

# Intracorporal injection of *hSlo* cDNA in rats produces physiologically relevant alterations in penile function

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**Christ, G. J., J. Rehman, N. Day, L. Salkoff, M. Valcic, A. Melman, and J. Geliebter.** Intracorporal injection of *hSlo* cDNA in rats produces physiologically relevant alterations in penile function. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H600–H608, 1998.—The Ca<sup>2+</sup>-sensitive K<sup>+</sup> channel (maxi-K<sup>+</sup>) is an important modulator of corporal smooth muscle tone. The goal of these studies was twofold: 1) to determine the feasibility of transfecting corporal smooth muscle cells in vivo with the *hSlo* cDNA, which encodes for the human smooth muscle maxi-K<sup>+</sup> channel, and 2) to determine whether transfection of the maxi-K<sup>+</sup> channel would affect the physiological response to cavernous nerve stimulation in a rat model in vivo. Intracorporal microinjection of pCMVβ/Lac Z DNA in 10-wk-old rats resulted in significant incorporation and expression of β-galactosidase activity in 10 of 12 injected animals for up to 75 days postinjection. Moreover, electrical stimulation of the cavernous nerve revealed that, relative to the responses obtained in age-matched control animals (*N* = 12), intracavernous injection of naked pcDNA/*hSlo* DNA was associated with a statistically significant elevation in the mean amplitude of the intracavernous pressure response at all levels of current stimulation (range 0.5–10 mA) at both 1 mo (*N* = 5) and 2 mo (*N* = 8) postinjection. Furthermore, qualitatively similar observations were made at 3 mo (*N* = 2) and 4 mo (*N* = 2) postinjection. These data indicate that naked *hSlo* DNA is quite easily incorporated into corporal smooth muscle and, furthermore, that expression is sustained for at least 2 mo in corporal smooth muscle cells in vivo. Finally, after expression, *hSlo* is capable of measurably altering nerve-stimulated penile erection. Taken together, these data provide compelling evidence for the potential utility of gene therapy in the treatment of erectile dysfunction.

corporal smooth muscle; neurostimulation; intracavernous pressure; relaxation; intracavernous injection

ERECTILE DYSFUNCTION is a common illness that is estimated to affect 10–30 million men in the United States (2, 14). From a mechanistic standpoint, erectile dysfunction can be viewed as a multifactorial neurovascular disease, with alterations in the tone and compliance of the corporal smooth muscle assuming a major role in impotence, regardless of the exact etiology. In this regard, heightened contractility and/or impaired relaxation of the corporal smooth muscle is a primary cause of erectile dysfunction in a large proportion of impotent men (1, 7, 9, 11, 23). Thus, with the exception of prosthetic devices, all effective forms of therapy for erectile dysfunction ultimately exert their actions by inducing relaxation of corporal smooth muscle. As such, it is clear that the improved treatment of erectile dysfunction in a vast majority of impotent men will

necessarily rely on more precise modulation of corporal smooth muscle tone.

In this regard, our recent studies have indicated that, as described for physiologically diverse vascular tissues (5, 19, 21, 25, 26, 28–30, 32, 34), hyperpolarization of corporal smooth muscle cells via activation of K<sup>+</sup> channels represents an important mechanism for controlling corporal smooth muscle tone (9–13, 18). In fact, the sustained contraction of human corporal smooth muscle in vitro (synonymous with flaccidity in vivo, the condition of the penis most of the time) is largely dependent on continuous transmembrane Ca<sup>2+</sup> flux through voltage-gated Ca<sup>2+</sup> channels (9). The activity of these voltage-dependent Ca<sup>2+</sup> channels in corporal smooth muscle cells is, in turn, closely modulated by hyperpolarizing currents, initiated by a variety of receptor- and non-receptor-mediated events, and carried mainly by K<sup>+</sup> channels. Among the several subtypes of K<sup>+</sup> channels present in human corporal smooth muscle, the ≈180-pS Ca<sup>2+</sup>-sensitive maxi-K<sup>+</sup> channel is one of the most prominent in cultured corporal smooth muscle cells (10, 12, 13). Furthermore, the activation of outward K<sup>+</sup> currents after both receptor (e.g., PGE<sub>1</sub>) and nonreceptor (e.g., pinacidil, nitric oxide, BAY K 8644, etc.) activation of cultured human corporal smooth muscle cells has been reported to cause hyperpolarization, alter intracellular Ca<sup>2+</sup> mobilization and transmembrane Ca<sup>2+</sup> flux, and, thus, affect smooth muscle relaxation (9, 11, 23).

Given the central role of the maxi-K<sup>+</sup> channel in modulating intracellular Ca<sup>2+</sup> levels and transmembrane Ca<sup>2+</sup> flux in corporal smooth muscle, modification of channel function is a logical target for molecular/pharmacological intervention in the treatment of erectile dysfunction. Therefore, as an initial test of the possible utility of genetic therapy for erectile dysfunction, we chose to examine the physiological and molecular consequences of transfection of corporal smooth muscle in vivo with the *hSlo* cDNA, which encodes the human smooth muscle maxi-K<sup>+</sup> channel (22). In short, this report documents that naked pcDNA/*hSlo* DNA is easily incorporated in, and expression is quite sustained in, rat corporal smooth muscle in vivo. Moreover, this prolonged expression of *hSlo* cDNA is capable of altering the physiologically relevant erectile response as measured by a significant increase in the intracavernous pressure response to stimulation of the cavernous nerve. As such, these data provide compelling evidence for the potential utility of gene therapy in the treatment of erectile dysfunction.

## MATERIALS AND METHODS

**Demographics of experimental animals.** A total of 74 male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used in these studies. Of these animals, 29 were 10–12 wk old, and 5 of these served as the young control animals. The remaining 45 animals were purchased as retired breeders and were all >9 mo old and ranged in weight from 500 to 700 g. All rats were fed Purina lab rodent chow ad libitum and housed individually with a 0700–1900 light cycle. The demographics of the animal population with respect to protocol enrollment are displayed in Table 1. The age-matched control animals were considered to represent a homogeneous population, because statistical analysis revealed that there was no significant difference between these animals 1–4 mo after receipt with respect to the mean amplitude of the intracavernous pressures (ICP) measured in response to all levels of current stimulation used in these studies. In addition, it should be noted that four retired breeders received an injection of the pcDNA/*hSlo* DNA (Table 1, group 3; pcDNA3 was from Invitrogen) as described in *Preparation of plasmids and transfection of rat corporal smooth muscle in vivo*, and then, 2 mo after this injection, the animals were killed and the corporal tissue was quickly excised and flash frozen in liquid N<sub>2</sub> for RT-PCR and Northern analyses (without the performance of any physiological experiments in vivo). Note again that five age-matched control animals were run in parallel and received injection of vehicle only.

**Microinjection of vectors/plasmids into rat corporal tissue.** Animals were anesthetized by intraperitoneal injection of pentobarbital sodium (35 mg/kg). An incision was made through the perineum, the corpus spongiosum was identified, and a window was made in the corpus spongiosum for identification of the corpus cavernosum. All microinjections consisted of a single bolus injection into the corporal tissue with an insulin syringe. The final volume of all microinjections was 200  $\mu$ l.

**Gene transfer of Lac Z into smooth muscle cells of rat corpus cavernosum in vivo.** Transfer of Lac Z DNA (coding for  $\beta$ -galactosidase) into rat corporal smooth muscle cells in vivo was accomplished by injecting the plasmid pCMV $\beta$  as naked DNA into the corporal tissue of 10-wk-old Sprague-Dawley

rats ( $N = 12$ ), and the corporal tissue was obtained from groups of three animals at each of four time points ranging from 2 to 11 wk postinjection (see Table 1 for details). An equivalent number of uninjected control animals at each time point postinjection were run in parallel. For these studies, 100  $\mu$ g of pCMV $\beta$  plasmid dissolved in 200  $\mu$ l PBS (containing 20% sucrose) were injected into the corpus cavernosum of 10-wk-old Sprague-Dawley rats (under anesthesia). Later (2–11 wk), the corporal tissue from injected and control rats was excised, fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 3 h, and then reacted with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal) for 15 h at 37°C (33). The efficacy of gene transfer was assessed, on the whole tissue level, by the conversion of the chromogenic substrate X-Gal into its blue breakdown product by the activity of  $\beta$ -galactosidase.

**Preparation of plasmids and transfection of rat corporal smooth muscle in vivo.** The human maxi-K<sup>+</sup> channel cDNA *hSlo* (~3,900 nucleotides; i.e., 3.9 kb) (22) was inserted into the *Xho* I-*Xba* I cloning site of the pcDNA3 vector, where expression is driven off of the cytomegalovirus (CMV) promoter. pcDNA/maxi-K<sup>+</sup> (100  $\mu$ g) in 200  $\mu$ l PBS (containing 20% sucrose) was injected into the corpus cavernosum of anesthetized 9-mo-old Sprague-Dawley rats. Control rats were either sham operated, sham operated with an intracorporal injection of 200  $\mu$ l PBS containing 20% sucrose, or sham operated with an intracorporal injection of 200  $\mu$ l PBS containing 20% sucrose and 100  $\mu$ g pcDNA vector DNA. Basal and nerve-stimulated ICP were measured between 1 and 4 mo after the intracavernous injection.

**Surgical preparation and placement of pressure-monitoring cannulas.** Details of this methodology have been recently described (27). Briefly, the rats were anesthetized by intraperitoneal injection (35 mg/kg) of pentobarbital sodium (Anpro Pharmaceuticals). Anesthesia was maintained during the course of the experimental protocol (2–3 h) by subsequent injection of pentobarbital (5–10 mg/kg) every 45–60 min. Animals were placed in the supine position, and the bladder and prostate were exposed through a midline abdominal incision. The inferior hypogastric plexus (i.e., the pelvic plexus or major pelvic ganglia), pelvic nerves, and the cavern-

Table 1. Demographics of gene transfer protocols in animal population

Group	N	Age at Initiation of Protocol, mo	Gene	Method of Gene Transfer	Time Course
1	24	2–3	Lac Z ( $N = 12$ )	Naked DNA	2 wk ( $N = 3$ ) 4 wk ( $N = 3$ ) 8 wk ( $N = 3$ ) 11 wk ( $N = 3$ )
			Age-matched controls ( $N = 12$ )	Uninjected	2 wk ( $N = 3$ ) 4 wk ( $N = 3$ ) 8 wk ( $N = 3$ ) 11 wk ( $N = 3$ )
2	34	9	<i>hSlo</i> ( $N = 17$ )	Naked DNA	1 mo ( $N = 5$ ) 2 mo ( $N = 8$ ) 3 mo ( $N = 2$ ) 4 mo ( $N = 2$ )
			Sham-operated control ( $N = 12$ )	No vehicle or vector, vector and vehicle, or vehicle alone	1 mo ( $N = 2$ ) 2 mo ( $N = 6$ ) 3 mo ( $N = 2$ ) 4 mo ( $N = 2$ )
			Young control ( $N = 5$ )	None	None
3	11	9	<i>hSlo</i> Sham	Naked DNA Vehicle only	2 mo ( $N = 5$ ) 2 mo ( $N = 6$ )

$N$ , no. of rats. Lac Z DNA codes for  $\beta$ -galactosidase activity; *hSlo* codes for human Ca<sup>2+</sup>-sensitive K<sup>+</sup> (maxi-K<sup>+</sup>) channel.

ous nerve were identified posterolateral to the prostate on both sides, and the stainless steel bipolar wire electrodes were placed around these structures for electrical stimulation. The penis was denuded of skin, and both crura were exposed by removing part of the overlying ischiocavernosus muscle. To monitor ICP, a 23-gauge cannula was filled with 250 U/ml of heparin solution, connected to PE-50 tubing (Intramedic7, Becton-Dickinson; Ref. 7), and inserted into the right corpus cavernosum. The tubing was then fixed to the tunica with a 7-0 Dermalon suture to ensure stability during measurement of ICP. Another 23-gauge cannula was connected to a 1-ml syringe and inserted into the left corpus cavernosum for intracavernous drug injection. Systemic arterial blood pressure (BP) was monitored via a 25-gauge cannula placed into the carotid artery.

Both pressure lines (BP and ICP) were connected to a pressure transducer, which was, in turn, connected via a transducer amplifier (ETH 400, CB Sciences) to a data acquisition board (MacLab/8e7, ADInstruments, Milford, MA). Real-time display and recording of pressure measurements was performed on a Macintosh computer (MacLab software v3.4). The pressure transducers and analog-to-digital board were calibrated in centimeters of H<sub>2</sub>O.

**Neurostimulation of cavernous nerve and recording of ICP.** Direct electrostimulation of the cavernous nerve 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 built-in constant current amplifier). Stimulation parameters were as follows: frequency, 20 Hz; pulse width, 0.22 ms; and duration, 1 min. The current protocol was the application of increasing current at the following intervals: 0.5, 1, 2, 4, 6, 8, and 10 mA. The changes in ICP and systemic BP were recorded at each level of neurostimulation.

**RNA preparation.** Total RNA was extracted from frozen tissue using the TRIzol method. Briefly, tissue was homogenized in TRIzol reagent by a Polytron homogenizer (Brinkman, Westbury, NY) for ~30 s. The tissue lysate was then transferred to a polypropylene 10 × 1.8-cm round-bottom tube (Falcon, Becton-Dickinson, Rutherford, NJ) and incubated for 5 min at room temperature. Chloroform was then added, and the solution was centrifuged at 12,000 *g* at 4°C for 15 min. RNA was extracted in the aqueous phase from the TRIzol-chloroform mixture, precipitated from the aqueous phase by mixing with isopropyl alcohol, and centrifuged at 12,000 *g* for 10 min at 4°C. RNA was stored in 0.08 M sodium acetate and 70% ethanol.

**Northern blot analysis.** Total RNA (20 µg) from each tissue sample was electrophoresed in 1% agarose containing 2.2 M formaldehyde and transferred onto nylon membranes by capillary transblotting. The positions of 28S and 18S rRNA bands on the ethidium-stained gels were observed under ultraviolet illumination before transblotting. RNA was fixed to the filter by heating for 2 h at 80°C. The *hSlo* cDNA was cut from the *Xho* I and *Xba* I sites of pCDNA3 and purified from an agarose gel, random primer biotin-labeled with the NE-Blot Phototope kit (New England Biolabs), and used as cDNA probes for Northern blots. Hybridization was carried out in Rapid-hyb buffer (Amersham, Arlington Heights, IL) at 68°C for 2 h. Filters were washed two times in 2.5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at room temperature, followed by two washes in 1× SSC and three washes in 0.1% SDS at 68°C for 20 min each. After the washes, the membranes underwent detection steps using streptavidin and biotin alkaline phosphatase with CDP-Star

substrate according to the manufacturer's instructions (New England Biolabs). After incubation with CDP-Star substrate, the membranes were removed and exposed to Hyperfilm (Amersham) in an intensifying screen with time adjustments, and the bands were analyzed.

**RT-PCR.** To further confirm and detect expression of *hSlo*, we also used two distinct PCR analysis strategies, one with primers specific to the plasmid sequences (T7 and SP6 promoters) and another with primers specific for the 5'-untranslated region. With respect to the latter strategy, oligonucleotide primers for PCR amplification of the 5'-untranslated region (~0.14 kb) were 5'-GCCGCCACCATT-GCCAT-3' (3' primer; coding for the first 6 amino acids of *hSlo*) and 5'-CCCTATAGTGAGTCGTATTA-3' (5' primer; specific to the T7 promoter). With respect to the first strategy, oligonucleotide primers for PCR amplification of the full *hSlo* insert (~4.2 kb) were the T7 promoter region (see primer for latter strategy) and the SP6 promoter region (5'-CTAGCATT-TAGGTGACACTATAG-3'). The primers for an endogenous maxi-K<sup>+</sup> region (base pairs 909–1074; 5'-GCTCTCCATATT-TATCAGCAC-3' and 5'-AACATCCCCATAACCAAC-3') were used as a control.

RT-PCR was performed using the SuperScript One-Step RT-PCR System (GIBCO-BRL, Grand Island, NY) with a final volume of 50 µl. The RT-PCR mixture included a buffer containing 1 µg of total RNA, 1 µM each of the sense and antisense oligonucleotide primers, 0.4 mM of each dNTP, SuperScript II RT/*Taq* mix, 80 units of RNaseOUT recombinant ribonuclease inhibitor (GIBCO-BRL), and an optimized concentration of MgSO<sub>4</sub> as per the manufacturer's instructions. First-strand cDNA was synthesized at 45°C for 30 min and denatured at 94°C for 5 min. PCR was performed with the cycle of annealing at 45°C for 1 min, extension at 72°C for 1.5 min, and denaturing at 94°C for 1 min for a total of 35 cycles, with a final extension step for 10 min. For amplification of the full insert, 1 µl of eLONGase was added to the reaction mixture at 68°C for 5 min for extension.

**Statistical analysis.** All statistical analyses were performed using the StatView 4.5 software (Abacus Concepts, Berkeley, CA). ANOVA with post hoc multiple comparisons (Tukey) or a two-tailed Student's *t*-test for unrelated samples was utilized as appropriate for comparison of group means for parameters of interest between gene therapy rats (i.e., maxi-K<sup>+</sup> transfected), age-matched control animals, and the young control rats. All differences were considered significant at *P* < 0.05. Unless otherwise stated, all data are expressed as means ± SE.

**Construction of stimulus-response curves.** Stimulus-response curves were generated for the effects of neurostimulation on ICP by expressing the change in ICP as a fraction of the mean systemic BP (expressed as ICP/BP) and plotting this fraction as a function of the magnitude of neurostimulation (1, 2, 4, 6, 8, or 10 mA). All data were plotted using SigmaPlot software for the Macintosh (SigmaPlot Mac v.5.0 Jandel Scientific, San Rafael, CA).

## RESULTS

**Transfection of pCMVβ/Lac Z into rat corpora in vivo.** Twelve rats were injected with pCMVβ/Lac Z and observed for up to ~11 wk (75 days) postinjection, with an equivalent number of age-matched control rats run in parallel (see Table 1). Histological evaluation of corporal tissue excised from rats receiving an intracorporal injection of naked Lac Z DNA revealed the presence of significant chromogenic material in 10 of 12 animals. Figure 1 shows a representative example of

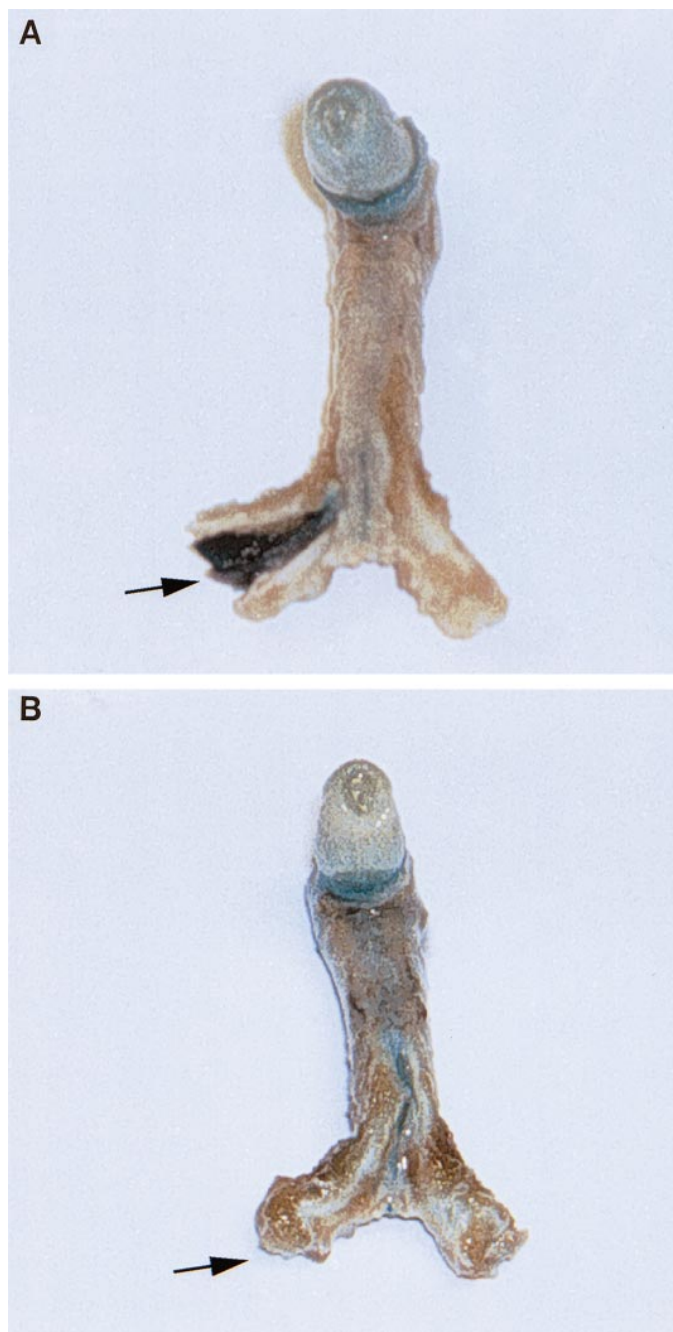


Fig. 1. Histological analysis of naked pCMV $\beta$ /Lac Z gene transfer into rat corpora in vivo. *A*: intact corpus cavernosum from rat injected with pCMV $\beta$  plasmid, containing Lac Z gene, leading to expression of  $\beta$ -galactosidase activity and commensurate formation of chromogenic substrate and characteristic blue color. Photograph was taken 60 days after original injection. Note that robust blue staining is largely confined to injected corpora, most heavily near injection site (arrow). *B*: corpus cavernosum from age-matched control rat that was not injected with plasmid. Note complete absence of any blue staining in corporal tissue (arrow) of uninjected control animal.

the presence of chromogenic material in the corpora of a rat 60 days postinjection. Thus incorporation of naked DNA appears sustained, over this time period, in this tissue. Similarly prolonged incorporation of gene transfer products has been reported in other vascular tissues (23).

*Resting and neurostimulation-induced ICP responses in maxi-K<sup>+</sup>-transfected and sham-operated control rats.* A one-way ANOVA revealed that there was a significant difference in the resting ICP in rats transfected with the pcDNA/*hSlo* DNA. There was no detectable difference in the mean resting ICP or mean arterial BP values among the three treatment groups ( $P > 0.07$ ; one-way ANOVA). The values for ICP and BP, respectively, were  $22.7 \pm 1.9$  and  $177.4 \pm 4.3$  cmH<sub>2</sub>O for the pcDNA/*hSlo* DNA rats ( $N = 17$ ),  $14.9 \pm 3.3$  and  $177 \pm 4.5$  cmH<sub>2</sub>O for the age-matched control animals ( $N = 12$ ), and  $19.8 \pm 3.3$  and  $168.6 \pm 7.4$  cmH<sub>2</sub>O for the young control animals ( $N = 5$ ).

The effects of current stimulation of the cavernous nerve on the ICP response in rats in vivo was utilized to evaluate the potential physiological relevance of overexpression of the maxi-K<sup>+</sup> channel after intracavernous injection of the pcDNA/*hSlo* DNA (see MATERIALS AND METHODS). For these studies, the rats were divided into distinct treatment groups as summarized in Table 1 (also see MATERIALS AND METHODS). All animals were examined using identical neurostimulation protocols with the magnitude of current stimulation ranging from 0.5 to 10 mA. Representative responses to current stimulation in pcDNA/*hSlo*-transfected, age-matched control, and young control animals are shown in Fig. 2 in response to 2-mA current stimulation.

For statistical comparison of treatment effects, the mean amplitude of the ICP response at each level of current stimulation was expressed as a fraction of the mean arterial BP (ICP/BP) during current stimulation. A two-way ANOVA revealed that there was a significant effect of treatment ( $P < 0.001$ ) on the mean ICP/BP, but

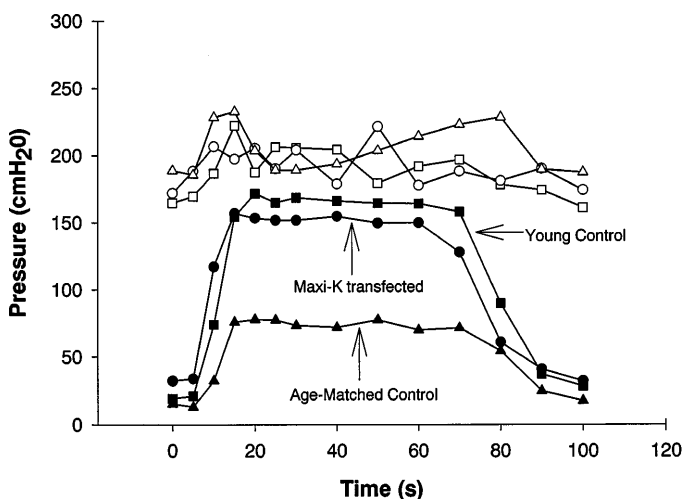


Fig. 2. Representative time course of changes in mean arterial blood pressure (BP; open symbols) and intracorporal pressure (ICP; closed symbols) during neurostimulation (2 mA) for an age-matched control animal (triangles), a maxi-K<sup>+</sup>-transfected animal (circles), and a young control animal (squares). As described in MATERIALS AND METHODS, maxi-K<sup>+</sup>-transfected animal was subject to a single intracavernous injection of pcDNA/*hSlo* naked DNA 3 mo before experiment, whereas age-matched control animal received pcDNA vector only in absence of *hSlo* cDNA. *hSlo* encodes for human smooth muscle Ca<sup>2+</sup>-sensitive K<sup>+</sup> (maxi-K<sup>+</sup>) channel. Note dramatic differences in mean amplitude of ICP responses between maxi-K<sup>+</sup>-transfected and age-matched control animals, despite similarity in mean arterial BP (see text).

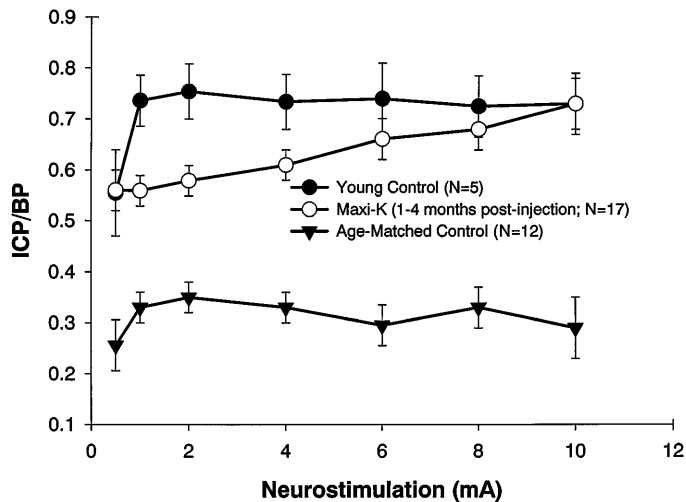


Fig. 3. Summary of all in vivo data for ICP measurements in response to electrical stimulation of cavernous nerve in maxi-K<sup>+</sup>-transfected, age-matched control, and young control animals. Values are means  $\pm$  SE for mean amplitude of ICP responses recorded in all animals in each treatment category, at every level of current stimulation examined in these studies. Note that, for purposes of comparison, all maxi-K-transfected and age-matched control animals were combined as a single population, where *N* refers to total number of animals in each treatment category.

there was no effect of current stimulation ( $P > 0.13$ ) and no treatment-current stimulation interaction ( $P = 0.26$ ). The mean response for all animals in each treatment group is graphically represented in Fig. 3, and the data are summarized in Table 2. Although post hoc analysis documented a significant difference in the mean amplitude of the ICP pressure response to all levels of current stimulation between the young control group and the maxi-K<sup>+</sup>-transfected groups ( $P < 0.05$ ), both groups had significantly elevated ICP responses compared with the age-matched control animals ( $P < 0.05$ ).

In addition, the pcDNA/*hSlo*-transfected animals were further subdivided according to whether the in vivo studies were conducted 1, 2, 3, or 4 mo posttransfec-

tion, with age-matched control animals run in parallel. Consistent with the overall trend for the entire animal population (see Fig. 4), two-way ANOVA revealed that intracorporal injection of the pcDNA/*hSlo* DNA was associated with significantly elevated intracorporal pressures at all levels of current stimulation for both the 1-mo (Fig. 4A) and 2-mo (Fig. 4B) postinjection time points. Statistically meaningful conclusions could not be drawn from the small number of observations at the 3- and 4-mo postinjection time points. However, Fig. 4C does show that apparently qualitatively similar results were obtained.

*Incorporation of hSlo into rat corporal smooth muscle.* The gene expression level of the maxi-K<sup>+</sup> channel in the transfected rat corporal smooth muscle was examined by both RT-PCR and Northern blot analyses. Total RNA from pcDNA/*hSlo*-transfected tissue and pcDNA-transfected control tissue was RT-PCR amplified with primers as described in MATERIALS AND METHODS and displayed in Fig. 5. As shown, the amplification of the 5'-untranslated region resulted in a significant cDNA band from the pcDNA/*hSlo*-transfected tissue (molecular size  $\sim 0.14$  kb) but not from the pcDNA-transfected control tissue (Fig. 5A). The full-length insert (molecular size  $\sim 4.2$  kb) was also RT-PCR amplified from the pcDNA/*hSlo*-transfected tissue but not from the pcDNA-transfected control (Fig. 5C). The quality of the RNA from all tissues was further examined with primers that amplified the endogenous maxi-K<sup>+</sup> channel. As indicated, the endogenous maxi-K<sup>+</sup> channel was amplified to a comparable level in all tissues (Fig. 5B). The presence of *hSlo* expression was further examined by Northern blotting with an *hSlo* insert as a probe (Fig. 6). Once again, RNA from the pcDNA/*hSlo*-transfected tissue, but not from the pcDNA-transfected control tissue, expressed a detectable level of maxi-K<sup>+</sup> channel. The *hSlo* band was detected between the 18S and 28S RNA bands, with an approximate molecular size of 4.2 kb. In light of the high-stringency conditions used in this assay (see MATERIALS AND METHODS), it is not

Table 2. Mean intracavernous pressure and blood pressure measurements made during cavernous nerve stimulation

Neurostimulation, mA	Maxi-K <sup>+</sup> (N=17)		Age-Matched Control (N=12)		Young Control (N=5)	
	ICP, cmH <sub>2</sub> O	BP, cmH <sub>2</sub> O	ICP, cmH <sub>2</sub> O	BP, cmH <sub>2</sub> O	ICP, cmH <sub>2</sub> O	BP, cmH <sub>2</sub> O
0.5	111 $\pm$ 7 (n=6)	198 $\pm$ 6	43 $\pm$ 11 (n=5)	178 $\pm$ 12	102 $\pm$ 16 (n=5)	183 $\pm$ 16
1.0	107 $\pm$ 7 (n=16)	192 $\pm$ 5	55 $\pm$ 5 (n=12)	178 $\pm$ 6	132 $\pm$ 10 (n=5)	180 $\pm$ 10
2.0	114 $\pm$ 11 (n=16)	198 $\pm$ 5	63 $\pm$ 5 (n=12)	177 $\pm$ 7	154 $\pm$ 10 (n=5)	186 $\pm$ 10
4.0	121 $\pm$ 7 (n=15)	196 $\pm$ 5	57 $\pm$ 5 (n=12)	177 $\pm$ 7	133 $\pm$ 10 (n=5)	181 $\pm$ 10
6.0	124 $\pm$ 5 (n=11)	190 $\pm$ 6	53 $\pm$ 6 (n=9)	180 $\pm$ 9	131 $\pm$ 13 (n=3)	176 $\pm$ 13
8.0	129 $\pm$ 5 (n=8)	191 $\pm$ 5	59 $\pm$ 7 (n=9)	181 $\pm$ 9	133 $\pm$ 11 (n=4)	184 $\pm$ 11
10.0	142 $\pm$ 6 (n=7)	195 $\pm$ 5	56 $\pm$ 13 (n=4)	191 $\pm$ 7	132 $\pm$ 11 (n=4)	180 $\pm$ 11

Values are means  $\pm$  SE of intracavernous pressure (ICP) and blood pressure (BP). *N*, no. of rats in treatment group; *n*, no. of observations for each level of stimulation.

surprising that the endogenous maxi-K<sup>+</sup> channel was not detected.

## DISCUSSION

Many recent advances have been made in the application of gene therapy to the treatment of human disease

(17). In particular, techniques for gene transfer into vascular smooth muscle cells have been developed in the hope of providing a novel therapeutic strategy for the treatment of several cardiovascular diseases (4, 8, 15, 24). Among these are atherosclerosis, vasculitis, and restenosis after balloon angioplasty. Such studies have provided important information on the efficiency and persistence of gene transfer methods in smooth muscle cells.

In light of these seminal observations, it was the explicit aim of these studies to begin to evaluate the feasibility of somatic gene transfer into corporal smooth muscle as a novel therapeutic strategy for the treatment of erectile dysfunction. The rationale for this approach is related to the fact that the tone of the corporal smooth muscle cells in the specialized vascular tissue of the penis plays a critical role in modulating the flow of blood to and from the penis, and thus in determining erectile capacity. This fact makes corporal smooth muscle cells a logical target for molecular intervention in the treatment of erectile dysfunction. Furthermore, given the central role of the maxi-K<sup>+</sup> channel in modulating human corporal smooth muscle tone (9–12), this report examined the physiological impact of transfection of corporal smooth muscle with *hSlo* cDNA in a rat model *in vivo*.

The major findings are as follows. First, after a single intracorporal injection of naked pCMVβ/Lac Z DNA (see MATERIALS AND METHODS) expression of β-galactosidase activity is sustained for at least 75 days. This clearly demonstrates that in the rat corpora, *in vivo*, relatively prolonged expression of extrachromosomal genes that encode physiologically detectable protein products is feasible (Fig. 1). Consistent with these observations, the mean amplitude of the nerve-stimulated ICP response is significantly augmented over a similar time course in rats, *in vivo*, after a single intracavernous injection of naked pcDNA/*hSlo* DNA (see Figs. 2–4). Moreover, both RT-PCR techniques and Northern blots revealed that, at least for the 2-mo time point, the observed augmentation in the nerve-stimulated ICP response is correlated with increased expression of the *hSlo* mRNA (Figs. 5 and 6). Presumably, the same holds true for the longer time points (i.e., 3–4 mo), although this was not directly evaluated in this series of experiments. Moreover, there were no detectable

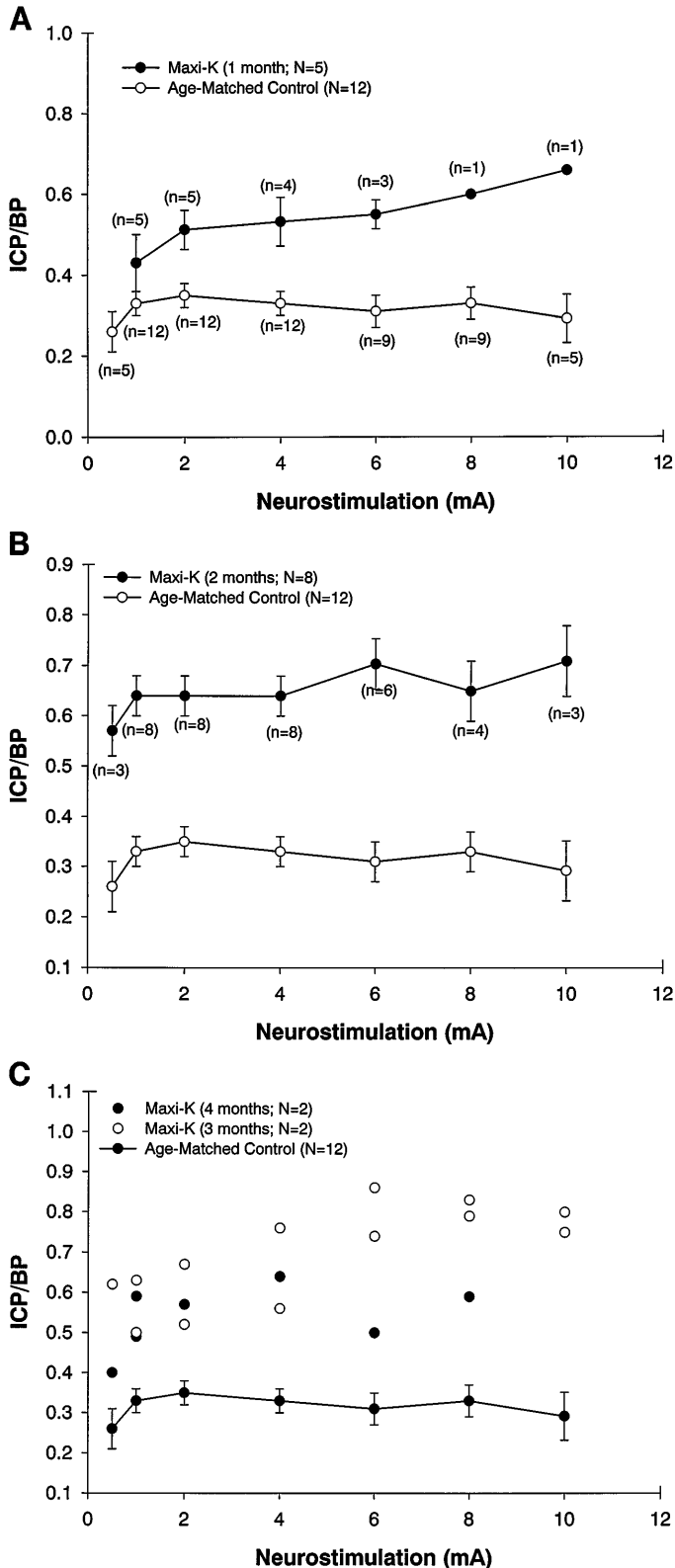


Fig. 4. Mean amplitude of ICP responses of maxi-K<sup>+</sup>-transfected animals (>9 mo old at time of injection) 1 mo (A), 2 mo (B), and 3–4 mo (C) after a single intracavernous injection of pcDNA/*hSlo* naked DNA. For purposes of statistical analysis, all age-matched control animals were considered to represent a single homogeneous population (see MATERIALS AND METHODS); *n* refers to total number of observations for each level of nerve stimulation, and *N* refers to total number of animals in particular treatment groups. Nos. of observations at each level of neurostimulation for age-matched control animals are shown only in A but were the same for B and C. All data are presented as means ± SE for mean amplitude of ICP response for each level of current stimulation. Two-way ANOVA revealed that there were significant differences in mean amplitude of ICP response at all levels of neurostimulation at 1- and 2-mo time points postinjection. A similar trend was observed even at 3–4 mo postinjection, although the smaller number of observations precluded statistical comparisons.

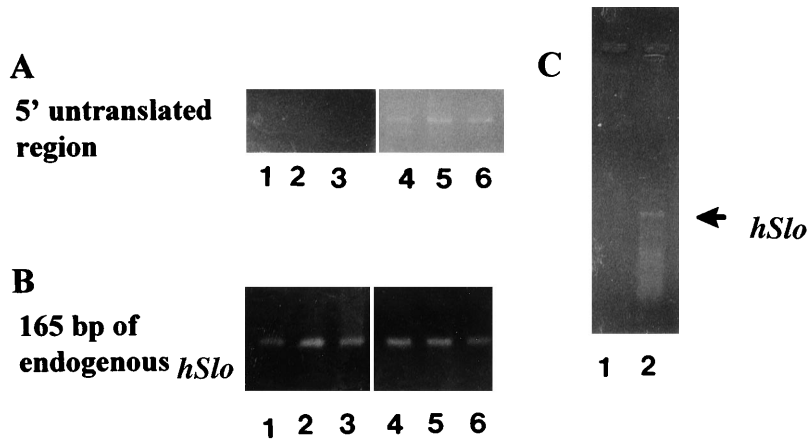


Fig. 5. RT-PCR assay for gene expression in corporal tissue excised from *hSlo*-transfected and control rats 2 mo after a single intracorporal microinjection of either pcDNA/*hSlo* or pcDNA alone (i.e., vector alone). Total RNA was then extracted from pcDNA/*hSlo*-transfected as well as pcDNA-transfected control tissues and was RT-PCR amplified with primers as described in MATERIALS AND METHODS. *A*: amplification to 5'-untranslated region resulted in a significant cDNA band from pcDNA/*hSlo*-transfected (lanes 4, 5, and 6) but not from pcDNA-transfected control tissue (lanes 1, 2, and 3). *C*: full-length insert with vector 5'- and 3'-untranslated sequences was also RT-PCR amplified from pcDNA/*hSlo*-transfected tissue (lane 2) but not from pcDNA-transfected control (lane 1). Quality of RNA from both groups of tissue was also examined with primers that amplified endogenous maxi-K<sup>+</sup>. *B*: endogenous maxi-K<sup>+</sup> was amplified to a comparable level in RNA from all tissues (pcDNA/*hSlo*-transfected tissue: lanes 4, 5, and 6; pcDNA control: lanes 1, 2, and 3). Sequences for these primers are described in MATERIALS AND METHODS. Note that each lane in *A* and *C* corresponds to a corporal tissue sample obtained from a distinct animal ( $N = 4$ ). However, samples run in *B* were obtained from same corporal tissues shown in *A*. As such, these data were obtained from a total of 4 distinct *hSlo*-transfected and 4 distinct vector-only rats at 2-mo time point.

effects of vector alone or sham surgery (see MATERIALS AND METHODS) on the nerve-stimulated ICP responses at any time point examined in these initial studies (see Figs. 2–4). Taken together, these data provide compelling evidence that the enhancement in the nerve-stimulated ICP responses observed in the maxi-K<sup>+</sup>-transfected animals, relative to the age-matched control animals, is most certainly related to the extrachromo-

somal expression of the *hSlo* cDNA and a nominally corresponding increase in expression of the maxi-K<sup>+</sup> channel protein.

To better judge the physiological meaning of the increase in ICP in the maxi-K<sup>+</sup>-transfected animals, a second group of young control (2–3 mo old) animals was also studied. As shown in Fig. 2, the increase in the mean amplitude of the nerve-stimulated ICP/BP ratio in the 10- to 13-mo-old maxi-K<sup>+</sup>-transfected animals approximates, but does not exceed, the response observed in the adolescent rats. This would suggest that the putative increased expression of the maxi-K<sup>+</sup> channel in the “older” animals is associated with a nerve-stimulated ICP response that is nominally equivalent to the best response expected under “normal” physiological conditions in younger animals.

With respect to the mechanistic basis for our current observations, it is clear that the injected *hSlo* cDNA is likely to be taken up into all cell types present in the rat corpora. In this regard, despite the fact that we cannot unequivocally exclude a role for uptake of the *hSlo* cDNA in the endothelial cell in mediating the observed increases in nerve-stimulated ICP, for the purposes of this report we will confine our discussion to putative effects resulting from uptake and expression in the corporal smooth muscle cell. This seems reasonable in light of the fact that the corporal smooth muscle cell makes up the vast majority of the corporal parenchyma and, moreover, that relaxation of the corporal smooth muscle is both necessary and sufficient for erection. A more precise analysis of the cellular disposition of the *hSlo* cDNA and the resulting expression of the  $\alpha$ -subunit of the maxi-K<sup>+</sup> channel, as well as the relative

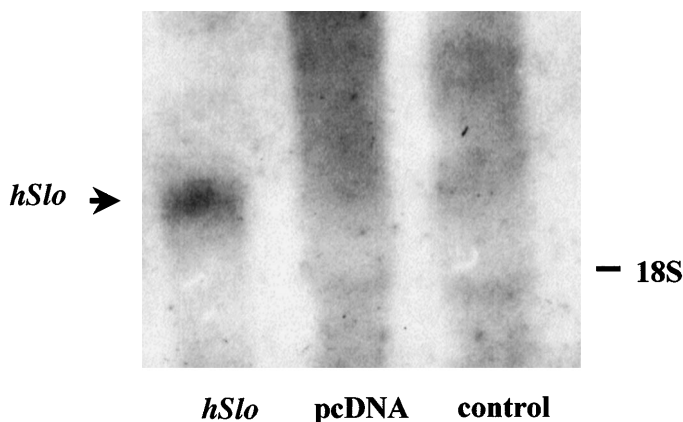


Fig. 6. Incorporation of *hSlo* into rat corporal smooth muscle examined by Northern blot analysis. Total RNA (20  $\mu$ g each) was obtained from pcDNA/*hSlo*-transfected, pcDNA-transfected control, or uninjected control rat corporal tissue and was hybridized with biotin-labeled *hSlo* cDNA full-length probe for human sequence. Full-length *hSlo* cDNA detects a band in pcDNA/*hSlo*-transfected rat tissue but not in pcDNA-transfected or uninjected control rat tissue. Size of *hSlo* mRNA is  $\sim$ 4.0 kb. Note that sequence homology of rat and human maxi-K<sup>+</sup> channels is  $\sim$ 90%, which corresponds to  $\sim$ 350 bp differences. Under high-stringency conditions used in this assay (see MATERIALS AND METHODS), therefore, we would expect that full-length probe would recognize only the recombinant maxi-K<sup>+</sup>, not the endogenous maxi-K<sup>+</sup> sequence.

percentage of cells thus affected, will necessarily be the province of future investigations.

Therefore, although a cause-effect relationship was not established, presumably the mechanistic basis for the increased maxi-K<sup>+</sup> channel activity would be related to the commensurate augmentation in the hyperpolarizing ability of the corporal smooth muscle cells. Moreover, given the exquisite dependence of sustained corporal smooth muscle contraction on continuous transmembrane Ca<sup>2+</sup> flux, it stands to reason that the increase in hyperpolarization is associated with a decreased transmembrane Ca<sup>2+</sup> flux through L-type voltage-dependent Ca<sup>+</sup> channels and a corresponding decrease in the free intracellular Ca<sup>+</sup> concentration, ultimately promoting greater corporal smooth muscle relaxation. Thus increasing the expression of the maxi-K<sup>+</sup> channel would logically dictate an increased sensitivity of the smooth muscle cells to the same level of neural stimulation.

As with all other *in vivo* gene therapy approaches, the potential utility of this genetic technique to the treatment of human erectile dysfunction heralds the following two considerations: 1) What is the likelihood of affecting only the desired cell type(s)? and 2) What percentage of target cells must be affected to indicate a physiologically relevant therapeutic effect? In light of such considerations, there are two main reasons for suspecting that gene therapy of erectile dysfunction may be inherently more successful than its proposed uses in other, more systemic, cardiovascular disorders such as atherosclerosis, vasculitis, and restenosis after balloon angioplasty (4, 8, 15, 24).

First, it is a well-documented fact that corporal smooth muscle cells are interconnected by a ubiquitously distributed population of intercellular channels known as gap junction proteins (6, 9–11, 23), with connexin 43 as the predominant isoform expressed in the human penis. These intercellular channels provide partial cytoplasmic continuity between adjacent smooth muscle cells, allowing the intercellular exchange of physiologically relevant ions (K<sup>+</sup> and Ca<sup>2+</sup>) and second messenger molecules (D-myo-inositol 1,4,5-trisphosphate, cAMP, cGMP). As such, the presence of gap junctions in the rat (27) and man (6, 9–11, 23) provides an important anatomic substrate for coordinating the syncytial contraction and relaxation responses that are a prerequisite to normal penile erection and detumescence; that is, intercellular communication among the smooth muscle cells permits cells that are not directly activated by a relevant neuronal/hormonal signal to be rapidly, albeit indirectly, recruited into the contraction or relaxation response.

To summarize, the main implication of gap junctions to the genetic therapy of erectile dysfunction is that their presence would ensure that only a fraction of the corporal smooth muscle cells would need to be genetically modified to effect rather global changes in corporal smooth muscle tone. This is of crucial importance, because it would minimize the necessity for utilizing more aggressive genetic incorporation strategies (e.g., adenoviral or retroviral incorporation) that have a

concomitantly greater number of side effects and concerns (e.g., insertional mutagenesis or immunological reactions).

Second, the proposed gene therapy is designed to take advantage of the fact that relatively subtle alterations in the balance between contracting and relaxing stimuli can result in profound alterations in erectile physiology and function (3, 9, 20, 31). The goal of gene therapy is therefore to restore a more normal balance between contracting and relaxing stimuli after expression of an exogenous gene(s) that codes for physiologically relevant proteins in corporal smooth muscle, in this case, the maxi-K<sup>+</sup> channel. In light of the multifactorial nature of erectile dysfunction in man, there may in fact be many distinct genetic therapy strategies that will be effective in the restoration of erectile potency. It is worth noting, for example, that qualitatively similar effects on ICP were observed after the intracavernous injection of an inducible form of the nitric oxide synthase enzyme in a rat model (16). Thus, if expression of these or other extrachromosomal genes can be maintained in humans for a period of weeks to months (as the preliminary data herein indicate), it is conceivable that a patient could obtain "normal" erections in the absence of any other exogenous manipulation during this time period. Clearly this would be a major advance over all currently available therapies. However, one should keep in mind that although we have arguably demonstrated "proof of principle" with regard to the potential utility of gene therapy, the real efficacy of this novel therapy certainly awaits clinical trials.

Taken together, these data are consistent with the supposition that increased maxi-K<sup>+</sup> channel activity after a single intracorporal injection of naked *hSlo* DNA is the result of the presence of a greater number of maxi-K<sup>+</sup> channels on some fraction of corporal smooth muscle cells. This, in turn, results in a greater hyperpolarization for any given level of neural stimulus, presumably altering intracellular Ca<sup>2+</sup> mobilization/homeostasis and thus promoting greater corporal smooth muscle relaxation. In conclusion, it seems reasonable to assume that the relatively stable transfection of corporal smooth muscle cells with the human smooth muscle maxi-K<sup>+</sup> channel cDNA represents an important and physiologically relevant strategy for the novel molecular manipulation of corporal smooth muscle tone in the treatment of organic erectile dysfunction.

The authors are grateful to Diane DiTrapani for excellent secretarial assistance.

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-42027 and DK-46379. G. J. Christ is a Ben Marden Distinguished Scholar in Urology.

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Received 24 October 1997; accepted in final form 13 April 1998.

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