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ORIGINAL ARTICLE

Gene transfer with a vector expressing Maxi-K from a smooth muscle-specific promoter restores erectile function in the aging rat

A Melman[1], G Biggs[1], K Davies[1], W Zhao[2], MT Tar[1] and GJ Christ[2]

[1]Department of Urology, Institute for Smooth Muscle Biology, Albert Einstein College of Medicine, Bronx, NY, USA and [2]Wake Forest Institute for Regenerative Medicine, Wake Forest University Baptist Medical Center, Winston-Salem, NC, USA

*Previous reports have demonstrated that gene transfer with the α, or pore-forming, subunit of the human Maxi-K channel (*hSlo*) restores the decline in erectile capacity observed in established rat models of diabetes and aging. Preliminary data from a human clinical trial also showed safety and potential efficacy in 11 men treated with the same plasmid construct expressing the Maxi-K channel. In all instances, the original plasmid was driven by the heterologous cytomegalovirus promoter which is broadly active in a wide variety of cell and tissue types. To more precisely determine the contribution of the corporal myocyte to the observed physiological effects* in vivo, *we report here our initial work using a distinct vector (pSMAA-*hSlo*) in which* hSlo *gene expression was driven off the mouse smooth muscle α-actin (SMAA) promoter. Specifically, older rats, with diminished erectile capacity, were given a single intracorporal injection with either 100 μg pVAX-*hSlo *or 10, 100 or 1000 μg pSMAA-*hSlo*, or vector or vehicle alone. Significantly increased intracavernous pressure (ICP) responses to cavernous nerve stimulation were observed for all doses of both plasmids encoding* hSlo*, relative to control injections. These data confirm and extend previous observations to document that smooth muscle cell-specific expression of* hSlo *in corporal tissue is both necessary and sufficient to restore erectile function in aging rats.*
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Keywords: *gene transfer; erectile dysfunction; pSMAA-*hSlo*; plasmid*

Introduction

Several epidemiological studies document that sexual dysfunction in aging men, manifesting as partial or complete erectile dysfunction, has a prevalence that is expected to increase in an aging US and world population.[1–4] A primary cause of erectile dysfunction associated with aging is a myopathy related to heightened contractility and/or impaired relaxation of the corporal smooth muscle and penile vasculature.[5,6] The result of these physiological changes is that endogenous relaxing mechanisms no longer are able to elicit a degree of smooth muscle relaxation sufficient to allow the increase in blood flow and enhanced intracavernous pressure (ICP) required for the initiation and maintenance of normal penile erection. Several changes at the anatomic and molecular level have been suggested to underlie these physiological changes, such as a reduction in smooth muscle content and increase in collagen content, reduction of nitric oxide production, associated with decreased eNOS and nNOS and increased iNOS expression, enhanced Rho-kinase activity, increased expression of tonic myosin isoforms and altered function, expression or regulation of ion channels.[7–13] Regardless of the precise cause, it is clear that enhancing corporal smooth muscle relaxation with on-demand pharmacotherapeutic drugs is insufficient to restore erectile capacity in many men with erectile dysfunction. Such observations suggest that gene transfer to correct relevant physiological pathways may be an attractive therapeutic strategy for the treatment of erectile dysfunction. The goal of gene transfer is to target molecular pathways that can restore the long-lasting capacity for spontaneous erections adequate for coitus in response to a natural endogenous signal.

In this regard, we previously reported that the intracavernous injection of naked plasmid DNA expressing the α or pore-forming unit of the human large-conductance, calcium-sensitive K channel, the Maxi-K channel (encoded by the *hSlo* gene), into the corpora of aged and streptozotocin-induced diabetic rats can ameliorate the decrease in erectile capacity normally observed in these *in vivo* rat models.[14–16] These studies were performed with a naked DNA vector, pcDNA-*hSlo*, in which *Slo* gene expression is driven by the cytomegalovirus (CMV) promoter, which functions in the majority of both human and animal tissues. A bacterial

Correspondence: Dr A Melman, Department of Urology, Institute for Smooth Muscle Biology, Albert Einstein College of Medicine/ Montefiore Medical Center, 3400 Bainbridge Avenue, Bronx, NY 10467, USA.
E-mail: amelman@aecom.yu.edu

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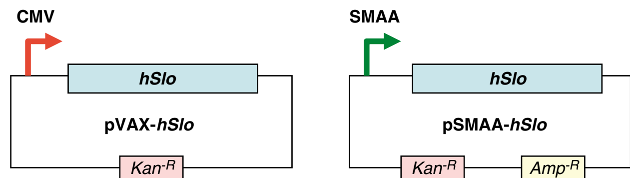

Figure 1 Diagram comparing the main features of pSMAA-*hSlo* and pVAX-*hSlo*.

selectable marker (the penicillin-resistance gene, *ampR*) is present in this plasmid. To address safety concerns related to penicillin sensitivity for the conduct of a Phase I human clinical trial, we recently subcloned *hSlo* into another commercially available vector, pVAX, which uses the same CMV promoter, but *ampR* is replaced by a kanamycin-resistance gene (*kanR*). In the recently completed Phase I study with pVAX-*hSlo* gene transfer there were no serious advents related to treatment and there were indications of potential efficacy.[17,18]

Despite these exciting preclinical and clinical results, the specific cell population targeted by pVAX-*hSlo* is uncertain. Therefore, to gain insight as to which cell type(s) is responsible for the *hSlo*-mediated restoration of erectile dysfunction, we designed a novel vector, pSMAA-*hSlo*, which contains a tissue-specific promoter (the mouse smooth muscle α-actin promoter (SMAA) Figure 1). The mouse SMAA promoter directed tissue-specific expression in all mammalian species thus far studied.[19–26] The purpose of this study was to evaluate the ability of this new construct to restore erectile function in an aging rat model of erectile dysfunction and to compare the results so obtained with that of our current construct, pVAX-*hSlo*. If the duration of action and efficacy are similar to preclinical studies with pVAX-*hSlo* and pcDNA-*hSlo*, then the use of pSMAA-*hSlo* may confer additional therapeutic and regulatory advantages, namely, enhancement of the safety profile of *Slo* gene transfer by limiting its action to smooth muscle cells only.

Results

In vitro *specificity of the SMAA promoter activity in human cell lines* in vitro

The SMAA promoter previously was shown to have smooth muscle-specific expression in rats and mice.[27,28] We extended these studies to look both at the expression of the *hSlo* gene from pSMAA-*hSlo* in a human-derived non-smooth-muscle human embryonic kidney (HEK) cell line and primary cultured corporal smooth muscle cells. As can be seen in Figure 2, the primers were specific to plasmid-derived *hSlo* since the control (pVAX transformed corporal cells) generated no product specific to *Slo*. Furthermore, the pSMAA-*hSlo* expresses the *hSlo* gene only in the corporal smooth muscle cell lines. In contrast, pVAX-*hSlo* drives expression of the gene in both cell types. Semi-quantitative determination of expression of plasmid-derived *hSlo* normalized to the housekeeping gene RPL19 and averaged for three replicates showed that relative to pVAX-*hSlo*, pSMAA-*hSlo* expressed approximately twice as much of the transcript in the corporal smooth muscle cells (Table 1).

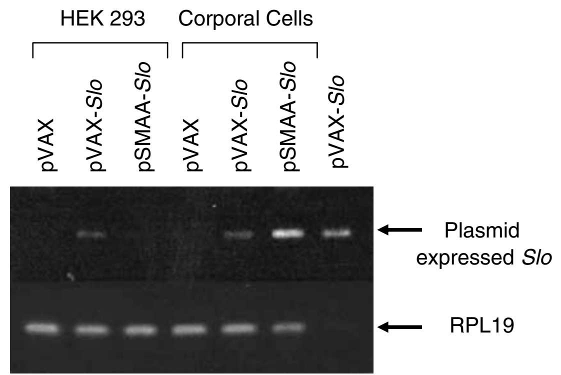

Figure 2 Expression of plasmid-derived *hSlo* in different cell lines. HEK293 and corporal cells were transfected with pVAX-*hSlo*, pSMAA-*hSlo* or the empty parent vector pVAX. RNA was derived from the cell lines and cDNA generated. Primers specific to plasmid-derived *hSlo* and the housekeeping gene were used in PCR, and the products were run on a 1.5% agarose gel and stained with ethidium bromide. Bands were visualized under ultraviolet illumination, images were captured and densitometry was carried out to quantitate relative absorbance.

Table 1 Densitometric analysis of plasmid-derived *Slo* expression in HEK293 and corporal smooth muscle cells

	HEK293	*Corporal cells*
pVAX	0.002 ± 0.001	0.001 ± 0.002
pVAX-*hSlo*	0.35 ± 0.02	0.37 ± 0.03
PSMAA-*hSlo*	0.002 ± 0.001	0.73 ± 0.03

Densitometry was performed for the expression of plasmid-derived *hSlo*, normalized to the housekeeping gene, RPL19. Average results are shown (three replicate experiments) ± s.d.

In vivo *effect of the SMAA promoter activity in rats with age-related erectile dysfunction*

Series 1 experiments. In the first series of experiments, the cavernous nerve (CN)-stimulated ICP responses were evaluated in rats given intracavernous injection with either 100 μg pSMAA-*hSlo* or 100 μg pVAX-*hSlo* or in age-matched control animals that received phosphate-buffered saline-20% sucrose alone. Representative tracings of the amplitude and duration of the CN-stimulated ICP response in individual rats from each experimental group are illustrated in Figure 3 for 60 s of stimulation at all levels of neurostimulation studied. In addition, Figure 4 shows representative examples of the ICP and blood pressure (BP) tracings observed following near maximal (that is, 4 mA) CN stimulation on a separate set of animals from each experimental group. As shown, there was little or no change in BP during CN stimulation, and furthermore, transfection with either pSMAA-*hSlo* or pVAX-*hSlo* produced equivalent effects. Mean data from all experiments in this series are depicted in Figure 5, which documents that both pSMAA-*hSlo* and pVAX-*hSlo* transfection were associated with virtually indistinguishable increases in the ICP/BP response, relative to age-matched control animals, at all levels of neurostimulation except 0.5 mA. At this level of stimulation, pSMAA-*hSlo* treated showed a significantly greater increase in the ICP/BP response.

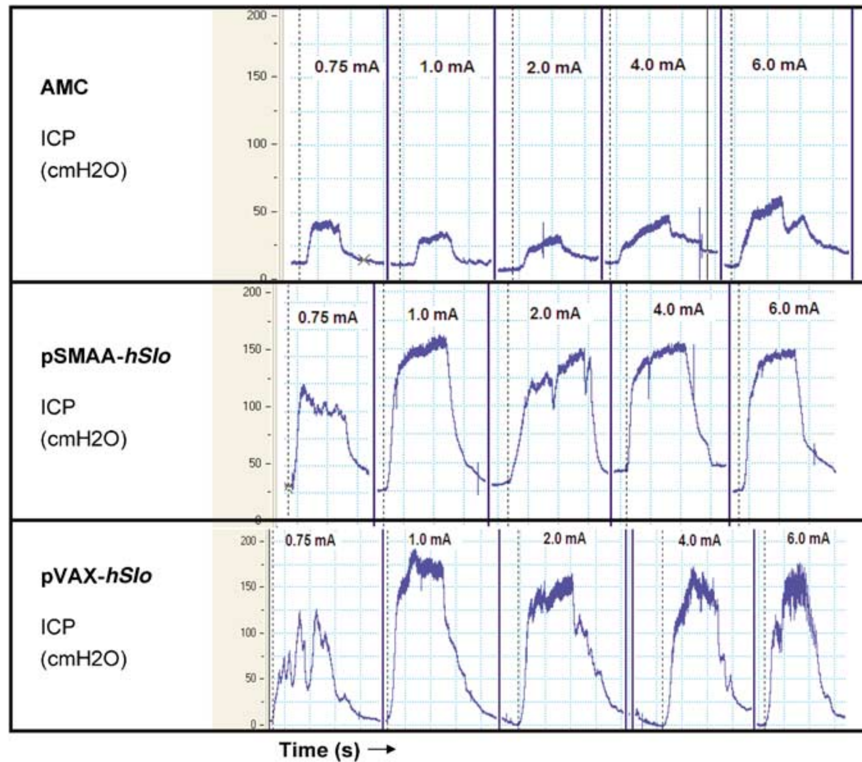

Figure 3 Representative tracings of the cavernous nerve (CN)-stimulated intracavernous pressure (ICP) responses obtained in an animal from each of the three treatment groups at all levels of current stimulation examined. AMC, age-matched controls with phosphate-buffered saline-20% sucrose. As illustrated, 100 μg doses of the pVAX-*hSlo* and pSMAA-*hSlo* produced relatively equivalent responses at all levels of current stimulation examined.

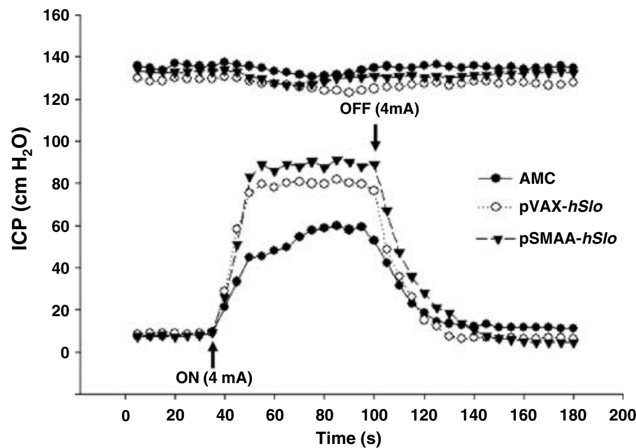

Figure 4 Representative pressure tracings of the cavernous nerve (CN)-stimulated intracavernous pressure (ICP) response (4 mA) in individual animals from control animals as well as the 100 μg doses of the pVAX-*hSlo* and pSMAA-*hSlo*. Note that both the pVAX-*hSlo* and SMAA-*hSlo* transfected rats displayed equivalent and significantly elevated ICP responses, in the absence of any detectable effect on blood pressure. The arrows denote when the CN stimulus was turned on and off.

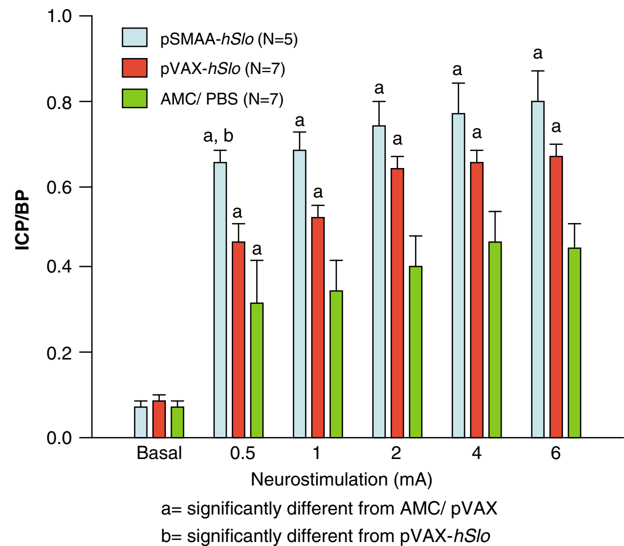

Figure 5 Statistical comparisons of the mean intracavernous pressure (ICP)/blood pressure (BP) data obtained following cavernous nerve stimulation in all experiments carried out on all treatment groups for series 1 experiments. Both pVAX-*hSlo* and pSMAA-*hSlo* (100 μg) transfected rats are significantly different from the age-matched controls at all levels of neurostimulation.

Series 2 experiments. In the second series of experiments, the CN-stimulated ICP responses were evaluated in rats injected with 10, or 1000 μg pSMAA-*hSlo*, or age-matched controls were given intracavernous injection of the plasmid backbone from which the gene was derived (1000 μg pSMAA-*EYFP*). The mean data from all experiments is summarized in Table 2. Transfer of both plasmids expressing *hSlo* (pSMAA-*hSlo* and pVAX-*hSlo*) resulted in significantly greater ICP/BP ratio as compared to the controls (see Materials and methods).

Table 2 Average ICP/BP response to varying levels of cavernous nerve stimulation after transfer of vector alone (pSMAA-EYFP and PBS) 10, 100 or 1000 μg of pSMAA-*hSlo* or 100 μg of pVAX-*hSlo*

Current (mA)	*Age-matched controls* (ICP/BP)*		*pVAX*-hSlo, *100 μg (ICP/BP);* n=7	*pSMAA*-hSlo, *10 μg (ICP/BP);* n=11	*pSMAA*-hSlo, *100 μg (ICP/BP);* n=5	*pSMAA*-hSlo, *1000 μg (ICP/BP);* n=9
	EYPF, N=7	*PBS-20% sucrose,* N=7				
0	0.10 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.0	0.07 ± 0.01	0.08 ± 0.01
0.75	0.14 ± 0.03	0.32 ± 0.10	0.47 ± 0.04[a]	0.12 ± 0.02	0.65 ± 0.03[b]	0.26 ± 0.1
1	0.20 ± 0.04	0.34 ± 0.08	0.52 ± 0.03[c]	0.33 ± 0.07	0.68 ± 0.05[d]	0.33 ± 0.1
2	0.33 ± 0.07	0.41 ± 0.07	0.63 ± 0.03[c]	0.54 ± 0.07	0.73 ± 0.07[c]	0.53 ± 0.1
4	0.40 ± 0.06	0.47 ± 0.07	0.65 ± 0.03[c]	0.58 ± 0.06	0.77 ± 0.07[c]	0.65 ± 0.05[e]
6	0.42 ± 0.05	0.45 ± 0.06	0.66 ± 0.03[c]	0.63 ± 0.05[e]	0.80 ± 0.07[c]	0.71 ± 0.04[c]

Abbreviations: BP, blood pressure; ICP, intracavernous pressure; PBS, phosphate-buffered saline.
*Seven animals received PBS-20% sucrose and seven received 1000 μg pEYFP in PBS; the controls are not statistically different from each other at any level of stimulation; each transfer group is significantly different from the combined controls by ANOVA at each level of stimulation; [a]indicates significant difference of pVAX-*hSlo* from control ($P<0.006$) and 1000 μg pSMAA-*hSlo* ($P<0.024$), [b]indicates significant difference of 100 μg of pSMAA-*hSlo* from control, 10 and 1000 μg pSMAA-*hSlo* ($P<0.001$), [c]indicates significant difference from control ($P<0.0001$), [d]indicates significant difference of 100 μg pSMAA-*hSlo* from control ($P<0.001$), 10 ($P<0.003$) and 1000 μg pSMAA-*hSlo* ($P<0.004$) and [e]indicates significant difference from control ($P<0.002$).

One-way analysis of variance (ANOVA) revealed evidence for a dose-related effect of pSMAA-*hSlo*, with an apparent maximal effect at 100 μg. Following gene transfection with the 100 μg dose of pSMAA-*hSlo*, the average ICP/BP ratio was higher than gene transfer of 100 μg pVAX-*hSlo* at all levels of stimulation. The 10, 100 and 1000 μg doses of pSMAA-*hSlo* were equivalent to the pVAX-*hSlo* at 6 mA of stimulation.

Discussion

Since the early 1990s, gene therapy has been forecast as a revolutionary treatment with the potential to prevent, correct or modulate genetic or acquired diseases. However, clinical trials that used viral vectors to allow the gene of interest to enter cells and integrate into the genome developed unforeseen, deleterious consequences, leading to restriction being imposed on gene transfer, particularly, for the treatment of nonfatal diseases.[29] One strategy for minimizing the theoretical potential risk of gene transfer therapy is to restrict the biodistribution and limit the expression and activity of the transferred gene to the targeted organ and cell type of interest using nonviral, nonintegrating cell-specific expression vectors (that is, naked DNA with a gene expressed from a tissue-specific promoter such as SMAA). However, these potential advantages could also reduce the efficacy of treatment if the tissue-specific promoters express lower levels of the gene, or expression in multiple cell types contributes to the efficacy. For example, with respect to erectile dysfunction, it is conceivable that uptake and expression of plasmids expressing *hSlo* in multiple cell types (such as endothelial and smooth muscle cells) may be necessary for the physiological process of erection. In this scenario, smooth muscle restricted expression may not improve erectile function as effectively.

To this point, the major finding of this report is that two independent lines of evidence suggest that, at least in the case of the SMAA promoter (that is, in the construct pSMAA-*hSlo*), this is not the case. Specifically, *in vitro* experiments in cultured cells, documented that expression of *hSlo* from pSMAA-*hSlo* was specific to corporal smooth muscle and that it was not expressed in a non-smooth muscle cell (that is, HEK293 cells; Figure 2). Moreover, there was approximately twice as much plasmid-derived gene expressed from the SMAA promoter as compared with that of the CMV promoter in smooth muscle cells (Table 1). Second, consistent with the *in vitro* data, the ability pSMAA-*hSlo* transfection to restore erectile function in the aging rat model of ED *in vivo* is similar to that observed with pVAX-*hSlo* under most conditions, and statistically better under some conditions (see Figures 2–4; Table 2). Thus, with respect to ED, despite the more limited smooth muscle expression profile, the CN-stimulated ICP response for pSMAA-*hSlo* compares quite favorably to that observed with *hSlo* expression driven by the heterologously expressed CMV promoter (the vector used in the recently completed Phase I human clinical trial),[17–20] as well as our prior preclinical observations with pcDNA/*hSlo*.[14–16] Again, it is important to keep in mind that gene transfer with both promoters produces an ICP/BP ratio commensurate with an erection; that is, an ICP/BP ratio of more than 0.6. As discussed elsewhere, the probability of visualizing an erection during CN stimulation increases dramatically as the ICP/BP ratio becomes 0.6 or more.[15,16] Stated another way, in both cases, the changes are not only statistically significant, but they are also physiologically relevant.

Another potential issue is that the pSMAA-EYFP vector-derived pSMAA-*hSlo* construct may have different expression characteristics as compared to the pVAX-derived vector. The backbone plasmid of pSMAA-EYFP has been evaluated previously by Dr Lessard for smooth muscle tissue-specific expression of EYFP.[30,31] EYFP (or other heterologous genes) are often used as markers for tissue distribution/expression when the gene of interest is difficult to detect or the endogenous protein is already present (as is the case with the *hSlo* gene product, Maxi-K). We believe that the replacement of EYFP with *hSlo* in the pSMAA-EYFP vector would have less impact on tissue-specific expression, than inserting the SMAA promoter into a new background vector.

So, how can low-level, cell-specific *hSlo* overexpression produce rather robust physiological effects? As described in detail elsewhere, physiological tolerance for the efficacy of low-level, cell-specific *hSlo* overexpression is presumably the result of the presence of a robust intercellular network of gap junction proteins among corporal smooth muscle cells.[6–8,15,16] In this scenario, the rapid intercellular distribution of second messenger molecules/ions from directly activated myocytes to adjacent, coupled cells restores the rapid and coordinated tissue responses required for erection and detumescence. In this scenario, presumably *hSlo* overexpression provides an enhanced hyperpolarizing K^+ current to ensure the syncytial spread of corporal smooth muscle relaxation, thus promoting erection.

Overall, these experiments show that overexpression of *hSlo* in corporal smooth muscle cell is both necessary and sufficient to restore erectile function in the aging rat. In addition, the use of a vector containing a smooth muscle-specific promoter may confer additional safety advantages to current gene transfer approaches to the treatment of human smooth muscle disorders, in this specific instance, age-related erectile dysfunction. Since these studies were conducted in a 1-week time frame, future experiments will be required to determine if the long-term expression of the SMAA promoter is equivalent to that observed with nonspecific CMV promoter in the same tissues (that is, 4–6 months). Nonetheless, these initial observations bode well for cell type-specific gene transfer as a novel method for the treatment of age-related erectile dysfunction.

Materials and methods

Study protocol

Retired breeder male Sprague–Dawley rats (⩾600 g body weight and 8–10 months old) were used in all studies according to a protocol approved by the Animal Institute Committee of the Albert Einstein College of Medicine. Two series of experiments were performed. In the series 1 experiments, rats were subjected to a single intracavernous 150 μl injection of either 100 μg pVAX-*hSlo*, 100 μg pSMAA-*hSlo* or phosphate-buffered saline-20% sucrose solution as control. Cavernosometry (that is, CN-stimulated ICP responses) was carried out 1 week after the injection.

In series 2, a single intracavernous 150 μl injection of either pSMAA-*hSlo* at doses of 10, or 1000, or 1000 μg of pSMAA-*EYFP* vector only was carried out. Again the CN-stimulated ICP responses were compared 1 week after injection.

Plasmid constructs

The pSMAA-*hSlo* was derived from the plasmid pSMAA-*EYFP* (eukaryotic yellow fluorescent protein, a kind gift from JL Lessard). This vector has a kanamycin-resistance gene identical to that found in pVAX. The *EYFP* gene was removed and *hSlo* cDNA was inserted in its place. To do this, pSMAA-*EYFP* was digested with *Bam*HI (klenowed) × *Xba*I to remove the *EYFP* gene and to replaced it with *hSlo* from pVAX-*hSlo* cut with *Xho*I (klenowed) × *Xba*I. Figure 1 shows the features of the two plasmids. In the case of pVAX-*hSlo* expression, the *hSlo* gene is driven by the CMV promoter, which functions in a wide variety of tissues, whereas the SMAA promoter has expression restricted to smooth muscle cells. Large-scale purification of plasmid for use in the gene transfer experiments, obtained from *Escherichia coli* grown in the presence of 200 μg ml^{-1} kanamycin, was accomplished using the Qiagen Giga Prep. Columns (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Surgical protocol

These protocols were performed as described in detail elsewhere.[14–16] Briefly, anesthesia was induced in all animals by the intraperitoneal injection (35 mg kg^{-1}) of sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA). Anesthesia was maintained during the course of the experimental protocol (1–2 h), as necessary, by subsequent injection of pentobarbital (5–10 mg kg^{-1}) every 45–60 min, as required. Animals were placed in the supine position and the bladder and prostate were exposed through a midline abdominal incision. The inferior hypogastric plexus (that is, the pelvic plexus or major pelvic ganglia), pelvic nerves and the CN were identified posterolateral to the prostate on both sides, and the stainless steel bipolar wire electrode was placed around these structures for electrical stimulation. The penis was denuded of skin; both crura (corpus cavernosum) were exposed by removing part of the overlying ischiocavernous muscle. To monitor ICP, a 23-gauge cannula was filled with 250 U ml^{-1} heparin solution connected to PE-50 tubing (Intramedic; Becton Dickinson, Sparks, MD, USA), which was inserted into the right corpus cavernosum (crus). The tubing then was fixed to the tunica with a 7-0 Dermalon suture to ensure stability during measurement of ICP. Systemic arterial BP was monitored via a 25-gauge cannula placed into the carotid artery.

Both pressure lines were connected to a pressure transducer, which was, in turn, connected via a transducer amplifier (ETH 400; CB Sciences Inc., Dover, NH, USA) to a data acquisition board (Mac Lab software version 8e; ADI Instruments Pty Ltd, Castle Hill, Australia). Real-time display and recording of pressure measurements was performed on a Macintosh computer (Mac Lab software 8e; ADI Instruments Pty Ltd). The pressure transducers and analogue/digital board were calibrated in centimeters of water before each experiment.

Cavernosometry: neurostimulation of cavernous nerve

Direct electrostimulation of the CN was performed with a delicate stainless steel bipolar hook electrode attached to the multijointed clamp. Each probe was 0.2 mm in diameter; the two poles were separated by 1 mm. Monophasic rectangular pulses were delivered by a signal generator (custom made and with a built-in constant current amplifier). Stimulation parameters were as follows: frequency, 20 Hz; pulse width, 0.22 ms; duration, ≈1 min increasingly from 0 to 6 mA of current.

Statistical analysis

The resting ICP value as well as the ICP measured during current stimulation of the CN (0, 0.75, 1.0, 2.0, 4.0 and 6.0 mA, in incremental values) was expressed as a fraction of the BP during neurostimulation. Unless otherwise stated, all values are expressed as the mean ± s.e.m. Statistical comparisons at each level of

stimulation between treatment groups in each series of experiments were subjected to a one-way ANOVA, with the Holm–Sidak method used for all pairwise multiple comparison procedures. Because increased electrical stimulation is well known to be associated with an increased ICP response, we limited our statistical analysis to examination of treatment effects at each level of stimulation for this initial investigation. The control groups were not statistically different from each other at any level of stimulation ($P>0.05$) and the groups were combined for analysis. Statistical analyses were carried out using Sigma Stat software (Chicago, IL, USA). All differences were considered statistically significant for unadjusted $P<0.05$.

In vitro *studies for tissue specificity of the SMAA promoter*

HEK fibroblast (HEK293; Clontech, Palo Alto, CA, USA) and human corporal smooth muscle cells were transfected transiently *in vitro* with pVAX-SVcyt and pVAX-SV0 using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The human smooth muscle cells were isolated from human corporeal tissues, washed and placed in physiological saline solution (137 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 0.42 mM Na_2HPO_4, 0.44 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM HEPES, pH adjusted to 7.4 with NaOH) with 0.1% bovine serum albumin. Tissue was cut into 1 mm pieces and placed in physiological salt solution with 0.1% bovine serum albumin, 45 U papain and 0.1% dithiothreitol and incubated at 37 °C for 35 min. At the end of incubation, this solution was replaced by physiological salt solution containing 0.1% bovine serum albumin, 0.1% collagenase type 4, 0.05% elastase and 0.1% soybean trypsin inhibitor and then incubated for an additional 25 min at 37 °C. At the end of incubation the enzyme-containing solution was washed twice with physiological salt solution containing albumin to obtain isolated smooth muscle cells. The freshly isolated cells were then transferred to 35 mm culture dishes for patch clamp experiments. HEK293 cells were obtained from a commercial source (Invitrogen).

Total RNA was extracted from transfected cell lines using the TRIzol (Invitrogen) method according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically. The reverse transcriptase reaction was primed with oligo-dT and cDNA generated using avian-enhanced AMV reverse transcriptase. The PCR was performed using RedTaq (Invitrogen) with the housekeeping gene ribosomal protein, large subunit, RPL19: RPL19R, 5′-CCTCATTCTCCTCATCC-3′; RPL19F, 5′-CGCCAATGCAACTCCCG-3′ and for the *Slo* gene expressed from the plasmid using primers that hybridize to theT7 promoter of plasmid sequences T7F-5′-CCCTATAGTGAGTCGTATTA-3′, and a primer specific to the first six amino acids of *hSlo* SloR-5′-GCCGCCACCATTTGCCAT-3′. Conditions for PCR were 95 °C for 60 s (denature), 72 °C for 60 s (anneal), 72 °C for 45 s (elongate), 25 cycles only, 72 °C for 5 min at the end to allow complete elongation of all product DNA. The PCR products were run on a 1.5% agarose gel and were stained with ethidium bromide to visualize products under ultraviolet illumination. The visualized bands were quantified using a model GS-700 imaging densitometer and molecular analyst program (Bio-Rad, Richmond, CA, USA). Results for three replicate experiments were averaged ± s.d. and are shown in Table 1.

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