CHAPTER 4

Properties and Mechanism of the Mechanosensitive Ion Channel Inhibitor GsMTx4, a Therapeutic Peptide Derived from Tarantula Venom

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I. OVERVIEW

Mechanosensitive ion channels (MSCs) are found in all types of cells ranging from *Escherichia coli* to morning glories to humans. They seem to fall into two families: those in specialized receptors such as the hair cells of the cochlea and

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those in cells not clearly differentiated for sensory duty. The physiological function of the channels in nonspecialized cells has not been demonstrated, although their activity has been demonstrated innumerable times *in vitro*. The only specific reagent to block MSCs is GsMTx4, a 4-kDa peptide isolated from tarantula venom. Despite being isolated from venom, it is nontoxic to mice.

GsMTx4 is specific for an MSC subtype, the nonselective cation channels that may be members of the TRP family. GsMTx4 acts as a gating modifier, increasing the energy of the open state relative to the closed state. Surprisingly, the mirror image D enantiomer of GsMTx4 is equally active, so mode of action is not via the traditional lock and key model. GsMTx4 probably acts in the boundary lipid of the channel by changing local curvature and mechanically stressing the channel toward the closed state.

Despite the lack of definitive physiological data on the function of the cationic MSCs, GsMTx4 may prove useful as a drug or lead compound that can affect physiological processes. These processes would be those driven by mechanical stress such as blood vessel autoregulation, stress-induced contraction of smooth muscle, and Ca^{2+} loading in muscular dystrophy.

II. INTRODUCTION

Mechanical sensitivity plays an essential role in cells and higher organisms. Specialized exteroceptors transduce external stimuli such as sound, vibration, touch, and local gravity. Interoceptors regulate for the voluntary musculature and the filling of the hollow organs, as in regulation of blood pressure. MSCs may serve as sensors for local control of blood flow, regulation of cell volume, deposition of bone, and so on (Sachs and Morris, 1998; Hamill and Martinac, 2001). The channels may also drive some of the hormonally coupled mechanical systems, such as renin-angiotensin and atrial natriuretic peptide that regulate fluid volume. They may also serve some of the autocrine and paracrine transducers that generate second messengers such as endothelin (ET) (Ostrow *et al.*, 2000; Ostrow and Sachs, 2005).

Mechanical transduction is ubiquitous and is present in cells of all phyla. In higher plants, mechanical transducers guide root, stem, and leaf growth in response to gravity. MSCs serve as sensory transducers in bacteria and other microorganisms where they may be the sensors for volume regulation (Martinac, 2001; Sachs, 2002). The fact that *E. coli* has as many as five different MSCs argues for their functional importance (Sachs, 2002). Mechanical transduction is presumed to have developed early in evolution, probably as a necessity for controlling cell volume when conducting metabolism in a membrane-limited compartment.

The pervasive nature of MSCs indicates that we will find genetic and environmental factors that create human pathologies related to MSC malfunction. For example, studies on dystrophic muscle cells show that the dystrophin mutations lead to weakening of the membrane, thereby activating a Ca^{2+} influx through MSCs (Patel *et al.*, 2001; Yeung *et al.*, 2003). This influx can be blocked by gadolinium (Yeung *et al.*, 2003) and the peptide GsMTx4 (Yeung *et al.*, 2005).

Although mechanical sensitivity of ion channels appears across phyla (Martinac and Kloda, 2003), there appears to be no homology associated with the primary structure. For example, in *E. coli*, the two dominant mechanosensitive channels MscL and MscS (generically noted MSCs) differ fundamentally in sequence and structure. MscL is a pentamer (Chang *et al.*, 1998) and MscS is a heptamer (Bass *et al.*, 2003), and the primary sequences have little homology. The only well-characterized MSC cloned from eukaryotes is the K^+ selective 2P channels such as TREK-1 (Patel *et al.*, 2001), and these channels have no sequence homology to the bacterial channels. Thus, mechanosensitivity, while universal, does not obey the delightful homologies of many of the voltage- and ligand-gated channels, an example of convergent evolution. Moreover, from a mechanistic viewpoint, bacterial MSCs are almost certainly different from eukaryotic channels given the difference in cytoskeletal structure that influences the mechanics. What we learn from bacteria does not necessarily apply to eukaryotic MSCs. Within the phenotypic MSC families, however, there appears to be a useful discriminator—channels that are stimulated by stress in the cytoskeleton and extracellular matrix (Corey, 2003a,b), as in the cochlea, and those that are stimulated by stress in the bilayer, as in bacterial MSCs.

The intrinsic mechanosensitivity of channels depends on dimensional changes between the closed and open states (Sachs and Morris, 1998; Sukharev *et al.*, 1999; Hamill and Martinac, 2001). One detailed kinetic study of MscL shows that these prototype channels require at least eight rate constants to characterize the gating reaction, but only a single rate constant is significantly sensitive to tension (Sukharev *et al.*, 1999). While most MSCs appear to be stretch-activated channels (SACs), stretch-inactivated channel (SIC) activity has also been described (Vandorpe *et al.*, 1994), although this may be an artifactual response from SACs subjected to stress at rest (Honore *et al.*, 2006). Only recently have cationic MSCs from nonspecialized tissues, TRPC1, been cloned or reconstituted (Maroto *et al.*, 2005).

Mechanosensitivity is not the domain of a particular class of ion channels. Any channel that changes dimensions between closed and open states may be mechanosensitive, in the same way that most ion channels are voltage sensitive. Ligand-gated and voltage-sensitive channels have been shown to be mechanically sensitive (Gu *et al.*, 2001; Calabrese *et al.*, 2002; Laitko and Morris, 2004;

Morris, 2004; see Chapter 11). The generality of mechanosensitivity poses an intriguing problem in evolution: how to design structures with the necessary flexibility to support large conformational changes (Jiang *et al.*, 2003a,b) while avoiding unnecessary mechanical activation.

MSCs are phenotypically described as channels whose kinetics are substantially altered by mechanical input. The key parameter that makes channels mechanosensitive is that they have large dimensional changes between the closed and open conformations (Howard and Hudspeth, 1988; Sachs *et al.*, 1998; Sukharev *et al.*, 1999; Hudspeth *et al.*, 2000; Hamill *et al.*, 2001; see chapter by Markin and Sachs in this series, vol. 58, pp. 87–119). MSCs are embedded in a heterogeneous, non-Newtonian mechanical structure consisting of the extracellular matrix, the bilayer and its embedded proteins, and the cytoskeleton (Garcia-Anoveros and Corey, 1996; Gillespie and Walker, 2001). The stress that activates MSCs may come from the lipid bilayer (Akinlaja and Sachs, 1998), but that tension depends on the cytoskeleton, the preparation geometry, and the boundary conditions (Suchyna and Sachs, 2004). Despite this complexity, it appears that MSCs from nonspecialized tissues are activated by tension in the lipid bilayer (Sukharev *et al.*, 1994; Suchyna *et al.*, 2004). The tension depends on the cortical structure, since the applied stresses are borne by cytoskeletal elements in parallel and in series with MSCs (Wan *et al.*, 1995; Mills and Morris, 1998). This is also true not only for patch clamp experiments but also for global stimuli such as hypotonic or shear stress. To define an absolute sensitivity of a channel requires working in lipid bilayers where the stress is reasonably well defined (Sukharev *et al.*, 1999; Suchyna *et al.*, 2004).

The physiological function of MSCs in nonspecialized tissues has not been demonstrated. One common ground (Sachs, 2002), however, may be volume regulation (Christensen, 1987), although preliminary data using GsMTx4 suggests that the volume sensor is not a cationic MSC (Hua, Gottlieb, and Sachs, in preparation). In general, to test the physiological role of a channel requires that one activate or inactivate the target by nonphysiological stimuli. Pharmacologic agents are one approach and genetic knockouts the other (Corey, 2003b). There is only one specific pharmacological agent for MSCs to date: GsMTx4 and its mutants (Suchyna *et al.*, 2000). The search has been hampered, in part, by technical difficulties in defining the stimulus (Hamill and McBride, 1995; Besch *et al.*, 2002). While stimulators for electrically gated and ligand-gated channels have long been available (ALA Scientific Instruments Inc., Westbury, NY), until recently none were available for mechanically gated channels. However, even with controlled pressure stimuli for patch clamp experiments, defining the stimulus that actually reaches the channel requires knowledge of preparation geometry and constitutive mechanical properties of the cell cortex (Sachs and Morris, 1998; see chapter by

Markin and Sachs in this series, vol. 58, pp. 87–119), factors that are generally unknown.

The focus of this chapter is the peptide GsMTx4 and how it affects MSCs. The initial part will describe our effort to characterize its chemical and structural properties. We then detail the biophysical properties as well as issue of specificity. These results have shown the peptide to work on MSCs in an unconventional manner. Finally, we survey some potential therapeutic uses that may emerge for this peptide and similar compounds which remain undiscovered.

III. PROPERTIES AND SPECIFICITY OF GsMTx4

A. Biochemical and Structural

A number of years ago we branched into natural products biochemistry with a blind search of invertebrate venoms to find anything that affects MSCs. Screening required an outside-out patch preparation that retained mechanical sensitivity, but most cell types lose mechanical activity after excision. Consequently, we developed an assay using primary rat astrocytes that were reasonably stable (Suchyna *et al.*, 2000). At the same time, we developed a pressure clamp to control cell stimulation (Besch *et al.*, 2002). These efforts led to the identification and isolated the peptide GsMTx4 from *Grammostola rosea*, the only peptide or other drug known to specifically affect cationic MSCs. The properties of this peptide were analyzed in detail and are summarized below. Interestingly, no scorpion venoms and only one other spider venom had an effect on MSCs.

The correct sequence for the peptide GsMTx4 was deduced by isolating the GsMTx4 gene. A cDNA copy was made from RNA extract derived from the glands of *G. spatulata* and was sequenced (Ostrow *et al.*, 2003). The protein exists in a pre-proform (Fig. 1) where the first 21 amino acids (light gray) are a predicted signal sequence and are removed during protein translocation (bimas.dcrt.nih.gov/molbio/signal). The last two amino acids are glycine-lysine (dark gray), a known site for amidation (Gomez *et al.*, 1984). The arginine adjacent to the active peptide molecule (prosequence in black) is presumably the cleavage site (indicated by arrow) to release active GsMTx4 peptide (gray).

Next, we chemically synthesized GsMTx4 peptide with a phenylalanine amide at the C-terminal and determined the conditions for folding (Ostrow *et al.*, 2003). Reduced peptide (10^{-4} M) is dissolved in 0.1 M Tris pH 7.8 containing glutathione (oxidized:reduced; 1:10 mM). The reaction is carried out at RT and completed within 24 h. Folding is easily achieved from the

FIGURE 1 cDNA of the gene encoding GsMTx4 with the open reading frame. The full length protein is processed. The first 21 amino acids are removed as a signal sequence. The protein is cleaved at an arginine (arrow) and the last two amino acids are removed during amidation (Ostrow *et al.*, 2003).

misfolded peptide as well. The synthetic peptide is indistinguishable from wild-type peptide in all physical-chemical properties. MALDI-MS reveals a mass of 4093.9 $[M^+H^+]$ while GsMTx4 from venom had a mass of 4094.0 $[M^+H^+]$. Reverse phase liquid chromatography shows identical retention times, and co-injection of the two compounds produced a single peak. Circular dichroism for both peptides is similar, having minima at around 192 and 202 nm. A comparison of the NMR spectra of the wild-type peptide with that of the synthetic peptide demonstrated that the structures were in good agreement (Ostrow *et al.*, 2003). Finally, the peptide produced the same physiological response on SACs when compared to the spider peptide.

The primary sequence of GsMTx4 has six cysteines. The spacing of these residues is identical to a family of peptides called inhibitory cysteine knot (ICK). These peptides adopt stable three-dimensional structures by forming three disulfide bonds (Pallaghy *et al.*, 1994). The structure was confirmed by NMR spectroscopy (Oswald *et al.*, 2002) and revealed that the peptide is amphipathic (Fig. 2).

GsMTx4 is shown with its hydrophobic face in green and the charged residues in red (negative) and blue (positive) and illustrates the hydrophobic surface (at bottom) surrounded by mainly positive charges. This architectural design suggests that the peptide binds to membranes using its hydrophobic face to penetrate the lipid bilayer. While the net charge of GsMTx4 is +5, the charge itself is not the essential component since polylysine has no effect on MSCs. The distribution of charge close to the membrane, however, is probably essential for activity.

GsMTx4 is homologous to other peptides derived from spider venoms. Figure 3 compares a number of peptides that have been recently isolated for

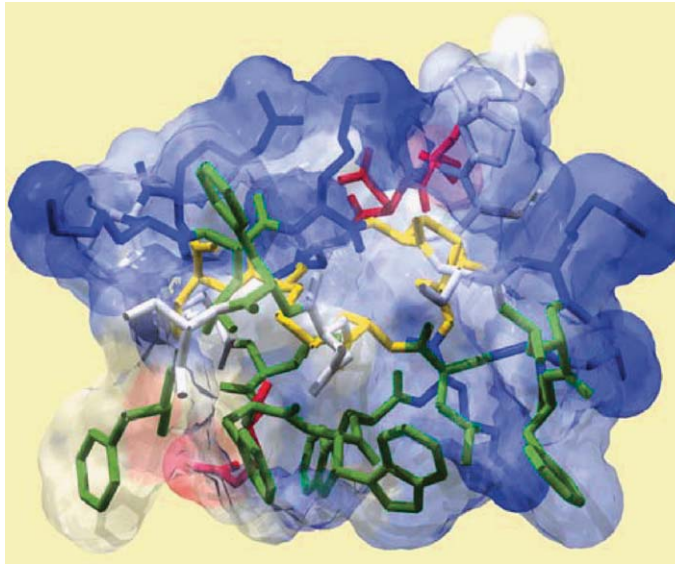


FIGURE 2 Solution structure of GsMTx4 determined by NMR spectroscopy. Disulfide bonds are shown in yellow, the hydrophobic residues are shown in green, the acidic residues in red, and the basic residues in blue. GsMTx4 has a predicted net charge of +5 at neutral pH (Suchyna *et al.*, 2004).

GsMTx4	GCLEFWWKCNPNDDKCCRPKLKCSKLFKLCNFSF-NH2
SgTx1	TCRYLFGGCKTTADCCKHLACRSAGKYCAWDGTF
HnTx1	ECRYLFGGCKTTSDCCKHLGCKFRDKYCAWDFTFS
VsTX1	ECGKFMWKCKNSNDCCKDLVCSSRWKWCVLASPF

FIGURE 3 Sequence comparison of four peptides derived from spider venoms. All peptides belong to the ICK structural family and they are all gating modifiers. Hydrophobic residues are indicated in green. Charged residues are shown with red for acidic residues and blue for basic. Cysteine residues are in yellow and boxed.

various spiders. While their targets are different, as they inhibit various voltage-gated channels, they nonetheless have features that are common. All the peptides listed belong to the ICK structural family. All of these peptides, including GsMTx4, are thought to be gating modifiers, and all of them have aromatic groups in hydrophobic regions at the C- and N-termini, which in the three-dimensional structure form a hydrophobic face, that enables them to interact with membranes.

The peptide's ability to bind to membranes was suggested as a means for understanding how GsMTx4 inhibits MSCs (Suchyna *et al.*, 2004). Simultaneously, the MacKinnon group demonstrated that the peptide VsTx1, a gating modifier for voltage-dependent K^+ channels, also partitions into membranes to effect inhibition of these channels (Lee and MacKinnon, 2004). Similar observations were made by the Swartz group for the SgTx1 peptide (Lee *et al.*, 2004; Wang *et al.*, 2004). The ability to partition into membranes appears to be an essential feature for these gating modifiers.

The peptide's ability to interact with membranes was measured using model systems of large unilamellar vesicles (LUVs). On the basis of previous work (White *et al.*, 1998), the Ladhokin group developed a sensitive method for determining partitioning of peptides into LUVs using differential iodine quenching (Posokhov *et al.*, 2006). They showed that the peptide partitions well into zwitterionic vesicles such as PC or PE ($\Delta G = -6$ kcal/mol) and to anionic vesicles (75%PS, $\Delta G = -8$ kcal/mol). Considering the charge of the peptide, the modest increase in binding in the presence of PS vs PE argues for a low effective charge and a lack of additivity between hydrophobic and charged interactions (Posokhov, Gottlieb, and Ladhokin, submitted for publication).

To answer the question of the peptide's orientation, the accessibility of tryptophanyl groups was examined in the absence and presence of vesicles. In solution, both Cs^+ and I^- quenched the fluorescence showing that the tryptophan residues were not shielded and that electrostatic interactions were negligible. This was similar to other soluble peptides containing tryptophan residues. However, in the presence of vesicles to the peptide's tryptophans were protected from quenching, consistent with the hydrophobic face being buried in the lipids.

The depth of the peptide penetration into the lipid bilayer was also measured using brominated lipids. Bromine, that quenches tryptophan fluorescence, was anchored in the acyl chains at different distances from the headgroup of the phospholipid, and the location of maximal quenching provided a measure of the depth of penetration-GsMTx4 penetrates about ~9 Å from the center of the lipid bilayer (Posokhov, Gottlieb, and Ladhokin, submitted for publication).

B. Biophysical and Mechanistic

The phenomenological dissociation rate constant was determined by averaging the current from several patches during washout, and curve fitting the recovery data (Fig. 4) (Suchyna *et al.*, 2000). To measure the association rate constant, the channels were activated with a three-second pressure step and GsMTx4 was rapidly applied after one second into the stimulus (<10-ms

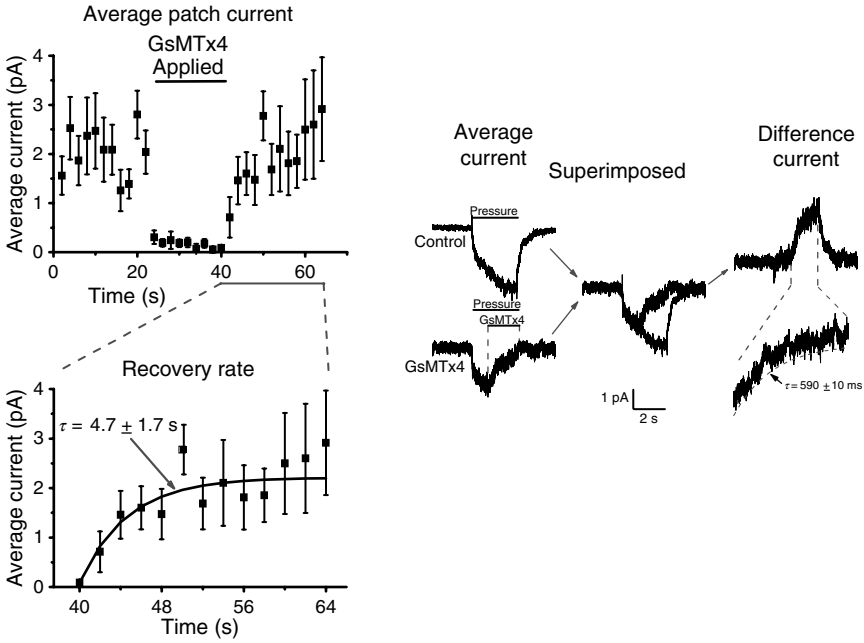
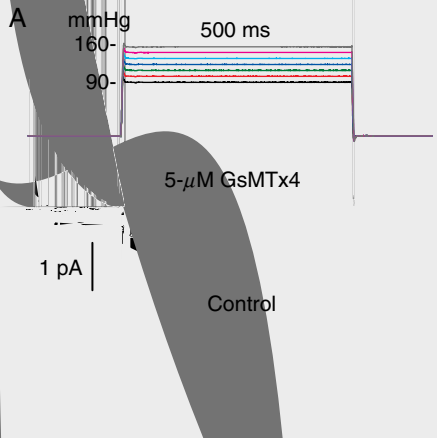


FIGURE 4 Left panel: dissociation rates of GsMTx4 from adult astrocyte SAC currents using steps of 500 ms at 2 s intervals and averaged ($n = 7$). The current recovered after ~5 s of washout. The data were fitted with a single exponential to obtain the dissociation rate. Right panel: association rates of GsMTx4 to astrocyte MSCs. Average SAC currents with GsMTx4 applied are subtracted from the control currents to generate a difference current. The difference current was fit with a single exponential to determine the association rate (Suchyna *et al.*, 2000).

switching time). The exponential decay of this current was taken to be the association rate (Fig. 4). We calculated the equilibrium constant from $K_D = k_d/k_a$. The kinetic method of determining K_D is important since it is hard to prevent rundown or run-up of the patch over time, particularly with the need to produce saturation of the starting stimulus. The association rate was $3.3 \times 10^5\ M^{-1}\ s^{-1}$, the dissociation rate $\sim 0.2\ s^{-1}$, providing $K_D = 0.5\ \mu M$. This is a lower affinity than many of the well-known channel-inhibiting peptides, but the association rate is similar to that for other peptides (Lewis and Garcia, 2003).

We then tried to determine the mechanism of action. There are two basic types of channel inhibition: kinetic (gating) and pore blocking (Suchyna *et al.*, 2004). Pore blockers will produce a reduction in current but retain the same time course. Gating modifiers will change the time course. Figure 5 shows the effect of GsMTx4 on SACs. GsMTx4 shifts the activation curve to the



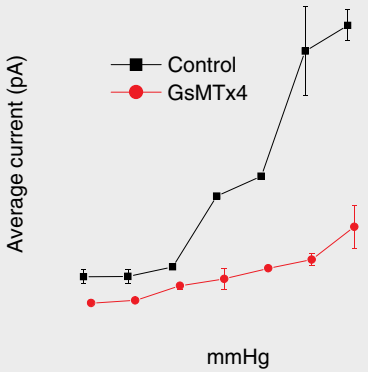


FIGURE 5 GsMTx4 is a gating modifier since the block can overcome inhibition by increasing suction. (A) Ensemble average current of an outside-out patch from rat astrocytes as a function of suction in the presence and absence of GsMTx4. (B) The peak average currents plotted as a function of suction showing a rightward shift for activation indicative of a gating modifier (Suchyna *et al.*, 2004).

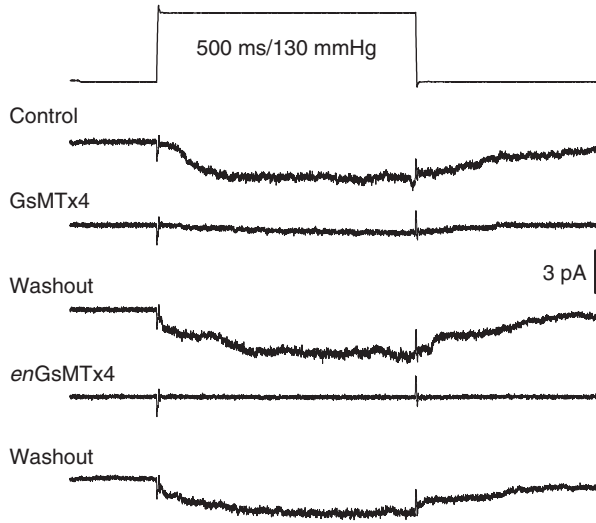


FIGURE 6 The effect of *en*GsMTx4 on MSCs from rat astrocytes. Outside-out patches were first treated with GsMTx4, then washed out and the enantiomeric peptide was then added and shown to have the same effect. The inhibition by *en*GsMTx4 was reversible (Suchyna *et al.*, 2004).

inhibitory response for MSCs. There are two basic models, global and local. The first is that the peptide alters the general properties of the lipid bilayer in which the channel is embedded. This would be analogous to the effect of Gd^{3+} on the lipid bilayer (Ermakov *et al.*, 1998, 2001), which in turn alters channels activity in the membrane (Yeung *et al.*, 2003). If this were the case, we might expect GsMTx4 to change membrane capacitance. Indeed, when Gd^{3+} was perfused onto rat astrocyte membrane, there was a substantial decrease in capacitance (Suchyna and Sachs, unpublished data). At a saturating dose concentration of GsMTx4, there was no effect on capacitance. These observations were supported by work on red blood cells, where their very soft bending rigidity makes them sensitive to the presence of amphipaths (Sheetz and Singer, 1974; Sheetz *et al.*, 1976). Iwasa (personal communication) showed that GsMTx4 at saturating doses did not alter the cells shape. This suggests that despite the partitioning of GsMTx4 into the membrane (above), the alteration in local shape or global number density is too small to significantly alter the general membrane structure.

A second possibility is that the peptide resides at the interface between the protein and lipid membrane. To ascertain whether the peptide was in proximity of the channel, we took advantage of the peptide's charge (+5). When the peptide is next to the channel, within a Debye length of the conducting pore, cations passing through the channel will experience the peptide's electric field and there should be a reduction in current as the local concentration of cations

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bilayers (Betanzos *et al.*, 2002). The simplest gating model is that the closed and open channels differ in size so that in a tension field T, the two conformations differ in energy by $T\Delta A$, where ΔA is the difference of in-plane area between close and open conformations. This change in dimensions need not be large; for the 2P channel TREK-1, it has been estimated to correspond to a change in radius of ~1 Å (Honore *et al.*, 2006). We think GsMTx4 alters the curvature of the boundary region of the channel. In a thermodynamic sense, GsMTx4 alters the line tension surrounding the channel or applies a torque that prestresses the channel in a manner that favors the closed state (see chapter by Markin and Sachs).

The effect of GsMTx4 on Gramicidin (gA) in lipid bilayers underscores the role of physical effects on drug activity. The gA is a tubular peptide having the thickness of a lipid monolayer (~1.5 nm). Two monomers, one from each leaflet of the bilayer, associate end to end forming a channel. The kinetics of channel creation and dissociation are affected by the thickness of the bilayer and the bending stiffness of the lipids (Andersen *et al.*, 1999) and these properties can be altered by amphipathic agents or by far field tension (Hwang *et al.*, 2003).

GsMTx4 affects gA gating (Fig. 8). Figure 8A shows that the addition of GsMTx4 to either AgA(15) or gA^{-}(13), two Gramicidin analogues, independently increased the opening rate, indicating that GsMTx4 increases dimer association as though the membrane is thinner. Similar to our observations for eukaryotic channels, GsMTx4 also decreased the unitary conductance for both gA analogues (Fig. 8B). The fact that the amplitude histograms display a monomodal distribution suggests a uniform association of GsMTx4 with the channels, but the concentration dependence of the unitary current further suggests that the average population of GsMTx4 around the channels is time dependent, but averaged at the observation time scale, that is there is rapid exchange.

The lifetime of the dimers (channels) increased 25-fold at 400-nM GsMTx4 (Fig. 8C). The qualitative effect of GsMTx4 was independent of the length of the gA as well as the chirality of the sequence (Fig. 8D). These results can be explained by the effect of GsMTx4 on local lipid curvature at the gA-lipid interface, reducing the hydrophobic mismatch by making the membrane look thinner.

C. *Specificity*

GsMTx4 is remarkably specific given its apparent achiral site(s) of action, although it may be useful to remember that phospholipids are chiral. GsMTx4 does not affect voltage-gated channels in rat astrocytes (Suchyna *et al.*, 2000) or rabbit heart (Baumgarten, 2004; Sachs, 2004a). As shown in

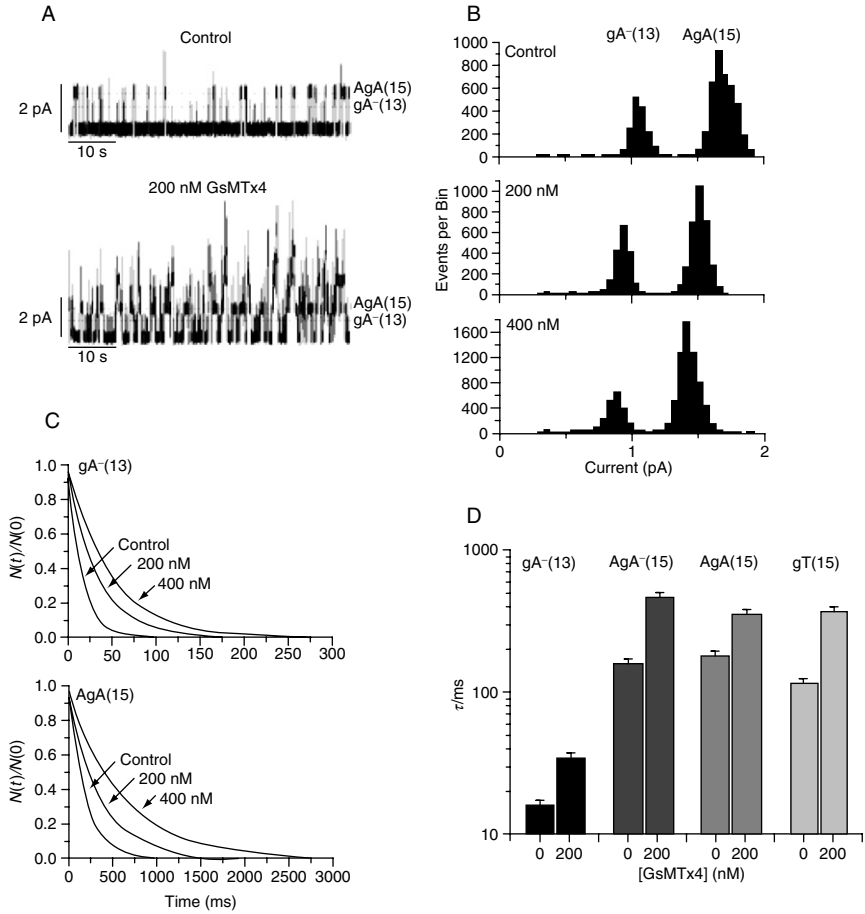

FIGURE 8 The effect of GsMTx4 on gramicidin gating. (A) Raw channel data showing GsMTx4 increased open channel activity. (B) GsMTx4 decreased unitary conductance. (C) Open channel lifetimes increased in the presence of GsMTx4, that is, a decrease in the closing rate. (D) GsMTx4 increases of the lifetime of gA channels that vary in structure, including enantiomers (Suchyna *et al.*, 2004).

Fig. 9, it has no measurable effect on the action potential of resting isolated atrial myocytes from the rabbit (Bode *et al.*, 2001) so that all of the channels and transporters responsible for the action potential are unaffected.

A residual question in the literature has been the background activity of MSCs in cells. This could not be tested with a patch since adhesion of the patch to the glass always produces significant tension (Akinlaja and Sachs, 1998). However, adding GsMTx4 to resting cells has little effect on cell electrophysiology,

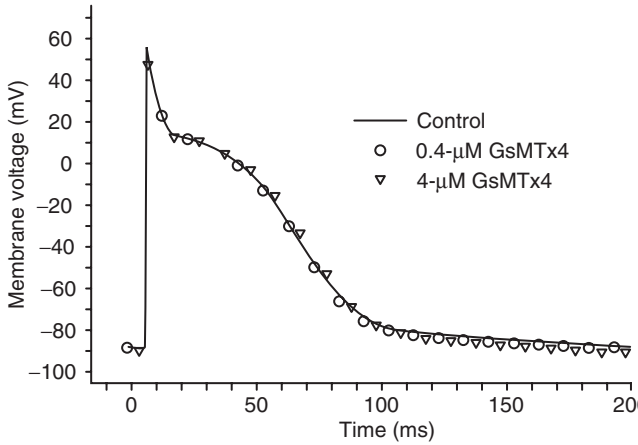

FIGURE 9 GsMtx4 does not affect the action potential of resting rabbit atrial cells at 8× the K_D. Perforated patch (amphotericin), $n = 5$, 37 °C (Sachs, 2004a,b).

although it does have effects on spontaneous contractions of *mdx* muscle cells (Suchyna and Sachs, 2007). It has no significant effect on the beating rabbit or sheep heart (Kalifa, Jalife, Gottlieb, and Sachs, unpublished data), unless the heart is distended (Bode *et al.*, 2001). Preliminary experiments have shown that intravenous injection of GsMTx4 into mice (at ~4× the K_d of MSCs in patch clamp) produces no behavioral change except for a slightly reduced water consumption over 24 h, perhaps by acting on the thirst center of the hypothalamus (Oliet and Bourque, 1996) or in the kidney.

Even among MSCs, GsMTx4 is specific as it does not affect auditory transduction (Marcotti *et al.*, 2001) that may originate in a TRPA1 channel (Lin *et al.*, 2005), or the activity of TREK channels (E. Honore, personal communication). The basis of this specificity is unclear, but probably arises from the channel itself; the only channels that respond to GsMTx4 are those for which local stress in the boundary layer couples to the energy of the closed or open states.

IV. CELLULAR SITES FOR GsMTx4

A. TRPC1 Channel

One of the major issues still outstanding is the identity of the channels affected by GsMTx4. Although we are able to study various aspects of mechanosensitive channels, the exact protein stimulated by membrane

tension is being studied by a number of laboratories. The work by Hamill, Martinac, and collaborators suggests the TRPC1 channel is mechanically activated (Maroto *et al.*, 2005), making it an excellent candidate for inhibition by GsMTx4. The transient response potential (TRP) ion channels were discovered in *Drosophila* when a mutation in the *trp* gene altered the organisms response to light (Minke, 1977; Montell *et al.*, 1985). Human homologues were soon identified and ~30 genes have been isolated, representing 6 families of ion channels (Clapham, 2003; Beech *et al.*, 2004). One of these families is called the TRPC (Classical) and is a class of channels found predominantly in smooth muscle. TRPC1 presumably can form a homomeric, nonselective, cation channel, although it may also form heteromers with different TRP subunits, making TRPC1 a critical ion channel in physiological function (Clapham, 2003; Beech *et al.*, 2004).

Maroto *et al.* (2005) introduced the human TRPC clone into oocytes, isolated the membrane proteins, fractionated them, and reconstituted an active fraction into liposomes. The mechanosensitivity they observed is associated with a protein whose molecular weight is consistent with that of TRPC1 and is inhibited by antibodies to TRPC1 and antisense RNA. The introduction of the TRPC1 gene into the Chinese hamster ovary (CHO) cell line seemed to support the ability of TRPC1 to be stretch sensitive.

A natural question is whether GsMTx4 inhibits TRPC1. Following Maroto *et al.* (2005), we transfected CHO channels with TRPC1 DNA and were able to observe stretch sensitivity. Outside-out patches from transfected cells were inhibited by 5 μM GsMTx4 acting as gating modifier (Gottlieb, Suchyna, Bowman, and Sachs, unpublished results; Hamill also found that GsMTx4 inhibits TRPC1, personal communication). However, more recent work on CHO and other cell lines in our laboratory has revealed that in all cell types there is a significant endogenous population of mechanosensitive channels exhibiting activity similar to the purported TRPC1. These channels may not be immediately visible in the recordings of native cells, but they are revealed by treatment with cytochalasin. As shown by Patel (Lauritzen *et al.*, 2005), transfection with the gene for GFP is not a good control for the effects of transfection-the cell cares about what it is expressing. Expression of active or inactive 2P ion channels causes extensive modification of the cytoskeleton, whereas transfection by GFP has no effect. To distinguish whether the channel under observation incorporates the subunit used for transfection requires the candidate be modified in a way that makes it different from the endogenous channels. As a start we made dual mutants of the pore domain of TRPC1 that has a different ion selectivity from the endogenous channel, and when expressed in COS7, these channels can be blocked by GsMTx4 (Gottlieb *et al.*, in preparation).

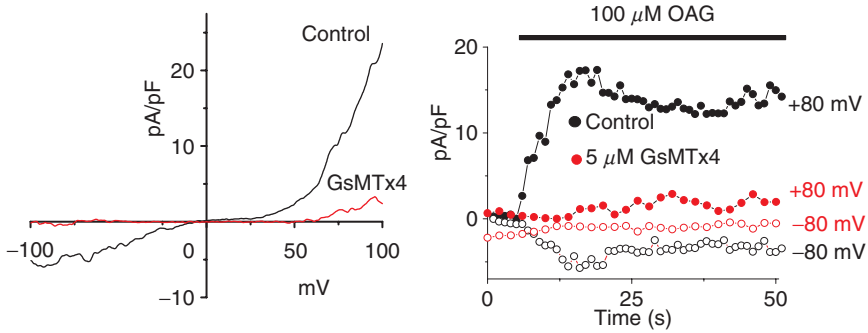

FIGURE 10 TRPC6 channel is blocked by GsMTx4 in whole-cell recording using voltage ramps. TRPC6 was overexpressed in CHO cells and activated by membrane permeable DAG derivative, OAG. Left panel shows the *I*/*V* curve taken near the peak of the OAG response and shows the inhibition by GsMTx4. The outward rectifying current is typical for TRPC6. The right panel shows the time course of OAG stimulation at ±80 mV. For illustrating GsMTx4 effects, the cells had been incubated for ~30 min in 5-μM GsMTx4 (red symbols) prior to OAG. Courtesy M. Spassova.

B. TRPC6 Channel

TRPC6 channels are abundant in cells of tissue exposed to hydrostatic pressure changes such as vascular smooth muscle and glomerular podocytes where they may play a role in modulating myogenic tone. Recent data from Don Gill's laboratory (Spassova *et al.*, 2006) shows that TRPC6, a stretch sensitive channel found in smooth muscle, is blocked by GsMTx4. TRPC6 is a nonselective cation channel that is activated by receptor-induced phospholipase C (PLC) activation probably via a direct effect of diacylglycerol (DAG). GsMTx4 inhibited both stretch- and DAG-activated currents that argue for a common mechanism of activation (Fig. 10) such as stress within the boundary lipids.

V. POTENTIAL THERAPEUTIC USES FOR GsMTx4

A. Cardiac Myocytes and Atrial Fibrillation

The connection between mechanical stress and excitability in the heart (known as mechanoelectric feedback or MEF) has been appreciated for over a century (Kohl and Sachs, 2002; Kohl *et al.*, 2003). The initiation of arrhythmias is a classic effect of stretch on the heart (Baumgarten, 2004;

Sachs, 2004b). MEF is not a trivial modulation as the ventricles of an intact heart can be reliably stimulated to repeatedly contract in phase with inflation of an intraventricular balloon (Franz *et al.*, 1992). Numerous studies have shown that stretch can affect both the cardiac action potential and whole-cell currents (Sachs, 2004a), and MSC activity (Bett and Sachs, 1997; Hu and Sachs, 1997). Recently, a mechanosensitive maxiK channel was cloned from chick heart (Tang *et al.*, 2003). Voltage-sensitive ion channels in the heart have also been shown to be mechanosensitive (see Chapter 11). Given the presence of MSCs, pharmacological agents that affect mechanosensitivity can produce therapeutic effects.

It has been known for a long time that atrial fibrillation is potentiated by mechanical stress. If that is driven by cationic MSCs, the stretch-induced effects may be inhibited by GsMTx4. In Langendorff-perfused rabbit hearts, GsMTx4 at 170 nM effectively blocked atrial fibrillation potentiated by atrial dilation (Fig. 11; Bode *et al.*, 2001). GsMTx4 provided complete protection against fibrillation up to a diastolic pressure of 15 cmH_2O, and shortened the duration of fibrillation at all pressures. In the unstressed rabbit heart, ionotropy and the action potential were unaffected by GsMTx4. At nearly 10× the K_d for MSCs, the action potential of resting atrial cells was unaffected suggesting that there is little MSC activity at rest (Sachs, 2004a).

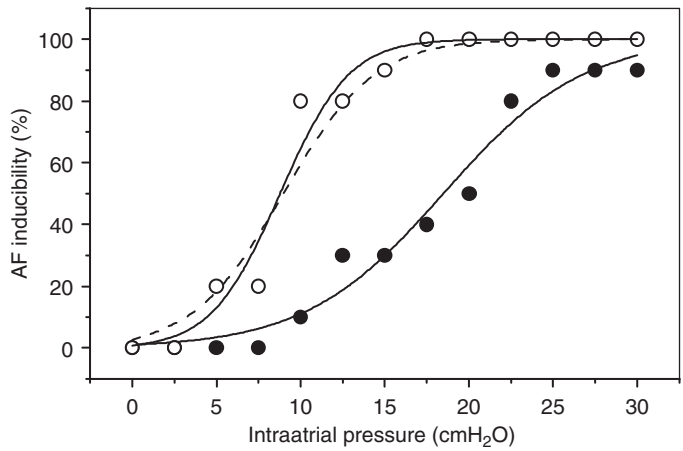

FIGURE 11 Block of dilation potentiated atrial fibrillation (AF) by 170-nM GsMTx4 in the Langendorff-perfused rabbit heart. AF was induced by a burst of rapid electrical stimulation, and we have plotted how often that resulted in AF lasting longer than 60 s. Inflation increased the probability of AF (open symbols), and GsMTx4 shifted the dose–response curve to higher pressures (filled symbols; Bode *et al.*, 2001).

The work on human heart tissue has also shown minimal effect of GsMTx4 on ionotropy (Kockskamper *et al.*, submitted for publication).

Marban's group described an arrhythmia with after depolarizations that occurred on termination of high-speed pacing, and these were not blocked by any inhibitors of known channels (Nuss *et al.*, 1999). Baumgarten and Clemo reproduced this result with isolated rabbit ventricular and atrial (Sachs, 2004a), and surprisingly found that GsMTx4 blocked the after depolarizations. Perhaps high-speed pacing overloads the SR causing spontaneous Ca^{2+} release, and in turn this causes local contractions. The nonuniform stresses then activate the cationic MSCs. This example of GsMTx4 diagnosis may be a prototype of how these compounds can be used for physiological diagnosis of MSC function, as well as serving as lead compounds for drug development.

B. Muscular Dystrophy

An intriguing connection between MSCs and physiological function exists for the muscular dystrophies that involve mutations in the dystroglycan complex (DGC), but not MSCs. The DGC is a multiprotein group that connects the internal actin cytoskeleton to the extracellular matrix through attachments to laminin (reviewed in Blake and Martin-Rendon, 2002), and presumably provide structural support to the bilayer. Mutations in this complex lead to different forms of muscular dystrophy that vary in severity, susceptible muscle groups, and the age of phenotypic onset (Khurana and Davies, 2003). Duchenne muscular dystrophy (DMD) is the most common of these disorders, caused by mutations to the large cytoskeletal protein dystrophin. Most dystrophies associated with mutations to proteins in the DGC are characterized by elevated internal Na^+ and Ca^{2+} levels, and excessive protein degradation (Ruegg *et al.*, 2002). Thus, a major strategy for acute therapy development can be to improve Ca^{2+} homeostasis (Khurana and Davies, 2003).

Franco-Obregon and Lansman (2002) have studied the effect of pressure stimuli on patches from mouse *mdx* myoblasts (a mouse DMD model). They demonstrated that the SACs observed in wild-type cells become hyperactive in *mdx* cells with prolonged stimulation, as though a reinforcing structure was being disrupted in the *mdx* cells. They proposed a decoupling mechanism whereby disruption of viscoelastic elements associated with the membrane led to a loss of membrane tension regulation and increased channel open probability. Suchyna and Sachs (Suchyna *et al.*, 2004) and others (Hamill *et al.*, 1992; Niu and Sachs, 2003) have shown that MSCs not only activate with stretch but inactivate with time (Honore *et al.*, 2006). In rat astrocytes, channel activation closely follows the change in membrane tension, but when inactivation is disrupted, the channels stay open during continued stress since

inactivation, which is only accessible through the open states, depends on cytoskeletal integrity (Suchyna *et al.*, 2004). The lack of membrane reinforcement by dystrophin can cause hyperactivity of SACs in dystrophic muscle. Yeung *et al.* (2003) linked the pathology of *mdx* muscle fibers with the influx of cations through SACs. They showed that both Gd^{3+} and streptomycin, nonspecific blockers of SACs, reduce elevated Na^+ levels in stretched *mdx* fibers, while having little effect on normal muscle fibers. They postulated that the cation selective SACs in *mdx* fibers are responsible for the increased influx of Na^+ and Ca^{2+} (Na^+ influx can lead to additional Ca^{2+} influx through the Na^+/Ca^{2+} exchanger; Bosteels *et al.*, 1999; Arnon *et al.*, 2000). Further work by Allen's group evaluated the effect of GsMTx4 on *mdx* muscle fibers and Ca^{2+} leakage currents that are presumed to be responsible for muscular atrophy (Yeung *et al.*, 2005). Single muscle fibers isolated from *mdx* mice were isolated and subjected to a series of stretched (eccentric) tetanic contractions while measuring intracellular calcium with flou-3 and confocal microscopy. In the absence of GsMTx4, there was a slow rise in resting intracellular Ca^{2+} levels after tetanic stimulation, and both Ca^{2+} influx and the force generated during tetanus were reduced. GsMTx4 reduced this effect by reducing Ca^{2+} influx through hyperactive SACs, and this in turn inhibited the increased force.

C. Astrocytes and Gliosis

Astrocytes are the most abundant cells in the CNS. They secrete substances that act on themselves, on other types of glia, vascular endothelial cells, and neurons (Araque *et al.*, 2001). They play an important role in the induction and maintenance of the blood-brain barrier (Zonta *et al.*, 2003) and communicate intracellular Ca^{2+} signals bidirectionally with neurons (Carmignoto, 2000; Zonta and Carmignoto, 2002; Burgo *et al.*, 2003).

SACs exist in fetal, neonatal, and adult astrocytes (Ding *et al.*, 1988; Bowman *et al.*, 1992; Sontheimer, 1994; Ostrow *et al.*, 2001) suggesting they may have a role in a variety of brain pathology including glial tumors (Ostrow and Sachs, 2005). Glial stretch not only activates SACs but also releases endothelin-1 (ET-1). ET is a peptide and potent autocrine mitogen produced by reactive and neoplastic astrocytes. The relationship between mechanical stretch and ET production in other cell types, combined with the observation that ET-1-positive reactive astrocytes appear in the *mechanically deformed* periphery of CNS pathology, led us to hypothesize that mechanical stress may regulate ET secretion in astrocytes through SACs.

Ostrow and Sachs demonstrated that mechanical stimulation of adult rat astrocyte cultures causes a dramatic increase in ET-1 production and secretion

into the culture medium (Ostrow *et al.*, 2000). These experiments represented the first demonstration that the *astrocytic* ET system can be directly stimulated by cell deformation (Fig. 12). Since virtually all brain pathology is associated with some degree of mechanical deformation of the surrounding parenchyma, and ET induces the proliferation of astrocytes (and glioma cells), the possibility arises that *mechanical induction of the ET system* represents a general pathway for activating/augmenting astroglial proliferation. ET-1 exerts its mitogenic effects in most cell types, including astrocytes and glioma cells, through Ca^{2+} homeostasis, possibly explaining how ET potentiates its own production and secretion (Supattapone *et al.*, 1989; Marsault *et al.*, 1990; Supattapone *et al.*, 1990; Marin *et al.*, 1991; Holzwarth *et al.*, 1992; Jacques *et al.*, 2000). Nonmechanical stimuli including growth factors and cytokines can also enhance ET expression in astrocytes (Masaki *et al.*, 1991; Tasaka *et al.*, 1991; Ehrenreich *et al.*, 1993; Brunner, 1995; Goto *et al.*, 1996). Regardless of the stimulus, alterations in cell Ca^{2+} seem to be the common link in regulating the ET system in all cell types (Yanagisawa *et al.*, 1989; Masaki *et al.*, 1991;

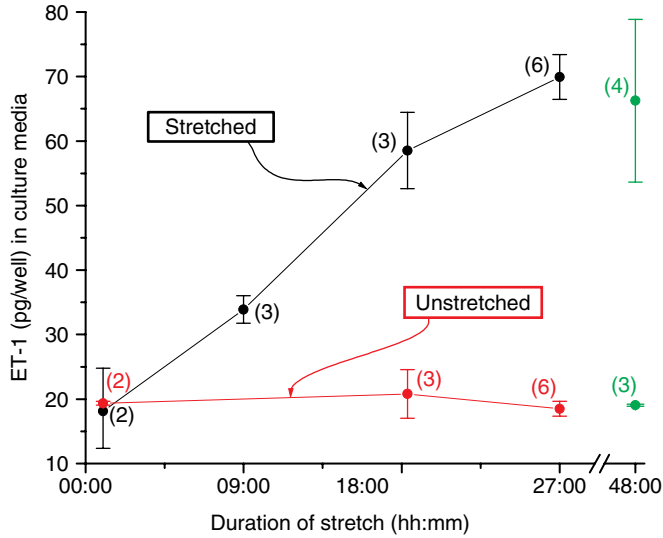

FIGURE 12 Effects of cyclic stretch (0.1 Hz, 0–20%) on ET-1 secretion. Both control and stretched cell cultures were incubated in *Starvation Medium* (0.1% serum) for 24 h prior to stretching. The media was aspirated and replaced with fresh *Starvation Medium* 5 min before stretching commenced. The green points represent data from a separate 2-day stretching protocol on cells of a similar age and passage number. (Numbers in parentheses are the number of culture wells per data point. Each well was run in duplicate in the ET-1 ELISA. Error bars = ±standard error; Ostrow *et al.*, 2000).

Kitazumi and Tasaka, 1992; Corder *et al.*, 1993; Brunner *et al.*, 1994; Morita *et al.*, 1994; Brunner, 1995). The Ca^{2+} effects seem to ultimately depend on activation of the mitogen-activated protein kinase (MAPK) pathway and the induction of PLC and protein kinase C (PKC) (Goto *et al.*, 1996).

An inhibitor of SACs could provide a novel therapeutic approach to gliomas by controlling a mitogen whose overproduction is associated with pathological states. Supportive of this view, Vaz *et al.* (1998) demonstrated that the degree of edema following head trauma is reduced by the nonspecific SAC inhibitors Gd^{3+}, amiloride, and gentamicin, implying that SACs are activated by swelling. Having already demonstrated that GsMTx4 blocks SACs in astrocytes, we showed it also inhibits the stretch-induced ET-1 production by astrocytes. It is striking that GsMTx4 does not alter basal ET secretion in the absence of stretch, so that its action is specific. As expected, stretch-induced ET secretion was also decreased by lowering extracellular Ca^{2+} (Ostrow, 2003).

Gliomas could grow in a positive feedback cycle where cell division increases local stress, causing ET-1 secretion and further division. An inhibitor of SACs might block or at least slow glioma development. Particularly appealing is the use of the D form of GsMTx4, since it is nonhydrolyzable, significantly hydrophobic, and might be able to be administered orally. It is not yet known whether GsMTx4 will cross an intact blood–brain barrier, but it is known that the barrier can be temporarily relaxed (Borlongan and Emerich, 2003).

D. Neurite Growth Extension

GsMTx4 was shown to stimulate neurite growth extension (Jacques-Fricke *et al.*, 2006). The importance of intracellular Ca^{2+} signals in the regulation of neurite outgrowth is well known, although the mode of entry is not well defined. To test whether calcium influx through MSCs is important for neurite growth, Gomez and colleagues used a series of agents, both specific and nonspecific, including GsMTx4, in an assay that directly measures neurite extension. Of all the reagents used, GsMTx4 was the most potent for stimulating neurite growth in *Xenopus* neurons. The role of GsMTx4 in this physiological response was shown to involve a reduction of Ca^{2+} influx through channels activated by cell swelling. The effective inhibition of GsMTx4 is not via global Ca^{2+} levels, meaning the mean cytoplasmic Ca^{2+} is unaffected by the presence of the peptide. Rather, GsMTx4 inhibits SACs in microdomains, a fact deduced by the sensitivity of the outgrowth to different Ca^{2+} buffers (Neher, 1998). The inactivation of these channels by GsMTx4 prevents activation of downstream processes that inhibit neurite outgrowth.

VI. CONCLUSIONS

GsMTx4 is the first compound known to specifically inhibit cationic MSCs in eukaryotes. The mechanism of action is a gating modifier, but it does not fit the familiar hand–glove model of peptide channel interactions since the D form is also active. The mode of action is probably to create stress within the boundary lipid region of the channel. GsMTx4 has begun to serve for the first time as a tool to study the physiological role of MSCs *in situ*. Since all physiology is subject to pathology, GsMTx4 may serve as a therapeutic tool or a lead compound to treat diseases resulting from defects in the mechanotransducing pathways.

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