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The Kv2.2 α Subunit Contributes to Delayed Rectifier K^+ Currents in Myocytes From Rabbit Corpus Cavernosum

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ABSTRACT: K^+ currents are known to regulate the excitability of corpus cavernosum myocytes and therefore to play a role in the control of penile erection and detumescence. We used electrophysiology and molecular cloning techniques to identify ion channel proteins that contribute to K^+ currents in rabbit cavernosal myocytes. Currents were recorded from freshly isolated myocytes using whole-cell patch clamp techniques. Cavernosal myocytes expressed a delayed rectifier voltage-gated K^+ current that appeared to contribute to the resting membrane potential. This voltage-gated K^+ (K_V) current was inhibited by the nonselective compounds 4-aminopyridine (1–10 mM), (+)-fenfluramine (10 μM–1 mM), and *Grammostola spatulata* venom (1:100) in a dose-dependent and reversible fashion. Hanatoxin-1 (1 μM), a selective Kv2 channel inhibitor, partially inhibited the current, but α-dendrotoxin (200 nM), a Kv1 channel blocker, had no effect. The nucleotide sequence of K^+ channel subunits was determined by polymerase chain reaction–based cloning techniques using RNA derived from cavernosal muscle strips and single identified myocytes. Molecular cloning techniques identified the full-length sequence of the rabbit ortholog of the Kv2.2 α subunit. This sequence contains 911 amino acid residues and is 92% identical to the recently revised human Kv2.2 sequence. Identified cavernosal myocytes of the type used in physiological recordings expressed Kv2.2 messenger RNA. We conclude that Kv2.2 α subunits contribute to whole-cell currents in rabbit cavernosal myocytes. Further, K_V currents play a role in regulating membrane potential and hence excitability in rabbit cavernosal myocytes.

Key words: Penile erection, potassium channels, patch clamp techniques, smooth muscle, molecular cloning.

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The contractile tone of cavernosal myocytes is a fundamental regulator of penile erection and detumescence (Andersson and Wagner, 1995; Christ, 1995), and is the target for many effective therapies for erectile dysfunction (Moreland et al, 2001; Padma-Nathan and Giuliano, 2001). As with all smooth muscle cells, membrane potential is a direct regulator of the tone of cavernosal myocytes. The experiments described in this paper investigated the pharmacological characteristics of a K^+ current that is a key contributor to cavernosal myocyte membrane potential and hence excitability. K^+ currents have varying sensitivity to voltage and the intracellular concentration of molecules such as adenosine triphosphate (ATP) and Ca^{2+} ions, which results in several classifications of the channels. In addition, the molecular composition of these proteins has provided a parallel but separate nomenclature that is summarized in Table 1.

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An increasing amount of literature is available on the actual ionic currents in cavernosal smooth muscle cells from both explant, cultured myocytes, and freshly dissociated cells. For example, the large-conductance Ca^{2+}-activated K^+ channel has been studied in some detail, particularly in cells from healthy rabbits (Malysz et al, 2001a) and from humans with erectile dysfunction (Fan et al, 1995). However, cavernosal myocytes express many types of ionic conductances and the description of these currents is still incomplete. Ion channels represent excellent potential targets for therapeutic intervention in treatment of erectile dysfunction because of their diverse molecular makeup. Voltage-dependent K^+ (K_V) currents have been identified and their properties have been studied in many different types of smooth muscle cells (Beech and Bolton, 1989; Edwards and Weston, 1990; Farrugia et al, 1993). K_V channels are important determinants of the membrane potential and have been shown to contribute to the regulation of contractility in smooth muscle cells (Thornbury et al, 1992; Zhang et al, 1993; Yuan et al, 1995).

Voltage-sensitive K^+ channels are composed of α and β subunits arranged in a tetrameric structure around a central ion-selective pore. The α subunits are typically sufficient to form a functional channel in expression sys-

Table 1. *Nomenclature used for K^+ channels identified in corpus cavernosum smooth muscle**

	Voltage-Dependent (K_v)	Large Conductance Ca^{2+}-activated (BK, Maxi K)	Inward Rectifiers (K_{IR}) (inc. ATP-sensitive (K_{ATP}))
Main regulators of ion currents	Voltage	Voltage and $[Ca^{2+}]_i$	Voltage, Inactive at resting $[ATP]_i$
Pore-forming α subunit families (mammals)	Kv1, Kv2, Kv3, Kv4	hSlo	Kir1, Kir2, Kir3, Kir6
Approved gene symbol families	KCNA, KCNB, KCNC, KCND	KCNM	KCNJ
Other names of pore-forming subunits	*Shaker, Shab, Shaw, Shal* (*Drosophila* homologues)	. . .	ROMK1, IRK, GIRK1, GIRK2

* See http://www.gene.ucl.ac.uk/nomenclature/genefamily/KCN.shtml for a comprehensive summary of K^+ channel nomenclature.

tems. The β subunits cannot form functional channels, but they modify the properties of coexpressed, functional α subunits. Work on flies and mammals has identified 4 distinct subtypes of Kv α subunits, and these have been given the systematic names of Kv1 (*shaker*), Kv2 (*shab*), Kv3 (*shaw*), and Kv4 (*shal*). A number of nomenclatures have been coined, with the Kv2 subunits also being referred to as *drk* or KCNB (Conley and Brammar, 1999). The identification of K_V channel subunits is further complicated by the cloning of several members of each family of α subunits with, for example, two different Kv2 genes identified in tissues of humans, mice, rats, and dogs. In addition, a number of so-called ''silent'' α subunits have been cloned from several different species, and these also appear to contribute to the ion channel proteins in a number of cell types. Until recently, Kv2 subunits from rabbits had not been identified, although a rabbit Kv2.1 sequence has recently been added to GenBank (accession number AF266507). The rabbit has been widely used as a model for studying the physiology and pathology of erectile function; therefore, one goal of the present investigation was directed at the necessary molecular identification of K^+ channel subunits in rabbit cavernosal myocytes.

Rabbit corpus cavernosum smooth muscle cells express a delayed rectifier voltage-sensitive K^+ current (Malysz et al, 2001a). The biophysical properties of the current have been characterized and it appears that the K_V current contributes to the membrane potential of cavernosal myocytes. Therefore, K_V channel regulation could be an important contributor to the process of penile erection.

Many compounds, in addition to their primary sites of action, inhibit K_V currents with varying degrees of selectivity. These compounds include the dissociative anesthetic phencyclidine (Frey et al, 2000) and the appetite-suppressant drug (+)-fenfluramine (Weir et al, 1996; Patel et al, 1997). In addition, K_V currents in many cell types can result from the expression of several different channel proteins within a given cell. Consequently, nonselective inhibitors, such as 4-aminopyridine (4-AP), have bimodal concentration response curves for blocking whole-cell currents (eg, Baker et al, 1993; Himmel et al, 1999). However, the differences in sensitivities to 4-AP cover a narrow concentration range and have required the identification of more selective compounds to make it feasible to do pharmacological profiling of K_V currents in a given cell type. Some such K_V-selective compounds have become available quite recently. These compounds, derived from snake and spider venoms, include α-dendrotoxin, which blocks some Kv1 channel subunits (Harvey, 2001), and hanatoxin, which blocks Kv2 channel subunits (Swartz and MacKinnon, 1997a; Kaczorowski and Garcia, 1999).

In this study, we tested the effects of several inhibitors of K_V currents on the delayed rectifier K^+ current in rabbit cavernosal myocytes. We used the information from these studies to further assess the contribution of K_V currents to the membrane potential of cavernosal myocytes. Furthermore, we have identified Kv channel α subunit messages that likely form ion channel proteins that contribute to these whole-cell currents. Molecular cloning techniques were used to confirm the conclusions of the physiological and pharmacological approaches, resulting in the identification of a rabbit ortholog of a mammalian K_V channel α subunit (Kv2.2) in corpus cavernosum smooth muscle. Some of this work has been previously presented in abstract form (Malysz et al, 2001b).

Materials and Methods

Preparation of Single Smooth Muscle Cells

The Institutional Animal Care and Use Committee at Mayo Clinic approved all animal-handling procedures in this study. The method for dissociating smooth muscle cells from rabbit corpus cavernosum has been described in detail previously (Malysz et al, 2001a). Briefly, the tissue was obtained from male white New Zealand rabbits aged 12–16 weeks (Covance Research Products, Denver, Pa and Myrtle's Rabbitry Inc, Thompson Station, Tenn) that had been killed using an overdose of pentobarbital sodium (Abbott Laboratories, North Chicago, Ill) administered through the ear vein. Single cells were prepared fresh each day by a papain-based enzymatic dissociation procedure from pieces of cavernosal tissue. Tissue pieces were incubated for 30–60 minutes at 32–35°C, washed with Hanks balanced salt solution, and then gently triturated to release single smooth muscle cells.

Table 2. *Sequence of oligonucleotide primers used to amplify Kv2 orthologs from rabbit tissue**

	Sense Primer	Antisense Primer
Kv2.1 deg	cayatgatggargaratgtg	aangcrtcyttcatrttcat
hKv2 deg	ctcaayacgctgcckgag	agcccaaytcattgtarctcc
GSP	ggagtacctcttacgcttcctgtcctc	ccgttgagtgtctggccagtttcagg
RbKv2_2out	tggaggctgtgtgtattgct	tcattgtagctccgcctcag
RbKv2_2in	ttacgcttcctgtcctcacc	tcattgtagctccgcctcag

* Degenerate residues are indicated with standard codes used by the International Union of Pure and Applied Chemistry.

Whole cell voltage clamp recordings were completed within 8 hours of the dissociation procedure. Single cells for reverse transcription and polymerase chain reaction (RT-PCR) amplification were collected immediately after dissociation and frozen directly on dry ice.

Whole-Cell Voltage Clamp Recordings

Conventional whole-cell patch clamp measurements were made at room temperature (22–24°C) in a custom-designed 0.5-mL recording chamber. Glass pipettes were coated with elastomer (R-6101; Dow Corning, Midland, Mich) and fire-polished to form electrodes with typical resistances of 2–6 MΩ. The seal resistance between the patch and the pipette was 2–10 GΩ. During recordings, the holding voltage was −70 mV, with voltage steps of between −80 mV and +30 mV for 200 msec using 10 mV increments. Iberiotoxin (100–200 nM) was included in the recording solution for every experiment to inhibit the large conductance K^+ current (BK) that is activated at membrane voltages more positive than +30 mV in these cells (Malysz et al, 2001a). Recordings were made from the cells characterized as type II cavernosal myocytes by virtue of the expression in the cells of not only a BK current, but also a significant component of outward, voltage-sensitive K^+ current (K_V) (Malysz et al, 2001a). We define type II myocytes as cells in which the outward current at +30 mV constitutes ≥6% of the outward current detected at +80 mV. This sets an objective standard for the choice of the cells under study and was chosen because this is the dividing point between the 2 populations of cells identified in our previous studies (see Malysz et al, 2001a for details). Type I myocytes express very little K_V-like current. Data were collected using an Axopatch 200A amplifier, Digidata 1200 interface, and pCLAMP 8 software from Axon Instruments (Union City, Calif). Data were sampled at 5–10 KHz and filtered at 2 KHz. A series resistance compensation of 70%–80% was applied to each recording. The pipette solution was a standard KCl-based intracellular solution buffered to pH 7.2 with Hepes-KOH and a osmolarity of 275 mOsm (135 mM KCl, 4 mM $MgCl_2$, 3 mM Na_2ATP, 2 mM Li_2GTP, 2 mM ethyleneglycotetraacetic acid, and 10 mM Hepes). The extracellular solution had an osmolarity of 270 mOsm and contained physiological concentrations of the standard solutes (146 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, and 5 mM Hepes) buffered to pH 7.35 with Hepes-KOH. All drugs and toxins were dissolved in the extracellular solution and applied directly to the bath.

Cloning of Rabbit Kv2 (Shab) Orthologs From Corpus Cavernosum

Rabbit Kv2 orthologs were cloned from total RNA extracted from strips of corpus cavernosum tissue and from brain. After the animals were killed, the corpus cavernosum was placed immediately into RNAlater (Ambion, Austin, Tex) to preserve RNA in experimental samples. The strips of tissue were removed and cut into small pieces for homogenization in the extraction buffer, then total RNA was extracted using a guanidinium thiocyanate-phenol-chloroform-based kit (Totally RNA, Ambion). First-strand complementary DNA was synthesized from 4 μg of total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies Inc, Gaithersburg, Md) with a mixture of random hexamers and oligo(dT) as the primers (Mayo Molecular Core, Rochester, Minn). Rabbit brain first-strand complementary DNA (cDNA) was synthesized by the same methods.

Two pairs of degenerate oligonucleotide primers were designed for PCR amplification of Kv2-like K^+ channel α subunits. One pair was based on conserved regions from an alignment of human, murine, rat, and canine Kv2.1 sequences (Kv 2.1deg; Table 2). The other pair of primers was derived from an alignment of human Kv2.1 with human Kv2.2 (hKv2 deg; Table 2). These primers were used to amplify products from both rabbit penis and rabbit brain RT-DNA using platinum *Taq* DNA polymerase (Life Technologies) and a touchdown PCR protocol that was run for 35 cycles of amplification. The products were separated on a low-melt agarose gel, and single bands were excised for cloning into the pCR2.1 plasmid vector using the TOPO-TA protocol (Invitrogen, Carlsbad, Calif). Automated DNA sequencing of both strands of the clones confirmed the identity of the partial sequences obtained by this method (Mayo Molecular Core).

The full-length sequence of rabbit Kv2.2 was obtained with the SMART-RACE technique (Clontech, Palo Alto, Calif) using gene-specific primers for PCR amplification from the RT reaction (GSP; Table 2). The PCR products were purified, cloned, and sequenced as described above for obtaining the initial, incomplete sequences. At least 4 independent overlapping clones were obtained and sequenced in order to confirm the correct sequence of the gene products.

Amplification of Rabbit Kv2.2 From Identified Single Cavernosal Myocytes

We tested for the expression of K^+ channel α subunits in identified single rabbit cavernosal myocytes by RT-PCR using oligonucleotide primers specific for the identified sequence of rabbit Kv2.2 (Rb2_2out and Rb2_2in; Table 2). Identified cells were collected in RNAase-free microcentrifuge tubes containing a carrier molecule (0.5 μg transfer RNA) and 10 μg of proteinase K (Lewis, 1999) and then immediately frozen on dry ice. Cells were lysed by incubation at 90°C for 10 minutes, 50°C for 30

minutes, then 95°C for 10 minutes. The RT was primed with a mixture of random hexamer and oligo(dT) primers, and used a modified MMLV RT (Superscript, Invitrogen). The product of the RT reaction was then diluted with a buffer containing the primers and polymerase enzyme for amplification (GeneAMP Gold, PE Applied Biosystems, Foster City, Calif). All PCR products were purified from the agarose gel using a standard procedure (Qiaquick, Qiagen, Valencia, Calif) and sent to the Mayo Molecular Core facility for sequencing to confirm their identity.

Materials

Iberiotoxin, (+)-fenfluramine, 4-AP, and α-dendrotoxin were purchased from Sigma Chemical Company (St Louis, Mo) and *Grammostola spatulata* venom was from Spider Pharm (Yarnell, Ariz). *G. spatulata* has been recently reassigned to the *Phrixotricus* genus, but we have used the nomenclature that is established in the biomedical literature. Hanatoxin was a gift from Dr Kenton J. Swartz at the National Institute of Neurological Disorders and Stroke in Bethesda, Md. The toxins were dissolved in extracellular solution at 1–2 μM and either diluted to the working concentration or frozen (−20°C) as stocks for future use. *G. spatulata* venom was diluted in the extracellular bath solution at the ratio of 1:100 (v/v). All other compounds were purchased from Sigma or Fisher Scientific (Fairlawn, NJ).

Statistical Analysis

Determination of statistically significant changes in K_V currents was done with the Student's *t* test for single comparisons or analysis of variance (ANOVA) using a Tukey-Kramer posttest for comparison of more than 2 data sets using the Graphpad Instat computer package (GraphPad Software Inc, San Diego, Calif). Data are shown as means ± SEM with $P < .05$ considered to be statistically significant. SEM is the standard error of the mean and n is the number of independent experiments, each from a different cell.

Results

The two major K^+ currents that contribute to outward currents in freshly isolated rabbit cavernosal myocytes are delayed rectifier K^+ (K_V) currents and BK currents (Malysz et al, 2001a). To study K_V currents in isolation, BK currents were blocked by iberiotoxin (100–200 nM) in all experiments. As previously described, iberiotoxin blocks the outward currents at voltages more positive than +30 mV and does not affect the membrane potential of myocytes. Charybdotoxin, another blocker of BK currents, had the same effect as iberiotoxin and did not appear to affect the K_V currents (Malysz et al, 2001a). The cells under study had the same properties as previously reported in that the resting membrane potential was in the −35 to −25 mV range. The cells had high input resistances (3 GΩ), and recordings could be continued for up to 1 hour with little apparent alteration in membrane integrity. Thus, it appears that the cells were healthy and metabolically stable.

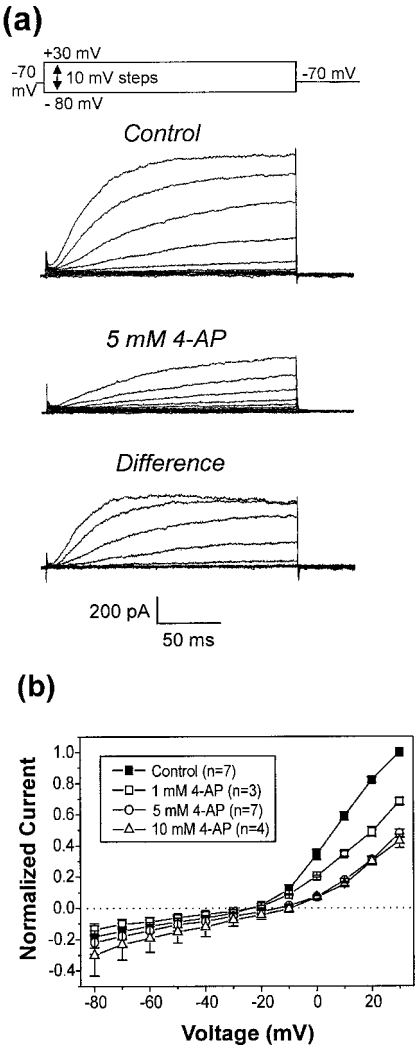

Figure 1. K_V currents were recorded from freshly isolated rabbit cavernosal myocytes using whole-cell voltage-clamp techniques. Recordings were made in the presence of 200 nM iberiotoxin to inhibit large conductance Ca^{2+}-activated K^+ (BK) currents. **(a)** 5 mM 4-AP inhibited the K_V current by approximately 55%. The current that was inhibited by 4-AP (difference) was obtained by subtracting the traces obtained in control experiments from the recordings obtained in the presence of 4-AP. This current activates slowly and does not inactivate significantly during the voltage step. **(b)** Data from several independent experiments normalized to the steady state outward current measured at +30 mV under control conditions. Each data point represents the mean ± SEM from n different cells.

To determine the effect of modulation of K_V channels on cavernosal outward currents and membrane potential, the responses to 4-AP (1–10 mM) were examined. 4-AP, a nonselective K^+ channel blocker, inhibited the K_V currents in cavernosal myocytes in a concentration-dependent manner (Figure 1). The effect of 4-AP was fully reversible and occurred equally at all membrane voltages. 4-AP at 5 mM inhibited 52.2% ± 2.6% of the outward

current at +30 mV (n = 7) and 10 mM inhibited 57.2% ± 3.2% (n = 4). 4-AP at 5 mM therefore appeared to be the maximally effective concentration (Figure 1b). The fraction of current that was inhibited by 4-AP (''difference,'' Figure 1a) is similar to the slowly activating, weakly inactivating current that we have described previously (Malysz et al, 2001a) and represents approximately 55% of the total K_V current in cavernosal myocytes. 4-AP (10 mM) significantly depolarized cavernosal myocytes from −27.2 ± 2.4 mV to −13.3 ± 4.1 mV (n = 4, $P < .05$). The concentration of 4-AP required to block the outward K^+ current in these studies is sufficient to inhibit many different types of voltage-sensitive K^+ currents (Mathie et al, 1998). These data therefore provide limited information about the identity of the K_V channel subunits contributing to the outward currents in rabbit cavernosal myocytes.

The K_V current observed in rabbit cavernosal myocytes is characteristic of the slowly activating, delayed rectifier current that has been described in other types of smooth muscle cells, including those from several different vascular tissues. In those studies, K_V currents were inhibited by the appetite-suppressant drug (+)-fenfluramine (Redux, American Home Products, Collegeville, Pa). Therefore, we tested this compound on cavernosal myocytes (Figure 2). (+)-Fenfluramine reversibly inhibited K_V currents in rabbit cavernosal myocytes in a concentration-dependent fashion between 10 μM and 1 mM (Figure 2a). The inhibition was rapid and was not sensitive to membrane voltage. At the highest tested concentration of 1 mM, (+)-fenfluramine inhibited the outward currents by 75.3% ± 2.2% at +30 mV and caused a significant depolarization of the cavernosal myocytes (control membrane potential, −30.8 ± 5.7 mV; (+)-fenfluramine, −22.9 ± 3.2 mV, n = 5, $P < .05$). These results demonstrate that the (+)-fenfluramine-sensitive current is a contributor to the regulation of membrane potential in cavernosal myocytes.

(+)-Fenfluramine is a poorly characterized modulator of K_V currents for further characterizing the currents; therefore, in cavernosal myocytes, we tested several peptide toxins that have been identified as selective blockers and gating modifiers of different types of K_V currents. α-Dendrotoxin, an inhibitor of some Kv1-type K^+ channel α subunits, did not affect the outward currents in cavernosal myocytes and had no effect on the membrane potential of the cells (control, −23.9 ± 4.0 mV; 200 nM α-dendrotoxin, −24.6 ± 4.4 mV, n = 7, Figure 3).

The weak effects of α-dendrotoxin prompted us to investigate the effects of other known toxins that inhibit K_V currents in native cells and heterologous expression systems. The venom from the Chilean tarantula *G. spatulata* contains a peptide inhibitor of a limited number of K_V currents. *G. spatulata* venom significantly inhibited the

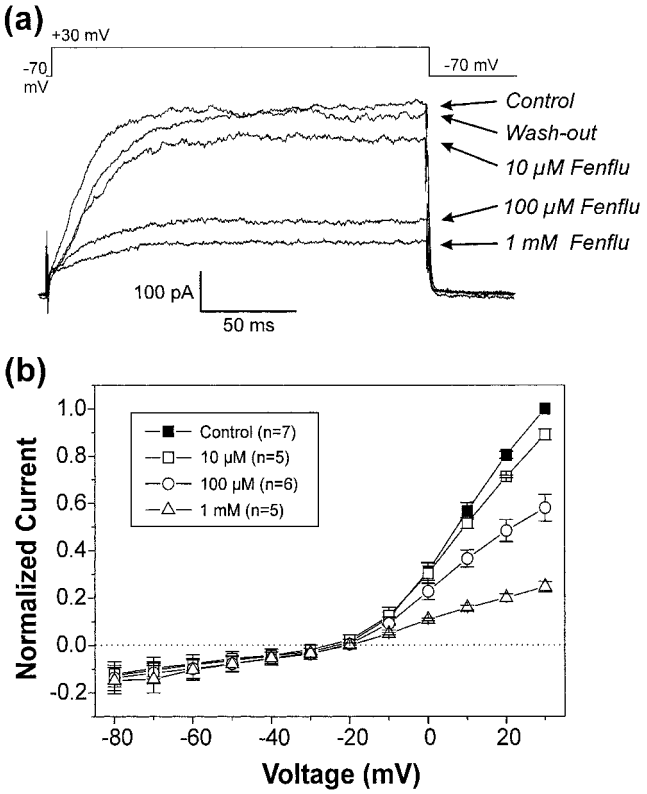

Figure 2. K_V currents were recorded from freshly isolated rabbit cavernosal myocytes using whole-cell voltage-clamp techniques. Recordings were made in the presence of 200 nM iberiotoxin to inhibit large conductance Ca^{2+}-activated K^+ (BK) currents. **(a)** An example of the dose-dependent inhibition of K_V currents following addition of (+)-fenfluramine to the bath perfusate for a voltage step from −70 mV to +30 mV. **(b)** (+)-Fenfluramine inhibits the steady state outward current at positive membrane voltages. Data are means ± SEM of n independent experiments and are normalized to the outward current measured at +30 mV under control conditions.

outward currents at membrane voltages more positive than −30 mV (Figure 4). At +30 mV, a 1:100 dilution of *G. spatulata* venom reduced the outward current by 54.8% ± 10% (n = 6). In addition, *G. spatulata* venom significantly depolarized the membrane potential from −32.3 ± 4.9 mV to −23.4 ± 4.9 mV (n = 6, $P < .05$, paired *t* test). The current that was inhibited by the venom was typical of a delayed rectifier K^+ current and exhibited weak voltage-dependent inactivation (difference current; Figure 4a). The effect of *G. spatulata* venom was rapid (less than 30 seconds) and could not be completely reversed by washing out the bath solution because a number of cells developed a large leak conductance after prolonged incubation.

G. spatulata venom contains a number of identified inhibitors of membrane ion channels, including hanatoxin-1, a known gating modifier of Kv2-like channels (Swartz and MacKinnon, 1995). Given that the Kv2 α subunits are components of K_V currents in several types of smooth muscle cells (Schmalz et al, 1998; Hulme et

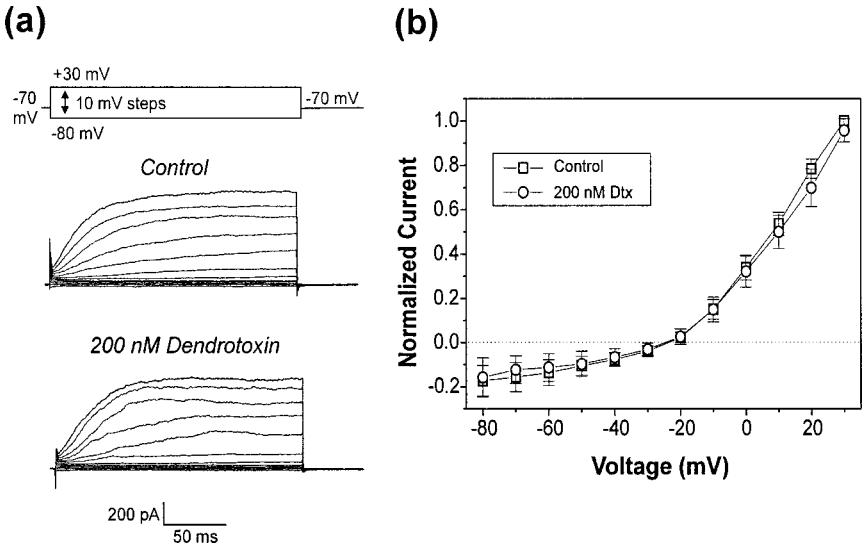

Figure 3. K_V currents were recorded from freshly isolated rabbit cavernosal myocytes using whole-cell voltage-clamp techniques. Recordings were made in the presence of 200 nM iberiotoxin to inhibit large conductance Ca^{2+}-activated K^+ (BK) currents. **(a)** α-Dendrotoxin (200 nM) had no effect on the size or time course of the evoked outward currents. **(b)** The size of steady state outward current is unaltered by 200 nM α-dendrotoxin in 7 independent experiments. The data are normalized to the steady state outward current measured at +30 mV under control conditions. Each data point represents the mean ± SEM from 7 different cells.

al, 1999; Patel et al, 1999; Xu et al, 1999), we tested a small fraction of hanatoxin-1 on rabbit cavernosal myocytes. In 7 cells, purified hanatoxin-1 (1 μM) caused a small, reversible inhibition of the outward current (Figure 5). An effect was observed at all voltages more positive than −10 mV ($P < .05$, repeated measures ANOVA) with 27.9% ± 4.2% (n = 7) of the outward current inhibited at +30 mV. Hanatoxin-1 is difficult to purify, and only

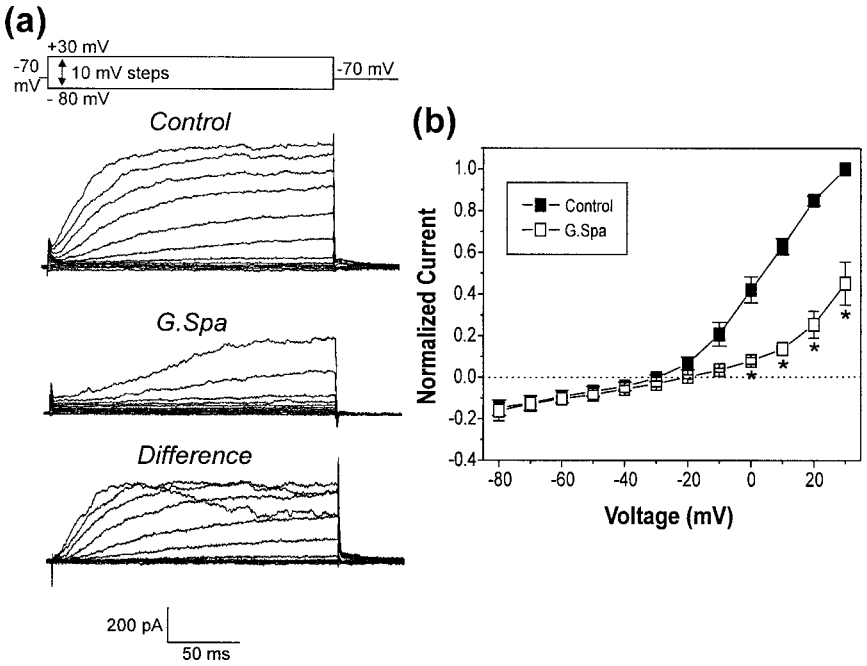

Figure 4. K_V currents were recorded from freshly isolated rabbit cavernosal myocytes using whole-cell voltage-clamp techniques. Recordings were made in the presence of 200 nM iberiotoxin to inhibit large conductance Ca^{2+}-activated K^+ (BK) currents. **(a)** *G. spatulata* venom (1:100) inhibited the K_V current by approximately 50%. The current that was inhibited by *G. spatulata* venom (difference) was obtained by subtracting the traces obtained in control experiments from the recordings obtained in the presence of the venom. The difference current activated slowly and did not inactivate significantly during the voltage step. **(b)** Data from 6 independent experiments normalized to the steady state outward current measured at +30 mV under control conditions. Each data point represents the mean ± SEM. *Significant inhibition of outward current vs control ($P < .05$, repeated measures ANOVA).

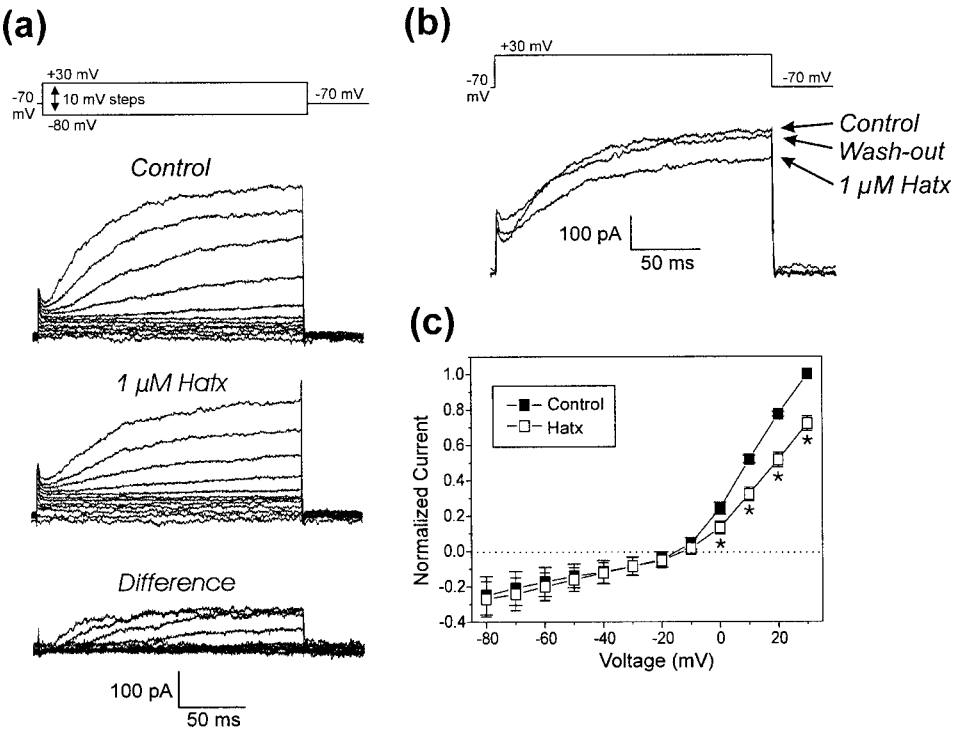

Figure 5. K_V currents were recorded from freshly isolated rabbit cavernosal myocytes using whole-cell voltage-clamp techniques. Recordings were made in the presence of 200 nM iberiotoxin to inhibit large conductance Ca^{2+}-activated K^+ (BK) currents. **(a)** Effect of 1 μM hanatoxin on a single cavernosal myocyte. Hanatoxin inhibited the K_V current by approximately 30%. The current that was inhibited by hanatoxin (difference) was obtained by subtracting the traces obtained in control experiments from the recordings obtained in the presence of hanatoxin. The current activated slowly and did not inactivate significantly during the voltage step. Inhibition was reversed during washout as shown in **(b)** for a single evoked current. **(c)** Data from 7 independent experiments normalized to the steady state outward current measured at +30 mV under control conditions. Each data point represents the mean ± SEM. *Significant inhibition of outward current vs control ($P < .05$, repeated measures ANOVA).

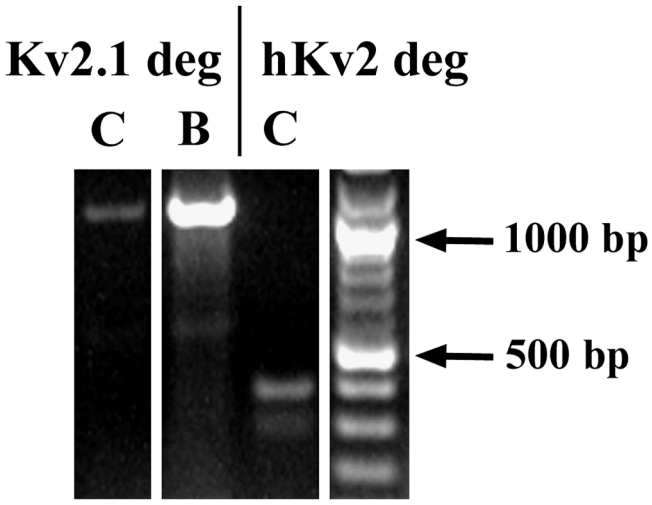

Figure 6. Kv2.1 and Kv2.2 α subunit cDNAs were amplified by PCR using degenerate oligonucleotide primers from rabbit brain (B) as a control and corpus cavernosum (C). The primers were designed using published sequences conserved in Kv2.1 from all species (Kv2.1 deg) or sequences conserved in the human Kv2 (hKv2 deg; see Table 2). The products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining. All PCR products were eluted from the gel, inserted into the TA cloning vector for cloning, and identified by sequencing individual clones. The 1028–base pair products in the Kv2.1 deg lanes were identified as rabbit Kv2.1. The smaller 400–base pair product in the hKv2 deg lane was identified as rabbit Kv2.2.

small quantities were available for these studies; therefore, we were unable to determine the effects of higher concentrations. The inhibition of outward currents by 1 μM hanatoxin-1 was not sufficient to significantly alter the membrane potential of the cells (control, −24.3 ± 6.2 mV; 1 μM hanatoxin-1, −21.6 ± 7.6 mV, n = 7).

The pharmacological sensitivity of K_V currents from rabbit cavernosal myocytes as described above, together with the biophysical properties of these currents described previously (Malysz et al, 2001a), suggest that at least a component of K_V current is mediated by a rabbit ortholog of Kv2 (*Shab*) K^+ channel α subunits. Because no rabbit Kv2 sequences were available when this work was initiated, we used degenerate oligonucleotide primers to amplify 2 products from rabbit brain and rabbit corpus cavernosum by RT-PCR. One 1028 nucleotide product had a 90% nucleotide sequence identity to human Kv2.1, whereas another 400 nucleotide product had a 91% identity to human Kv2.2 (Figure 6a). The full-length sequence for rabbit Kv2.1 was published in GenBank shortly after these data were obtained, and our 1028 nucleotide clone has >99% sequence identity to this sequence (accession number AF266507). However, the cloning and sequencing of the rabbit ortholog of Kv2.2 has not been reported.

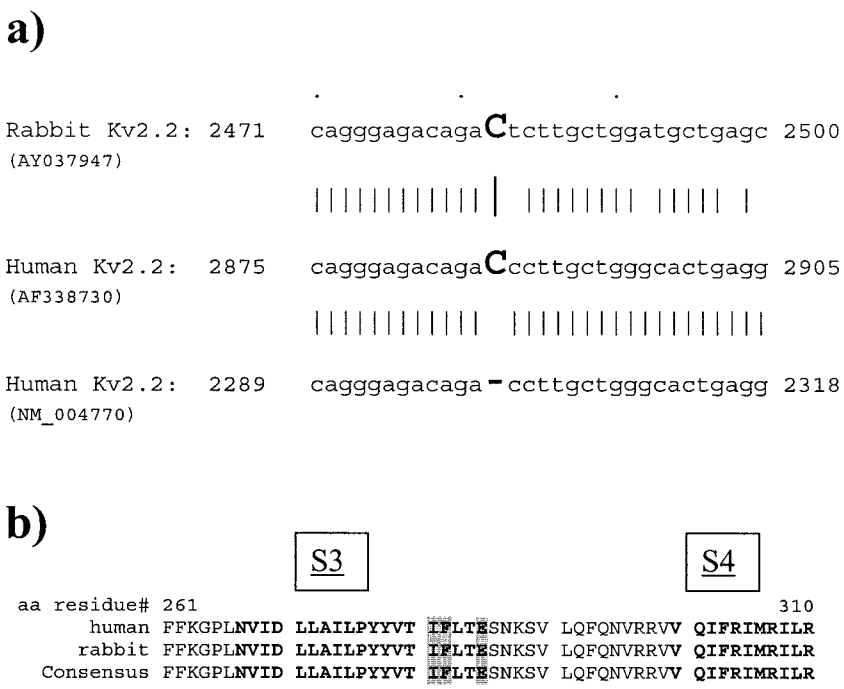

Figure 7. **(a)** An alignment of the rabbit Kv2.2 sequences with the published human Kv2.2 sequences indicating the presence of a cytosine residue in the 3′ end of the nucleotide sequence for rabbit Kv2.2. This residue results in an open reading frame that corresponds to the recently revised human Kv2.2 sequence (GenBank accession number AF338730) but which is longer than the previously published sequence (GenBank accession number NM_0047770). The rabbit sequence has been deposited in GenBank under accession number AY037947. **(b)** An alignment of part of the predicted peptide sequence highlighting the amino acid residues identified as critical for binding of hanatoxin.

We obtained the full-length sequence of rabbit Kv2.2 by 5′ and 3′ rapid amplification of cDNA ends using gene-specific oligonucleotide primers based on the sequence of the 400 nucleotide product. Several independent overlapping clones were obtained and sequenced to produce a full-length open reading frame of 2736 nucleotides, which has been submitted to GenBank under accession number AY037947. This nucleotide sequence has 90% identity to the revised sequence for human Kv2.2 (accession number AF338730) and translates into a protein of 911 amino acid residues. The revised sequence for human Kv2.2 is significant in one important respect; namely, the presence of an additional cytosine nucleotide near the 3′ end of the messenger RNA (mRNA) sequence. This results in a longer open reading frame than was initially reported for the human Kv2.2 (accession number NM004770; Schmalz et al, 1998). We have obtained independent confirmation that the Kv2.2 sequence published as AF338730 is correct by the sequencing of RT-PCR–amplified cDNA from human jejunum mRNA (data not shown). The residue corresponding to this position in our rabbit sequence is also a cytosine (see alignment in Figure 7a). In rabbits and humans alike, the change results in a significantly different amino acid sequence starting at the 768th residue. Further analysis of the predicted rabbit Kv2.2 peptide sequence revealed that it contains the amino acid residues identified as necessary for high-affinity binding of hanatoxin (Figure 7b; Swartz and MacKinnon, 1997b). These residues are located on the extracellular face of the channel between the third and fourth transmembrane spanning regions.

The cloning of Kv2.2 from rabbit corpus cavernosum indicated that this K^+ channel α subunit was expressed in the tissue but gave no indication as to which cell type contained the actual protein. To specifically determine whether rabbit cavernosal myocytes express Kv2.2, we used single-cell RT-PCR amplification of mRNA from identified individual myocytes. The cells that were collected were typical of the cells that were used for patch clamp recording. Using 10 cells as a template, we amplified a PCR product that was identified as Kv2.2 by sequencing (Figure 8). This result was duplicated in 2 additional experiments.

Discussion

We have shown that the K_V current in rabbit cavernosal myocytes is sensitive to 4-AP, (+)-fenfluramine, *G. spatulata* venom, and hanatoxin. This pharmacological profile is consistent with the contribution of a Kv2 α subunit to the K_V channel proteins in rabbit cavernosal myocytes. Furthermore, we have identified and cloned the rabbit Kv2.2 α subunit cDNA from cavernosal myocytes. These

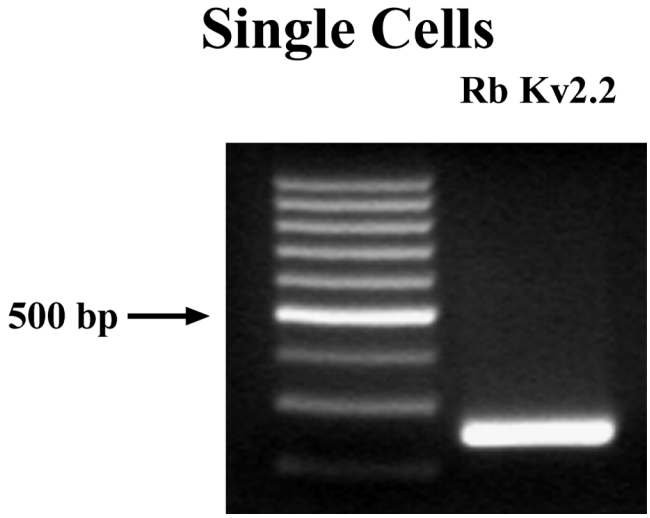

Figure 8. Ten identified cavernosal myocytes were collected using a patch clamp pipette into a single tube then processed using a one-tube RT-PCR technique. Kv2.2 α subunit cDNA was amplified by PCR with the specific oligonucleotide primers Rb Kv2_2out and Rb Kv2_2in (see Table 2). All products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining. Automatic sequencing confirmed the identities of the purified cDNA products. This figure is representative of a total of 3 independent experiments.

results are a step toward a fuller understanding of the electrical properties of cavernosal myocytes. Previously, this understanding was limited to biophysical descriptions of the K_V, BK, and K_{ATP} currents in freshly dissociated and cultured cells (Christ et al, 1993; Fan et al, 1995, 1999; Lee et al, 1999; Malysz et al, 2001a). The Kv2.2 α subunit identified in rabbit cavernosal myocytes is a potential molecular target, and hanatoxin is a selective inhibitor that can be exploited in further studies on the contribution of K_V currents to cavernosal contractility. The investigation proved that cavernosal myocytes do not express some Kv channel subunits (eg, Kv1.1, Kv1.2, and Kv1.6), but there remains the likelihood that other subunits are present and remain to be identified.

In a previous study, we determined in whole-cell voltage clamp recordings from type II cavernosal myocytes that the K_V currents in these myocytes likely regulate the resting membrane potential (Malysz et al, 2001a). In the present study, we have found further evidence for the importance of K_V currents to cavernosal myocyte excitability. 4-AP, *G. spatulata* venom, and (+)-fenfluramine all caused inhibition of K_V currents and significant depolarization of the membrane potential. Hanatoxin also blocked the K_V current, but the block was small and did not result in membrane potential depolarization, likely due to the low concentration used as a result of the limited availability of the toxin. The cells that were treated with hanatoxin were slightly but not significantly more depolarized than those that were treated with the other compounds, and this may also have decreased the likelihood of seeing an effect of the drug at these low concentrations. The concentration of hanatoxin used in our studies corresponded to the approximate EC_{50} value for inhibition of ion channel subunits expressed as homo-oligomers in heterologous expression systems, so the observed effect likely represents inhibition of less than 50% of the total hanatoxin-sensitive current. The effects of K^+ channel inhibitors on membrane potential are not surprising given that K_V currents have been identified in most types of smooth muscle cells and inhibition of K_V currents alters the function of both vascular and gastrointestinal smooth muscle (eg, Michelakis et al, 1999; Frey et al, 2000). The data presented here establish that K_V currents can regulate electrical excitability in single cavernosal myocytes.

The pharmacological sensitivity of K_V currents in rabbit cavernosal myocytes is consistent with the expression of a Kv2-like (*drk* or *Shab*) subunit in the cells. In particular, hanatoxin is a selective inhibitor of voltage-sensitive K^+ channels that contain certain identified amino acid residues (Li-Smerin and Swartz, 2000), and those residues are restricted to certain Kv2 and Kv4 (*Shab* and *Shal*) orthologs. The toxin is known to have a very low affinity for Kv1.1, Kv1.3, Kv1.6, and Kv3.1, and is predicted to have low affinity for all other Kv1 and Kv3 channels based on mutagenesis studies (Li-Smerin and Swartz, 2000). Hanatoxin is not a classical pore-blocking inhibitor but is characterized as a gating modifier that appears to bind to a region of the ion channel on the extracellular surface close to the voltage-sensing transmembrane spanning region (S4) (Swartz and MacKinnon, 1997b). Hanatoxin was tested on the whole-cell current in cavernosal myocytes because *G. spatulata* venom blocked the current. *G. spatulata* venom contains several toxins that alter ion channel function, including the voltage-sensitive Ca^{2+} channel blocker, ω-grammotoxin-S1A, and the recently characterized inhibitor of cation-selective, stretch-activated channels, GsMTx-4 (Suchyna et al, 2000). The amount of hanatoxin in the venom is variable and was not determined for the fractions of venom that we tested. It is also possible that there is an additional, unidentified toxin in this venom that inhibited the K_V currents in cavernosal myocytes; therefore, the data obtained with purified hanatoxin are important to our observations.

The conclusion that Kv2 α subunits contribute to the currents in cavernosal myocytes is supported by the ineffectiveness of α-dendrotoxin. α-Dendrotoxin did not affect the whole cell currents and did not depolarize the membrane potential of cells. The effects of α-dendrotoxin have been well characterized and the concentration used in these studies (200 nM) is sufficient to completely inhibit native currents in neurones and glial cells as well as expressed homomeric Kv1.1, Kv1.2, and Kv1.6 channels in heterologous expression systems (Harvey, 2001). These data therefore suggest that at least some types of

Kv1 channel subunits do not contribute to the K_V currents in cavernosal myocytes.

The effects of (+)-fenfluramine were intriguing because this compound is known to block currents mediated by the Kv2.1 α subunit (Patel et al, 1997) in addition to effects on a number of other types of K_V currents (eg, Perchenet et al, 2001), probably including other Kv2 α subunits. (+)-Fenfluramine can have significant effects on smooth muscle function. When used as an appetite suppressant, the serum concentrations of (+)-fenfluramine are sufficient to substantially inhibit K_V currents (Michelakis et al, 1999). Several studies have implicated K_V channel inhibition in the effects of (+)-fenfluramine on systemic blood pressure and primary pulmonary hypertension (Abenhaim et al, 1996). There are no published reports of the peripheral effects of (+)-fenfluramine on erectile function, but clearly, therapeutic agents that affect K_V channel function also have the potential to affect erectile function.

The pharmacological data presented here suggest the presence of Kv2-like α subunits in rabbit cavernosal myocytes. The biophysical data obtained in previous studies support these conclusions. The delayed rectifier K_V current in type II myocytes has been characterized in detail and its properties are consistent with the expression of a Kv2-like α subunit. The current activates in response to depolarization at rather positive voltages ($V_{ON} > -30$ mV), and activation is comparatively slow compared to neuronal K_V currents or expressed homomeric Kv α subunits (τ of activation ≈30 msec at +30 mV) (see Coetzee et al, 1999 and Malysz et al, 2001a for comparison). The current also exhibits slow and incomplete inactivation for very positive voltage steps (35.1% inactivation after 5 seconds at +30 mV) and rapid deactivation after membrane repolarization. It is not possible to absolutely correlate the biophysical properties of a native current with studies on cloned channels expressed in heterologous systems, but some general conclusions can be drawn. Fast-activating currents (τ of activation <20 msec) are usually observed in cells that express Kv1, Kv3, or Kv4 α subunits. Rapid, complete, voltage-dependent inactivation of K_V currents is characteristic of some channels containing Kv1 and all Kv4 α subunits. Slow deactivation is typical of cells that express Kv3 α subunits. Slowly activating, weakly inactivating currents are typical of currents carried by expressed Kv2 α subunits. Therefore, the slowly activating, weakly inactivating, and fast-deactivating current observed in cavernosal myocytes most resembles currents in cells that express Kv2 α subunits (Patel et al, 1997; Archer et al, 1998; Schmalz et al, 1998). This represents a clear overlap between the pharmacological, molecular, and biophysical data. At least part of the K_V current has the biophysical properties of a Kv2-like subunit, at least part of the K_V current is inhibited by a selective blocker of Kv2 channels, and a Kv2 α subunit has been cloned from identified cavernosal myocytes. In these respects, a putative regulator of cavernosal smooth muscle contractility has been identified and additional, similarly detailed studies can identify other channel proteins that have the same function.

The amplification of Kv2.2 mRNA from isolated cavernosal myocytes similar to the cells used in the electrophysiological recordings provides further evidence for the expression of Kv2 α subunits in cavernosal smooth muscle. The full-length Kv2.2 mRNA was cloned from tissue that probably contained many different cell types, including fibroblasts and endothelial cells; therefore, it was important to perform single-cell PCR on typical cavernosal myocytes in order to specifically target the cells of interest and to exclude this possible source of contamination. The only conclusive way to demonstrate the contribution of Kv2.2 to the current would be to knock out the gene in an animal model or to transiently reduce expression in cultured cells with antisense oligonucleotides. This approach is not possible in the freshly isolated cells that were studied in our experiments; therefore, our analysis is restricted to pharmacological studies and single-cell PCR analysis.

It is likely that a Kv2-containing channel protein is not the only Kv channel expressed in cavernosal myocytes because the maximally effective doses of 4-AP and (+)-fenfluramine do not inhibit all of the whole-cell outward current. It is doubtful that the native channel is a homomeric Kv2.2 assemblage. With respect to Kv2 α subunits, they have been shown to form hetero-oligomers with several of the ''silent'' Kv α subunits such as Kv5, Kv6, or Kv9 orthologs (Patel et al, 1997; Salinas et al, 1997; Zhu et al, 1999). Our initial investigations using oligonucleotide primers to amplify known rabbit Kv α subunits from corpus cavernosum tissue, indicated that one of these silent subunits, Kv9.3, is expressed in the tissue as a whole (data not shown). We have not confirmed the expression of this message in single myocytes and the full elucidation of the molecular composition of Kv channels in cavernosal myocytes requires further investigation. Smooth muscle cells in general often express the Kv1.5 α subunit, which has many properties in common with Kv2 channels. However, Kv1.5 is not sensitive to hanatoxin, and the currents mediated by Kv1.5 usually show much more rapid activation kinetics than the whole-cell current in cavernosal myocytes. This does not definitively exclude Kv1.5 as a contributor to whole-cell currents in cavernosal myocytes, and proper investigation of this possibility would require the use of different selective pharmacological agents.

The sequence of rabbit Kv2.2 is informative with respect to the conservation of the residues critical for hanatoxin binding. Otherwise, the main observation is that

the recently revised sequence for human Kv2.2 is preserved in this rabbit sequence. The core channel-forming sequence is almost completely conserved, including a consensus protein kinase C-phosphorylation site and the long C terminal amino acid sequence typical of a Kv2-like α subunit. It is interesting that the previously published clones (NM_004770) that translate into proteins with shorter C-terminus sequences do not appear to be functionally affected by the truncation. The proteins form functional currents and can be coexpressed with other Kv channel subunits in heterologous systems (Hwang et al, 1992; Schmalz et al, 1998). However, some of the immunolocalization data should be reconsidered given that the revised nucleotide sequence results in a C-terminus peptide sequence that is very different from the previously published sequence of Kv2.2. It has been indicated that the Kv2.1 C-terminus contains a signal that results in the proximal restriction and clustering of these subunits in hippocampal neurons, and that this signal was reported to be absent in Kv2.2 (Lim et al, 2000). Our data would affect these conclusions because the effect of the additional amino acids on the behavior of the proximal restriction and clustering signal has not been tested. These observations are particularly significant for the results obtained with the antibody raised to C-terminus amino acid residues (eg, Hwang et al, 1993a,b). The immunolocalization data obtained for native channels using antibodies to the N-terminus amino acids should not be affected by this revised peptide sequence (eg, Epperson et al, 1999).

In summary, our data indicate that Kv2 α subunits contribute to the whole-cell currents in cavernosal myocytes. We show that blocking K_V currents results in membrane depolarization consistent with a role for these currents in the regulation of membrane potential. The pharmacological and physiological data indicate that Kv2-like channels mediate a substantial fraction of the Kv current, and these data are supported by the molecular biology showing the presence of Kv2.2 in single cavernosal myocytes. This Kv2.2 protein is therefore one of the likely subunits in the Kv2-like channel protein and may play a role in regulating the contractility of the corpus cavernosum in health and disease.

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