but local changes of $[Ca^{2+}]_i$ along with a strategic assembly of proteins in signal complexes.

A successful paradigm to study *in vitro* calcium-dependent axon pathfinding is the analysis of growth cone turning responses to guidance cues. Local Ca^{2+} signals regulate growth cone steering toward or away from a source of specific neurotransmitters, neurotrophic factors, or components of ECM and cell surface. Thus, well-defined responses have been established for different classes of neurons. For example, among others, brain-derived neurotrophic factor (BDNF) and netrin-1 are chemoattractants for several classes of developing neurons, such as *Xenopus* spinal neurons and rat cerebellar granule cells (Li *et al*., 2005; Wang and Poo, 2005), whereas, myelin-associated glycoprotein (MAG) is a chemorepellent for *Xenopus* spinal neurons (Song *et al*., 1998).

This experimental approach was also exploited to highlight the unexpected role of intracellular cAMP ($[cAMP]_i$) in determining the direction of growth cone turning to a particular guidance cue. Low levels of $[cAMP]_i$ in growth cones lead to the conversion of netrin-induced attraction into repulsion (Song *et al*., 1997). Interestingly, both laminin-1 (Hopker *et al*., 1999) and N-cadherin or L1 (Ooashi *et al*., 2005), via cAMP, regulate the steering of growth cones.

Therefore, both ECM molecules and CAMs modify the growth cone response to guidance cues. Other cyclic nucleotides, such as cGMP, can play such a role and also the reverse shift, that is repulsive factors converted to attraction, can occur (Song *et al.*, 1998). Thus, a general implication here is that a given guidance cue may act to either repel or attract growth cones, depending on the cellular context. The discovery of spontaneous changes of $[cAMP]_i$ in neurons (Bacskai *et al.*, 1993; Hempel *et al.*, 1996) and, particularly, the demonstrated interdependence of $[Ca^{2+}]_i$ and $[cAMP]_i$ oscillations (Gorbunova and Spitzer, 2002) open the possibility that cell uses specific dynamics of intracellular second messengers to encode complex environmental stimuli, converging onto a limited number of intracellular signals (Zaccolo *et al.*, 2002). Many potential sites of cross talk between the $[Ca^{2+}]_i$ and the $[cAMP]_i$ signaling pathways have been found. Among the main ones, Ca^{2+} activates some isoforms of adenylyl cyclase and phosphodiesterases, while cAMP, in turn, modulates Ca^{2+} channels and pumps (Bruce *et al.*, 2003).

The mechanisms of growth cone guidance exhibit adaptation. High concentration of a given cue reversibly desensitizes the turning response, not only to the same guidance cue but also to those sharing common cytosolic transduction mechanisms (Ming *et al.*, 2002). Such adaptive behavior enables growth cones to modulate the gain of their guidance signal transduction.

IV. REQUIREMENT OF TRP CHANNELS IN CALCIUM-DEPENDENT AXON PATHFINDING

VDCCs have been found only partly responsible for the netrin-1-mediated Ca^{2+} influx into *Xenopus* growth cones. Furthermore, the mechanisms of their activation by netrin remained elusive (Hong *et al.*, 2000; Nishiyama *et al.*, 2003). Investigations have provided conclusive evidence that some cationic channels, belonging to the TRP superfamily (for reviews see Clapham *et al.*, 2001; Minke and Cook, 2002; Montell *et al.*, 2002; Clapham, 2003; Moran *et al.*, 2004; Lin and Corey, 2005; Pederson *et al.*, 2005; Owsianik *et al.*, 2006; Ramsey *et al.*, 2006), included in the canonical family (TRPC), are involved in chemotropic axon guidance. Although all TRPCs are tetramers of six transmembrane polypeptide subunits, are nonselective cation channels, and share invariant sequences in both C-terminal and part of N-terminal tails, their selectivity ratio varies significantly.

Members of different subfamilies of TRPCs are reported to be required for growth cone guidance in various neuronal types. TRPC5 have been found to regulate length and morphology of hippocampal neurites, since neurite growth is inhibited or enhanced by overexpression of TRPC5 or a dominant-negative (DN) pore mutant, respectively (Greka *et al.*, 2003). Furthermore, in rat

cerebellar granule cells, the block of influx through endogenous TRPC3 by siRNA or by overexpression of DN-TRPC3 or DN-TRPC6 destroys growth cone attraction toward BDNF (Li *et al.*, 2005). Parallel studies demonstrated that TRPC1 is essential for netrin-induced axon turning in *Xenopus* spinal neurons, because this response is abolished by pharmacological inhibition with SKF-96365 or La^{3+}, knockdown of protein by morpholino injection or expression of DN-TRPC1 (Wang and Poo, 2005).

In summary, these results demonstrate that netrin or BDNF stimulates its receptor, DCC or TrkB, respectively, which in turn promotes through activation of a different phospholipase C the production of inositol-1,4,5-triphosphate (IP_3), a messenger causing the release of Ca^{2+} from internal stores. While in amphibian spinal neurons, the consequent activation of TRPCs produce membrane depolarization and additional Ca^{2+} influx through VDCCs, this step does not seem to be involved in the response to BDNF of mammalian neurons in which only TRPCs are activated (Li *et al.*, 2005; Wang and Poo, 2005). However, interesting points emerging from these remarkable findings are: (1) TRPCs are activated by guidance cues such as netrin or BDNF; (2) their local applications produce localized and asymmetrical increases of $[Ca^{2+}]_i$; (3) both Ca^{2+} influx and Ca^{2+} release are necessary for the turning response; (4) TRPCs, with or without VDCC coactivation, have a clear-cut role in the amplification of the Ca^{2+} response. As far as the activation mechanism of TRP is concerned, these channels have often been associated with the store-operated Ca^{2+} entry (SOCE; Parekh and Putney, 2005), also reported as capacitative Ca^{2+} entry because it allows stores to be replenished, but the process responsible for TRP activation is still a matter of intense debate (Clapham, 2003; Parekh and Putney, 2005).

The physiological role of TRPC1 channels has been confirmed both *in vitro* and *in vivo*, demonstrating that they are essential for the proper formation of commissural axon tracts in *Xenopus* spinal cord (Shim *et al.*, 2005).

Different mechanisms can be considered to account for the ability of cyclic nucleotides to shift the growth cone turning direction in response to a given guidance cue. Cyclic nucleotides might modulate proteins involved in voltage-dependent Ca^{2+} influx, in ATPase activities, as well as in Ca^{2+} buffering or exchange, but the possibility that TRPs themselves might be potential targets of modulation should be taken into account.

Four main findings concerning these channels are relevant in this context. The first is that *Xenopus* TRPC1, which can be activated by netrin and BDNF, was also found to form MscCa (Maroto *et al.*, 2005), appearing as a molecular integrator of chemical and physical stimuli. Interestingly, other members of TRPs have been found mechanosensitive (MS); TRPA1 has been proposed as a candidate for the transduction channel of vertebrate hair cells

(Corey *et al.*, 2004, but see Kwan *et al.*, 2006). The second outcome is that TRPC1 and TRPC3 coimmunoprecipitate and colocalize with caveolins, and all members of TRPCs have a conserved motif, adjacent to the first transmembrane domain in the cytosolic N-terminus, which is similar to the caveolin-1 binding region. TRPCs have also binding domains for calmodulin, as well as for PLC and scaffolding proteins (Kiselyov *et al.*, 2005), indicating that they can be localized within Ca^{2+} signaling microdomains (Ambudkar, 2006). The third interesting finding is that regulation of TRPC5 in response to growth factors involves rapid (within few minutes) and reversible insertion of vesicles containing constitutively active channels into the plasma membrane. In principle, this phenomenon enables the membrane to accomplish a tight spatiotemporal control of Ca^{2+} influx (Bezzerides *et al.*, 2004). The last relevant outcome is the ability of TRPCs to exist as heterotetramers, both when heterologously expressed and *in vivo*, resulting a wide range of channel subtypes (Hofmann *et al.*, 2002; Strubing *et al.*, 2003).

V. PHYSICAL GUIDANCE CUES AND ROLE OF MECHANOSENSITIVE ION CHANNELS

The means by which physical interactions at the cell–substrate interface can guide cell movement has been investigated for many years. Cells show different morphologies and motility rates on substrates of given chemical properties but different rigidities (Pelham and Wang, 1997; Lo, 2006). Moreover, it has been shown that substrate topography alone contains neurite guidance information (Rajnicek *et al.*, 1997). The complexity of the cell responses to mechanical stimuli can be appreciated in some excellent reviews (Hamill and Martinac, 2001; Ingber, 2006), which present mechanotransduction as a cascade of multiscale events in which forces are unevenly distributed in order to force specific target molecules, while protecting most other cellular components. Prestress of membrane domains appears as a key factor that enables rapid responses to mechanical deformation. Environmental mechanical stimuli can directly affect intracellular targets since communication by means of physical signals seems to be as important as that carried out by chemical messengers, in mechanisms of cellular interaction with the substrate (Wang *et al.*, 1993). On the other hand, mechanical stimuli from the substrate can be transduced via MS proteins (receptors, channels, and enzymes). The adhesive interactions, mediated by cell surface receptors that bind to ligands in the ECM and on other cells, trigger intracellular signaling events that regulate different cellular functions, including cell growth and differentiation (Clark and Brugge, 1995; Condic and Letourneau, 1997). There is increasing evidence that signaling via adhesion takes two forms:

inside-out signaling (regulation of expression, conformation, and affinity of the receptors) and outside-in signaling (triggering of intracellular responses by ligand occupation of surface adhesion receptors; Hynes, 1992). Dynamics of integrin-mediated adhesions depends on local tension. Thus, application of force to focal adhesions strengthen the cytoskeletal anchorage, increasing the activation of integrin signaling (Geiger and Bershadsky, 2002). In keeping with this, a substrate-dependent calcium dynamics has been reported (Gomez *et al.*, 2001). MS channels appear to be involved in both cell–substrate and cell–cell interactions. On the one hand, MscCa of epithelial keratocytes regulate cell movement, mediating detachment of the cell margin (Lee *et al.*, 1999). On the other hand, mechanical forces applied to adherens junctions of human gingival fibroblasts activate MscCa and increase actin polymerization (Ko *et al.*, 2001).

To better understand the mechanisms by which cells transduce changes in membrane tension into different biochemical responses which regulate growth, we should explain how different signals are integrated inside the cell. In particular, growth cone membranes undergo large changes in tension during their dynamics (Lamoureux *et al.*, 1989) and a link between pulling mechanical tension and neurite elongation has been demonstrated in cultured hippocampal neurons (Lamoureux *et al.*, 2002).

The conceivable involvement in neurite elongation of MS channels, as tension-dependent modulators of membrane voltage, has already been put forward (Sigurdson and Morris, 1989). However, the failure to evoke macroscopic K^+ currents, in response to various mechanical stresses applied to the growth cones, raised doubts about the physiological relevance of single-channel MS currents (Morris and Horn, 1991). Cytoskeleton may provide a mechanoprotective shock absorber and this can be one of the factors which accounts for the failure to elicit MS currents from cells expressing MS channels (Wan *et al.*, 1999; Ko and McCulloch, 2000). More recently, the hypothesis of a role of MS channels in neurite growth has been newly supported. For example, gentamicin, which blocks single-channel currents of MscCa in leech neurons, was found to increase their axon outgrowth in culture, as shown in Fig. 1 (Calabrese *et al.*, 1999). In the last few years, the hypothesis was strongly supported by the convergence of two fields of investigation: the study of the molecular basis for calcium-dependent axon pathfinding and that concerning the molecular identification of MscCa. On the one hand, as reported above, TRPC cation channels have been found to play essential roles in the control of neurite length and growth cone morphology (Greka *et al.*, 2003) as well as in the growth cones guidance (Li *et al.*, 2005; Shim *et al.*, 2005; Wang and Poo, 2005). On the other hand, TRPC was reported to form MscCa of vertebrate cells. In frog oocytes, the protein responsible for MscCa was identified as TRPC1, and liposome

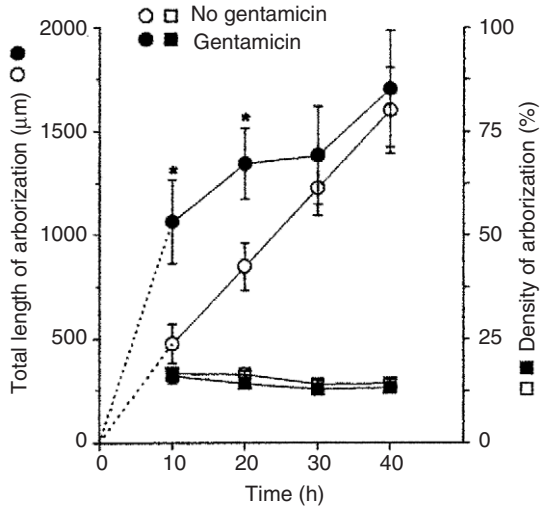

FIGURE 1 Effects of 200-μM gentamicin on the total length and density of arborization of leech AP neurons, at different times after plating. Each point of the plot is expressed as mean values of at least 11 cells ± SEM. The statistical significance of the differences between the means ($p < 0.025$ with the Mann-Whitney test) is denoted by an asterisk. Reproduced with permission from Calabrese *et al.* (1999).

reconstitution showed that this channel can be gated by tension developed purely in the lipid bilayer (Maroto *et al.*, 2005). TRPCs are activated in response to G-protein–coupled receptor activation and/or depletion of intracellular Ca^{2+} stores (Minke and Cook, 2002; Montell *et al.*, 2002). Three activation mechanisms have been hypothesized. First, a direct link between IP_3R and TRP channel; second, the action of a diffusible second messenger released from the Ca^{2+}-depleted endoplasmic reticulum; third, a fusion of vesicles containing constitutively active TRPs, induced by Ca^{2+} release. Which mechanism is used by which channel subtype is a matter of intense debate (Putney and McKay, 1999; Beech, 2005). Whatever the mechanism involved in the TRPC1 response to G-protein–coupled receptor activation, Maroto *et al.* (2005) demonstrated that membrane stretch alone can activate TRPC1. Thus, the multiplicity of activation makes these channels suitable to integrate chemical and physical stimuli, meeting the requirements for a context-dependent sensor.

Other findings are consistent with the idea that also other MS ion channels are involved in neurite growth. It has been demonstrated that NGF-TrkA regulates the ENaC expression in PC12 cells and that blocking protein transcription blunts neurite formation (Drummond *et al.*, 2006).

VI. ION CHANNELS AS MOLECULAR INTEGRATORS

The previous section pointed out the ability of TRPC1 to integrate different stimuli. The multimodal activation is emerging as a notable common feature of MS, TRP channels. In the nervous system of the leech *Hirudo medicinalis*, we have identified large conductance cation channels, activated by negative pressure applied to the membrane in inside-out configuration or by hypotonic swelling in the cell attached (Pellegrino *et al.*, 1990). These channels are expressed by mechanosensory cells as well as by neurons not involved in sensory mechanotransduction. Both selectivity and $[Ca^{2+}]_i$-imaging studies have shown that these channels admit cations and exhibit a substantial Ca^{2+} permeability (Calabrese *et al.*, 1999; Barsanti *et al.*, 2006b). Their pharmacological features are similar to those of typical MscCa of vertebrate cells, in particular, gentamicin produces a complete voltage-dependent block (Calabrese *et al.*, 1999). Both cell bodies and growth cones of leech neurons growing in culture express MscCa. Interestingly, two activity modes differing in kinetics and single-channel subconductances were identified. The first, denoted as spikelike (SL) mode, was mainly displayed in membrane patches excised from freshly desheathed quiescent cell bodies, while the second, called multiconductance (MC) mode, was commonly found in cultured cell bodies and mainly in growth cones (Pellegrini *et al.*, 2001). As previously reported, we found that addition to the culture medium of gentamicin, a nonspecific blocker of MscCa, which does not affect voltage-dependent Ca^{2+} currents in the leech neurons, increased the neurite extension in culture (Calabrese *et al.*, 1999). MS channels of leech neurons have been further characterized as polymodal cation channels: both SL and MC increase their open probability with depolarization (Menconi *et al.*, 2001) and with intracellular acidosis, while only SL was activated by intracellular calcium in the range 1–10 μM (Barsanti *et al.*, 2006b). MC activity is quickly and robustly increased by intracellular ATP in excised membrane patches, whereas intracellular cAMP is capable to slowly overcome the ATP activation to reach a complete inhibition, as illustrated in Fig. 2 (Barsanti *et al.*, 2006a). Thus, these channels exhibit typical biophysical and pharmacological features of TRPs, with the clear-cut ability to integrate. In this context, the major new finding is the powerful antagonistic modulation by intracellular ATP and cAMP. The different time dependence of the two regulations might enable these channels to participate in the interplay between intracellular Ca^{2+} and cAMP.

Other ion channels which are MS and display multimodal properties might be involved in cell motility. For example, recombinant N-type VDCCs expressed in HEK cells have been reported to be MS (Calabrese *et al.*, 2002). Among the K^+ channels expressed in neuronal tissues, it has been also hypothesized that some members (TREK-1 and TRAAK) of the 2P/4TM

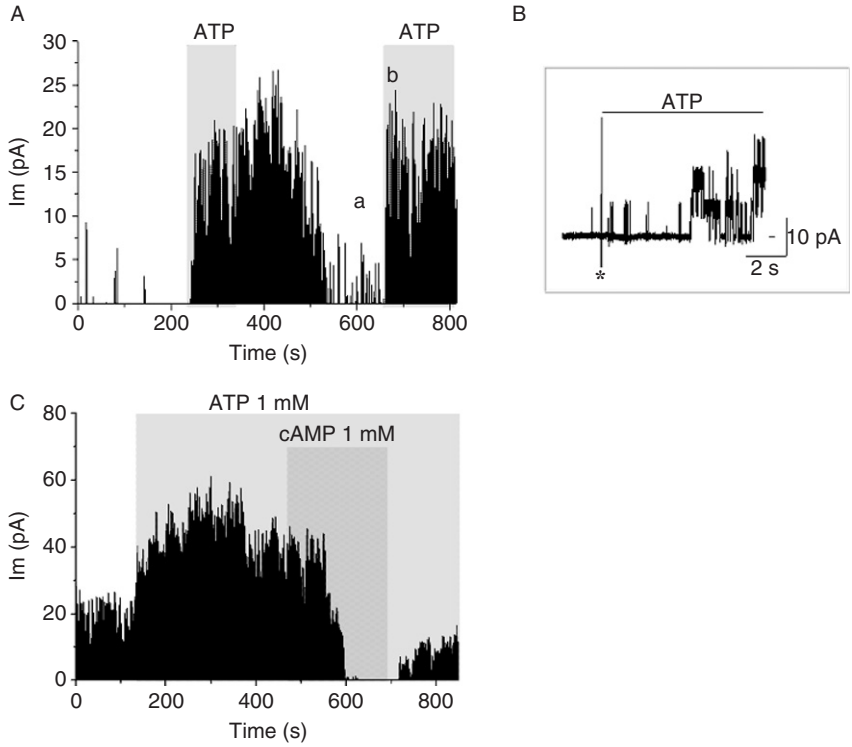

FIGURE 2 The cytoplasmic side of a membrane patch containing four MscCa of leech AP neurons was perfused twice with 1-mM MgATP. The membrane potential was held at +80 mV. Columns in the plot (A) represent the mean patch current calculated from 1-s-long consecutive data segments. Trace (B) illustrates the transition between a and b to estimate the activation delay. The asterisk (*) marks the opening of the electrovalve in the solution changer. (C) Addition of cAMP during sustained activation by ATP produced a slow, complete, and reversible inhibition of eight MscCa. The membrane potential was held at +80 mV. The plot shows the mean patch current measured at consecutive intervals of 1 s. Modified from Barsanti *et al.* (2006a).

structural class of mammalian K^+ channels might be involved in cell growth (Maingret *et al.*, 1999). These channels are MS and exhibit polymodal activation (Patel and Honoré, 2001).

VII. CONCLUDING REMARKS

It is clear from this chapter that TRPCs are involved in neurite growth, with distinct mechanisms in different species, playing the general role of Ca^{2+} signal amplifier. Since the Ca^{2+} release from intracellular stores is involved

in signal amplification, the question arises whether cells need an additional amplifier in the plasma membrane. It is tempting to speculate that TRPCs because of their capabilities of integration and translocation are suitable for the special function of developing powerful Ca^{2+} responses in restricted membrane domains of temporary structures such as lamellae and filopodia. Much of the work discussed in this chapter indicates that MS ion channels should be included in the list of molecules that control the $[Ca^{2+}]_i$ homeostasis in the growing neurites. Excessive activation of MscCa is potentially cytotoxic; therefore, it is conceivable that they are controlled by various mechanoprotective mechanisms. These can consist in the cytoskeletal action, both as absorber structure and as regulator of prestress, as well as in metabolic modulation of the channels themselves. In addition, the multimodal activation of these channels can produce local effects, enabling them to work in a context-dependent mode, in membrane microdomains where different stimuli can be integrated. Although the field has recently made impressive progress, major questions remain to be answered. The next stages will be to clarify how MscCa contribute to the integration of different signals, such as Ca^{2+} and cyclic nucleotides, and how distinct patterns of oscillation of these messengers can be decoded by downstream effector molecules, to determine the growth cone behavior. It will be also important to investigate the relationships between the fusion of vesicles containing TRPCs and integrin dynamics, in order to explore the possible participation of MS ion channels in the reorganization of focal adhesions during cell movement.

NOTE ADDED IN PROOF

B. T. Jacques-Fricke, Y. Seow, P. A. Gottlieb, F. Sachs, and T. Gomez (*J. Neurosci*. **26,** 5656–5664, 2006) showed that inhibition of Ca^{2+} influx through stretch-activated channels, with gentamicin or GsMTx4, a peptide isolated from *G. spatulata* spider venom, enhances the rate of neurite extension of *Xenopus* neurons. These results are in keeping with those obtained with leech neurons. These authors also found that SKF-96365, which blocks TRPCs, slows neurite outgrowth in *Xenopus*. This suggests that different TRP channels can antagonistically modulate neurite growth.

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