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STRETCH-SENSITIVE ION CHANNELS OF NEURONAL GROWTH CONES

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INTRODUCTION

Different regions of the cell surface have spatially segregated functions and the physiological properties of each cell membrane domain are largely determined by quality and density of its ion channels. These protein molecules form persistent and selective aqueous pores, which allow currents of some inorganic ions to flow into or out of the cell. Ion channels fluctuate between closed and open states and their open probability is affected by different stimuli. Based on the source of free energy that controls gating, we know of three kinds of channels: voltage-, ligand- and mechanically-sensitive.

Mechanosensitive (MS) channels respond to membrane stress changing their open probability. In 1984 Guharay and Sachs (15) first described stretch-activated (SA) channels that mediate mechano-electric transduction. Then, various SA channels, differing from one another in conductance, ion selectivity and sensitivity to other forms of energy, have been found in most plant and animal cells, as reported in a number of comprehensive reviews available in the literature (29, 47, 48).

Besides the mechano-electrical transduction, other cellular functions, such as volume regulation, electrolyte homeostasis and motility involve SA channels.

This paper is intended to give an overview of the involvement of SA channels in cell motility, focusing attention on the experimental results that suggest a functional role of these mechanotransducers in neurite elongation.

I. *Classification of mechanosensitive ion channels.*

Mechanosensitive channels can be classified in four principal sub-groups, as regards their ion selectivity: K^+-selective, cation-selective, anion-selective and non-selective (47). As one might expect, also in the family of mechanosensitive channels there are some members which display some voltage- and ligand-sensitivity. Thus, stretch sensitivity was found in three channels that we know by other names, such as serotonin-inactivated K channels of *Aplysia* neurons (55), maxi K channels of the apical membrane of rat cortical collecting tubule (39) and NMDA channels of mouse central neurons (40).

Besides SA channels, stretch inactivated (SI) channels have been identified in several different tissues, such as distrophic muscle from mdx mice (13), snail

neurons (31) atrial myocytes (59), toad gastric smooth muscle (19) and astrocytes (9). SI channels decrease their open probability with membrane stretch. It has been demonstrated that SI channels enable neurosecretory cells of supraoptic nuclei to behave as intrinsic osmoreceptors (34). SA and SI often coexist in the same membrane domain and this complex can express peculiar functional properties.

II. *Activation mechanisms.*

Activity of MS ion channels can be affected by applying negative pressure of ~ 40-100 mmHg through the patch pipette or by inducing cell swelling, during hypotonic perfusion (Fig. 1 and 2).

Most MS channels have been studied with single-channel recording technique in at least one of the patch-clamp configurations (cell-attached, inside-out, outside-out), however, macroscopic mechanosensitive currents have been measured only in few preparations (10, 16, 17). In particular, Morris and Horn (30), performing both single-channel and whole-cell experiments on cultured molluscan neurons, were unable to elicit macroscopic mechanosensitive currents, although they could readily demonstrate mechanosensitive activity in the single channel experiments. These authors used the perforated patch technique, that minimizes both cytoplas-

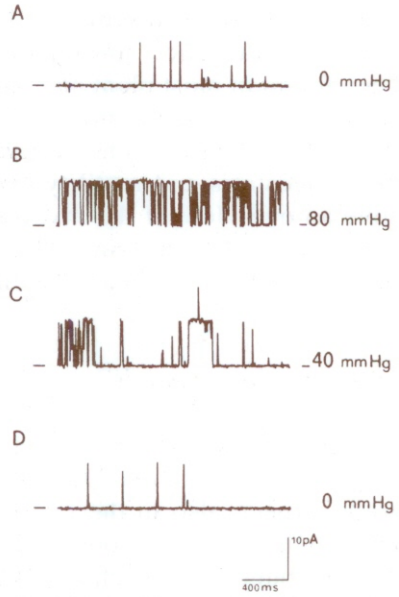

Fig. 1 - *Activation by negative pressure of two MS channels in a inside-out patch excised from a leech neuron.*

Recordings were performed in symmetrical 120 mM K^+ solutions, at a membrane potential of + 50 mV. Horizontal bar on the left of each record indicates the closed level. Outward currents are displayed as upward deflections. Records filtered at 1 KHz. (Modified from Ref. 41).

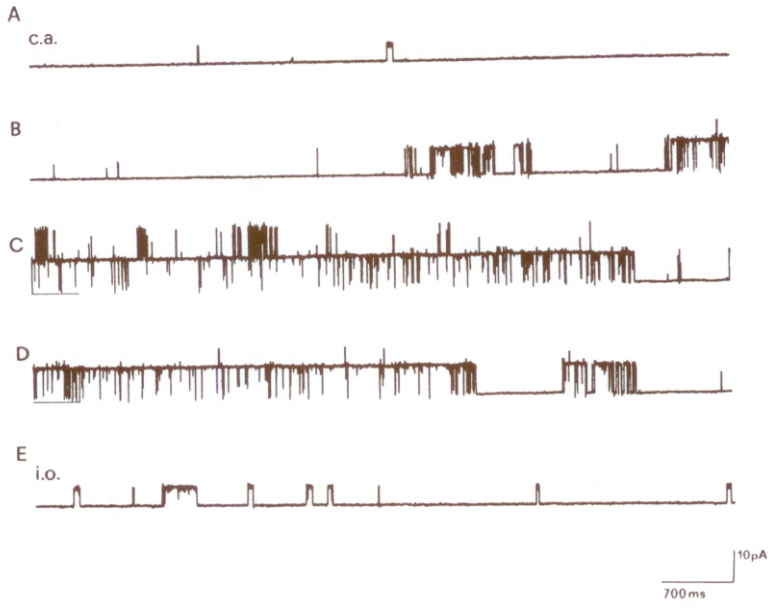

Fig. 2 - *Effect of hypotonic perfusion on two MS channels in a cell attached patch from the cell body of a leech neuron.*

The activity was recorded in isotonic condition (A), during hypotonic perfusion (B-D) and after excision (E), at a membrane potential of + 50 mV. After patch excision, MS channels became silent. The small conductance channel in traces A and E is a stretch insensitive leak channel. The signal was filtered at 1 KHz, outward currents are displayed as upward deflections. c.a.: cell-attached; i.o.: inside-out. (Modified from Ref. 41).

mic disruptions and cell dialysis. Accordingly, the failure to evoke macroscopic mechanosensitive currents was interpreted as a suggestion that MS ion channels become hypersensitive to mechanical perturbations only when the membrane-cytoplasm interface is modified by gigaohm seal formation. Therefore, while the existence of ion channels identified as MS is not in doubt, the relevance of their mechanosensitivity is not clear.

On the other hand, three main experimental results argue against the hypothesis that mechanosensitivity is an artifact of the patch-clamp technique (30). First, macroscopic MS currents can be consistently recorded in some preparations such as, for example, rabbit cardiac myocytes (17), bovine chromaffin cells (10) and mouse central neurons (40). Second, most identified channels such as ACh channels, Na channels and ATP-inhibited K channels, are insensitive to stretch (47). Third, SA channels of *Escherichia coli* cells can be reconstituted into different lipid membranes, independently of an underlying cytoskeletal network (53, 54).

At present it is not possible to provide a comprehensive scheme for the activation of MS channels. Based on recent findings (35), we must update the theory of MS activation, taking account of indirect mechanisms.

As far as the direct mode of activation is concerned, the mechanics of stretch

activation is widely discussed in a recent review (48). The membrane tension is the relevant parameter that controls the channel open probability and a tension of about 0.07 dyne/cm is sufficient to turn on SA channels. The force gating the MS channels is from the lipid bilayer, as clearly demonstrated by Martinac *et al.* (25). These authors found that amphipathic compounds slowly activate MS channels. These molecules would preferentially insert into the outer or inner lipid leaflet, producing a microscopic bending of the bilayer. This would increase the membrane tension (T), by increasing the radius (r) of the patch calotte, under a given applied pressure (p), as governed by the Laplace's law, T=rp/2.

In a recent paper on MS channels of the toad smooth muscle (35) the hypothesis of an indirect mode of activation has been put forward. It has been reported that SAK channels, stimulated by a train of suction pulses, gradually increase their activity and show increased baseline activity between pulses. Application of exogenous fatty acids, at micromolar concentration, to the cell surface, outside the patch pipette, increases stretch activation of these channels. Application, during continued stretch stimulation, of albumin, a sink for fatty acids, dramatically and reversibly decreases both the stretch activation and the persistent activity of the channel between suction pulses. The authors present their data as consistent with the hypothesis that fatty acids, produced by mechanically activated phospholipases, are the intermediaries in SA channels activation. Alternatively, fatty acids might simply modulate the stretch-induced activity of the channels.

Fatty acids have been recently proposed as a new class of first or second messenger molecules (36). They are covalently linked to other molecules to form the phospholipids that comprise a major portion of the lipid bilayer. Sources of fatty acids include those released from phospholipid by agonist-stimulated and basal phospholipase activities, as well as those delivered to cells via the circulation. A variety of fatty acids regulate the activity of specific ion channels both indirectly and directly. The indirect regulation involves enzymatic pathways, such as lipoxygenase pathway, that convert arachidonic acid to oxygenated metabolites (32). The direct action of fatty acids on ion channels has been clearly demonstrated with straightforward patch-clamp experiments, for a number of channel types, in a variety of preparations (1, 6, 20, 21, 37). In particular, the application of arachidonic acid or myristic acid to outside-out patches, excised from toad smooth muscle cells, promptly activates SAK channels. This action, observed in the absence of nucleotides, cyclic nucleotides and Ca^{2+}, can be also obtained in inside-out and cell-attached configurations, with slower onset and recovery of the response (37).

The direct action of fatty acids might be due to an interaction with the channel itself or to alterations of the lipid bilayer.

III. *MS ion channels as components of the growth cone membrane.*

The pattern of regional distribution of ion channels correlates with function, thus the density of most channels varies by several orders of magnitude in different

regions of the same cell. A salient feature of SA channels is that they tend to be ubiquitous and uniformly distributed, on the order of 1 per μm^2 (46). According to Sachs, the ability of MS channels to collect mechanical energy would be optimal at a density of about 1.5 per μm^2 (46).

Neurons not specialized as mechanosensors possess MS channels. They have been found both in somadendritic and in growth cone membranes. Various subtypes of MS channels have been reported in different preparations. Cultured snail neurons (49) contain K^+ selective SA and SI channels, of about 40 pS in physiological solutions. It is noteworthy that in the range of pressure values between -30 and -50 mmHg most SA are not activated, while most SI are inactive, so that the stretched region of the membrane has a minimum K^+ permeability. SA channels with large unitary conductance (200 pS in symmetrical K^+ solutions) have been identified in leech neurons (41). These channels are cation selective, with preferential selectivity for K^+. The activation threshold is about -40 mmHg, when suction is applied to the patch pipette (Fig. 1). In cell-attached configuration, these cation channels can be activated by cell swelling (Fig. 2). Using single neurone microcultures (42), SA channels have been localized in both cell-attached and inside-out patches of growth cone membrane (unpublished results).

At present, it is not known whether snail and leech SA channels respond to other forms of energy. On the other hand, both NMDA channels of mouse central neurons (40) and KS channels of *Aplysia* neurons (56) exhibit mechanosensitivity. The multiple mode of activation enables mechanosensitive channels to integrate endogenous and environmental stimuli. However, NMDA receptors differ from other SA channels as they cannot open in the absence of an agonist. Accordingly, they should be classified as ligand-sensitive, stretch-modulated channels.

Besides the general features, such as activation mechanism, ion selectivity and conductance, the kinetic behavior represents a fingerprint of each ion channel. A conventional kinetic analysis, that yields the minimum number of kinetic states, can be performed from the dwell time distributions (50). Most MS channels exhibit rapid bursting activity between sustained shut periods. In the proposed kinetic scheme there are one open state, with a mean open time that is relatively unaffected by stretch, and three closed states, in a linear arrangement; only the rate constant governing transitions out of the longest closed state is stretch sensitive (48). Although amphibian and leech SA channels display two closed and three open states, respectively, the main effect of increasing the membrane tension is a decrease of the third shut time constant (41).

Furthermore, the kinetic analysis can be implemented, in order to provide advanced tools to refine kinetic schemes (26, 43, 44). Recently, Vandorpe *et al.* (57) have used a set of such advanced computer programs to compare the kinetically identifiable states of KS channels, induced by FMRFamide and by stretch. These authors have established that FMRFamide and stretch effects converge on a single gating mechanism that is indistinguishable from that of the serotonin-sensitive K channel.

Both KS (3) and NMDA (40) channels have been localized in growth cones.

IV. *Functional role of MS ion channels in cell motility.*

MS channels are involved in typical phenomena of cell motility . A good example comes from studies by Medina and Bregestovski (27). They found that oscillatory changes in resting potential, associated with the cell-cleavage cycle of fish embryos, correlate with periodical changes in open probability of SAK channels. Furthermore, in plant physiology, it has been demonstrated that the transductive step of both geotropism and thigmotropism of roots is the opening of stretch-activated ion channels, which permits flow of Ca^{2+} across the plasmalemma (28).

Although direct evidence of a precise role of MS channels in neurite extension is still missing, a wealth of experimental evidence now supports the idea that key steps of the process are controlled by these mechanotransducers.

The typical sequence of events in neurite elongation includes three steps (14). In the first step, protrusion, filopodia form and the plasma membrane advances as flat veils between filopodia. In the second step, engorgement, membranous organelles and cytoplasm move forward into some of veils, moving forward the central region. The third step, consolidation, converts the spread central region into the cylindrical neurite, displaying axonal transport. Growth cone contains a pool of intracellular vescicles and membrane sacs which provide a source of membrane for insertion into the axolemma (7). The distinctive movements of a growth cone seem to involve a continual stream of surface material from its leading edge, a region specialized in producing cellular extensions like filopodia, back to its base, a region exhibiting micropinocytosis (4). The steady retrograde movement is due to actin associated with the plasma membrane (12). The protrusion and retraction of filopodia are mediated by polymerization and disassembly of actin filaments and by their translocation away from the leading edge.

Growth cone filopodia contain signal transduction mechanisms that allow autonomous responses, as demonstrated by intracellular Ca^{2+} increase and shortening, induced in isolated filopodia by depolarizing fields and excitatory neurotransmitters (8). Moreover, it has been put on a firm quantitative basis that growth cones in tissue culture have the capacity to develop mechanical tension and to pull against the axon (23). Using glass needles of known compliance, Lamoureux and colleagues made direct measurements of neurite force as a function of growth cone advance; they found a linear relationship between these two parameters and concluded that pulling growth cone provides a stimulus for growth, under standard culture conditions (23). Tension in the growth cone has been suggested to act as a trigger of assembly of structures such as microtubules and new actin cortex. In support of this view, a growth cone fixed at one end and towed passively produces many hundreds of microns of axon, apparently normal both ultrastructurally and functionally (5).

Of particular interest are some observations reported in papers that do not specifically deal with MS channels. First, interaction of growth cones with polycationic microbeads can trigger site-directed actin filament assembly (11). Second, dilution of culture medium produces a sudden proliferation of rapidly growing branches

from growth cones, while a hyperosmotic shift results in an increase in filopodial length (4), rather than a decrease, as expected from an osmotic mechanism providing the driving force for filopodia (38). Both polycationic microbeads and dilution are expected to affect MS channels.

Different stimuli which affect neuronal growth cone dynamics, such as neurotransmitters and depolarization, converge on the mechanisms of regulation of intracellular Ca^{2+} (2, 33, 33 bis, 61). Transient changes of $[Ca^{2+}]_i$, in turn, regulate the filopodial morphology (45). It is also clear that Ca^{2+}-sensitive α–actinin and actin filaments are involved in Ca^{2+}-dependent filopodial movements (52). A key role in the growth cone motility is played by a growth-associated protein, designated GAP-43 (51), whose expression increases dramatically in most neurons during axon development and regeneration. GAP-43 is neuron-specific and is one of the most abundant proteins in growth cone membranes. It is initially synthesized as a soluble protein that becomes attached to membranes post-translationally, after addition of fatty acids chains, through thioester linkages to cysteine residues 3 and 4. GAP-43 molecules are dynamically deacylated and reacylated in growth cones. Fatty acylation provides a hydrophobic domain and enables the protein to anchor (51). Since the fatty acids derive from endogenous membrane phospholipids by the actions of phospholipases, this raises the possibility that the modification of the protein and its redistribution may be modulated by transmembrane signals, mediated by phospholipases. In addition, neutralization by divalent cations of some charged domains on GAP-43 enlarges its hydrophobic region (51). Thus, fluctuations of $[Ca^{2+}]_i$ that occur in growth cones can modulate the GAP-membrane interaction.

Several lines of evidence demonstrate that GAP-43 directly contributes to filopodial extension. Experiments of transient transfection of non-neuronal cells resulted in the expression of membrane associated GAP-43 and in the abnormal formation of filopodial processes (62). Moreover, the diffusible intercellular messenger nitric oxide, that rapidly and reversibly inhibits the growth of neurites, inhibits long-chain fatty acylation of GAP-43 (18). Finally, GAP-43 can bind to CAM (cell-adesion molecules) as a function of $[Ca^{2+}]_i$ in the physiological range (10^{-7} - 10^{-5} M) (24). A role of GAP-43 in membrane fusion during neurite outgrowth is suggested by both properties and subcellular localization of this protein.

Another key role is played by microtubule-associated proteins (MAPs). Binding of calcium/calmodulin to MAPs competitively inhibits the association of MAPs with tubulin (22, 58). Accordingly, the free-calcium concentration can favour or suppress microtubule instability, in response to signal transduction pathways from those extracellular cues, such as growth factors, neurotransmitters and adhesion molecules, that control calcium level fluctuations.

We can now take account of the above evidence to suggest a possible role for MS channels in growth cone motility. MS channels could profoundly affect local $[Ca^{2+}]_i$. Cation channels would act to increase $[Ca^{2+}]_i$, by depolarizing the cell membrane and by passing Ca^{2+} ions, while activation of SAK channels, would decrease influx of Ca^{2+} through its voltage-gated channels, by stabilizing the

membrane potential near the potassium equilibrium potential (E_k). Changes of $[Ca^{2+}]_i$, in turn, regulate growth cone movements. There is a wide consensus that the membranes of growth cones experience large changes in tension during their extension and retraction. However, it is worth pointing up that the developed tension is not expected to be uniform for at least two reasons. First, the components of the cytoskeleton display a differential distribution in the growth cone domains; second, the adhesive features of the substratum determine a discrete pattern of interaction with growth cone. It follows that MS channels, unevenly activated, migth finely perform a local and contingent regulation of $[Ca^{2+}]_i$. In addition, not only can MS channels be regulators of a second messenger, but they can also transduce both membrane tension and those environmental stimuli that affect phospholipase activity.

SUMMARY

This paper unifies and outlines some important points made by recent experimental work, dealing with MS ion channels and growth cone motility. The considerable work carried out in cell biology, neurochemistry and membrane biophysics in the past decade allows us to imagine the neurite growth in term of known molecules and chains of simple mechanisms. On the other hand, it is becoming increasingly clear the real complexity of the growth cone behavior.

There remain important gaps in our understanding of the role of MS channels in growth cone motility. At present, a major limitation for physiological work is the lack of specific blockers. An encouraging development of molecular genetic studies of *Drosophila* SAK channels (60) will probably provide valuable tools for straightforward experiments.

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