



Comparative studies of the maxi-K (K_{Ca}) channel in freshly isolated myocytes of human and rat corpora

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Patch clamp techniques in freshly isolated myocytes from human corpora have documented that the large conductance calcium-sensitive K channel (K_{Ca}) subtype represents an important convergence point for the modulation of corporal smooth muscle tone, and therefore, erectile capacity. Other recent studies indicate a similar role for the K_{Ca} channel in the modulation of smooth muscle tone in the rat penis. Therefore, the explicit aim of this investigation was to evaluate and compare the characteristics of the K_{Ca} channel subtype present in freshly isolated myocytes from rat and human corpora. In short, myocytes isolated from rat and human corpora retain their characteristic morphology and contractility *in vitro*, as evidenced by light microscopic studies of their respective responses to activation of the α_1 -adrenergic receptor subtype by phenylephrine (PE). Large conductance K^+ currents commensurate with the presence of the K_{Ca} channel were readily apparent in myocytes from both preparations. I–V curves constructed from cell-attached patches utilizing symmetric KCl solutions revealed the presence of a single channel slope conductance of ≈ 200 pS for both rat and human myocytes. 1 mM TEA applied in the bath solution reversibly diminished whole cell outward K^+ currents by $\approx 50\%$, and also blocked the unitary K_{Ca} channel activity observed in the outside-out patch mode. Addition of 2 mM 8-bromo-cAMP elicited a TEA-sensitive (1 mM) ≈ 2 –3 fold increase in the magnitude of the whole cell outward K^+ currents in rat myocytes. Taken together, these data confirm and extend previous observations and provide strong evidence that the rat corporal smooth muscle K_{Ca} channel has many similarities to its counterpart in the human penis. *International Journal of Impotence Research* (2000) 12, 9–18.

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Introduction

Potassium (K) channels are key regulators of membrane potential, and therefore, of transmembrane Ca^{2+} flux, and subsequently, the degree of contraction of many types of smooth muscle, including human corporal smooth muscle.^{1–3} Corporal smooth muscle tone, in turn, modulates penile blood flow and intracavernous pressure, and as such, affects both penile rigidity and erectile capacity.^{4–6} In this regard, recent studies indicate that at least four subtypes of K channels are present in human corporal myocytes, with the large conductance calcium-sensitive potassium channel (maxi-K or K_{Ca} channel) and the metabolically regulated potassium channel (K_{ATP}) apparently being the most physiologically relevant.^{7–11}

With respect to the K_{Ca} channel subtype, cyclic AMP-dependent phosphorylation has been reported to stimulate channel activity in several smooth muscle cell types.^{1,12} A recent publication has documented that prostaglandin E_1 (PGE₁) relaxes human corporal smooth muscle, in large part, by activation of the K_{Ca} channel subtype, therefore increasing K^+ efflux, hyperpolarizing the smooth muscle cell, and ultimately, decreasing transmembrane Ca^{2+} -flux and the cytosolic Ca^{2+} concentration. The putative mechanistic for the effects of PGE₁ on the K_{Ca} channel presumably relates to a G-protein mediated activation of the adenylate cyclase/cAMP/PKA (protein kinase A) pathway.^{10,20}

In this regard, recent publications indicate that the rat is a relevant animal model for gaining greater insight into many aspects of human erectile physiology/dysfunction.^{2,24} Importantly, the vast majority of these studies have been conducted *in vivo*. Therefore, it is not surprising that there is little or no mechanistic information available concerning the identification or function of K channels present on rat corporal myocytes. However, recent

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molecular biological studies have verified that an endogenous K_{Ca} channel subtype is present in rat corporal smooth muscle. Moreover, utilization of an *in vivo* transfection technique (gene therapy) has demonstrated that increased expression of the human smooth muscle K_{Ca} channel in the rat penis increases the sensitivity and maximum responsiveness of the smooth muscle cells to cavernous nerve stimulation, therefore preventing the normally observed age-related decline in the intracavernous pressure response to cavernous nerve stimulation.

Taken together, these data indicate that K_{Ca} channels may be as relevant to erectile function and capacity in the rat, as they are in the human. The goal of the current investigation, therefore, was to begin to study the characteristics of K channels in freshly isolated, viable myocytes derived from rat corporal smooth muscle. Another explicit aim of these studies was to begin to compare the characteristics of the K_{Ca} channel present on rat corporal myocytes to that of freshly isolated myocytes from human corpora. In short, these initial investigations confirm and extend our previous studies in the rat model to indicate that there are many biophysical and pharmacological similarities in K_{Ca} channel physiology and function in corporal myocytes obtained from the rat and human penis. As such, the extant evidence continues to indicate that the rat is a good model for better understanding and evaluating at least some aspects of human erectile physiology and dysfunction.

Material and methods

Procurement of human corporal tissue

Human erectile tissue was obtained from the corpus cavernosum of patients undergoing surgery for implantation of penile prostheses as previously described.⁴ All studies were performed according to a protocol approved by the Internal Review Board of the Albert Einstein College of Medicine/Montefiore Medical Center.

Procurement of rat corporal tissue

Rat erectile tissue was obtained from Sprague-Dawley retired breeders (≈ 500 – 600 g). Rats were sacrificed by an over-dose of Pentobarbital and the whole penis was cut and placed in ice-cold DMEM solution. The corporal tissue was dissected from the rat penis as previously described.^{10,11}

Cell isolation protocol

The same protocol was utilized for isolating myocytes from human and rat corporal tissue. In addition, this protocol was identical to that previously described.^{10,11} Briefly, human or rat corporal tissues were washed and placed in 100 mm culture dishes containing 20 ml of physiological saline solution (PSS in mM: NaCl 137, KCl 5.6, $MgCl_2$ 1, Na_2HPO_4 0.42, NaH_2PO_4 0.44, $NaHCO_3$ 4.2, HEPES 10, pH set to 7.4 with NaOH) with 0.1% bovine serum albumin. Tissues were then cut into 1 mm^3 . Approximately 10 pieces of tissue were placed into 2 ml plastic tubes containing 1 ml PSS with 0.1% bovine serum albumin, 45U Papain, 0.1% dithioerythritol (DTT). Tissues were then incubated at 37°C for 35 min. This solution was removed at the end of incubation period, and replaced by 1 ml PSS containing 0.1% bovine serum albumin, 0.1% Collagenase Type 4, 0.05% Elastase, and 0.1% Soybean trypsin inhibitor. The tissue was then incubated for an additional 25 min at 37°C . Once again, the enzyme containing solution was gently removed, and replaced by 2 ml of a fresh PSS solution containing albumin. The tissue was then allowed to settle down for 10 min, after which the solution was gently replaced by 2 ml of fresh PSS solution containing albumin. The tissue was then gently and repeatedly pipetted with a Pasteur pipette. It was during this final washing that the individual smooth muscle cells were isolated. The freshly isolated cells were then transferred to 35 mm culture dishes for the patch clamp experiments.

Evaluation of cellular viability (that is, retention of contractile ability)

Freshly dispersed myocytes were plated onto 35 mm culture dishes and allowed to settle for ≈ 10 min. Perfusion was then started with PSS solution containing 1 mM Ca^{2+} . Cells were examined with an inverted microscope (Nikon TMS). Spindle shaped smooth muscle cells were readily identified and a control picture was taken of this presumably largely relaxed state. Subsequently, $10\text{ }\mu\text{M}$ (final concentration of phenylephrine (PE)) PE was added to the buffer solution, and several sequential pictures were taken. The documented changes in shape were assumed to reflect increased smooth muscle cell contraction.

Electrophysiological recordings and K channel current analysis

The conventional tight-seal patch clamp method was used in both the cell-attached and whole-cell

patch modes. The seal resistance between the patch and the pipette was typically 10–50 G Ω . Liquid junction potentials were corrected for by ensuring that the bath and pipette solutions contained sufficient chloride concentrations; previous studies have shown that under these conditions the potential liquid junction artifacts are reduced to ≤ 3 mV.¹³ Symmetric KCl solutions were used in both bath and pipette for the cell-attached and whole-cell patch modes. Patch and bath solutions contained (in mM): 140 KCl, 1 CaCl₂, 1 EGTA, 10 HEPES, pH 7.2.¹⁰

Experimental protocols

For whole-cell current measurements, all protocols were run using pCLAMP software (version 6.4; Axon Instruments, Inc., Foster City, California) running on a DELL Pentium PC. Initially, cells were held at -70 mV and the membrane potential was stepped to ± 100 mV in 10 mV increments. The pulse duration was 200 ms. For the single channel recordings, manual voltage steps were applied with membrane potentials ranging from ± 100 mV, and step duration ranging from 30 s to 3 min. All current and voltage recordings were digitized using a Neurocorder (Model DR 384, Neuro Data Instruments Corp., NY) analog-to-digital converter and stored on magnetic videotape using a VCR recorder for latter off-line analysis.

Analysis of the single channel current data

All analog signals were low pass filtered (8-pole Bessel, LPF-30; WPI, Inc., Sarasota, FL) at 1 kHz and digitized at 4 kHz using a DT2801A A/D board (Data Translation, Inc., Marlboro, MA) installed in a DELL Pentium computer, and running pCLAMP6 software. Further off-line analysis of all digitized current traces was performed using the PATCH program developed by Drs SV Ramanan and PR Brink. Amplitude histograms were generated for single channel data analysis to allow computation of channel open probability (P_o). For any one histogram the ground state (0 current) represented the closed state. The remaining peak(s) in the Gaussian distribution represent the open state(s). By placing a discriminator halfway between the closed and open state for each level, and determining the area of each distribution, the open probability (P_o), channel mean open time (MOT) and channel mean closed time (MCT, if only one channel exists), as well as channel transitions can be estimated.^{9,13–15}

Results

Phenylephrine-induced contraction

The viability of the freshly isolated corporal myocytes from rat and human was assessed by cellular responsiveness to the addition of phenylephrine (PE), an α_1 -adrenergic agonist. Figure 1A (human) and B (rat) illustrate the representative examples of the typical spindle shaped corporal myocytes yielded by our cell isolation protocol (see Methods). Note, however, that when 10 μ M PE was added to the bath solution, the smooth muscle cells exhibited quite dramatic change in shape, that is, the development of a robust contractile responses (Figure 1C and 1D, respectively). These observations clearly indicate that the contractile response to a major endogenous modulator of corporal smooth muscle tone *in vivo* is preserved using our cell isolation protocol.

Identification of large-conductance K_{Ca} channels in human and rat corporal smooth muscle cells

Consistent with our previous observations,^{10,11} in the cell-attached patch mode, large amplitude, outward single-channel currents were observed in the majority of freshly isolated human corporal myocytes in symmetrical K⁺ solutions. Figure 2A depicts a representative example of a single K_{Ca} channel current trace in the cell-attached patch mode at a membrane potential 50 mV. The corresponding all points amplitude histogram compiled from the entire 60 s recording is shown Figure 2B. As illustrated, relatively little channel activity (only 30 total events) was observed under these conditions. This is reflected by the fact that the calculated channel P_o (channel open probability) was 0.5%, with corresponding MOT (mean open time) and MCT (mean close time) values of 6 ms and 1306 ms, respectively. The average slope conductance value obtained from analysis of the current-voltage (I–V) curve in a total of five experiments was 202 pS ($n = 5$ cells).

Similar observations were made on freshly isolated rat corporal myocytes in the cell-attached patch mode. This is well illustrated in the representative example shown in Figure 3A, which depicts a single K_{Ca} channel current trace at a membrane potential of 80 mV. As shown, once again there was a predominant 0 current peak and a small, but significant, open channel current peak, based on a total 30 channel events. The calculated channel P_o was 0.22%, with corresponding MOT and MCT values of 4 ms and 1849 ms, respectively. The average slope conductance value obtained from analysis of the I–V curve in a total of three

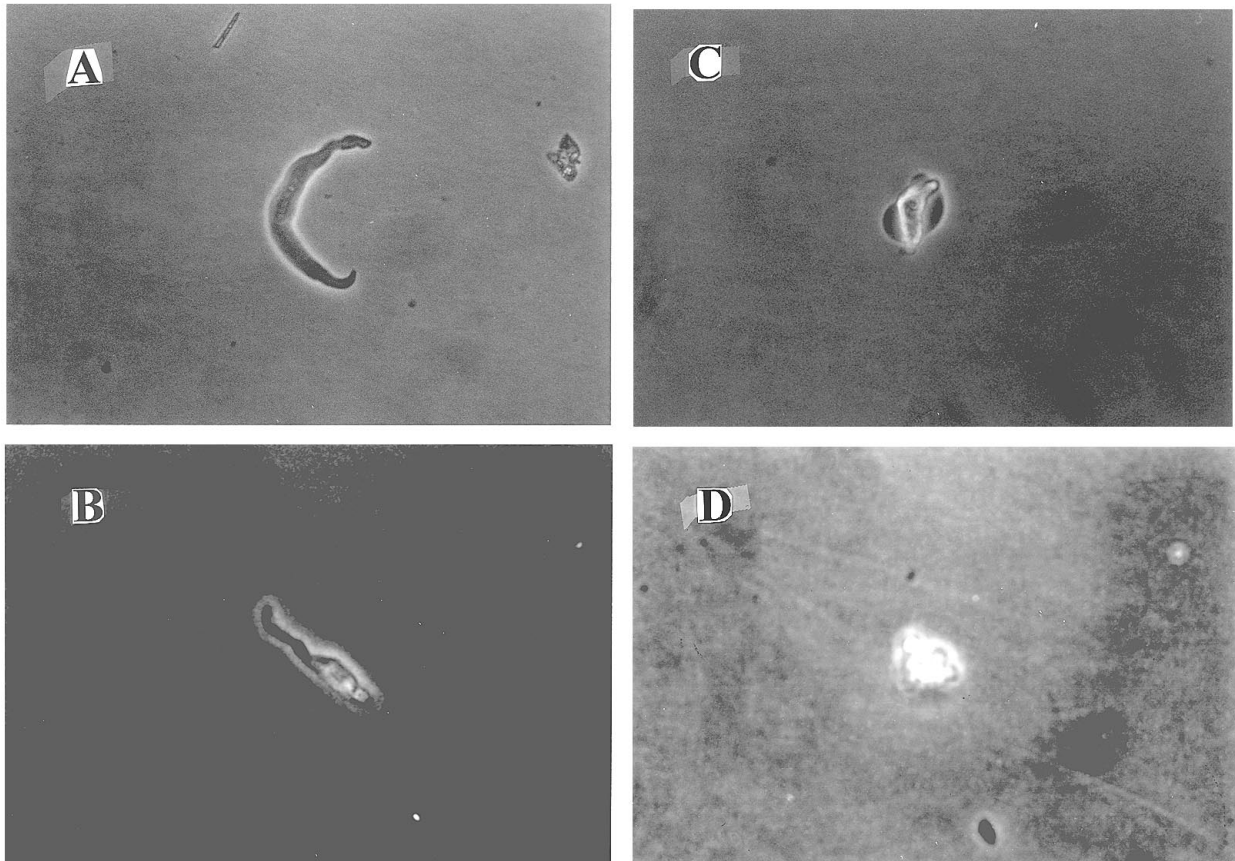


Figure 1 Characteristic morphology of freshly isolated human (A) and rat (B) corporal myocytes. Shown are cells that are representative of the spindle shaped myocytes routinely used in these studies. Panels C (human) and D (rat) show the effects of application of 10 μ M PE to the bath solution (magnification is 200 \times).

experiments was 204 pS ($n=3$ cells). Note that the single channel conductance values, for both human and rat myocytes, are similar to those previously reported in other enzymatically isolated vascular myocytes.^{10,16,17}

Voltage and Ca^{++} sensitivity of single channel recordings in the inside-out detached patch configuration

In addition to the characteristically large single channel conductance, the K_{Ca} channel is known to be sensitive to both the membrane potential and the intracellular Ca^{2+} concentration. As such, preliminary experiments were conducted to evaluate the voltage and Ca^{2+} sensitivity of freshly isolated rat corporal myocytes, using symmetrical KCl solutions in inside-out patches. Shown in Figure 4A is a 50 s recording of unitary K_{Ca} channel activity with 1 mM Ca^{2+} in the bath solution, at a membrane potential of 30 mV. The right panel in Figure 4A shows the all points amplitude histogram compiled from the same 50 s of data. The channel P_o calculated from a total

of 193 events was 98.4%, with corresponding MOT and MCT values of 279 ms and 6 ms, respectively. As shown in Figure 4B, removal of calcium from the bath solution resulted in a nearly complete cessation of unitary activity (see Figure 4B), such that P_o could not be accurately estimated (a total of only four events were observed). When the membrane voltage was increased to 60 mV, on the same patch of membrane (still in the absence of calcium), the channel activity increased significantly (see Figure 4C). Under these latter recording conditions, 27 channel events were observed during the same time period. The calculated channel P_o was 1% and corresponding MOT and MCT values of 27 ms and 2719 ms, respectively.

TEA block of the K_{Ca} channel

To further verify the nature of the observed K channel activity, we examined the ability of TEA (1 mM; a concentration known to be selective for the blockade of the K_{Ca} channel) to block channel activity when applied to the bath solution in an

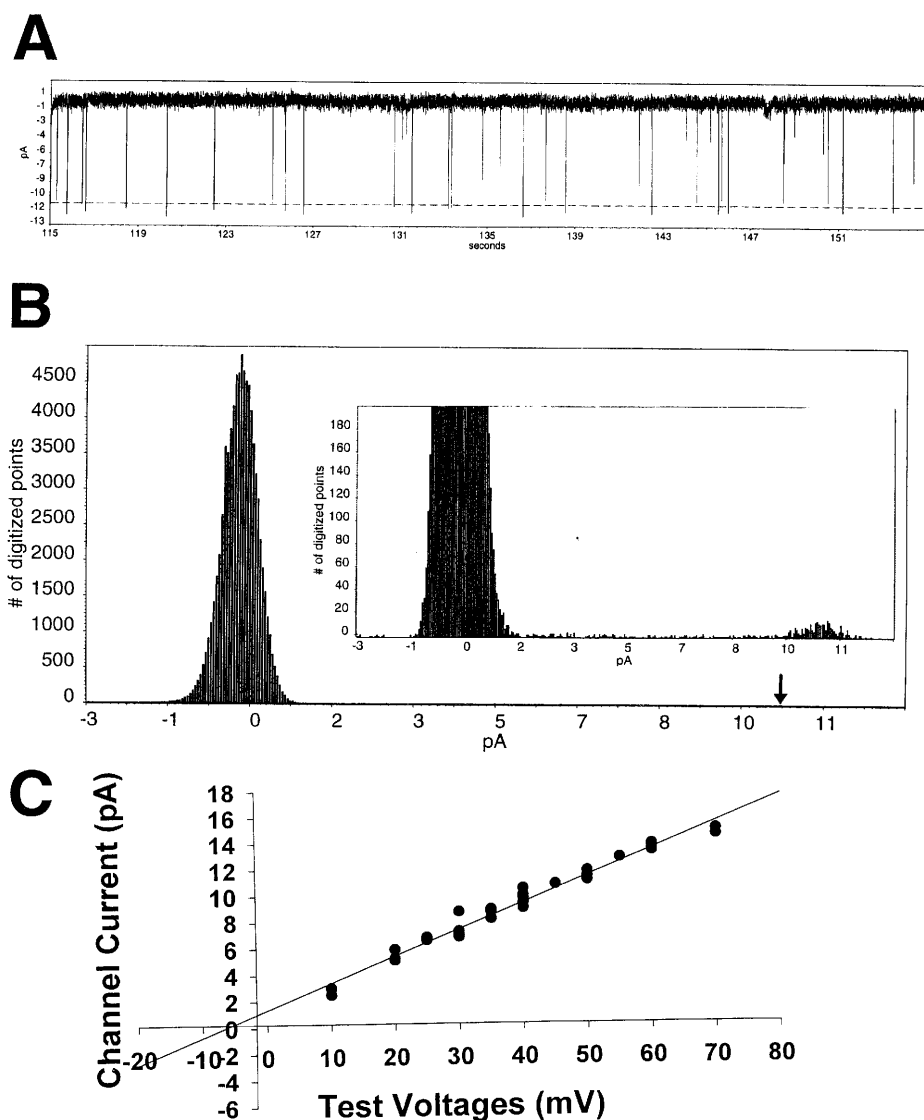


Figure 2 Unitary K channel activity in freshly isolated human myocytes. **A.** Single K_{Ca} channel current trace in the cell-attached mode at a membrane potential of 50 mV. The dashed lines indicate the 0 current and fully open channel current levels. **B.** All points amplitude histogram compiled from the entire 60 s recording. There is a predominant 0 current peak and a small (see insert) open channel current peak. The calculated channel P_o (channel open probability) was 0.5%, with corresponding MOT (mean open time) and MCT (mean close time) values of 6 ms, and 1306 ms, respectively. A total of 30 channel events were observed. **C.** Linear regression analysis of the composed single channel current-voltage (I–V) relationship yielded a single-channel slope conductance of 202 pS ($n = 5$ cells). Note the slight offset potential in the I–V curve (≈ 5 mV).

outside-out patch. Figure 5A shows a 4 min recording of single channel activity at a membrane potential of 30 mV. Initially, there were four stable and active channels. Removal of Ca^{2+} from the bath solution had no apparent effect on unitary activity under these conditions. However, addition of 1 mM TEA to the bath solution resulted in a rapid and complete blockade of channel activity.

The effect of addition of 1 mM TEA to the bath solution in the whole cell recording configuration was also examined. The results of a representative are shown in Figure 5B–E. As illustrated in panels B–D, TEA caused a very rapid and reversible diminution in the amplitude of the whole cell outward K^+ currents.

Panel E depicts the I–V derived from plots of the steady-state current tracings depicted in panels B–D. In a total of three experiments conducted in symmetrical 140 mM KCl solution, addition of 1 mM TEA blocked an average of $46 \pm 13\%$ of the measured whole cell outward current ($n = 3$).

Activation of large-conductance K_{Ca} channels by cAMP in the whole cell configuration

To further examine the physiological relevance of the freshly isolated rat corporal myocytes, 2 mM

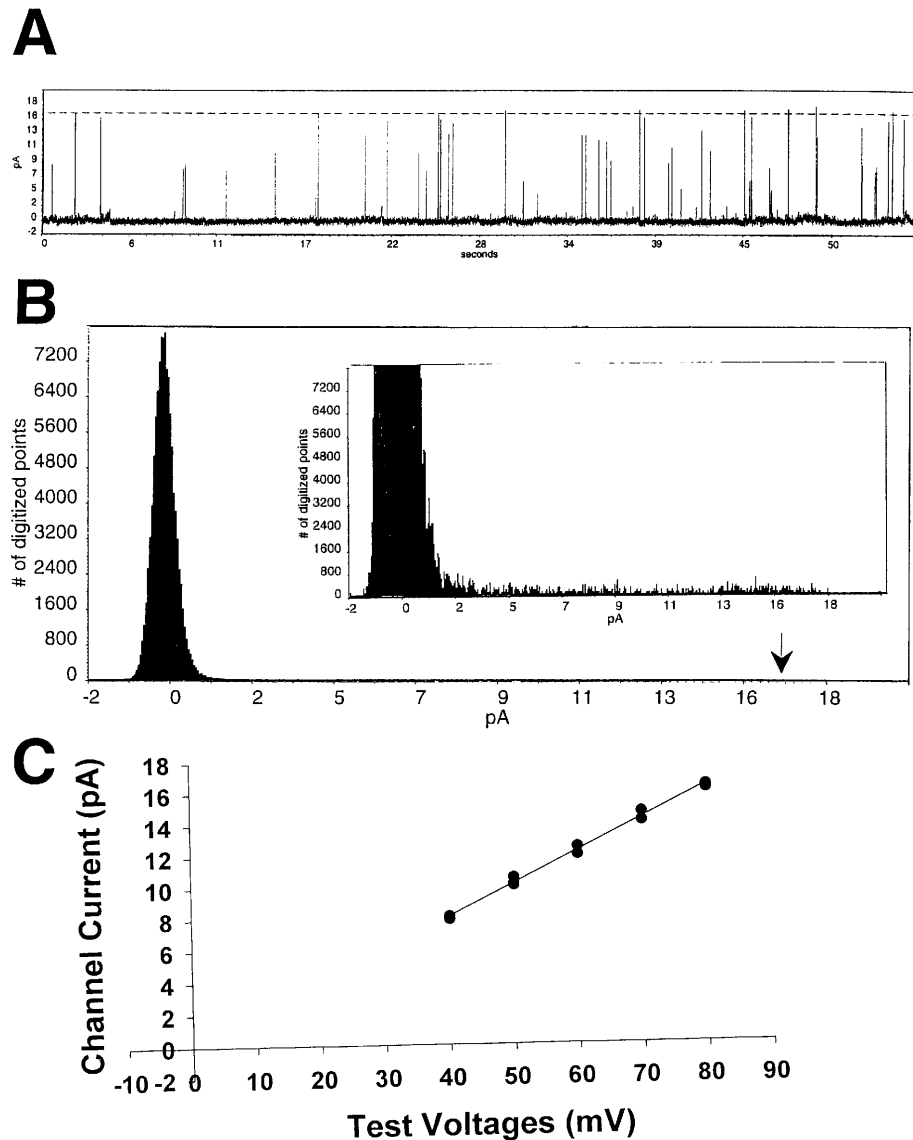


Figure 3 Unitary K channel activity in freshly isolated rat myocytes A. Single K_{Ca} channel current trace in the cell-attached mode at a membrane potential of 80 mV. The dashed lines indicate the 0 current and fully open channel current levels. B. All points amplitude histogram compiled from the entire 60 s recording with a calculated channel P_o of 0.22%, and corresponding MOT and MCT values of 4 ms and 1849 ms, respectively. These calculations were based on the total of 30 channel events observed during the recording period. C. Linear regression analysis of the composed single channel I–V curve yielded single-channel slope conductance of 204 pS ($n = 3$ cells).

8-bromo-cAMP (a membrane permeant, nonhydrolyzable analog of cAMP) was applied to the bath solution in the whole cell recording mode. Figure 6A shows the outward current tracings obtained under control conditions. As illustrated in Figure 6B, application of 2 mM 8-bromo-cAMP resulted in a quite dramatic more than 2-fold increase in the magnitude of the whole cell outward currents. In Figure 6C, the application of 1 mM TEA to the bath solution was associated with a similarly rapid diminution in the magnitude of the whole cell outward currents, to current levels near or below the control current levels. The I–V curves from the tracings shown in Figure 6A–C are shown in Figure

6D. Similar results were obtained in two additional experiments.

Discussion

This present study demonstrates that freshly isolated myocytes from human and rat corpora retained their expected morphology (Figure 1A and B), contractility (Figure 1C and D), tolerance to alterations in extracellular calcium levels (Figure 5A), and responsiveness to phenylephrine stimulation (Figure 1C and D). Such observations illustrate that the

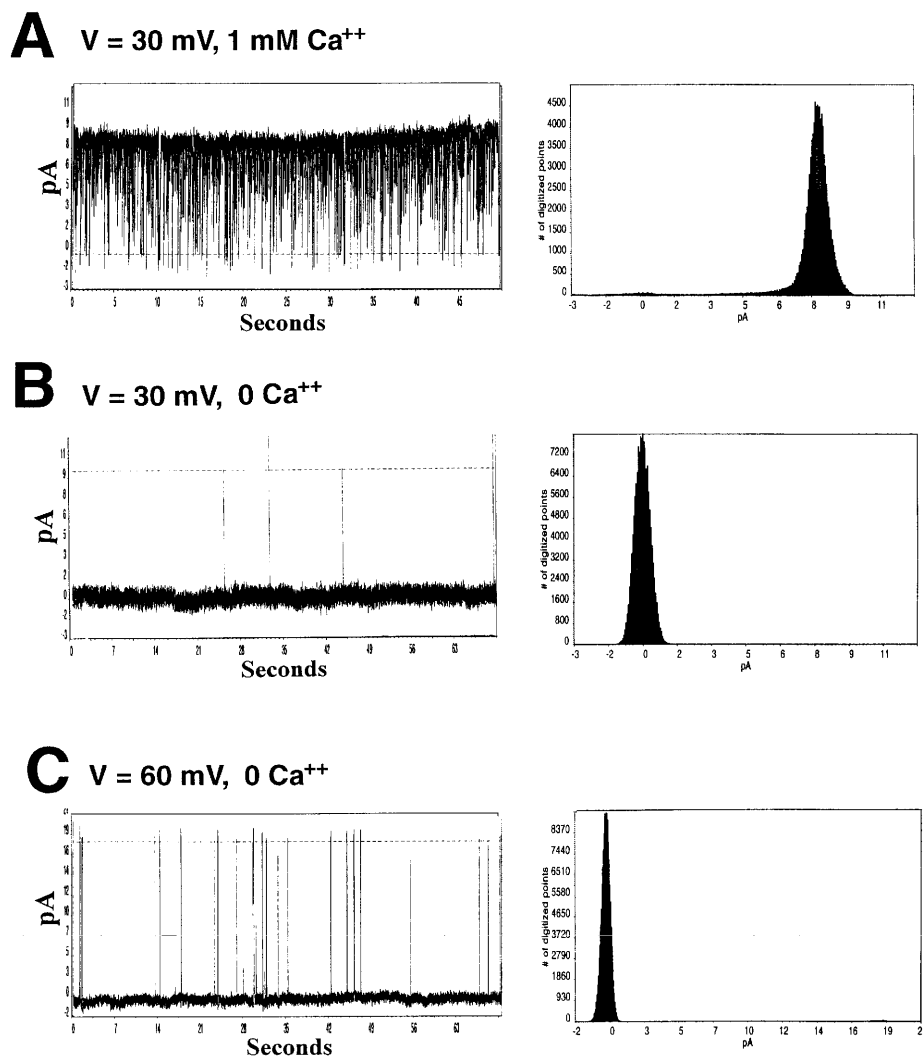


Figure 4 Voltage and calcium-sensitivity of K^+ currents in rat myocytes. A. 50 s recording of unitary K_{Ca} channel activity in the inside-out detached patch configuration. As shown, unitary activity is quite robust in the presence of 1 mM Ca^{2+} , at a membrane potential of 30 mV. The dashed lines indicate the 0 current and fully open channel current. The channel was in open state almost all the time under these conditions. The all points amplitude histogram (A, right panel) shows a small 0 current peak and a predominant open channel current peak. The calculated channel P_o was 98.4%, with corresponding MOT and MCT values of 279 ms and 6 ms, respectively, based on a total of 193 channel events. B. 70 s recording of unitary K_{Ca} channel activities in the absence of Ca^{2+} (0 mM Ca^{2+}), and at a membrane potential of 30 mV. There were only four channel events observed in the entire 70 s recording. As such, the channel P_o could not be estimated due to the very limited channel activity. In fact, there was no detectable open channel current peak (see B, right panel). C. Another segment of the same patch (again, a 70 s recording) of unitary K_{Ca} . Under these conditions, the calculated channel P_o was 1%, with corresponding MOT and MCT values of 27 ms and 2719 ms, respectively, based on a total of 27 channel events. In this latter case, there was a small, but detectable, open channel current peak (C, right panel).

integrity of the cell membrane, as well as the expected phenotypic characteristics are well preserved following our cell isolation protocol. The other major implication of the current studies is that freshly isolated myocytes of human and rat corpora are quite similar to each other with respect to many physiologically relevant cellular parameters.⁸⁻¹¹ These properties are reviewed below.

Firstly, as previously revealed for outward currents studied in cultured⁹ and freshly isolated¹⁰ human myocytes, patch clamp experiments now reveal that the K_{Ca} channel subtype is also a

predominant component of the outward currents observed in freshly isolated rat myocytes. More specifically, analysis of unitary events recorded in the attached patch mode from freshly isolated human (Figure 2) and rat (Figure 3) corporal myocytes revealed a nearly identical single channel slope conductance value of ≈ 200 pS in myocytes from both species. As previously reported for human corporal myocytes, the K channel recorded in these experiments was very sensitive to intracellular calcium (Figure 4) and low concentrations of TEA (1 mM; Figure 5). Based on these initial

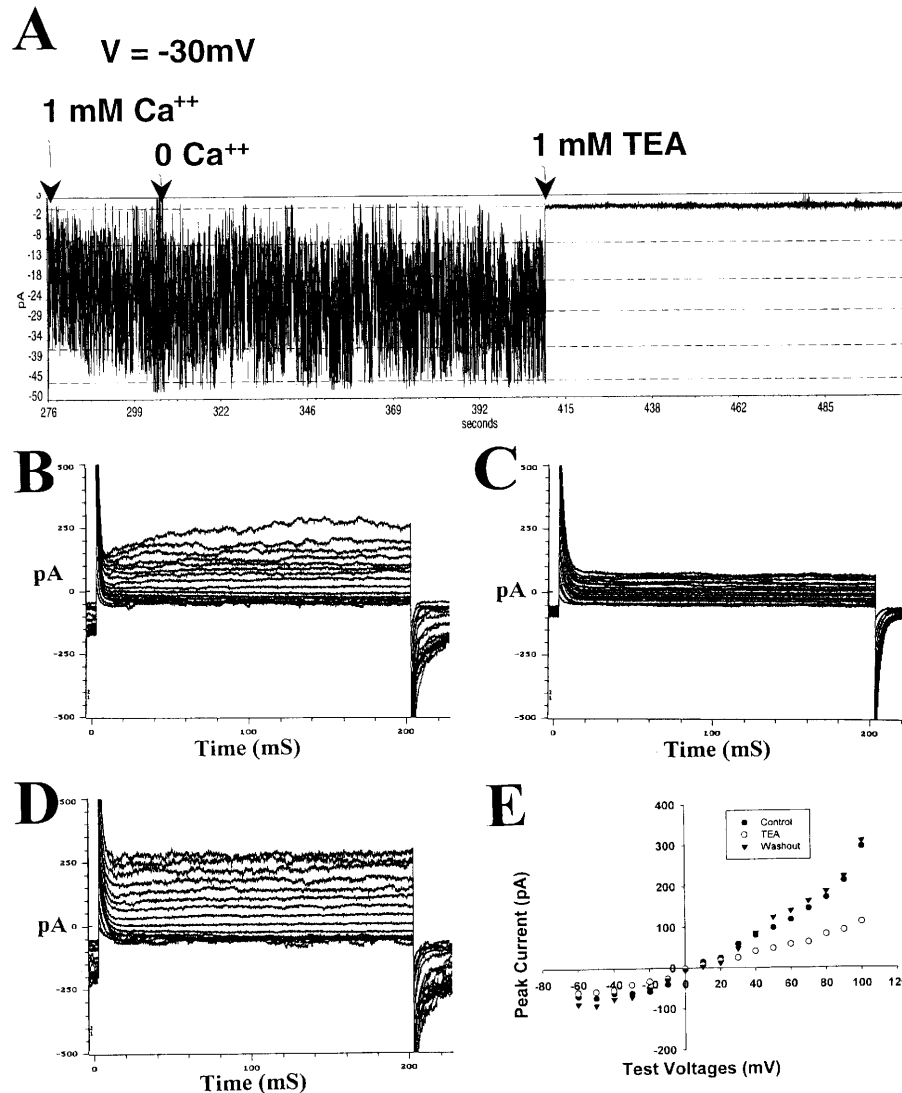


Figure 5 TEA reversibly blocks K_{Ca} channel activity in both the detached patch and whole cell recording modes. A. Single channel current recording of TEA blockade of the K_{Ca} channel in the outside-out detached patch recording configuration. Shown is a 4 min record at a membrane potential of -30 mV. As illustrated, four stable and active channels were observed. Replacement of the bath solution with 0 Ca^{2+} (indicated by arrow), had little detectable effect on the observed channel activity. However, when 1 mM TEA was introduced to the bath solution, channel activity was rapidly and completely blocked (as indicated by the arrow). B. A set of typical whole cell outward K^+ currents observed under control conditions (140 mM KCl and 1 mM Ca^{2+} in both bath and pipette solutions). C. Significant diminution in whole cell currents is observed after application of 1 mM TEA to the bath solution. D. After washout of TEA, there is a restoration of the whole cell currents to near control levels. E. The plotted $I-V$ curve compiled from the data displayed in panels B, C, and D.

observations and their similarities to the characteristics of K_{Ca} channels as reported in many other smooth muscle cell types from different tissues and species,^{12,16-18} it is clear that the K_{Ca} channel subtype is also present and predominant in freshly isolated rat corporal myocytes. Consistent with this supposition, a previous report has confirmed the presence of the K_{Ca} channel subtype transcript in rat corporal tissue.

To further characterize the presence and physiological relevance of the K_{Ca} channel subtype in freshly isolated rat corporal myocytes, we examined

second messenger regulation of whole cell outward K^+ currents. The rationale for so doing is related to the observation that second messengers are well documented modulators of K_{Ca} channel activity in physiologically diverse smooth muscle cell types.²⁰⁻²⁴ In this regard, for example, we have previously reported that PGE_1 (prostaglandin E_1) activates the K_{Ca} channel present in both freshly isolated and cultured human corporal myocytes, and furthermore, that the PGE_1 -induced activation of the K_{Ca} channel involves stimulation of PKA.^{10,19} This investigation provides evidence consistent

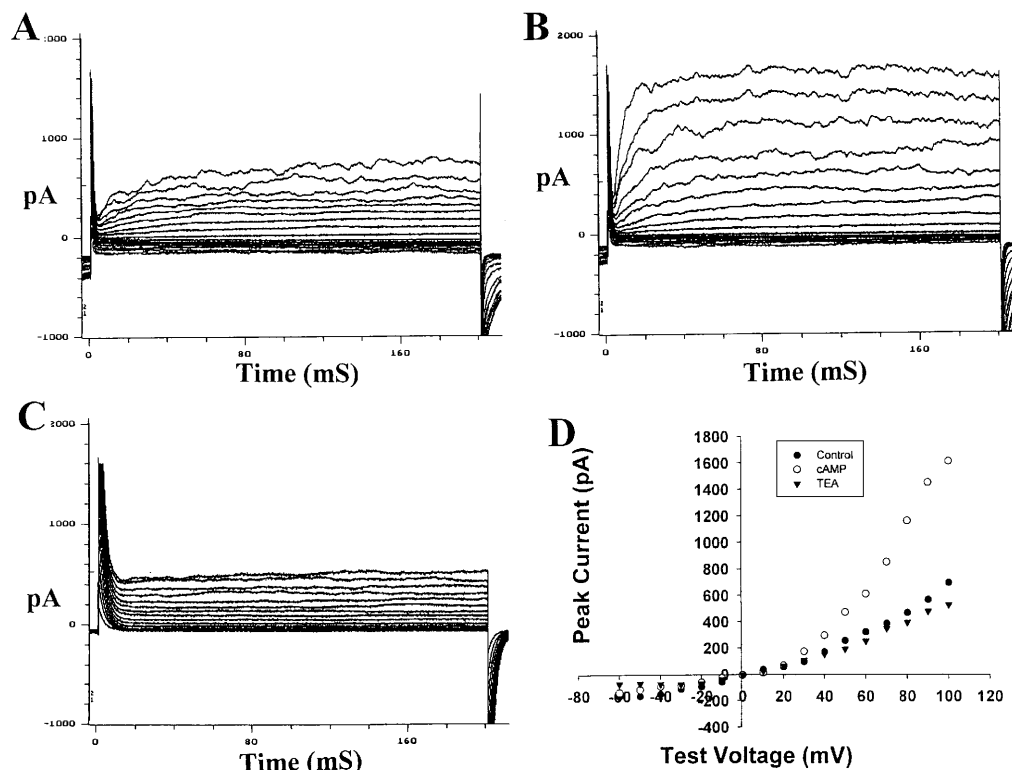


Figure 6 Activation of the cAMP pathway increases the activity of the large-conductance K_{Ca} channels recorded in the whole cell configuration. A. Another set of typical whole cell outward K^+ currents under control conditions (that is, symmetrical 140 mM KCl and 1 mM Ca^{2+} solutions). The cell was stepped in 10 mV increments from ± 100 mV. B. 5 min after 2 mM 8-bromo-cAMP was applied to the bath solution. C. After 1 mM TEA was applied in the bath solution. D. The current-voltage relationships compiled from the data displayed in A, B, and C.

with the presence of similar regulatory elements in rat corporal smooth muscle. More specifically, Figure 6 clearly documents that application of 8-bromo-cAMP (2 mM) to the bath solution elicited a greater than 2-fold increase in the magnitude of the whole cell outward K^+ currents in freshly isolated rat corporal myocytes. Moreover, the observed 8-bromo-cAMP-induced increase in the magnitude of the whole cell outward K^+ currents was completely reversed by the addition of the K_{Ca} -subtype channel blocker TEA to the bath solution (TEA concentrations ≤ 1 mM are nominally thought to be selective for blockade of the K_{Ca} channel subtype; see Figure 6).

Conclusions

These data provide first direct electrophysiological evidence for the presence of the K_{Ca} channel in freshly isolated rat corporal myocytes. Moreover, it is clear that there are numerous morphological, electrophysiological and pharmacological similarities between the K_{Ca} channel identified in freshly isolated myocytes from human and rat corporal

tissue. As such, a reasonable conclusion would seem to be that the physiological/electrophysiological regulation of smooth muscle tone in the rat corpora is likely to be remarkably similar to that observed in the human corpora. In light of these overt similarities, it would seem that the rat corporal myocyte provides an excellent model for assessing the putative effects of age- or disease-related alterations in K_{Ca} regulation, expression or function on the physiology of erection and the etiology of erectile failure. By extension then, given the central importance of the K_{Ca} channel to the modulation of erectile capacity^{2,9,20} the rat should also provide a good *in vivo* and *in vitro* model system for better understanding the effects of pharmacological and/or molecular manipulation of the K_{Ca} channel on human erectile function.

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