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Regulation of the sperm calcium channel CatSper by endogenous steroids and plant triterpenoids

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The calcium channel of sperm (CatSper) is essential for sperm hyperactivated motility and fertility. The steroid hormone progesterone activates CatSper of human sperm via binding to the serine hydrolase ABHD2. However, steroid specificity of ABHD2 has not been evaluated. Here, we explored whether steroid hormones to which human spermatozoa are exposed in the male and female genital tract influence CatSper activation via modulation of ABHD2. The results show that testosterone, estrogen, and hydrocortisone did not alter basal CatSper currents, whereas the neurosteroid pregnenolone sulfate exerted similar effects as progesterone, likely binding to the same site. However, physiological concentrations of testosterone and hydrocortisone inhibited CatSper activation by progesterone. Additionally, testosterone antagonized the effect of pregnenolone sulfate. We have also explored whether steroid-like molecules, such as the plant triterpenoids pristimerin and lupeol, affect sperm fertility. Interestingly, both compounds competed with progesterone and pregnenolone sulfate and significantly reduced CatSper activation by either steroid. Furthermore, pristimerin and lupeol considerably diminished hyperactivation of capacitated spermatozoa. These results indicate that (i) pregnenolone sulfate together with progesterone are the main steroids that activate CatSper and (ii) pristimerin and lupeol can act as contraceptive compounds by averting sperm hyperactivation, thus preventing fertilization.

CatSper | steroids | lupeol | triterpenoids | pristimerin

Steroid hormones control fundamental organism functions such as development, metabolism, inflammation, ion homeostasis, and reproduction. According to the conventional model, steroid hormones signal through a corresponding genomic receptor, which upon binding to a steroid hormone initiates the translocation of the hormone–receptor complex to the nucleus. There it acts as a transcription factor by altering gene expression, and such a process can take hours (1). However, there is another, much faster pathway that is initiated by steroid hormone binding to its membrane receptor on the extracellular side of the cell. The latter signaling involves second messengers and signal-transduction cascades, which often result in the activation of ion channels (1). The female hormone progesterone (P4) activates the principal calcium channel of sperm (CatSper) (2–5) and blocks the potassium channel K_{Sper} (6) via these nongenomic pathways (7). The modulation of ion channel functions by steroid hormones has been reported in the heart (8, 9), neurons (10–12), smooth muscle (13), and pancreatic beta cells (14). In marine invertebrates, the sulfated steroid cholestanol acts as a chemoattractant for sea squirt sperm, which also happens via the nongenomic pathway (15). The latter represents an unconventional chemoattractant for ascidian sperm, as such factors are usually proteins or peptides and not steroids. In humans, pregnenolone sulfate (PregS), a sulfated steroid hormone similar in structure to P4, is a nongenomic activator of the transient receptor potential cation channel subfamily M member 3 (TRPM3) channel in pancreatic beta cells (14). However, the precise mechanism of this activation is unknown. Here, we report that PregS also activates the sperm calcium channel

CatSper in a manner similar to P4 and explore the possibility of PregS binding to the same sperm receptor as progesterone.

As spermatozoa travel through the male and female reproductive tract, they are exposed to a variety of steroid hormones, such as testosterone and estrogen. The rising levels of hydrocortisone (HC) in the body as a result of stress can impact fertility (16) by interfering with spermatogenesis and/or sperm functions. Therefore, we have also explored what influence testosterone, estrogen, and HC have on CatSper activation. The structural precursor of all steroid hormones in animals is the triterpenoid lanosterol, which is formed upon cyclization of the linear triterpene squalene. Structurally related plant triterpenoids have been shown to exhibit antifertility properties in mice and rats. The most extensively studied plant triterpenoid is lupeol, which is found in mangoes, grapes, and olives. It has been shown to possess antifertility properties when orally administered to rats, resulting in a significantly reduced number of motile sperm (17), although the underlying mechanisms are not understood. A plausible explanation is that lupeol blocks CatSper, which is indispensable for sperm motility (4). Another plant triterpenoid, pristimerin, which is found in *Tripterygium wilfordii* (also known as “Thunder God Vine”) and *Celastrus regelii*, acts as a monoacylglycerol lipase (MAGL) inhibitor. It inhibits the hydrolysis of 2-arachidonoylglycerol (2-AG) to arachidonic acid (AA) and glycerol (18). In sperm, 2-AG acts as an endogenous CatSper inhibitor, and 2-AG degradation is mediated by the α/β hydrolase domain-containing protein 2 (ABHD2) in a P4-dependent manner (7). Because it is likely that pristimerin, lupeol, as well as

Significance

The calcium channel of sperm—CatSper—is vital for male fertility. CatSper is activated by the hormone progesterone, but its pharmacological profile is not well studied. By exploring steroid selectivity of CatSper activation, we found one additional agonist—pregnenolone sulfate—and the two plant-derived inhibitors pristimerin and lupeol. By averting sperm hyperactivation, both inhibitors can prevent fertilization, thus acting as contraceptive agents. Additionally, by exploring CatSper regulation by endogenous steroids, we explain why CatSper is silent within the male reproductive tract and is only activated in close proximity to the egg. Interestingly, both testosterone and hydrocortisone antagonize the action of progesterone at physiological concentrations, which may explain why elevated levels of these steroids in the female organism affect fertility.

Author contributions: N.M. and P.V.L. designed research; N.M., M.R.M., and P.V.L. performed research; N.M. contributed new reagents/analytic tools; M.R.M. helped with pilot experiments; P.V.L. led the research study; N.M. and P.V.L. analyzed data; and N.M. and P.V.L. wrote the paper.

Conflict of interest statement: P.V.L. and N.M. are inventors on a patent application filed by University of California, Berkeley related to the work presented in this paper.

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endogenous steroids can interfere with ABHD2 activity based on their structure similarities, we have explored the ability of these triterpenoids and steroids to prevent CatSper activation and fertilization.

Results

Testosterone, 17 beta-Estradiol, HC, and PregS Exert Distinct Effects on CatSper. Progesterone (P4) and its closest analog 17-OH-P4 have been shown to trigger calcium influx into the sperm flagellum (19) via activation of the sperm-specific calcium channel CatSper (3, 5). However, CatSper specificity toward other steroid hormones has not been evaluated. To reveal CatSper regulation by endogenous steroids and steroid-like molecules, we have analyzed inward monovalent currents through CatSper ($I_{CatSper}$) of human sperm. Cells were first stimulated with either 1 μ M testosterone, 17 beta-estradiol (E2), HC, or PregS alone. Subsequently, each preexposed cell was stimulated with 1 μ M P4 in combination with the corresponding steroid (testosterone,

E2, or HC). Testosterone, E2, or HC did not alter basal CatSper activity, whereas P4, as expected, caused a sevenfold potentiation of inward CatSper currents (Fig. 1 A–D and Table S1). However, coadministration of testosterone and P4 to spermatozoa, which were preexposed to testosterone, completely prevented $I_{CatSper}$ potentiation by P4 (Fig. 1 A and B and Table S1). E2 and HC partially reduced CatSper activation by P4 to 3.6- and 4.1-fold, when applied simultaneously with P4 (Fig. 1 C and D and Table S1). To explore the efficiency of P4 inhibition by either testosterone, E2, or HC, we have determined the IC_{50} values for each of them by measuring CatSper activation by 1 μ M of P4 in the presence of various concentrations of the corresponding steroid. Human spermatozoa were first preexposed to corresponding concentrations of either testosterone, E2, or HC. Subsequently, cells were exposed to both the individual steroid concentration from the first stimulus plus 1 μ M P4. The respective IC_{50} values were 429 ± 73 nM (testosterone; Fig. 1 F and I and Table S1), 833 ± 280 nM (E2; Fig. 1 G and I and Table S1), and 153 ± 90 nM

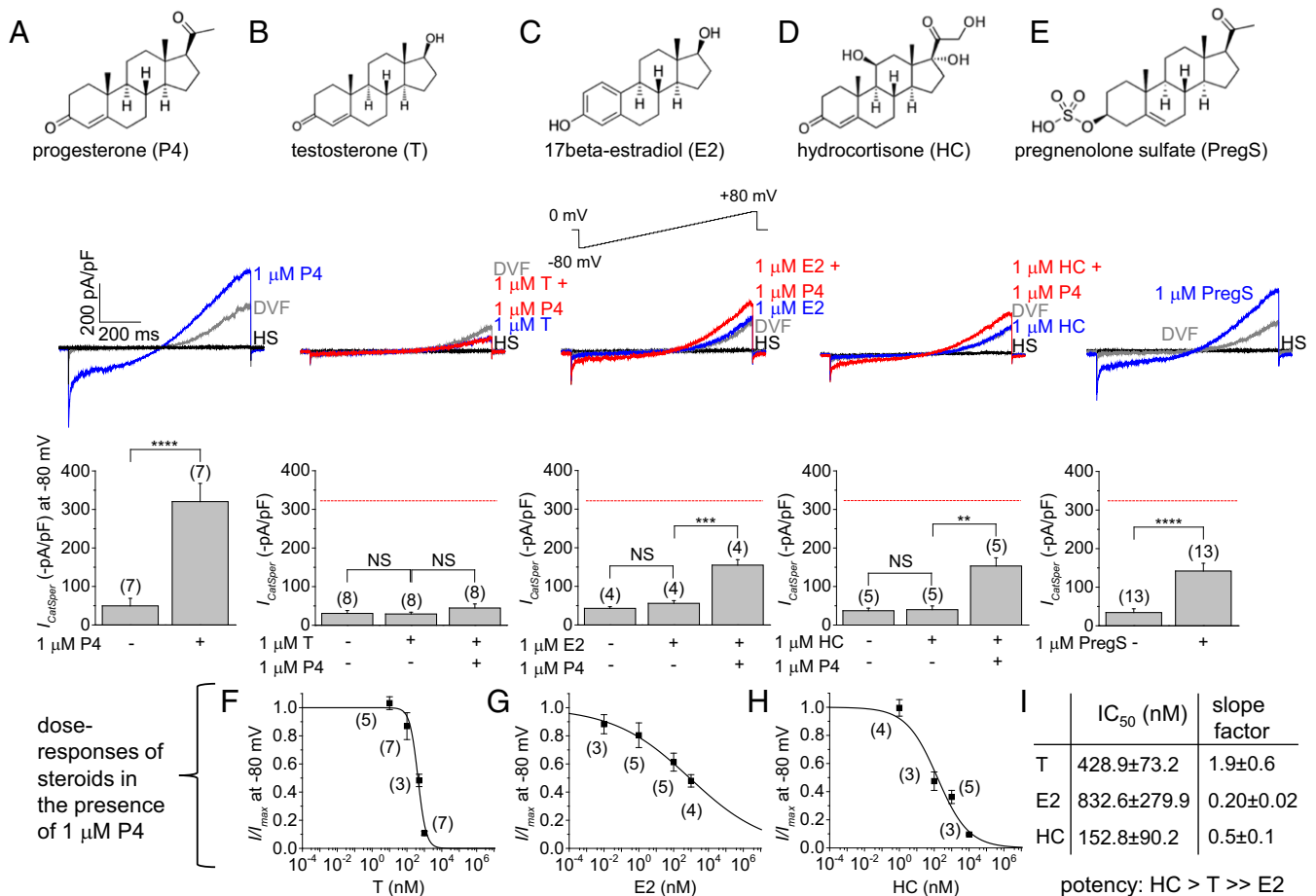


Fig. 1. Testosterone, E2, hydrocortisone, and PregS exert distinct effects on CatSper. The *Top* row of A–E displays the chemical structures of steroid tested. *Middle* row shows representative $I_{CatSper}$ recordings in response to the indicated voltage ramp (same voltage protocol was used for all traces). *Bottom* illustrates averaged $I_{CatSper}$ densities obtained at –80 mV as shown in the corresponding *Middle* panels. The red dotted line in B–E refers to the maximal CatSper activation in response to progesterone in A, *Bottom*. F–H, *Bottom* illustrate dose–response curves for testosterone (T), estradiol (E2), and hydrocortisone (HC) in the presence of progesterone. Sperm cells were stimulated via continuous perfusion. (A, *Middle*) The black trace refers to baseline currents in HS solution, and the gray trace indicates CatSper control currents in divalent-free (DVF) solution. The blue trace shows $I_{CatSper}$ in the presence of 1 μ M progesterone (P4). (*Bottom*) Averaged inward current densities under control conditions (DVF) and in response to 1 μ M P4. (B, *Middle*) Representative $I_{CatSper}$ with 1 μ M testosterone (T, blue) and 1 μ M T + 1 μ M P4 (red). (C, *Middle*) Representative $I_{CatSper}$ with 1 μ M 17 beta-estradiol (E2, blue) and 1 μ M E2 + 1 μ M P4 (red). (D, *Middle*) Representative $I_{CatSper}$ with 1 μ M hydrocortisone (HC, blue) and 1 μ M HC + 1 μ M P4 (red). (E, *Middle*) Representative $I_{CatSper}$ with 1 μ M PregS (blue). (F) Dose–response of inward $I_{CatSper}$ inhibition by testosterone in the presence of 1 μ M P4. (G) Dose–response of $I_{CatSper}$ inhibition by E2 in the presence of 1 μ M P4. (H) Dose–response of $I_{CatSper}$ inhibition by HC in the presence of 1 μ M P4. $I_{CatSper}$ densities obtained at –80 mV were averaged and fitted with a Hill-based equation. (I) Shown are IC_{50} values and slope factors from the dose–response curves in F–H. All bar graphs are presented as the mean \pm SEM, and *n* indicates number of individual cells analyzed. ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$.

(HC; Fig. 1 *H* and *I* and Table S1). These data indicate that exposure to physiological concentrations of either testosterone or hydrocortisone prevents CatSper activation by P4, and therefore, human spermatozoa must be free from testosterone or hydrocortisone influence to achieve full CatSper activation. Estrogen, however, requires much higher concentrations, which are outside of the physiological range, to antagonize CatSper activation (Fig. 1*I*).

In contrast to the aforementioned steroids, PregS alone stimulated inward currents fourfold, which resembled $I_{CatSper}$ properties (Fig. 1*E* and Table S1). To test whether PregS-elicited currents were identical to $I_{CatSper}$, we conducted the following experiments.

PregS Activates CatSper Channel Through a Molecular Mechanism, Which Is Similar to the P4-Mediated CatSper Activation. To evaluate whether PregS activated CatSper, we inhibited PregS-elicited currents with 1 μ M NNC 55-0396, a known CatSper inhibitor (3) (Fig. 2*A* and *B* and Table S2). Indeed, 1 μ M NNC 55-0396 reduced PregS-elicited currents below basal levels indicating that PregS is another steroid capable of activating CatSper. The potentiation of $I_{CatSper}$ by PregS was dose-dependent, with an EC_{50} of 15.2 ± 5.0 nM (Fig. 2*C* and *D*, black line and Table S2), which is close to the EC_{50} obtained with P4 [7.7 ± 1.8 nM (3)]. The serum concentration of PregS in adults reaches levels of 130 nM (women) and 140 nM (men) (14). It is also abundant in testes, in which the total PregS concentrations can reach 2.8 μ mol/kg (3.8 μ M), whereas it drops to 0.76 μ mol/kg (190 nM) in the epididymis (14). Such high PregS concentrations may result in premature CatSper activation, while spermatozoa are still in the male reproductive tract, which would be detrimental for sperm fertility. Because testosterone is also abundant in the male reproductive tract, we have explored whether it can block CatSper

activation by PregS. Human sperm cells were first preincubated with 1 μ M testosterone alone followed by 1 μ M testosterone plus various concentrations of PregS (Fig. S1). Under such conditions, the EC_{50} of PregS increased 10-fold and reached 176.0 ± 71.0 nM, indicating that testosterone indeed interferes with PregS activation of CatSper (Fig. 2*D*, red line and Table S2). In human sperm, P4 activates CatSper indirectly via binding to and then activating its membrane receptor ABHD2 (7). ABHD2 acts as a serine hydrolase, thus depleting the endogenous CatSper inhibitor 2-AG from the plasma membrane. Removal of 2-AG subsequently leads to the opening of CatSper. To determine whether the potentiation of $I_{CatSper}$ by PregS was ABHD2-mediated, we inactivated ABHD2 by exposing human spermatozoa to 2 μ M methyl arachidonoyl fluorophosphate (MAFP), a serine hydrolase inhibitor (Fig. 2*E* and *F* and Table S2). As expected, MAFP exposure ablated CatSper activation by PregS, whereas basal $I_{CatSper}$ was not affected. Taken together, these results suggest that in addition to P4, ABHD2 can also be activated by PregS. Because both P4 and PregS influence ABHD2 activity, we tested whether these compounds compete for the binding site. To do so, human sperm cells were stimulated with saturated concentrations of PregS (Fig. 3*A*, blue trace) followed by costimulation with a combination of saturated concentrations of P4 and PregS (Fig. 3*A* and *C*, red trace and Table S3). The experiments were repeated in the reverse order (Fig. 3*B* and *C* and Table S3). The increase of CatSper currents by PregS was not further potentiated upon addition of P4. The same result was obtained when sperm cells were first stimulated with P4 and then with a combination of P4 and PregS. These experiments suggest that P4 and PregS likely compete for the same ABHD2 binding site and that CatSper activation by PregS follows the same mechanism as CatSper activation by P4.

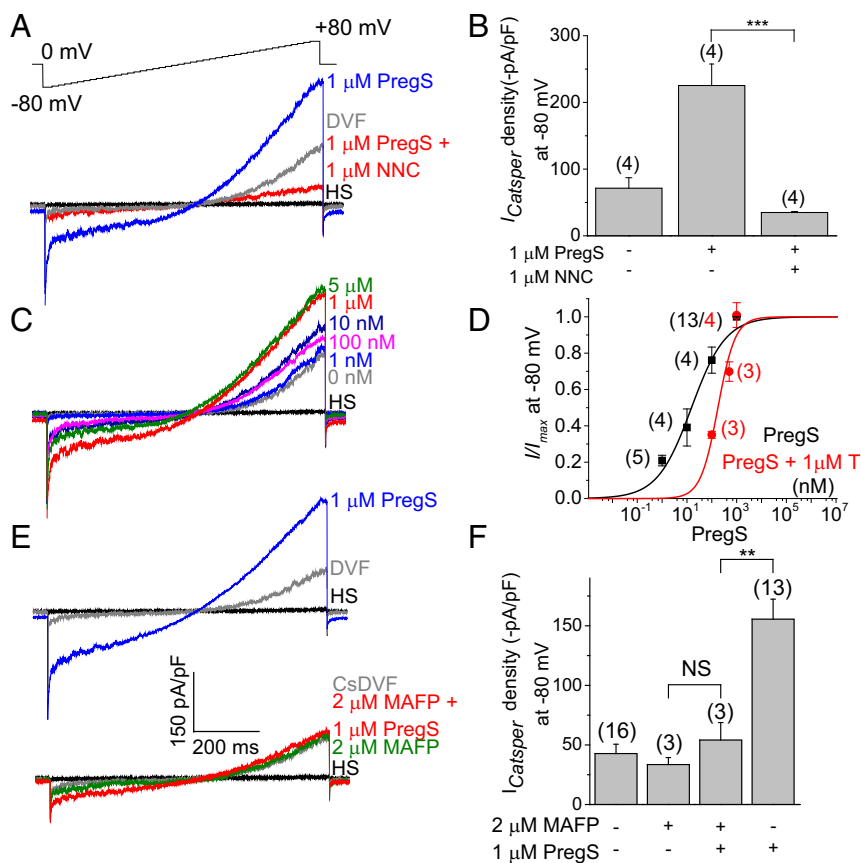


Fig. 2. PregS activates CatSper through a molecular mechanism, which is similar to the P4-mediated CatSper activation. (A) Representative $I_{CatSper}$ traces in response to stimulation with 1 μ M PregS (blue) or 1 μ M PregS + 1 μ M NNC 55-0396 (red). The same voltage protocol was used for all traces. (B) Averaged inward $I_{CatSper}$ densities of control and test conditions. (C) Representative $I_{CatSper}$ with different concentrations of PregS (1 nM, light blue; 10 nM, dark blue; 100 nM, magenta; 1 μ M, red; 5 μ M, green). (D) Dose-response of $I_{CatSper}$ activation with PregS alone (black trace) or with various concentrations of PregS in the presence of 1 μ M T (red trace). The data were fitted with a Hill-based equation. For PregS alone, the EC_{50} was 15.2 ± 5.0 nM with a slope factor of 0.6 ± 0.1 . The EC_{50} in the presence of 1 μ M T with various concentrations of PregS was 176.0 ± 71.0 nM with a slope factor of 1.2 ± 0.5 . (E) Representative control $I_{CatSper}$ in response to 1 μ M PregS (blue, Top). Bottom shows recording from a cell preincubated with 2 μ M MAFP (green) and with 2 μ M MAFP + 1 μ M PregS (red). The scaling of the axes refers to all graphs in A, C, and E. (F) Averaged inward $I_{CatSper}$ densities of control recordings and in response to MAFP and/or PregS. All bar graphs are presented as the mean \pm SEM, and *n* indicates number of individual cells analyzed. ***P* < 0.005; ****P* < 0.001.

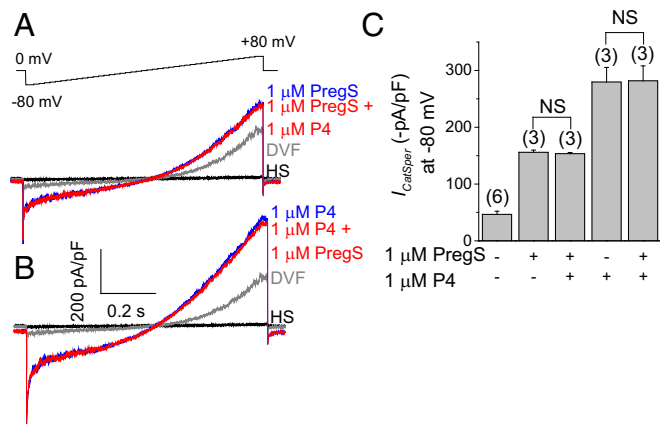


Fig. 3. PregS and P4 compete for the ABHD2 binding site. (A) Representative $I_{CatSper}$ in response to the indicated voltage ramp in the presence of 1 μ M PregS (blue) or 1 μ M PregS + 1 μ M P4 (red). (B) $I_{CatSper}$ recording with 1 μ M P4 (blue) followed by 1 μ M P4 + 1 μ M PregS (red). (C) Averaged $I_{CatSper}$ densities obtained at -80 mV and presented as mean \pm SEM; *n* indicates number of individual cells analyzed.

The Two Plant Triterpenoids Pristimerin and Lupeol Significantly Reduce the Activation of CatSper by Either P4 or PregS.

2-AG hydrolysis by monoacyl lipases, such as ABHD2, is required for CatSper activation. The plant triterpenoid pristimerin was reported to inhibit the activity of the ABHD-related enzyme monoacylglycerol lipase (MAGL) (18). If pristimerin can also inhibit ABHD2, then sperm exposure to this compound should prevent CatSper activation by P4 or PregS. To test this hypothesis, human spermatozoa were stimulated with pristimerin, followed by exposure to either a mixture of pristimerin and P4 or to a mixture of pristimerin and PregS. The mere presence of pristimerin did not affect basal $I_{CatSper}$ (Fig. 4A, Fig. S2 A–D, and Table S4), which indicates that pristimerin did not target the channel directly. However, coapplication of pristimerin with P4 or PregS significantly reduced CatSper activation. Compared with stimulation with P4 or PregS alone, $I_{CatSper}$ stimulation was reduced by 63% (pristimerin and P4) and by 48% (pristimerin and PregS; Fig. 4A, Fig. S2 A–D, and Table S4). To determine the IC_{50} value of pristimerin's inhibitory effect on CatSper activation by P4, human sperm cells were first preexposed to various concentrations of pristimerin. Then, cells were exposed to the respective pristimerin concentration of the first stimulus plus 1 μ M P4. The IC_{50} of pristimerin in the presence of 1 μ M P4 was 116 ± 77 nM (Fig. 4B and Table S4).

Another pharmacologically active plant triterpenoid, which affects sperm functions when orally administered to rats, is lupeol (17). Comparable to pristimerin, lupeol alone did not affect basal $I_{CatSper}$ (Fig. 5A, Fig. S2 E and F, and Table S5). However, administration of lupeol with P4 or PregS led to an even stronger inhibition of $I_{CatSper}$ than pristimerin. Compared with CatSper stimulation elicited by P4 or PregS alone, CatSper currents were reduced by 71% and 68% in combination with P4 or PregS, respectively (Fig. 5A). The IC_{50} value of lupeol to inhibit CatSper activation by 1 μ M P4 was 109 ± 56 nM (Fig. 5C and Table S5).

Pristimerin and Lupeol Decrease Sperm Hyperactivation in the Presence of P4.

Hyperactivated sperm motility is characterized by a highly asymmetrical bending of the sperm tail due to a CatSper-mediated rise in flagellar calcium concentration (20). Curvilinear velocity (VCL) is the average velocity of the sperm head through the sperm trajectory, which increases during capacitation. A CatSper-mediated calcium rise promotes hyperactivation and makes the sperm trajectory less linear, which results in an increase of VCL. Capacitation increases CatSper activity, which results in a higher percentage of sperm with hyperactivated

motility. Thus, capacitated spermatozoa tend to have higher VCL values than noncapacitated spermatozoa (Fig. S3A and Table S6). Because both P4 and PregS activate CatSper, whereas pristimerin and lupeol inhibit it, we explored whether VCL values of human sperm were affected in the presence of steroid hormones or plant triterpenoids. As expected, neither of the compounds changed VCL values of noncapacitated sperm cells (Fig. S3, Bottom panels and Table S6). In capacitated spermatozoa, P4 stimulation increased VCL by 12%, comparable to numbers reported earlier (7, 21), but VCL values remained unchanged in the presence of PregS, pristimerin, or lupeol alone (Fig. S3A, Top and Inset and Table S6). When spermatozoa were stimulated with P4 + pristimerin or P4 + lupeol, VCL values were reduced by 39% and 48%, respectively, in comparison with VCL values obtained in the presence of P4, and the reduced VCL values were comparable to those of noncapacitated cells (Fig. S3B and Table S6). Stimulating capacitated sperm with PregS in combination with pristimerin or lupeol decreased VCL by 18% and 9%, respectively, compared with sperm, which were treated with PregS alone (Fig. S3C, Top and Table S6). VCL values of noncapacitated sperm remained unchanged under such conditions (Fig. S3C, Bottom and Table S6). To explore whether pristimerin and lupeol also affect basal sperm motility, we assessed the effect of these compounds on motility of capacitated and noncapacitated sperm cells. The number of both capacitated and noncapacitated motile spermatozoa remained unchanged when stimulated with either compounds (Fig. S4A and Table S7). However, when P4 was coapplied with pristimerin or lupeol, the percentage of motile capacitated sperm decreased by 19%, whereas the values of noncapacitated cells did not change (Fig. S4B and Table S7). Costimulation with PregS and pristimerin or lupeol, however, did not result in a significant reduction of motile capacitated or noncapacitated sperm (Fig. S4C and Table S7). These results demonstrate that both pristimerin and lupeol have the capacity to significantly reduce sperm hyperactivation by blocking the P4-mediated activation of CatSper but also have a minor effect on basal sperm motility of capacitated cells.

Discussion

Our results demonstrate that testosterone, estrogen, and hydrocortisone alone did not activate CatSper but that they reduce or prevent CatSper activation by progesterone (P4). In the case of testosterone and hydrocortisone, the effects were the strongest with a significant inhibition of P4-mediated CatSper stimulation under physiological concentrations of either steroid. One possibility for this effect could be that testosterone binds with a

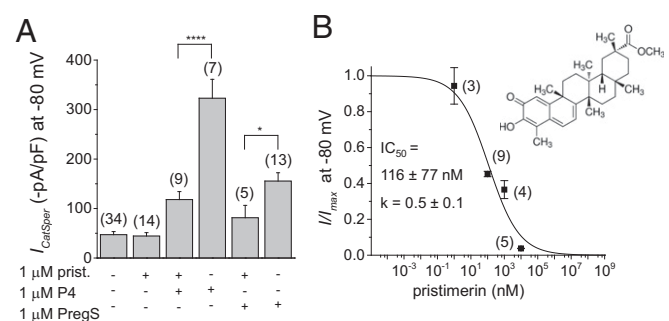


Fig. 4. The plant triterpenoid pristimerin significantly reduces the activation of CatSper by P4 and PregS. (A) Averaged $I_{CatSper}$ inward current densities obtained at -80 mV and presented as the mean \pm SEM; *n* indicates number of individual cells analyzed. * $P < 0.05$; **** $P < 0.0001$. (B) Dose-response of pristimerin inhibition of $I_{CatSper}$ in response to P4. Data were averaged and fitted with a Hill-based equation with *k* as the slope factor. The chemical structure of pristimerin is shown as inset.

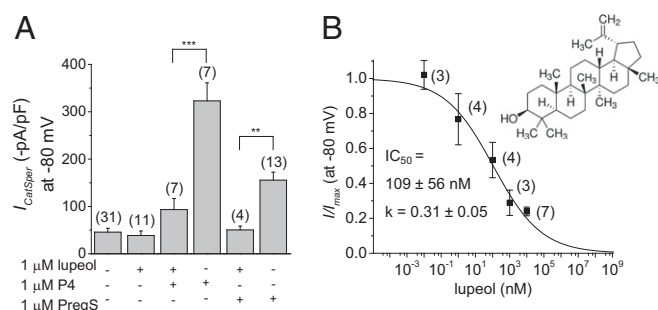


Fig. 5. The plant triterpenoid lupeol mimics the effect of pristimerin. (A) Averaged $I_{CatSper}$ inward current densities obtained at -80 mV and presented as the mean \pm SEM; n indicates number of individual cells analyzed. $**P < 0.005$; $***P < 0.001$. (B) Dose–response of lupeol inhibition of $I_{CatSper}$ in response to P4. Data were averaged and fitted with a Hill-based equation with k as the slope factor. The chemical structure of lupeol is shown as *Inset*.

much higher affinity to ABHD2 than P4, thereby preventing CatSper activation. The blood serum concentration of P4 in men is about 2 nM (22), whereas the minimum concentration needed for CatSper activation is 10 pM, with an EC_{50} of 7.7 nM (3). Testosterone blocked CatSper activation even in the presence of 1 μ M P4 with an IC_{50} of 429 nM, which is within the physiological range, as testosterone concentrations reach 2 μ M in the blood plasma of men (23). CatSper can also be activated by PregS, which can reach high concentrations in male reproductive tissues. However, testosterone also inhibits CatSper response to PregS by shifting its EC_{50} 10-fold to 172 nM. Therefore, even if sperm are exposed to elevated concentrations of PregS, high concentrations of testosterone in the male genital tract prevent premature CatSper activation by P4, or even PregS. It is therefore possible that testosterone acts as an anticapacitation factor by preventing CatSper activation until it is removed in the female reproductive tract, presumably by chelation with albumin.

Resting serum E2 levels in women are about 110 pM, which peak at ovulation to concentrations of around 403 pM (24). Therefore, sperm encounter an E2-enriched milieu in the uterus and the fallopian tube. Our results show that E2 did not alter resting CatSper currents but that it also did not allow full channel activation by P4. We assume that elevated levels of E2 during ovulation render CatSper in its closed state to prevent premature calcium influx and thus sperm activation. However, the significantly weaker IC_{50} for E2 of 833 nM indicates that E2 acts as a much less potent P4 antagonist. Right after ovulation, E2 levels decrease, whereas P4 concentrations surge to ~ 7 nM (24) in the blood. Because cumulus cells surrounding the oocyte also secrete P4 (25–28), sperm travel through a P4 gradient with maximal concentrations in close proximity to the egg. It is therefore possible that P4 outcompetes E2, leading to CatSper activation, as P4 is the natural E2 antagonist (29).

Various conditions, such as stress and elevated levels of glucocorticoids, particularly hydrocortisone, are known to impair male fertility (16) by either inhibiting spermatogenesis (30) or reducing sperm counts and sperm motility (31). Stress and elevated hydrocortisone levels can also affect female fertility. One study shows that in women who underwent in vitro fertilization, baseline urine cortisol levels increased from ~ 230 nM to ~ 500 nM (32). According to our results, hydrocortisone blocked CatSper activation by P4 with an IC_{50} of 153 nM. It is therefore possible that elevated levels of stress hormones in the female genital tract impair not only early stages but also late stages of sperm acquisition of their fertilizing potential, thus significantly contributing to infertility.

Another interesting candidate among the hormones tested in this study was the sulfated neurosteroid PregS, as it stimulates

CatSper currents via the P4-related pathway. Although significant, the response to PregS was not as pronounced as the response to P4 and the EC_{50} of PregS was twofold higher than the EC_{50} of P4 [15 nM vs. 7 nM (3)]. Nevertheless, we show that PregS acted via an ABHD2 mechanism to activate CatSper—the same mechanism of channel activation as demonstrated for P4 (7). These findings identify PregS as the third steroid hormone to exert nongenomic actions on CatSper apart from P4 and its close analog 17-OH-P4 (3, 5, 33) and demonstrate the importance of sulfated steroids to regulate physiological processes in human sperm. Even though the concentrations of PregS in human testes are higher than those of pregnenolone (51 μ g/100 g tissue) (34), it is unclear whether elevated concentrations of PregS exist within the entire testis or only in specific domains. Because testosterone inhibits the response to PregS with an IC_{50} of 172 nM, it will prevent CatSper activation by PregS even if sperm cells are exposed to high PregS concentrations. Interestingly, the plasma concentration of PregS in women is about 14 nM (35). This concentration matches the EC_{50} we determined for PregS to activate CatSper. It is therefore possible that once testosterone is removed from sperm cells within the female genital tract, both P4 and PregS can bind to ABHD2, resulting in CatSper activation. These two compounds indeed compete for the ABHD2 binding site(s), but further studies are needed to reveal whether PregS and P4 act synergistically or independently to activate CatSper.

Our earlier findings identified the acylglycerol lipase ABHD2 as the P4 binding partner (7). Therefore, we tested whether the monoacylglycerol lipase inhibitor pristimerin, a plant triterpenoid (18), can inhibit both the P4- and the PregS-mediated activation of CatSper of human sperm. Although basal CatSper currents were not affected, both P4 and PregS-mediated CatSper potentiation was significantly reduced. Lupeol, another plant triterpenoid, had similar effects on $I_{CatSper}$ as pristimerin. It is possible that both triterpenoids occupy the steroid binding site of ABHD2, thus preventing CatSper activation by P4 via a competitive antagonist-type mechanism. Both compounds were also able to inhibit sperm hyperactivation and slightly reduced basal motility of capacitated sperm cells, as evident from computer-assisted sperm analyses (CASAs). Interestingly, pristimerin and lupeol had no effect on sperm motility of noncapacitated cells, which indicates their low toxicity effect toward spermatozoa. These results correlate with our electrophysiological data, which showed a significant reduction of $I_{CatSper}$ with pristimerin + P4 and lupeol + P4, respectively. Because CatSper is indispensable for hyperactivated sperm motility (36–40), it is evident that the reduction of sperm hyperactivation by both triterpenoids may significantly impair sperm ability to fertilize an egg.

CASA experiments also revealed that although P4 increased hyperactivated motility, PregS failed to do so. This could be due to the fact that PregS is a charged molecule, which cannot pass the plasma membrane (41). It is therefore possible that the nonpolar P4 can activate additional intracellular pathways, contributing to a more pronounced activation of CatSper. Indeed, for full sperm hyperactivation in vitro, two events must be met at the same time: (i) CatSper must be relieved from inhibition by 2-AG, and (ii) the sperm plasma membrane needs to be depolarized. Because CatSper is a voltage-dependent channel, it requires at least +30 mV for half-activation (3). The latter can be achieved via the P4-mediated inhibition of the potassium channel K_{Sper} (6), which creates membrane depolarization required for full CatSper activation. If P4 inhibits K_{Sper} from the intracellular side, which PregS fails to do, as it cannot cross plasma membrane, then P4 is able to cause a more pronounced hyperactivation.

In conclusion, our findings show that apart from P4, PregS is another steroid hormone that can activate CatSper via ABHD2 in human spermatozoa, whereas testosterone, E2, and HC may bind to ABHD2 competitively to modulate the response to P4 and PregS. In addition, we describe two plant triterpenoids that

can serve as promising candidates for contraception, as they reduce the number of hyperactive spermatozoa, thus preventing sperm from reaching and fertilizing an egg.

Materials and Methods

Reagents. Progesterone and pristimerin were purchased from Calbiochem (EMD Millipore). MAFP was from Cayman Chemical Company, and NNC 55-0396 was from Tocris. All other compounds were from Sigma Aldrich. Testosterone was purchased in accordance with the controlled substance protocol (CS084484), as a collaborative effort with Yuriy Kirichok (University of California, San Francisco).

Donors and Purification of Human Ejaculated Spermatozoa. The participation of four healthy human sperm donor volunteers was approved by the Committee on Human Research at the University of California, Berkeley (protocol number 2013-06-5395). All donors provided informed consent. Freshly ejaculated semen samples were obtained by masturbation. Sperm were purified with the swim-up technique (3) using artificial human tubal fluid solution (HTF), containing (in mM) 21 Hepes, 21 lactic acid, 98 NaCl, 4.7 KCl, 3 glucose, 2 CaCl₂, 0.3 KH₂PO₄, 0.3 sodium pyruvate, and 0.2 MgSO₄, pH 7.4 (adjusted with NaOH).

Electrophysiology. All recordings were performed as described in ref. 3. Briefly, gigaohm seals between patch pipette and spermatozoa were formed at the cytoplasmic droplet in high saline (HS) solution containing (in mM) 135 NaCl, 20 Hepes, 10 lactic acid, 5 KCl, 5 glucose, 2 CaCl₂, 1 MgSO₄, 1 sodium pyruvate, pH 7.4 (adjusted with NaOH), and ~320 mOsm/L. Transition into whole-cell mode was achieved by applying suction and short voltage pulses. For CatSper recordings, the divalent-free bath solution contained (in mM) 140 Cs-methanesulfonate, 40 Hepes, 1 EDTA, pH 7.4 (adjusted with CsOH), and ~325 mOsm/L. Pipettes (10–15 MΩ) were filled with 130 mM Cs-methanesulfonate, 70 mM Hepes, 3 mM EGTA, 2 mM EDTA, 0.5 mM Tris-HCl, pH 7.4 (adjusted with CsOH), and ~335 mOsm/L. Access

resistance was 42–60 MΩ. Cells were stimulated every 5 s, and data were sampled at 2–5 kHz and filtered at 1 kHz. All experiments were performed at room temperature and currents elicited by a voltage ramp from –80 mV to 80 mV with a holding potential of 0 mV. Data were analyzed with Clampfit 9.2 and OriginPro 9.0. To build dose–response curves, data were fitted with the Hill-based equation: $y = I_{\min} + (I_{\max} - I_{\min}) / (1 + (x / IC_{50})^k)$, where I_{\max} is close to 100% activation, I_{\min} is close to inactivation, and k is the Hill slope factor.

CASA. Purified human spermatozoa were capacitated for 3.5 h at 37 °C and 5% CO₂ in capacitation media (HS supplemented with 15 mM NaHCO₃ and 5% BSA) as reported in ref. 7. Aliquots of the cell suspension were preincubated for 15 min with 3 μM pristimerin or 3 μM lupeol before exposure to steroids (3 μM progesterone or 3 μM PregS). Sperm motility was analyzed at 37 °C with an HTM-IVOS sperm analysis system (version 12.3, Hamilton Thorne Biosciences). We pipetted 10 μL of sperm suspension into a two-chamber slide (Leja), and sperm movement of a minimum of 300 cells was recorded. Parameters measured were the four motility classes (A–D), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), and VCL (μm/s). Measurements on a given day were performed in duplicates and defined as one experiment.

Data Analysis. Statistical data were calculated as the mean ± SEM, and n indicates number of individual cells analyzed unless stated otherwise. Statistical significance (unpaired t test) is indicated by * P < 0.05, ** P < 0.005, *** P < 0.001, and **** P < 0.0001.

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