Published-Ahead-of-Print April 1, 2006, DOI:10.2164/jandrol.05152
Journal of Andrology, Vol. 27, No. 4, July/August 2006

DOI: 10.2164/jandrol.05152

Journal of Andrology, Vol. 27, No. 4, July/August 2006
Copyright © American Society of Andrology

SAMMA Induces Premature Human Acrosomal Loss by Ca^{2+} Signaling Dysregulation

ROBERT A. ANDERSON*, KENNETH A. FEATHERGILL*, DONALD P. WALLER† AND LOURENS J. D. ZANEVELD*

*From the Program for the Topical Prevention of Conception and Disease (TOPCAD) and * Departments of Obstetrics and Gynecology, Rush University Medical Center, Chicago, Illinois; and the † Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, Illinois.*

Correspondence to: Robert A. Anderson Jr, Ob/Gyn Research, Rush Medical Center, Chicago, IL 60612 (e-mail: randerso{at}rush.edu).

Received for publication August 29, 2005; accepted for publication February 10, 2006.

Abstract

SAMMA is licensed for development as a contraceptive microbicide. Understanding mechanisms of its biological activity is prerequisite to designing more active second generation products. This study examined Ca^{2+} involvement in SAMMA-induced premature acrosomal loss (SAL) in noncapacitated human spermatozoa. SAMMA causes acrosomal loss (AL) in a dose-dependent manner (ED_{50} = 0.25 µg/mL). SAL requires extracellular Ca^{2+} (ED_{50} = 85 µM). SAL is inhibited by verapamil (nonspecific voltage-dependent Ca^{2+} channel blocker; IC_{50} = 0.4 µM), diphenylhydantoin and $NiCl_2$ (T-type [$Ca_v3.x$] channel blockers; IC_{50} 210 µM and 75 µM, respectively). Verapamil blockade of L-type ($Ca_v1.x$) channels is use-dependent; activated channels are more sensitive to inhibition. However, verapamil inhibition of SAL does not increase after repeated SAMMA stimulation. SAL is unaffected by 10 µM nifedipine (selective L-type channel blocker). This contrasts to 40% inhibition ($P < .001$) of AL induced by 1 µM thapsigargin (Ca^{2+}-ATPase inhibitor; releases intracellular Ca^{2+} stores, promotes capacitative Ca^{2+} entry). SAL is unaffected by 1 µM BAPTA-AM (intracellular Ca^{2+} chelator), and 50 µM 2-APB (blocks InsP3 receptors and store-operated channels). This contrasts with thapsigargin-induced AL, inhibited nearly 65% by BAPTA-AM ($P < .005$) and 91% by 2-APB (P, .001). The results suggest that SAL is mediated by Ca^{2+}

entry through channels pharmacologically similar to the T-type ($Ca_V3.2$) class. This process appears distinct from that caused by physiological stimuli such as progesterone or zona pellucida-derived proteins. SAMMA's contraceptive activity may be caused by induction of premature AL through dysregulation of Ca^{2+} signaling.

Key words: Topical contraceptive microbicide, signal transduction, spermatozoa, mechanism, calcium channels

---

Introduction

Global statistics on the spread of human immunodeficiency virus (HIV) and other sexually transmitted pathogens, as well as the continued growth of the world population, underscore the importance of initiatives for providing protection against sexually transmitted infections and unplanned conception. Approximately half of over 40 million adults infected with HIV are women (UNAIDS, 2004). Young women are 3 times more likely than men of the same age to become infected (UNFPA UNAIDS and UNIFEM, 2004). In the United States, AIDS is the leading cause of death among African Americans between the ages of 25 and 34 (UNAIDS, 2003).

More than 1 in 5 individuals over the age of 12 in the United States are infected with the herpes simplex virus (HSV-2) (Lafferty, 2002). HSV and other genital tract infections enhance HIV transmission (Cohen, 1998; Mbopi-Keou et al, 2000). Gonorrhea rates among adolescents are high in several countries, including the United States (Barberis et al, 1998; Panchaud et al, 2000). Chlamydial infections are higher among female teenagers than among males of the same age (Panchaud et al, 2000).

Unplanned pregnancies present additional risk to women's reproductive health. Nearly half of these in the United States are terminated (Henshaw, 1998). Safe contraceptive microbicides will improve the reproductive health of women and the population at large.

Sulfuric acid modified mandelic acid (SAMMA) is a carboxylated oligomeric (average molecular weight of less than 2 kd) compound from the Topical Prevention of Conception and Disease (TOPCAD) Program, under development as a contraceptive microbicide. A license has been secured (Yaso Biotechnology Inc, Coppell, Tx) for its commercialization under US patent no. 5932619 (Zaneveld et al, 1999). It is active against HIV, HSV, and *Chlamydia trachomatis*, among other sexually transmitted pathogens (Zaneveld et al, 2002). SAMMA is active against spermatozoa; it inhibits hyaluronidase and acrosin (required for fertilization), causes premature acrosomal loss (AL), and is contraceptive in the rabbit. However, sperm motility is unaffected, suggesting that SAMMA is not acting as a spermicide (Zaneveld et al, 2002).

SAMMA's relatively simple method of synthesis and its low molecular weight lends itself to chemical modifications that could improve efficacy and selectivity of action. Prerequisite to more active SAMMA derivatives is the knowledge of its mechanism(s) of action. SAMMA disrupts acrosomes in noncapacitated spermatozoa.

The acrosome overlies the sperm head and contains enzymes required for fertilization (eg, Yanagimachi, 1988). Normally, acrosomes of capacitated spermatozoa are disrupted (acrosome reaction) upon contacting the zona pellucida, that surrounds the oocyte. Premature AL during the residence of spermatozoa in the vagina would hinder or prevent fertilization, and be contraceptive. The acrosome

reaction requires entry of extracellular calcium ions (Ca^{2+}) (Breitbart and Naor, 1999; Guraya, 2000). This work was carried out to evaluate pathways of Ca^{2+} entry in SAMMA-induced acrosomal loss (SAL).

The results show that SAL, similar to the acrosome reaction, occurs by a Ca^{2+}-dependent mechanism. However, unlike acrosome reactions, entry of Ca^{2+} in response to SAMMA does not involve capacitative entry through store-operated channels, but rather may only be through T-type voltage-dependent channels.

Materials and Methods

- Top
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- References

Materials

Calcium ionophore A23187, verapamil, nifedipine, 2-APB (2-aminoethoxydiphenylborate), and BAPTA-AM (tetraacetoxymethyl ester of (1,2-*bis*(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) were purchased from CalBiochem Corporation (La Jolla, Calif). Thapsigargin, diphenylhydantoin, Rose Bengal, Bismarck Brown Y, and Ficoll (type 400-DL) were purchased from Sigma Chemical Company (St Louis, Mo). All Ca^{2+} modulating agents were dissolved in DMSO before use. The final concentration of DMSO did not exceed 1%. All other reagents were of the highest quality commercially available. A proprietary method was used to synthesize SAMMA by reacting D,L-mandelic acid with concentrated sulfuric acid. Additional details regarding the synthesis have been provided (Zaneveld et al, 1999). The product is a mixture of polyphenylacetic acid oligomers with an average of approximately 10–12 repeating subunits. The sodium salt was prepared by reacting the free acid with alcoholic NaOH, yielding an off-white powder with an average molecular weight of approximately 1550–1860.

Human Subjects— All subjects gave informed consent to participate in this study, which was carried out in compliance with and approved by the institutional review board. In each experiment, fresh semen was collected from 2 to 3 individuals out of a total pool of 9 healthy donors (mean age = 32 + 3.5 [SEM] years). All subjects were free from illness requiring medical intervention for at least 1 year before their participation in this study; they were not taking prescription, over-the-counter, or recreational drugs at the time of the study. Alcohol consumption was low to moderate, with no subject consuming more than 4 to 7 drinks per week. No subject reported urinary infection, prostatic inflammation, or contraction of sexually transmitted infection within 3 years of the study. Inclusion criteria for participation included maximum allowable abnormal sperm forms of 30% (World Health Organization, 1992), minimum proportion of spermatozoa responding to hypoosmotic medium (HOS test; Jeyendran et al, 1984) of 60% and minimum of 30% of capacitated spermatozoa that undergo acrosome reaction in response to the calcium ionophore A23187 (De Jonge et al, 1989). Semen quality was consistently high, with average volume, sperm count and motility within 1 hour of collection of 3.7 ± 0.43 mL, 70 (90% confidence limits = 57.5–84.2) x 10^6/mL and 71% (68.4–74.4), respectively (n = 30).

Methods

In this context, acrosomal loss refers to the disruption of the sperm acrosome in response to exposure to a treatment or chemical entity. No inference is made as to whether this response is identical to the physiological acrosome reaction, during which the acrosome is also lost.

Preparation of Spermatozoa and Induction of Acrosomal Loss— Semen was allowed to liquify for at least 30 minutes after collection. Within 90 minutes, semen was layered over buffered 11% Ficoll,

and spermatozoa were sedimented and separated from seminal plasma by centrifugation. Spermatozoa were resuspended (5 x 10^6 cells/mL) in a modified BWW medium (Biggers et al, 1971), less bovine serum albumin (Anderson et al, 1994). Composition of the medium is as follows: 94.6 mM NaCl, 4.8 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.1 mM $NaHCO_3$, 0.25 mM sodium pyruvate, 23.3 mM sodium lactate, 5.6 mM glucose, and antibiotics (100 IU penicillin, 50 µg/mL streptomycin). The sperm suspension was divided into 0.5-mL portions. A small portion (approximately 10 µL) was reserved for sperm motility assessment by light microscopy.

After 5 minutes of equilibration at 37 °C, modulators were added as indicated. Ten minutes thereafter, acrosomal loss was induced by SAMMA at different concentrations as indicated. Fifteen minutes after addition of SAMMA, 10 µL of the suspension were removed for motility assessment. Spermatozoa were fixed with buffered glutaraldehyde (Anderson et al, 1992) and stained for acrosome visualization with Bismarck Brown and Rose Bengal (De Jonge et al, 1989). Results are expressed as percent of maximal acrosomal loss induced by a maximally stimulating concentration of the calcium ionophore, A23817 (Anderson et al, 1992).

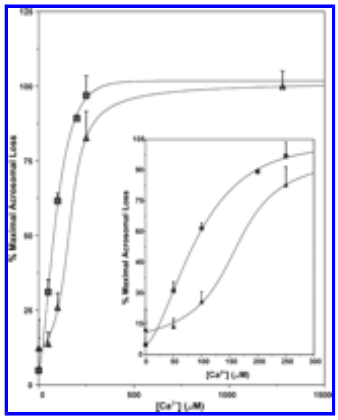
Data Analysis— All frequency (%) data were subjected to arcsine transformation before further analysis (Sokal and Rohlf, 1981). Values are presented as average percentage of maximal acrosomal loss, with the 90% confidence limits. Analysis of variance and the Newman-Keuls multiple range test were used to identify differences among treatment groups within individual experiments. Dose–response data were best fit to transitional-type curves (eg, Sigmoidal) with TableCurve 2D curve-fitting software (SPSS Statistical Software, Chicago, Ill), from which appropriate constants (eg, ED_{50} values) were derived. Differences among treatment groups were considered significant at $P < .05$. Differences were not considered significant at $P > .10$.

Results

- Top
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- References

SAMMA induces acrosomal loss in a dose-dependent manner at concentrations ranging from 0.025–2.0 µg/mL. SAMMA produces a 50% maximal response (90% confidence limits = 42.2%–59.1%) at 0.25 µg/mL; 1.7 µg/mL SAMMA induces a nearly maximal (99.9%) response (data not shown).

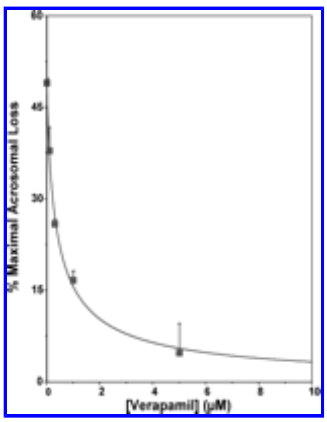
SAL is Ca^{2+}-dependent. When SAMMA is present at 2 µg/mL, acrosomal loss increases with increasing concentration of added extracellular Ca^{2+}. The concentration of extracellular Ca^{2+} required for half-maximal effect is 85 µM. This is similar to the concentration of extracellular Ca^{2+} required for half-maximal response to a maximally stimulating concentration of the calcium ionophore, A23187 (Figure 1). Nearly maximal (99.9%) response to SAMMA is observed at 0.55 mM Ca^{2+}.



View larger version (18K): [in this window] [in a new window]

Figure 1. SAMMA-induced acrosomal loss is Ca^{2+}-dependent. SAMMA-induced and A23187-induced acrosomal losses are represented by the closed rectangles and closed triangles, respectively. Sperm suspensions were mixed with either 2 µg/mL SAMMA or 1 nM A23187 and allowed to equilibrate at 37°C for 10 minutes. These concentrations yield maximal acrosomal loss when added to spermatozoa in Ca^{2+}-complete medium. Maximal acrosomal loss induced by A23187 is 32% (31.4–32.5) of the total spermatozoa (n = 24). Acrosomal loss was induced by addition of $CaCl_2$, as indicated. After 15 minutes, sperm were fixed and stained for acrosome visualization (see "Materials and Methods"). Values are presented as averages of 4 replicates at each concentration of Ca^{2+}, with 90% confidence limits. Data were fit to the following curves with TableCurve 2D curve-fitting software: SAMMA (r^2 = 0.9995), $y = a + b(1 - \exp[-\{x - d\ln(1 - 2^{0.5}/2) - c\}/d])^2$, where a = 4.472633, b = 97.298247, c = 84.926268, and d = 72.494712; A23187 (r^2 = 0.9991), $y = a + b(a\tan[\{x - c\}/d] + \pi/2)/\pi$, where a = 20.79925596, b = 102.41362, c = 158.05548, and d = 59.672928. These curves were used to estimate the concentration of added extracellular Ca^{2+} required for 50% maximal acrosomal loss (0.08 mM and 0.16 mM for SAMMA and A23187, respectively). Figure inset represents a clearer representation of response to Ca^{2+} up to 300 µM.

SAL is blocked by verapamil, a relatively nonselective voltage-dependent Ca^{2+} channel blocker (Carboni and Wojcik, 1988; Dobrev et al, 1999; De Paoli et al, 2002b). Inhibition of SAL by verapamil is dose-dependent (Figure 2), with an IC_{50} of 0.4 µM when SAMMA is present at 0.25 µg/mL (ED_{50}). Because Ca^{2+} influx promoted by the calcium ionophore A23187 is independent of channel activity, it is not surprising that there is no effect of 5 µM verapamil on A23187-induced AL (2.7 [-1.88–7.22]% reduction; t = 0.80, df = 6; $P > .10$).

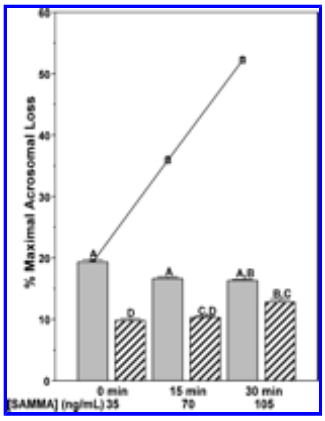


View larger version (11K): [in this window] [in a new window]

Figure 2. Verapamil inhibits SAMMA-induced acrosomal loss. Verapamil was added to sperm suspensions at the indicated concentrations. After 10 minutes, acrosomal loss was initiated by adding 0.25 µg/mL SAMMA. This concentration of SAMMA yields 50% maximal acrosomal loss. Acrosomes were visualized and data presented as described in the legend to Figure 1. Verapamil (5 µM) alone has a negligible effect on acrosomal loss (3% less than control reactions with no additions; t = 1.15, df = 6; $P > .1$). Data were fit to the following curve (r^2 = 0.9941) with TableCurve 2D curve-fitting software: $y = a + b/(1 + [x/c]^d)$, where a = 23.65 x 10^{-5}, b = 49.514364, c = 0.38441295, and d = 0.80860556. This curve was used to estimate the concentrations of verapamil required to inhibit SAMMA-induced acrosomal loss by 50% (0.4 µM) and 99.9% (2 mM).

The effectiveness of verapamil to block voltage-dependent L-type calcium channels is "use-dependent" (ie, its potency increases during repetitive channel stimulation; Reuter, 1983; Godfraind, 1984). However, prior stimulation with submaximal concentrations of SAMMA does not increase sensitivity of acrosomal loss to inhibition by verapamil. In fact, the response becomes refractory to inhibition by verapamil. At 0 time, 0.4 µM verapamil inhibits SAMMA-induced acrosomal

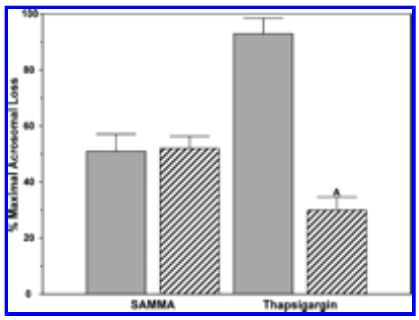
loss by 49% ($P < .05$, Newman-Keuls multiple range test). Inhibition decreases to 38% ($P < .05$) and 22% ($P > .10$) at 15 minutes and 30 minutes, respectively (Figure 3). The concentration of verapamil used was predicted to inhibit the response by 50% (Figure 2). These results argue against L-type calcium channels mediating SAMMA-induced acrosomal loss. The approximate ED_{50} (0.1 µg/mL) is somewhat lower than that obtained when SAMMA is added as a single bolus (0.25 µg/mL).



View larger version (20K): [in this window] [in a new window]

Figure 3. Inhibition of SAMMA-induced acrosomal loss by verapamil is not increased by repeated stimulation with SAMMA. Bars represent the incremental AL response at each time point. Shaded bars are responses to SAMMA alone and cross-hatched bars are responses to SAMMA in the presence of verapamil. The curve is the cumulative AL response. Submaximally stimulating concentrations of SAMMA (35 ng/mL increments) were added to sperm suspensions starting at 0 minutes and every 15 minutes thereafter until 30 minutes. The 0-minute group received one addition of SAMMA, the 15-minute group received 2 additions of SAMMA and the 30-minute group received 3 additions of SAMMA. Five minutes before the last addition of SAMMA to each group, 0.4 µM verapamil was added, as indicated. Five minutes after the last addition of SAMMA in each time group, sperm were fixed and examined for acrosomal loss (see "Materials and Methods"). Data are presented as average (n = 4) maximal acrosomal loss with upper 90% confidence limits.**A–D** Values with different letter designations are different ($P < .05$, Newman-Keuls multiple range test).

SAL is not blocked by 10 µM nifedipine, an L-type voltage-dependent Ca^{2+} channel blocker (Figure 4). However, nifedipine inhibits acrosomal loss induced by 1 µM thapsigargin by 40% ($t = 11.06$; $P < .001$; $df = 6$). Thapsigargin is a Ca^{2+}-ATPase inhibitor, and is thought to act by releasing intracellular Ca^{2+} stores (Meizel and Turner, 1993; Dragileva et al, 1999; O'Toole et al, 2000).

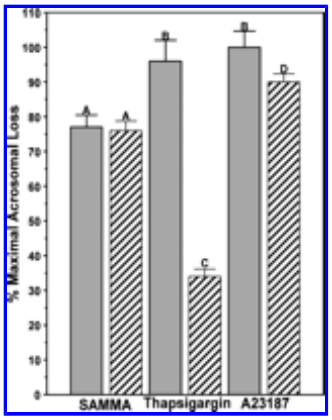


View larger version (22K): [in this window] [in a new window]

Figure 4. Nifedipine blocks thapsigargin- but not SAMMA-induced acrosomal loss. Shaded bars show effects of the indicated stimulus without nifedipine. Hatched bars show the effect of 10 µM nifedipine. Nifedipine (10 µM) was added to sperm suspensions, followed 7 minutes later by either 0.25 µg/mL SAMMA or 10 µM thapsigargin. Acrosomes were visualized and data presented as described in the legend to Figure 1. The concentration of SAMMA was chosen to yield 50% maximal acrosomal loss. Nifedipine alone had a negligible effect on acrosomal loss (1% maximal loss; 90% confidence limits = -2.3%–9.8%).**A** Nifedipine inhibits verapamil-induced acrosomal loss by 41% ($t = 11.06$; $P < .001$; $df = 6$).

The intracellular Ca^{2+} chelator, BAPTA-AM (1 µM), does not inhibit SAL; this is independent of SAMMA concentrations that yield from approximately 20%–80% maximal AL. Responses to 0.035 µg/mL, 0.25 µg/mL, and 0.55 µg/mL SAMMA in the absence and presence of 1 µM BAPTA-AM are 19% (16.0–22.4) and 24% (22.8–25.4); 44% (42.0–46.9) and 44% (40.2–47.2); and 76% (72.3–80.5) and 76% (73.4–79.2) maximal AL, respectively. In contrast, BAPTA-AM inhibits by nearly 65% (Figure 5; $P < .005$, Newman-Keuls multiple range test) AL induced by thapsigargin. Although substantially less effective, BAPTA-AM also inhibits AL induced by the calcium ionophore A23187 by approximately 10% (Figure 5; P

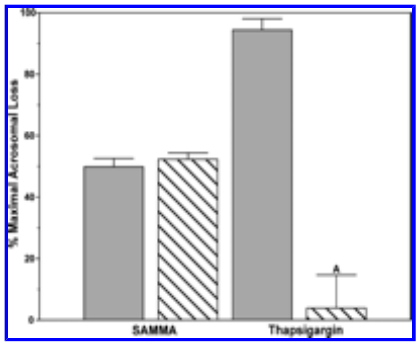
< .05). These data further argue against involvement of release of intracellular Ca^{2+} stores as part of the mechanism of SAL.



View larger version (37K):
[in this window]
[in a new window]

Figure 5. Depletion of intracellular Ca^{2+} stores inhibits thapsigargin- and A23187-, but not SAMMA-induced acrosomal loss. Shaded bars show effects of the indicated stimulus without BAPTA-AM. Hatched bars show the effect of 1 µM BAPTA-AM. BAPTA-AM was added to sperm suspensions, followed 7 minutes later by either 0.55 µg/mL SAMMA, 1 µM thapsigargin, or 1 nM A23187. Acrosomes were visualized and data presented as described in the legend to Figure 1.[A–D] Values with different letter designations differ ($P < .05$, Newman-Keuls multiple range test).

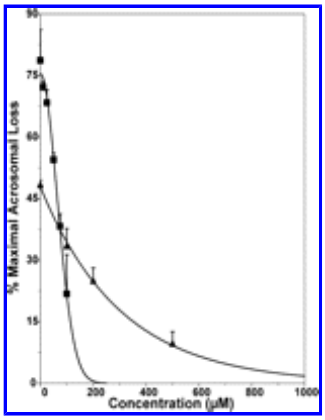
2-Aminoethoxydiphenylborate (2-APB), an inositol-1,4,5-trisphosphate (InsP3) receptor antagonist and store-operated Ca^{2+} channel blocker (Bilmen and Michelangeli, 2002), is without effect on SAL. This is in contrast to nearly 91% inhibition by 2-APB ($t = 13.51$, $df = 7$; $P < .001$) of AL induced by 1 µM thapsigargin (Figure 6).



View larger version (16K):
[in this window]
[in a new window]

Figure 6. 2-APB inhibits thapsigargin-, but not SAMMA-induced acrosomal loss. Shaded bars show effects of the indicated stimulus without 2-APB. Hatched bars show the effect of 50 µM 2-APB. 2-APB (2-aminoethoxydiphenyl borate; 50 µM) was added to sperm suspensions, followed 10 minutes later by either 0.25 µg/mL SAMMA or 1 µM thapsigargin. After 15 minutes, sperm were fixed and stained for visualization of acrosomes (see Materials and Methods). Data are presented as average (n = 4) maximal acrosomal loss, with 90% confidence limits. 2-APB alone had a negligible effect on acrosomal loss (0.0% maximal loss; 90% confidence limits = 0.00%–0.36%).[A] Value differs from its respective control ($t = 13.51$, $df = 7$; $P < .01$).

SAL is inhibited by diphenylhydantoin, a relatively selective blocker of T-type Ca^{2+} channels (Suzuki et al, 1990; Perez-Reyes, 2003). Inhibition by this channel blocker is dose-dependent (Figure 7). The approximate IC_{50} is 210 µM, with nearly complete (99.9%) inhibition at 2.1 mM. Similarly, SAL is inhibited by the T-type channel inhibitor, $NiCl_2$ (Lee et al, 1999; Perez-Reyes, 2003), with 50% and 99.9% inhibition observed at 75 µM and 236 µM, respectively (Figure 7).



View larger version (9K): [in this window] [in a new window]

Figure 7. T-type calcium channel blockers inhibit SAMMA-induced acrosomal loss. Either diphenylhydantoin (closed triangles) or $NiCl_2$ (closed squares) was added to sperm suspensions at the indicated concentrations. After 10 minutes, acrosomal loss was initiated by adding either 0.25 μg/mL SAMMA (for diphenylhydantoin) or 0.5 μg/mL SAMMA (for $NiCl_2$). These concentrations of SAMMA were selected to yield 50% and 80% maximal acrosomal loss, respectively. Acrosomes were visualized and data presented as described in the legend to Figure 1. Diphenylhydantoin (500 μM) and $NiCl_2$ (50 μM) alone have minimal effects on acrosomal loss (9% [6.7–12.3] and 0% [-1.39–0.65], respectively). Data were fit to the following curves with TableCurve 2D curve-fitting software: diphenylhydantoin ($r^2 = 0.9982$), $y = a\exp(-x/b)$, where $a = 47.91584212$ and $b = 303.3345568$; NiCl ($r^2 = 0.9921$), $\ln y = a + bx^2$, where $a = 4.322852889$ and $b = 20.00012419$. These curves were used to estimate the concentrations of diphenylhydantoin and $NiCl_2$ required to inhibit SAMMA-induced acrosomal loss by 50% (210 μM and 75 μM, respectively) and 99.9% (2.1 mM and 236 μM, respectively).

In no instance was average sperm motility reduced more than 5.2% (15 minutes after addition of 5 μM verapamil) from initial values as a result of treatment. Average motility loss caused by 0.25 μg/mL SAMMA (concentration required to produce 50% maximal acrosomal loss) is 1.8% (-8.6–10.0). Further, of the several agents evaluated, no relation exists between AL and the change in percentage of motile spermatozoa (Kendall's rank order correlation coefficient $\tau = .098$; $n = 7$; $P > .1$). Because motility is unaffected, changes in acrosomal loss are not likely secondary to decreased cell viability.

Discussion

- Top
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- References

The present study extends previous data showing that SAL occurs in noncapacitated spermatozoa (Zaneveld et al, 2002). The acrosome reaction is a prerequisite to successful fertilization. It must occur at the appropriate time and in proximity to the oocyte. Spermatozoa that have prematurely lost their acrosomes are short-lived and are less likely to fertilize (Jones, 1990; Brucker and Lipford, 1995; Herrero and Gagnon, 2001). We propose that at least part of the contraceptive activity of SAMMA is caused by its ability to induce premature AL.

SAL requires extracellular Ca^{2+} (Figure 1). The Ca^{2+} requirement of SAL agrees well with that for stimulus-induced acrosome reactions of capacitated spermatozoa (eg, Thomas and Meizel, 1989). SAL inhibition by verapamil (Figure 2) suggests that it may be mediated through voltage-dependent Ca^{2+} channels.

Voltage-dependent Ca^{2+} channels consist of four or five subunits. Electrophysiological and pharmacological properties are determined primarily by the α_1 subunit (Catterall et al, 2003). Evidence exists for α_{1A} (P/Q-type channels), α_{1E} (R-type channels) (Lievano et al, 1996), α_{1G}, α_{1H} (T-type channels), α_C (L-type channels) (Espinosa et al, 1999), and α_{1B} (N-type channels) (Wennemuth et al, 2000) in mouse, α_{1C} in rat (Goodwin et al, 1998), and α_{1B}, α_{1E}, α_{1G}, α_{1H}, and α_{1I} (T-type channels) in human (Goodwin et al, 1997; Park et al, 2003) spermatogenic cells and spermatozoa. Any

or all of these channels may allow stimulus-induced Ca^{2+} influx into the cell.

Ca^{2+} influx in response to the zona pellucida has been attributed to activation of T-type Ca^{2+} channels and subsequent activation of store-operated channels, possibly through transient receptor potential protein 2 (Trp2) (Darszon et al, 2001; Primakoff and Myles, 2002; Felix, 2005). These conclusions are based primarily on studies carried out with mouse spermatozoa and spermatogenic cells (Arnoult et al, 1999; O'Toole et al, 2000; Jungnickel et al, 2001). Ca^{2+} transients, measured over a period of approximately 20–200 milleseconds, and inhibition by 1 μM pimozide and 50 μM Ni^{2+}, suggest involvement of T-type channels (Arnoult et al, 1999). However, peak Ca^{2+} levels are inhibited by 0.1 μM PN200-110 (a dihydropyridine [DHP]). Although T-type channels are not completely insensitive to DHPs (Bean, 1989; Perez-Reyes, 2003), this concentration of PN200-110 is generally considered selective for L-type channels (Lacinova et al, 2000; Grumann et al, 2003). Further, although not definitive, work by Kobori et al (Kobori et al, 2000) suggests that inhibition of Ca^{2+} influx into mouse spermatozoa by pimozide may be secondary to L-type channel blockade.

Inhibition of sustained Ca^{2+} increase by PN200-110, nifedipine, and verapamil is attributed to their initial effects on T-type currents (O'Toole et al, 2000). However, nifedipine is also generally regarded as selective for L-type channels and has little effect on T-type channels (Morita et al, 2002). Some T-type currents are inhibited by low concentrations of nifedipine (K_d = 5 μM) (Akaike et al, 1989). However, this current is largely insensitive to Ni^{2+} (K_d = 0.6 mM), suggesting that they are formed by either α_{1G} or α_{1I} (Perez-Reyes, 2003). Recent evidence favors expression of primarily α_{1H} channels in mouse spermatocytes (Stamboulian et al, 2004), which are about 20 times more sensitive to Ni^{2+} (Perez-Reyes, 2003). Our data demonstrate that processes inhibited by the T-type channel blockers and by nifedipine appear to be different, because nifedipine inhibits thapsigargin-induced AL, but not SAL (Figure 3). Thus, the role of T-type currents in DHP-sensitive spermatozoal processes remains open to question, and participation of high voltage activated (HVA) channels can not be excluded. Splice variants of DHP-sensitive channels in spermatozoa may have electrophysiological properties of T-type channels (Benoff, 1998).

Inhibition of SAL by verapamil suggests that SAL occurs through activation of voltage-dependent Ca^{2+} channels. However, this antagonist is relatively non-selective. For example, verapamil has similar effects on L- and T-type channels (Bean, 1989; De Paoli et al, 2002a). Inhibition of L-type channels by phenylalkylamines such as verapamil is use-dependent. Upon repeated stimulation of L-type channels, their sensitivity to inhibition by channel blockers increases (Reuter, 1983; Godfraind, 1984; Emanuel et al, 1998). Absence of use-dependent inhibition of SAL by verapamil (see Results; Figure 3), failure of nifedipine to inhibit SAL (Figure 4), and SAL sensitivity to Ni^{2+} (Zamponi et al, 1996) make it unlikely that L-type channels are involved.

Although there are no selective T-type channel antagonists (Catterall et al, 2003), the pharmacological profile of SAL is more consistent with involvement of T-type channels than other sperm-derived channels. SAL inhibition by diphenylhydantoin and Ni^{2+} is similar to inhibition by these agents of Ca^{2+} current in T-type channels in other cell types (McCarthy et al, 1990; Suzuki et al, 1990; Viana et al, 1997). Other channels are considered below.

Verapamil inhibits R-type (α_{1E} or $Ca_V2.3$) channels, although apparently much less than either L- or T-type channels. The IC_{50} against R-type channels is much higher (170 μM (Benquet et al, 1999) than against SAL (0.4 μM). The IC_{50} for Ni^{2+} against SAL (75 μM; Figure 7) is higher than generally required for R-type currents (Tottene et al, 2000); it is in the same range as concentrations used

in other tissues to inhibit T-type channel activity (Wolfart and Roeper, 2002; Watanabe et al, 2004). Our data argue against a role of R-type channels in SAL.

The α_{1A} and α_{1B} subunits form part of the P/Q-type ($Ca_V2.1$) and N-type ($Ca_V2.2$) channels, respectively. The relative insensitivity to verapamil (<1 μM) of α_{1A} (Hirasawa et al, 2001; Miranda et al, 2001) and α_{1B} (Molderings et al, 2000; Ichida et al, 2005) and to Ni^{2+} (,100 μM) of α_{1A} (Zamponi et al, 1996; Hirasawa et al, 2001) and α_{1B} (Zamponi et al, 1996; Hirasawa et al, 2001) argue against their involvement in SAL.

Sustained entry of Ca^{2+} is required for AL. For zona- and progesterone-induced AL, this sustained entry is thought to be mediated by the activation of store-operated channels (Breitbart et al, 2005; Felix, 2005). However, the present data do not support involvement of store-operated channels in SAL (see below).

T-type channels, under appropriate circumstances, may allow sustained Ca^{2+} entry. T-channel inactivation can be slow and incomplete, leading to sustained Ca^{2+} influx (Bean, 1989). Window currents produced in T-type channels by potentials from -50 to -20 mV can lead to sustained entry of Ca^{2+} (Jensen et al, 2004). Noncapacitated spermatozoa have resting potential of around -26 mV (Arnoult et al, 1999), making these cells candidates for T-type window currents, which may account for Ca^{2+} influx responsible for SAL.

Findings in this study differentiate AL in response to SAMMA from that caused by either physiological stimuli or to thapsigargin. Unlike AL induced by physiological stimuli (Luconi et al, 1996; O'Toole et al, 2000), SAL is unaffected by agents that modulate intracellular Ca^{2+} stores or store-operated channels. BAPTA (introduced as BAPTA-AM) is an intracellular Ca^{2+} chelator that depletes Ca^{2+} released from intracellular stores and inhibits Ca^{2+} influx occurring by capacitative entry (Ng et al, 1990; Cloutier et al, 1993). Intracellular BAPTA reverses increases in Ca^{2+} secondary to release of intracellular Ca^{2+} stores (O'Toole et al, 2000; Yeromin et al, 2004). Further, intracellular BAPTA completely inhibits the Ca^{2+} response to progesterone in human spermatozoa (Luconi et al, 1996), but has no effect on SAL. In contrast, BAPTA inhibits thapsigargin-induced AL (releases Ca^{2+} stores). BAPTA has a smaller effect on ionophore A23187-induced AL (Figure 5). This is consistent with release of Ca^{2+} stores by A23187 (Pittman et al, 1994; Roy and Lee, 1995), which represents a minor contribution to increased intracellular Ca^{2+}. SAL is unaffected by 2-APB (Figure 7). 2-APB blocks InsP3 receptors (responsible for release of intracellular Ca^{2+} stores) and store-operated channels. This provides additional evidence against involvement of store-operated channels or release of Ca^{2+} stores by InsP3 in the mechanism of SAL. The concentration of 2-APB is within the range of effective concentrations of 2-APB used in other cell types (Chen et al, 2002; Yeromin et al, 2004). 2-APB inhibits thapsigargin-induced AL; this agrees with the ability of 2-APB to inhibit store-operated channels and thapsigargin-induced capacitative Ca^{2+} entry (Enfissi et al, 2004; Dedos et al, 2005). Our findings are inconsistent with Ca^{2+} influx through store-operated channels. Further, SAL is clearly different from AL induced by thapsigargin.

Thapsigargin and ZP3 activate the same Ca^{2+} influx pathway in spermatozoa (O'Toole et al, 2000). The similarity of the Ca^{2+} pathway promoted by ZP3 and thapsigargin is of particular importance regarding the present study. Thapsigargin-induced AL differs from SAL (Figures 3, 5, and 6). By inference, the Ca^{2+}-dependent pathway leading to SAL differs from that leading to ZP3-induced AL. Further, involvement of Trp2 in zona pellucida-induced AL (Jungnickel et al, 2001) suggests that SAL is not likely mediated by Trp2. Jungnickel and coworkers suggest that Trp2 may be identified as or

closely related to store-operated channels. Store-operated channels do not mediate SAL (Figures 5 and 6).

Activities of SAMMA as a broad-spectrum contraceptive microbicide suggest common underlying mechanisms for these effects. As a microbicide, SAMMA is classified as an entry inhibitor (Herold et al, 2002; Cheshenko et al, 2004). Signal transduction perturbations may be partly responsible for the multiple activities of this contraceptive microbicide. Infection of target cells by HSV and HIV are associated with Ca^{2+} signaling in the target cells (Haughey et al, 1999; Dellis et al, 2002; Cheshenko et al, 2003). SAMMA may exert some of its microbicidal properties through altered Ca^{2+} signaling.

Voltage-activated Ca^{2+} ions are typically identified by their biophysical and pharmacological properties and by their transcripts. However, assigning a channel to a biological process is complicated by the presence of several channel types, by association of the α_1 subunit with different combinations of auxiliary subunits, and by the occurrence of splice variants. These variables contribute to blurring the distinction of channel types based on pharmacological and physical properties; they give rise to channel variants that may have atypical properties (Bean, 1989; Benoff, 1998; Publicover and Barratt, 1999; Morita et al, 2002; Jensen et al, 2004). Further, T-type channels, although usually responsible for transitory currents, can also support sustained Ca^{2+} entry (Bean, 1989; Jensen et al, 2004). This problem is further complicated in spermatozoa, cells that are not amenable to characterization by conventional electrophysiological techniques and that do not uniformly respond to stimuli. The molecular identity of Ca^{2+} channel(s) participating in zona pellucida-induced AL has not been established (Darszon et al, 2001). In spite of these limitations, pharmacological characterization of relevant responses such as AL remains useful. Results from the present provide evidence that the pharmacological sensitivity of SAL, although supporting involvement of T-type channels, is not the same as that of zona pellucida-induced AL; SAL and zona pellucida-induced AL likely occur through different Ca^{2+}-mediated pathways. We propose that SAMMA's contraceptive activity lies in its ability to induce premature AL, caused by dysregulation of Ca^{2+} signaling.

Footnotes

This work was supported in part by grants from the National Institute for Allergy and Infectious Diseases (grant PO1 A137940) and the National Institute of Child Health and Human Development (grant PO1 HD41763).

DOI: 10.2164/jandrol.05152

References

Anderson RA, Feathergill KA, De Jonge CJ, Mack SR, Zaneveld LJD. Facilitative effect of pulsed addition of dibutyryl cAMP on the acrosome reaction of noncapacitated human spermatozoa. *J Androl*. 1992;3: 398 –408.

Anderson RA, Feathergill KA, Drisdel RC, Rawlins RG, Mack SR, Zaneveld LJD. Atrial natriuretic peptide (ANP) as a stimulus of the human acrosome reaction and a component of ovarian follicular fluid: correlation of follicular ANP content with in vitro fertilization outcome. *J Androl*. 1994;15: 61 –70. [Abstract/Free Full Text]

Top
Abstract
Introduction
Materials and Methods
Results
Discussion
References

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, Florman HM. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proc Nat Acad Sci U S A*. 1999;96: 6757 –6762.[Abstract/Free Full Text]

Barberis IL, Pajaro MC, Godino S, Pascual L, Rodriguez I, Aguero M, Ordonez C. Survey of sexually transmitted diseases in the region of Rio Cuarto [Spanish]. *Medicina*. 1998; 58: 469 –473.

Bean BP. Classes of calcium channels in vertebrate cells. *Ann Rev Physiol*. 1989; 51: 367 –384. [CrossRef][Medline]

Benoff S. Voltage dependent calcium channels in mammalian spermatozoa. *Front Biosci*. 1998; 3: D1220 –D1240.[Medline]

Benquet P, Guen JL, Dayanithi G, Pichon Y, Tiaho F. omegaAgaIVA-sensitive (P/Q-type) and -resistant (R-type) high-voltage-activated Ba(2+) currents in embryonic cockroach brain neurons. *J Neurophysiol*. 1999; 82: 2284 –2293.[Abstract/Free Full Text]

Biggers J, Whitten W, Whittingham D, Freeman W. The culture of mouse embryos in vitro. In: Daniel J, ed. *Methods In Mammalian Embryology*. San Francisco: WH Freeman; 1971: 86 –116.

Bilmen JG, Michelangeli F. Inhibition of the type 1 inositol 1,4,5-trisphosphate receptor by 2-aminoethoxydiphenylborate. *Cell Sig*. 2002;14: 955 –960.[CrossRef][Medline]

Breitbart H, Cohen G, Rubinstein S. Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. *Reproduction*. 2005; 129: 263 –268.[Abstract/Free Full Text]

Breitbart H, Naor Z. Protein kinases in mammalian sperm capacitation and the acrosome reaction. *Rev Reprod*. 1999; 4: 151 –159.[Abstract]

Brucker C, Lipford GB. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. *Hum Reprod Upd*. 1995;1: 51 –62.[Abstract/Free Full Text]

Carboni E, Wojcik WJ. Dihydropyridine binding sites regulate calcium influx through specific voltage-sensitive calcium channels in cerebellar granule cells. *J Neurochem*. 1988; 50: 1279 –1286. [Medline]

Catterall WA, Striessnig J, Snutch TP, Perez-Reyes E. International Union of Pharmacology. XL. Compendium of voltage-gated ion channels: calcium channels. *Pharmacol Rev*. 2003; 55: 579 –581. [Abstract/Free Full Text]

Chen NX, O'Neill KD, Niwa T, Moe SM. Signal transduction of beta2m-induced expression of VCAM-1 and COX-2 in synovial fibroblasts. *Kidney Int*. 2002; 61: 414 –424.[CrossRef][Medline]

Cheshenko N, Del Rosario B, Woda C, Marcellino D, Satlin LM, Herold BC. Herpes simplex virus triggers activation of calcium-signaling pathways. *J Cell Biol*. 2003; 163: 283 –293. [Abstract/Free Full Text]

Cheshenko N, Keller MJ, MasCasullo V, Jarvis GA, Cheng H, John M, Li J-H, Hogarty K, Anderson RA, Waller DP, Zaneveld LJD, Profy AT, Klotman ME, Herold BC. Candidate topical microbicides bind herpes simplex virus glycoprotein B and prevent viral entry and cell-to-cell spread. *Antimicrob Agent Chemother*. 2004; 48: 2025 –2036.[Abstract/Free Full Text]

Cloutier MM, Guernsey L, Sha'afi RI. Duramycin increases intracellular calcium in airway epithelium. *Mem Biochem*. 1993;10: 107 –118.

Cohen MS. Sexually transmitted diseases enhance HIV transmission: no longer a hypothesis. *Lancet*.

1998; 351: 5 –7.[CrossRef][Medline]

Darszon A, Beltran C, Felix R, Nishigaki T, Trevino CL. Ion transport in sperm signaling. *Develop Biol*. 2001; 240: 1 –14.[CrossRef][Medline]

Dedos SG, Wicher D, Fugo H, Birkenbeil H. Regulation of capacitative Ca2+ entry by prothoracicotropic hormone in the prothoracic glands of the silkworm, Bombyx mori. *J Exp Zool Pt A, Comp Exp Biol*. 2005;303: 101 –112.

De Jonge CJ, Mack SR, Zaneveld LJD. Synchronous assay for human sperm capacitation and the acrosome reaction. *J Androl*. 1989;10: 232 –239.[Abstract/Free Full Text]

Dellis O, Gangloff SC, Paulais M, Tondelier D, Rona JP, Brouillard F, Bouteau F, Guenounou M, Teulon J. Inhibition of the calcium release-activated calcium (CRAC) current in Jurkat T cells by the HIV-1 envelope protein gp160. *J Biol Chem*. 2002; 277: 6044 –6050.[Abstract/Free Full Text]

De Paoli P, Cerbai E, Koidl B, Kirchengast M, Sartiani L, Mugelli A. Selectivity of different calcium antagonists on T- and L-type calcium currents in guinea-pig ventricular myocytes. *Pharmacol Res*. 2002b;46: 491 –497.[CrossRef][Medline]

De Paoli P, Cerbai E, Koidl B, Kirchengast M, Sartiani L, Mugelli A. Selectivity of different calcium antagonists on T- and L-type calcium currents in guinea-pig ventricular myocytes. *Pharmacol Res*. 2002a;46: 491 –497.[CrossRef][Medline]

Dobrev D, Milde AS, Andreas K, Ravens U. The effects of verapamil and diltiazem on N-, P- and Q-type calcium channels mediating dopamine release in rat striatum. *Brit J Pharmacol*. 1999; 127: 576 –582.[CrossRef][Medline]

Dragileva E, Rubinstein S, H.B. Intracellular Ca(2+)-Mg(2+)-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. *Biol Reprod*. 1999; 61: 1226 –1234.[Abstract/Free Full Text]

Emanuel K, Mackiewicz U, Pytkowski B, Lewartowski B. Effects of mibefradil, a blocker of T-type Ca2+ channels, in single myocytes and intact muscle of guinea-pig heart. *J Physiol Pharmacol*. 1998; 49: 577 –590.[Medline]

Enfissi A, Prigent S, Colosetti P, Capiod T. The blocking of capacitative calcium entry by 2-aminoethyl diphenylborate (2-APB) and carboxyamidotriazole (CAI) inhibits proliferation in Hep G2 and Huh-7 human hepatoma cells. *Cell Calcium*. 2004; 36: 459 –467.[CrossRef][Medline]

Espinosa F, Lopez-Gonzalez I, Serrano CJ, Gasque G, de la Vega-Beltran JL, Trevino CL, Darszon A. Anion channel blockers differentially affect T-type Ca(2+) currents of mouse spermatogenic cells, alpha1E currents expressed in Xenopus oocytes and the sperm acrosome reaction. *Dev Genetics*. 1999;25: 103 –114.[CrossRef][Medline]

Felix R. Molecular physiology and pathology of Ca^{2+}-conducting channels in the plasma membrane of mammalian sperm. *Reproduction*. 2005; 129: 251 –262.[Abstract/Free Full Text]

Godfraind T. Calcium modulators: tools for cell biology. *Adv Cyclic Nucl Prot Phos Res*. 1984; 17: 551 –555.

Goodwin LO, Leeds NB, Hurley I, Cooper GW, Pergolizzi RG, Benoff S. Alternative splicing of exons in the alpha1 subunit of the rat testis L-type voltage-dependent calcium channel generates germ line-specific dihydropyridine binding sites. *Mol Hum Reprod*. 1998; 4: 215 –226.[Abstract/Free Full Text]

Goodwin LO, Leeds NB, Hurley I, Mandel FS, Pergolizzi RG, Benoff S. Isolation and characterization of the primary structure of testis-specific L-type calcium channel: implications for contraception.

Mol Hum Reprod. 1997;3: 255 –268.[Abstract/Free Full Text]

Grumann JA, Alves RV, Calixto JB. The role of calcium channels in substance P-induced contractile response in the rat iris. *Peptides*. 2003;24: 1985 –1991.[CrossRef][Medline]

Guraya SS. Cellular and molecular biology of capacitation and acrosome reaction in spermatozoa. *Int Rev Cytol*. 2000; 199: 1 –64.[CrossRef][Medline]

Haughey NJ, Holden CP, Nath A, Geiger JD. Involvement of inositol 1,4,5-trisphosphate-regulated stores of intracellular calcium in calcium dysregulation and neuron cell death caused by HIV-1 protein tat. *J Neurochem*. 1999;73: 1363 –1374.[CrossRef][Medline]

Henshaw SK. Unintended pregnancy in the United States. *Fam Plan Perspect*. 1998; 30: 24 –29. [CrossRef][Medline]

Herold BC, Scordi-Bello I, Cheshenko N, Marcellino D, Dzuzelewski M, Francois F, Morin R, Mas Casullo V, Anderson RA, Chany C, Waller DP, Zaneveld LJD, Klotman ME. Mandelic acid condensation polymer: novel candidate microbicide for prevention of human immunodeficiency virus and herpes simplex virus entry. *J Virol*. 2002; 76: 11236 –11244.[Abstract/Free Full Text]

Herrero MB, Gagnon C. Nitric oxide: a novel mediator of sperm function. *J Androl*. 2001; 22: 349 –356.[Medline]

Hirasawa M, Kombian SB, Pittman QJ. Oxytocin retrogradely inhibits evoked, but not miniature, EPSCs in the rat supraoptic nucleus: role of N- and P/Q-type calcium channels. *J Physiol*. 2001; 532(pt. 3): 595 –607.[Abstract/Free Full Text]

Ichida S, Abe J, Komoike K, Imanishi T, Wada T, Masuko T, Minami T. Characteristics of omega-conotoxin GVI A and MVIIC binding to Cav 2.1 and Cav 2.2 channels captured by anti-Ca^{2+} channel peptide antibodies. *Neurochem Res*. 2005; 30: 457 –466.[CrossRef][Medline]

Jensen LJ, Salomonsson M, Jensen BL, Holstein-Rathlou NH. Depolarization-induced calcium influx in rat mesenteric small arterioles is mediated exclusively via mibefradil-sensitive calcium channels. *Brit J Pharmacol*. 2004; 142: 709 –718.[CrossRef][Medline]

Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fert*. 1984; 70: 219 –228.[Abstract/Free Full Text]

Jones R. Identification and functions of mammalian sperm-egg recognition molecules during fertilization. *J Reprod Fert*. 1990; 42(suppl): 89 –105.

Jungnickel MK, Marrero H, Birnbaumer L, Lémos JR, Florman HM. Trp2 regulates entry of Ca^{2+} into mouse sperm triggered by egg ZP3. *Nat Cell Biol*. 2001; 3: 499 –502.[CrossRef][Medline]

Kobori H, Miyazaki S, Kuwabara Y. Characterization of intracellular Ca^{2+} increase in response to progesterone and cyclic nucleotides in mouse spermatozoa. *Biol Reprod*. 2000; 63: 113 –120. [Abstract/Free Full Text]

Lacinova L, Klugbauer N, Hofmann F. Regulation of the calcium channel alpha(1G) subunit by divalent cations and organic blockers. *Neuropharmacology*. 2000; 39: 1254 –1266.[CrossRef][Medline]

Lafferty WE. The changing epidemiology of HSV-1 and HSV-2 and implications for serological testing. *Herpes*. 2002; 9: 51 –55.[Medline]

Lee JH, Gomora JC, Cribbs LL, Perez-Reyes E. Nickel block of three cloned T-type calcium channels:

low concentrations selectively block alpha1H. *Biophys J*. 1999; 77: 3034 –3042.[Medline]

Lievano AS, C.M., Serrano CJ, Trevino CL, Bellve AR, Hernandez-Cruz A, Darszon A. T-type Ca2+ channels and alpha1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. *FEBS Lett*. 1996; 388: 150 –154.[CrossRef][Medline]

Luconi M, Krausz C, Forti G, Baldi E. Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol Reprod*. 1996; 55: 207 –216.[Abstract]

Mbopi-Keou FX, Gresenguet G, Mayaud P, Weiss HA, R.G, Matta M, Paul JL, Brown DW, Hayes RJ, Mabey DC, Belec L. Interactions between herpes simplex virus type 2 and human immunodeficiency virus type 1 infection in African women: opportunities for intervention. *J Infect Dis*. 2000; 182: 1090 –1096. [CrossRef][Medline]

McCarthy RT, Isales CM, Bollag WB, Rasmussen H, Barrett PQ. Atrial natriuretic peptide differentially modulates T- and L-type calcium channels. *Amer J Physiol*. 1990; 258: F473 –F478.

Meizel S, Turner KO. Initiation of the human sperm acrosome reaction by thapsigargin. *J Exp Zool*. 1993; 267: 350 –355.[CrossRef][Medline]

Miranda DM, Romano-Silva MA, Kalapothakis E, Diniz CR, Cordeiro MN, Moraes-Santos T, De Marco L, Prado MA, Gomez MV. Spider neurotoxins block the beta scorpion toxin-induced calcium uptake in rat brain cortical synaptosomes. *Brain Res Bull*. 2001; 54: 533 –536.[CrossRef][Medline]

Molderings GJ, Likungu J, Gothert M. N-Type calcium channels control sympathetic neurotransmission in human heart atrium. *Circulation*. 2000; 101: 403 –407.[Abstract/Free Full Text]

Morita H, Shi J, Ito Y, Inoue R. T-channel-like pharmacological properties of high voltage-activated, nifedipine-insensitive Ca^{2+} currents in the rat terminal mesenteric artery. *Brit J Pharmacol*. 2002;137: 467 –476.[CrossRef][Medline]

Ng J, Gustavsson J, Jondal M, Andersson T. Regulation of calcium influx across the plasma membrane of the human T-leukemic cell line, JURKAT: dependence on a rise in cytosolic free calcium can be dissociated from formation of inositol phosphates. *Biochem Biophys Acta*. 1990;1053: 97 –105. [Medline]

O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. Ca(2+) entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Mol Biol Cell*. 2000; 11: 1571 –1584.[Abstract/Free Full Text]

Panchaud C, Singh S, Feivelson D, Darroch JE. Sexually transmitted diseases among adolescents in developed countries. *Fam Plan Perspect*. 2000;32: 24 –32.[CrossRef][Medline]

Park JY, Ahn HJ, Gu JG, Lee KH, Kim JS, Kang HW, Lee JH. Molecular identification of Ca2+ channels in human sperm. *Exp Mol Med*. 2003;35: 285 –292.[Medline]

Perez-Reyes E. Molecular physiology of low-voltage-activated T-type calcium channels. *Physiol Rev*. 2003; 81: 1415 –1459.

Pittman DD, Tomkinson KN, Kaufman RJ. Post-translational requirements for functional factor V and factor VIII secretion in mammalian cells. *J Biol Chem*. 1994; 269: 17329 –17337. [Abstract/Free Full Text]

Primakoff P, Myles DG. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. *Science*. 2002; 296: 2183 –2185.[Abstract/Free Full Text]

Publicover SJ, Barratt CL. Voltage-operated Ca^{2+} channels and the acrosome reaction: which channels are present and what do they do? *Hum Reprod*. 1999; 14: 873 – 879.[Abstract/Free Full Text]

Reuter H. Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature*. 1983; 301: 569 – 574.[CrossRef][Medline]

Roy B, Lee AS. Transduction of calcium stress through interaction of the human transcription factor CBF with the proximal CCAAT regulatory element of the grp78/BiP promoter. *Mol Cell Biol*. 1995; 15: 2263 – 2274.[Abstract]

Sokal R, Rohlf FJ. *Biometry*. 2nd ed. San Francisco, Calif: WH Freeman and Company; 1981: 400 – 453.

Stamboulian S, Kim D, Shin H, Ronjat M, De Waard M, Arnoult C. Biophysical and pharmacological characterization of spermatogenic T-type calcium current in mice lacking the $Ca_V3.1$ (α1G) calcium channel: $Ca_V3.2$ (α1H) is the main functional calcium channel in wild-type spermatogenic cells. *J Cell Physiol*. 2004; 200: 116 – 124.[CrossRef][Medline]

Suzuki N, Kudo Y, Takagi H, Yoshioka T, Tanakadate A, Kano M. Participation of transient-type Ca2+ channels in the sustained increase of Ca2+ level in GH3 cells. *J Cell Physiol*. 1990; 144: 62 – 68. [CrossRef][Medline]

Thomas P, Meizel S. Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca2+ influx. *Biochem J*. 1989; 264: 539 – 546. [Medline]

Tottene A, Volsen S, Pietrobon D. α1E Subunits Form the Pore of Three Cerebellar R-Type Calcium Channels with Different Pharmacological and Permeation Properties. *J Neurosci*. 2000; 20: 171 – 178. [Abstract/Free Full Text]

UNAIDS. *AIDS epidemic update: 2003*. Report UNAIDS/03.39E. Geneva: UNAIDS/WHO, 2003.

UNAIDS. *AIDS epidemic update, 2004*. Report UNAIDS/04.45E. Geneva: UNAIDS/WHO, 2004.

UNFPA, UNAIDS and UNIFEM. *Women and HIV/AIDS: confronting the crisis*. Report ISBN: 0-89714-708-1. New York, Geneva: 2004.

Viana F, Van den Bosch L, Missiaen L, Vandenberghe W, Droogmans G, Nilius B, Robberecht W. Mibefradil (Ro 40-5967) blocks multiple types of voltage-gated calcium channels in cultured rat spinal motoneurones. *Cell Calcium*. 1997; 22: 299 – 311.[CrossRef][Medline]

Watanabe M, Sakuma Y, Kato M. High expression of the R-type voltage-gated Ca^{2+} channel and its involvement in Ca^{2+} -dependent gonadotropin-releasing hormone release in GT1–7 cells. *Endocrinology*. 2004; 145: 2375 – 2383.[Abstract/Free Full Text]

Wennemuth G, Westenbroek RE, Xu T, Hille B, Babcock DF. $Ca_V2.2$ and $Ca_V2.3$ (N- and R-type) Ca^{2+} channels in depolarization-evoked entry of Ca^{2+} into mouse sperm. *J Biol Chem*. 2000; 275: 21210 – 21217.[Abstract/Free Full Text]

Wolfart J, Roeper J. Selective coupling of T-type calcium channels to SK potassium channels prevents intrinsic bursting in dopaminergic midbrain neurons. *J Neurosci*. 2002; 22: 3404 – 3413. [Abstract/Free Full Text]

World Health Organization. In: *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. Cambridge University Press; 1992: 56 – 59.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill J, Ewing LL, Greenwald GS, Markert CL, Pfaff DW, eds. *The Physiology of Reproduction*. New York, NY: Raven Press; 1988: 135–185.

Yeromin AV, Roos J, Stauderman KA, Cahalan MD. A store-operated calcium channel in Drosophila S2 cells. *J Gen Physiol*. 2004; 123: 167–182.[Abstract/Free Full Text]

Zamponi GW, Bourinet E, Snutch TP. Nickel block of a family of neuronal calcium channels: subtype- and subunit-dependent action at multiple sites. *J Membr Biol*. 1996; 151: 77–90.[CrossRef][Medline]

Zaneveld LJD, Anderson R, Diao X-H, Waller DP, Chany C, Feathergill K, Doncel D, Cooper MD, Herold B. Use of mandelic acid condensation polymer (SAMMA), a new antimicrobial contraceptive agent, for vaginal prophylaxis. *Fertil Steril*. 2002; 78: 1107–1115.[CrossRef][Medline]

Zaneveld LJD, Anderson RA, Diao XH, Young PR, Waller DP, Garg S, Chany CJ, Kim DSHL. *Method for preventing sexually transmitted diseases*. Patent no. 5,932,619; Assignee, Rush-Presbyterian, St Luke's Medical Center; 1999.