

Dual regulation of the T-type Ca^{2+} current by serum albumin and β -estradiol in mammalian spermatogenic cells

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Abstract This study provides evidence for a novel mechanism of voltage-gated Ca^{2+} channel regulation in mammalian spermatogenic cells by two agents that affect sperm capacitation and the acrosome reaction (AR). Patch-clamp experiments demonstrated that serum albumin induced an increase in Ca^{2+} T current density in a concentration-dependent manner, and significant shifts in the voltage dependence of both steady-state activation and inactivation of the channels. These actions were not related to the ability of albumin to remove cholesterol from the membrane. In contrast, β -estradiol significantly inhibited Ca^{2+} channel activity in a concentration-dependent and essentially voltage-independent fashion. In mature sperm this dual regulation may influence capacitation and/or the AR. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: T- Ca^{2+} channel; Spermatogenic cell; Serum albumin; Bovine serum albumin; β -Estradiol; Sperm capacitation

1. Introduction

Ca^{2+} channels in the mammalian sperm plasma membrane govern Ca^{2+} uptake and thus regulate intracellular signaling. Capacitation, a poorly understood maturational process, renders sperm responsive to physiological agents such as the zona pellucida or progesterone leading to the acrosome reaction (AR). This exocytotic event is required for successful fertilization. Capacitation, while occurring in vivo in the female reproductive tract, can also be accomplished in vitro by incubating sperm in defined medium containing appropriate concentrations of three key components: Ca^{2+} , NaHCO_3 and serum albumin [1,2]. In vitro studies have demonstrated that capacitation of mouse sperm is accompanied by metabolic alterations, a time-dependent and cAMP-regulated increase in the protein tyrosine phosphorylation of a subset of proteins [2,3], and changes in the distribution and composition of plasma membrane lipids and phospholipids. The requirement for serum albumin in capacitation has been related to its ability to

remove cholesterol from the sperm membrane [4,5]. It has been proposed that cholesterol efflux then leads to changes in the membrane architecture and fluidity that give rise to the capacitated state [6,7]. In addition, the presence of a functional 29 kDa estrogen receptor on the human sperm surface has been recently documented. Its activation results in a rapid and sustained increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), dependent on the presence of extracellular Ca^{2+} , that causes increased tyrosine phosphorylation of sperm proteins and reduced progesterone-induced AR [8].

Extracellular Ca^{2+} appears also to be necessary for the completion of capacitation and for the increase of $[\text{Ca}^{2+}]_i$ required during capacitation [2]. In line with this, electrophysiological studies carried out in spermatogenic cells, a developmental precursor stage of mature sperm, have revealed that the voltage-gated T-type Ca^{2+} channel in the plasma membrane [9,10] is a major mediator of Ca^{2+} entry into these cells [11]. The pharmacology of T channels from spermatogenic cells resembles that of the Ca^{2+} entry mechanisms thought to be responsible for the AR in sperm [12]. These findings suggest that the Ca^{2+} T channels play an important role in the AR and could participate in the regulation of $[\text{Ca}^{2+}]_i$ during capacitation. Unfortunately, the size, complex geometry and highly differentiated and motile nature of sperm preclude its systematic electrophysiological characterization. In this report we used spermatogenic cells as a model system to study whether compounds that regulate capacitation and the AR are able to modulate T channels. Our results show that albumin and β -estradiol can regulate Ca^{2+} T channels in spermatogenic cells, and suggest that in mature sperm they may influence capacitation and the AR.

2. Materials and methods

2.1. Materials

Bovine albumin (essentially fatty acid-free prepared from bovine albumin fraction V), 17 β -estradiol (1,3,5[10]-estratriene-3,17 β -diol), and cholesterol 3-sulfate (sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

2.2. Cell preparation

Spermatogenic cells were obtained as described previously [13]. Briefly, testes from adult CD1 mice were excised and suspended in ice-cold dissociation solution. The tunica albuginea was removed and the seminiferous tubules were separated. Tissue fragments were extruded from a single tubule and dispersed into individual cells or

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symplasts (aggregates), collected by centrifugation, resuspended in external recording solution (see below) without Ca^{2+} and stored on ice until assayed. Spermatogenic cells or symplasts at two different stages of differentiation (pachytene spermatocytes and round spermatids) were preferentially observed and used in electrophysiological recordings. Inasmuch as similar results were obtained from both stages, data were pooled for presentation.

2.3. Electrophysiology

Ca^{2+} currents were recorded using the whole-cell patch-clamp technique [14] at room temperature using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2–4 M Ω micropipettes. Cells were clamped at a holding potential (HP) of -90 mV and capacity transients were electronically compensated. Currents were evoked by 50–125 ms depolarizing voltage steps to test potentials ranging from -70 to $+20$ mV. To measure Ca^{2+} channel inactivation at steady state, cells were held for 210 ms at potentials ranging successively from -75 through -36 mV prior to a 90-ms step depolarization to a test potential of -25 mV. Linear leak and residual capacity currents were subtracted on-line using a P/4 standard protocol. Current records were captured on-line and digitized at a sampling rate of 5 kHz following filtering of the current record (2 kHz). Series resistance was compensated by $\geq 50\%$. Pulse protocols, data capture

and analysis of recordings were performed using pCLAMP software (Axon). To isolate Ca^{2+} currents, cells were bathed in a solution containing (in mM): CaCl_2 10; NaCl 130; KCl 3; MgCl_2 2; NaHCO_3 1; NaH_2PO_4 0.5; HEPES 5; glucose 10 (pH 7.3). The internal (patch pipette) solution consisted of (mM): CsCl 100; CsF 10; EGTA 5; HEPES 5; ATP- Mg_2 4; phosphocreatine 4 (pH 7.3). Bovine serum albumin (BSA) was diluted in the bath solution to give the desired final concentration. Cholesterol 3-sulfate and 17β -estradiol were prepared as 100 mM stocks in dimethylsulfoxide (DMSO) and stored at -20°C . A fresh aliquot was diluted to its final concentration in the bath solution for each experiment. Final concentration of DMSO typically was $<0.001\%$.

3. Results and discussion

The possibility that serum albumin, a required component in media that supports capacitation, may modulate sperm Ca^{2+} channels has not been directly examined. Inasmuch as electrophysiological experiments are very difficult to perform in mature sperm, we used spermatogenic cells as a model system to determine the influence of albumin on their Ca^{2+}

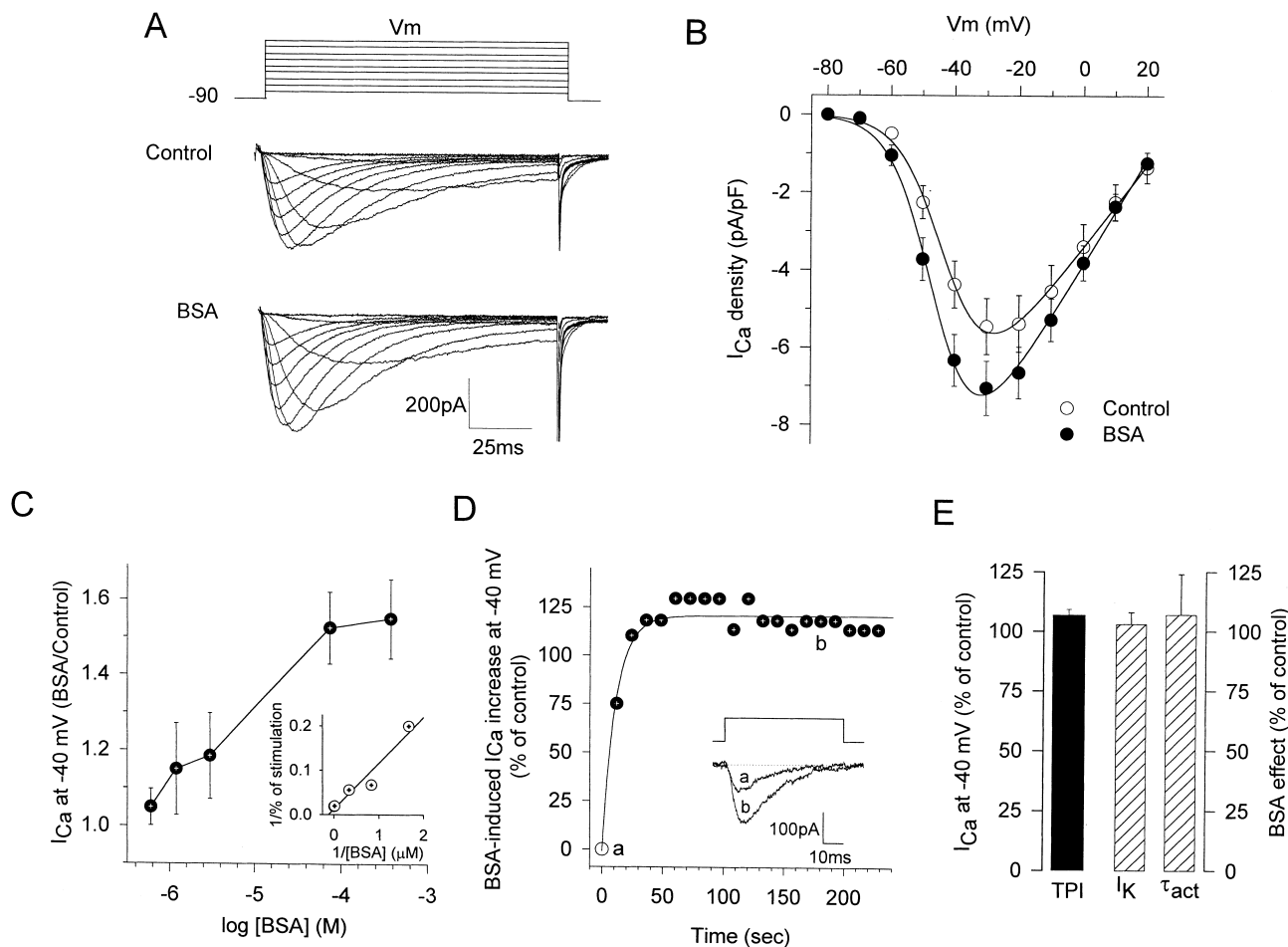
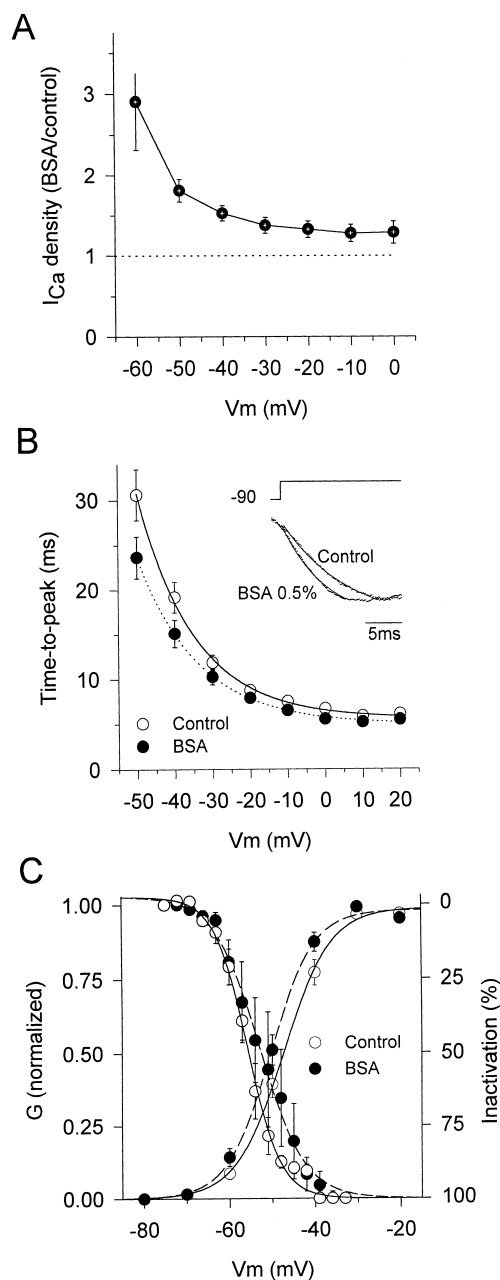


Fig. 1. BSA enhances Ca^{2+} current in mouse spermatogenic cells. A: Voltage-gated T currents (I_{Ca}) recorded, according to the protocol shown above, from a representative cell in the absence (upper traces) and in the presence of 0.5% BSA (lower traces). B: Current-voltage ($I-V$) relationships for steady-state activation of I_{Ca} before and after BSA application. Values represent mean $\pm$ S.E.M. from peak currents normalized by cell capacitance. C: Semilogarithmic plot of current density increase as a function of BSA concentration. Data were obtained in response to 100 ms depolarizations to -40 mV from a HP of -90 mV. Inset: Lineweaver-Burk plot derived from the BSA concentration-response data. D: Representative plot of peak current as a function of time. Data were approximated by an exponential function (solid line). Inset: Individual traces taken from the time course shown; letters designate the regions of the curves represented. E: BSA specifically increases T current in spermatogenic cells. Application of 75 μM triosephosphate isomerase (TPI) was not effective in increasing Ca^{2+} current amplitude. The solid bar corresponds to the summary of normalized peak current in cells subjected to TPI ($n=4$). Hatched bars summarize a comparison of current amplitude (I_{K}) and time constant of activation (τ_{act}) before and after BSA (75 μM) application ($n=4$). Currents were recorded in response to 200 ms depolarizing pulses from -90 to $+50$ mV.



T currents. A pachytene synplast subjected to the patch-clamp technique showed typical Ca^{2+} currents (Fig. 1A) in response to 10 mV voltage steps from a HP of -90 mV before (upper traces) and 2 min after incubation with $75 \mu M$ of BSA (lower traces). Test pulses elicited a rapidly activating and inactivating inward current (I_{Ca}) whose amplitude was significantly increased in the BSA-treated cells. The magnitude of these currents was normalized by cell capacitance and the averaged results plotted as a function of membrane potential in Fig. 1B. Having established significant differences in T current density, we next determined the concentration dependence and time course of the BSA action. The effects of BSA were concentration-dependent with a maximal increment of T current occurring at $75 \mu M$. This increase was normalized with respect to the control, averaged and plotted against the logarithm of concentration (Fig. 1C). A concentration–

Fig. 2. Bovine serum albumin affects the kinetics of T currents in spermatogenic cells. **A**: Voltage dependence of the relative increase in current density induced by BSA. The ratios of current density after BSA application over the control at different voltages from the same cells in Fig. 1B are shown. **B**: Time-to-peak–voltage relationship for activation of I_{Ca} . Smooth lines correspond to exponential fits of data ($n=11$). Inset: Example traces taken from a representative cell included in the time-to-peak–voltage relationship. Currents are shown normalized and only the first 15 ms are displayed to allow comparison of kinetics. The activating phase of the currents was fitted with single exponential equations (superimposed lines). **C**: BSA significantly increases the window current in spermatogenic cells. Comparison of steady-state activation and inactivation in control and BSA-treated cells. The fraction of inactivated channels was plotted as a function of prepulse voltage and data were fitted with Boltzmann equations (control, solid line; BSA-treated cells, dotted line). Symbols represent mean $\pm$ S.E.M. values of 3–5 cells in each condition. Conductance (G) was calculated from the Goldman–Hodgkin–Katz equation with an equilibrium potential for Ca^{2+} calculated using the MaxChelator (MAXC v5.0) software. Data were averaged and then fitted with Boltzmann equations (smooth curves). Symbols represent mean $\pm$ S.E.M. values of 11 cells in each condition.

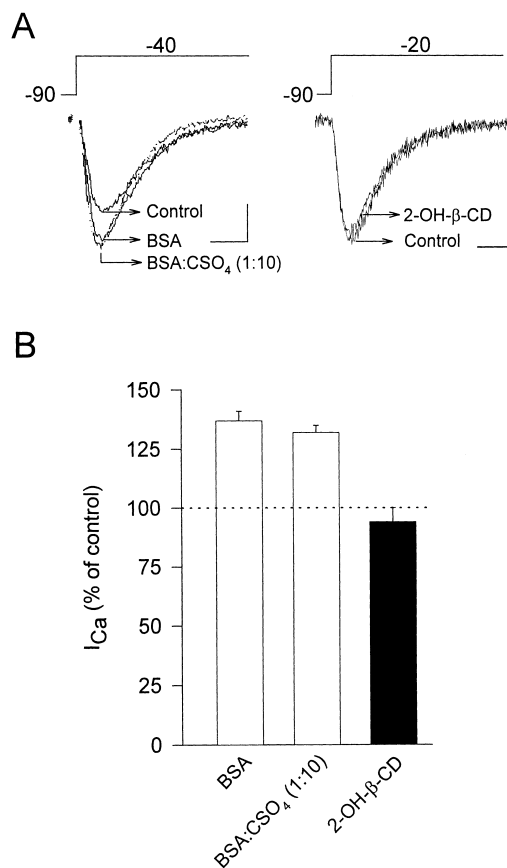


Fig. 3. The effects of BSA on T-channels in spermatogenic cells are not related to its ability to serve as cholesterol acceptor. **A**: Montage of current traces obtained first in the absence of BSA (control) and then treated with $15 \mu M$ BSA presaturated with cholesterol, followed by a third incubation in the presence of BSA alone. The scale bars represent 50 pA and 25 ms (left panel). The right panel shows representative superimposed current traces obtained before and after application of $500 \mu M$ of 2-OH- β -cyclodextrin (2-OH- β -CD). **B**: Histograms comparing the effect of a 2 min incubation with BSA alone, BSA presaturated with cholesterol sulfate (CSO₄), and 2-OH- β -CD on Ca^{2+} current amplitude ($n=4-6$).

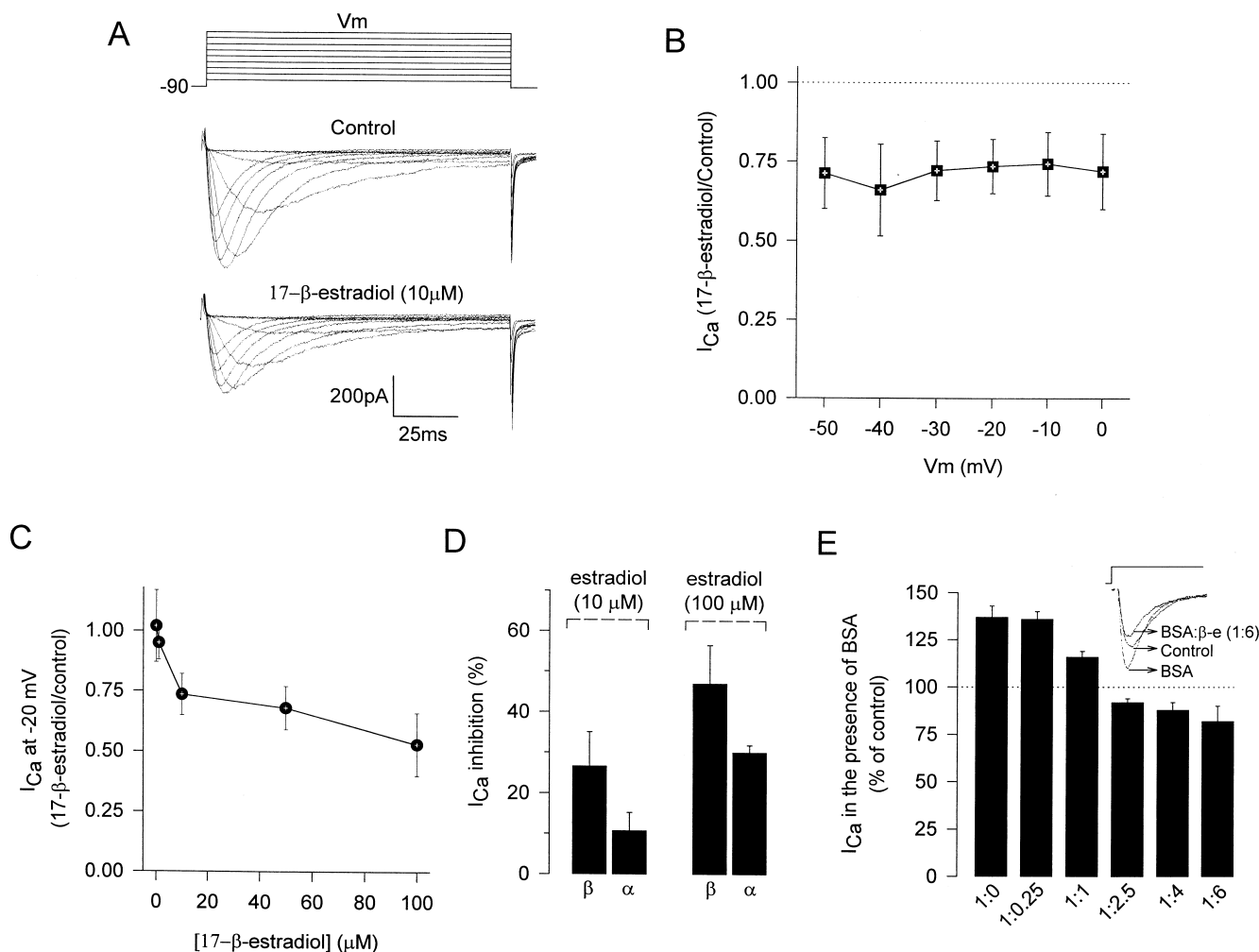


Fig. 4. 17 β -Estradiol inhibits Ca^{2+} current in spermatogenic cells. A: T currents recorded in a representative cell before (upper panel) and after a 2 min incubation in 17 β -estradiol. B: Peak currents after β -estradiol application at each test potential were averaged and normalized to control. C: Currents measured in response to 200 ms depolarizing pulses from a HP of -90 mV to a test potential of -20 mV before and after application of different concentrations of estradiol normalized to the control. Data represent mean $\pm$ S.E.M. of 3–8 experiments. D: Comparison of current inhibition after a 2 min incubation in the presence of β or α isomers ($n=3$ and 8, respectively). Currents were evoked with depolarizing pulses from a HP of -90 to a test potential of -20 mV and the concentration of estradiol was 10 (left bars) or 100 μ M (right bars). E: The ratio of current before and after presaturating BSA with various concentrations of 17 β -estradiol is shown. Inset: Superimposed current traces evoked by a voltage step from -90 to -40 mV. 17 β -Estradiol-presaturated BSA was added at a concentration of 15 μ M. After incubation for 2 min, cells were treated with BSA alone. Bars denote pooled results showing the effect of BSA presaturated with increasing concentrations of 17 β -estradiol on the T current.

response curve was generated (Fig. 1C, inset), where the reciprocal of the normalized average current in response to BSA was plotted against the reciprocal of the BSA concentration. Data were then fitted using a Lineweaver–Burk equation and a K_D of ~ 8 μ M was calculated. Fig. 1D illustrates that BSA increased the Ca^{2+} T current within seconds of administration. Peak current was measured every 12 s in response to depolarizations from a HP of -90 mV to a test potential of -40 mV (inset), and the time course was approximated by a single exponential expression with $\tau \approx 11$ s. This modulation was specific since the use of a non-related protein such as triosephosphate isomerase did not significantly affect Ca^{2+} current amplitude (Fig. 1E, solid bar). Furthermore, control experiments demonstrated that a 2 min incubation with BSA had no effect on the magnitude and kinetics of whole-cell K^+ currents recorded in spermatogenic cells (Fig. 1E, hatched bars).

The enhancement of Ca^{2+} current by BSA was voltage-de-

pendent. Fig. 2A compares current–voltage (I – V) curves obtained before and after BSA application and shows that albumin increased Ca^{2+} current density at negative voltages most markedly (from -60 to -40 mV). We next examined current activation kinetics. Records were obtained at different potentials and both the time-to-peak and time constant of activation were measured. In the control cells, time-to-peak decreased monotonically with voltage from 30.6 ± 2.8 ms at -50 mV to 6.1 ± 0.4 ms at +20 mV, reaching a voltage-independent minimum of about 6 ms at 0 mV. Treatment with BSA decreased the time-to-peak to 23.6 ± 2.3 and 5.5 ± 0.3 ms at -50 and +20 mV, respectively, with values statistically different in the voltage range below -30 mV (Fig. 2B). In addition, the time constant of activation after BSA decreased 1.3-fold on average with respect to the control at -40 mV. In these experiments currents were recorded in response to depolarizations from a HP of -90 mV to a test potential of -40 mV and the traces were fitted to single exponential equations

(Fig. 2B, inset) with time constants of 9 ± 1.1 and 6.9 ± 1.1 ms for the cells before and after BSA treatment, respectively.

Calculating whole-cell conductance and fitting the average normalized data with Boltzmann equations (Fig. 2C) we found that T currents after BSA treatment activated at lower test potentials ($V_{1/2} = -47.5$ in the control and -50.5 mV in the treated cells). In addition, inactivation was studied by applying 210 ms pulses followed by a test pulse to -25 mV, as described in Section 2, to measure channel availability. Average data were fitted to Boltzmann equations and comparison of the T current before and after BSA treatment indicated significant differences in the voltage-dependent inactivation at steady state ($V_{1/2} = -58.5$ versus -52.9 mV). Notably, there were voltages at which channels were activated during the prepulse but they were not completely inactivated, as evidenced by currents evoked during the test pulses. This activity is referred to as a window current and is typically illustrated by the overlap in the steady-state activation and inactivation curves (Fig. 2C). It can be clearly seen that T currents after treatment had a larger window region. At the peak of the window (~ -52 mV) 28 and 44% of the channels are available to be open in the control condition and after BSA administration, respectively.

To gain insight into the mechanisms underlying the albumin regulation of T channels, we next investigated whether this effect was a consequence of its well-known ability to serve as a cholesterol binding molecule. Fig. 3A (left panel) shows that the addition of BSA presaturated with a high concentration (1:10 molar ratio) of an analog of cholesterol (cholesterol- SO_4^-) or cholesterol (not shown) produced a significant increase in current amplitude (~ 1.3 -fold) that was not modified by a second application of BSA alone. Neither cholesterol nor cholesterol- SO_4^- by itself modified T current properties (not shown). In addition, a 2 min incubation with 2-OH- β -cyclodextrin, a cyclic heptasaccharide able to promote a rapid cholesterol efflux from the sperm membrane and to induce an increase in tyrosine phosphorylation patterns similar to those seen in response to BSA during sperm capacitation [7], had no effect on the magnitude and kinetics of the T current (right panel). Together these results, summarized in Fig. 3B, provide evidence that BSA is not functioning here through its ability to remove cholesterol from the cell membrane.

Previous studies have shown that acute application of β -estradiol inhibits T-type channels in smooth muscle cells [17] as well as L-type channels in neurons [15], and cardiac and smooth muscle myocytes [18–22]. Thus it is possible that serum albumin could be enhancing T currents by removing a hydrophobic molecule such as β -estradiol. To test this hypothesis, spermatogenic cells were first directly superfused with increasing concentrations of 17β -estradiol and patch-clamp Ca^{2+} currents were recorded (Fig. 4A). As anticipated, currents in treated cells were smaller at all voltages tested (Fig. 4B). A small inhibition ($\sim 10\%$) was observed at nM concentrations and became significant at μM concentrations (Fig. 4C). This inhibition was voltage-independent (Fig. 4B) and too fast (< 2 min) to be attributed to genomic activation. Unlike what has been reported for high voltage-activated channels [15,16], this inhibition probably does not involve the activation of a G protein signaling pathway since spermatogenic T channels are basically G protein-independent [17]. A hormonally inactive isomer of 17β -estradiol (17α -estradiol) caused less reduction in T channel activity (Fig. 4D) indicat-

ing some specificity in the response. We next explored whether BSA stimulation could be antagonized by 17β -estradiol. As shown in Fig. 4E, BSA presaturated with 17β -estradiol did not increase peak current but induced a small reduction of current amplitude which was reversed by a second addition of BSA alone (inset). This finding is consistent with the hypothesis that the enhancement of T current caused by BSA might be due to the removal of 17β -estradiol rather than cholesterol from the plasma membrane. However, as shown in Fig. 4E, a progressive increase in 17β -estradiol inhibited T channel activity only at molar ratios $> 1:2.5$, raising the question whether this effect was caused by the presence of free 17β -estradiol. In addition, since BSA affects the T currents in a voltage-dependent manner while estradiol does not, it is likely that their effects are independent. However, these results suggest a potential physiological regulation of estrogens on spermatogenic cells. On the other hand, recently it was shown that 17β -estradiol can increase $[\text{Ca}^{2+}]_i$ by activating a novel functional estrogen receptor on the cell surface [8]. Our data suggest that this effect of estradiol is not mediated through T channels.

Mature sperm have only a single secretory vesicle (acrosome) and exocytosis must be coordinated with egg contact for efficient fertilization. Our results suggest that T-type Ca^{2+} channels, due to their window current, may contribute to setting $[\text{Ca}^{2+}]_i$ at the resting potential and therefore influence sperm capacitation. Since BSA increases the window current by modifying the voltage dependence of activation and inactivation, it could increase Ca^{2+} influx. As there is evidence that T channels are present in mature sperm [12] where resting potential is about -50 mV [23], BSA could facilitate an increase in Ca^{2+} entry, a prerequisite to capacitation. It is also possible that the actions of BSA on the T current may result from the removal of a hydrophobic molecule present in the sperm plasma membrane which suppresses premature secretion until the completion of capacitation, at which time sperm have arrived near the site of fertilization. The signaling mechanism by which 17β -estradiol reduces Ca^{2+} currents must await further investigation. Interestingly, numerous studies have shown that the function of membrane-spanning proteins can be affected by the material properties of the lipid bilayer through hydrophobic interactions between the protein and the bilayer [24]. Since 17β -estradiol is present in the sperm plasma membrane [6], the question arises as to whether changes in its concentration may be translated into an increase in the structural stress in the bilayer, and this force may be transmitted to sperm Ca^{2+} channels residing therein.

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