

# Nitric Oxide–Dependent Human Acrosomal Loss Induced by PPCM (SAMMA) and by Nitric Oxide Donors Occurs by Independent Pathways: Basis for Synthesis of an Improved Contraceptive Microbicide

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**ABSTRACT:** PPCM (previously designated sulfuric acid–modified mandelic acid [SAMMA]) is a contraceptive microbicide in preclinical development. Its contraceptive activity is attributable in part to its ability to promote premature acrosomal loss. Prior studies showed that PPCM-induced human acrosomal loss (PAL) is  $\text{Ca}^{2+}$ -dependent. This study was carried out to determine transduction elements downstream from  $\text{Ca}^{2+}$  entry. PAL is inhibited by inhibitors selective for endothelial-type nitric oxide synthase. PAL is completely inhibited by 0.1  $\mu\text{M}$  ODQ (soluble guanylate cyclase inhibitor). PAL is inhibited by protein kinase G inhibitors with selectivity for the type II isotype. Several inhibitors of the nitric oxide/cyclic guanosine monophosphate (cGMP)/protein kinase G pathway induce  $\text{Ca}^{2+}$ -dependent acrosomal loss when added alone. These responses are inhibited by nifedipine, a blocker of  $\text{Ca}_{v1,x}$  voltage-dependent channels. Acrosomal loss induced by the nitric oxide donor SNAP (SNAL) does not require added  $\text{Ca}^{2+}$ . Sperm production of nitric oxide is increased by PPCM, an effect inhibited by nitro-L-arginine (nitric oxide synthase inhibitor). Although inhibited by ODQ, SNAL and acrosomal loss induced by other nitric oxide donors are unaffected by KT5823 (protein kinase G inhibitor). Unlike PAL, SNAL is partially inhibited by KT5720 (protein kinase A inhibitor) and genistein (protein tyrosine

kinase inhibitor). Acrosomal loss response to PPCM and SNAP added in combination suggests that these agents act by independent mechanisms. A PPCM derivative was synthesized, in which a nitric oxide donor was esterified to PPCM (NOSPPA-23). NOSPPA-23 induces acrosomal loss with or without added  $\text{Ca}^{2+}$ . The  $\text{ED}_{50}$  of NOSPPA-23 (4.8 nM) in the presence of  $\text{Ca}^{2+}$  is 35-fold less than that of PPCM. These findings suggest the following: 1) elements responsible for PAL include endothelial nitric oxide synthase, soluble guanylate cyclase, and type II protein kinase G; 2) the resting state of the nitric oxide/cGMP/protein kinase G pathway is a determinant of acrosomal status; 3) PPCM and nitric oxide donors induce acrosomal loss via nitric oxide, but through independent pathways; and 4) covalent attachment of a nitric oxide donor to PPCM provides synergistic efficacy as a stimulus of acrosomal loss. Further studies with this novel prototype as an improved contraceptive microbicide are warranted.

Key words: Signal transduction, mechanism, nitric oxide synthase, cGMP, guanylate cyclase, protein kinase G, protein kinase A, protein tyrosine kinase.

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In 2007, there were over 1 million cases of human immunodeficiency virus (HIV)/AIDS in North America, with one-quarter of these undiagnosed. Over 46 000 people were newly diagnosed with HIV. A disproportionate

number of women are infected with HIV in some geographic locations, such as sub-Saharan Africa (UNAIDS, 2007). Similar gender-biased data have been reported for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Panchaud et al, 2000).

Unplanned pregnancies and abortion procedures are another risk factor to women's reproductive health. Nearly half of unplanned pregnancies in the United States are terminated (Henshaw, 1998), at a cost of more than \$5 billion (Trussell, 2007). Improved methods for contraception and prevention of sexually transmitted disease will help to reduce population growth and demand for natural resources, and will improve the general health of both genders.

Microbicidal products (microbicides), with or without contraceptive properties, are being developed (D'Cruz and Uckun, 2004). These products are intended for

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prophylactic topical use. In 2004, about 60 candidates had entered various phases of development (Mantell et al, 2005). Recent late-stage failures (European AIDS Treatment Group, 2006; Alliance for Microbicide Development, 2007; BBC News, 2007; Lirri, 2007) of several microbicides and one vaccine underscore the importance of continued discovery.

Sulfuric acid–modified mandelic acid (SAMMA) is a carboxylated oligomer with a molecular weight of 1.5 to 1.9 kDa. It is contraceptive, with activity against several pathogens, including among others, HIV, HSV, *N gonorrhoeae*, and *C trachomatis* (Zaneveld et al, 2002).

SAMMA is an acronym that describes several possible products formed from reaction of D,L-mandelic acid with sulfuric acid. The exact nature of the reaction (hence, the product) depends on reaction conditions. Among possible reaction products are sulf(on)ated ring structures (Roberts and Caserio, 1964b), ethers (Roberts and Caserio, 1964a), cyclic dimers, and acid-catalyzed polyesters (Whitesell and Pojman, 1990). A proposed ether product with activity against HIV-1 and a polyester (with weak activity against HIV-1) have been described (Ward et al, 2008). The product described herein, formed under proprietary reaction conditions, and distinct from those mentioned above, will be referred to as PPCM, a code name assigned by Yaso Biotechnology Inc, the licensee for this material (Zaneveld et al, 1999). The form of SAMMA used to describe its microbicidal properties (Zaneveld et al, 2002; Cheshenko et al, 2004; Chang et al, 2007) and effects on spermatozoa/conception (Zaneveld et al, 2002; Anderson et al, 2006) is PPCM, a carboxylated oligomer. Structural characterization of this compound will be considered in a separate document (Krunic et al, unpublished).

PPCM commercialization and design of second generation products based on the PPCM prototype require understanding of its mechanisms of action. PPCM exerts its antiviral activity in part by preventing binding and entry of viruses to their target cells (Cheshenko et al, 2004). Its prevention of HIV transmission by dendritic cells (Chang et al, 2007) suggests other mechanisms of antiviral activity as well. Mechanisms for its antibacterial activity are unknown.

Part of PPCM's contraceptive activity may be attributable to its induction of premature acrosomal loss (AL; Zaneveld et al, 2002). This process is  $\text{Ca}^{2+}$ -dependent, with  $\text{Ca}^{2+}$  entry into the spermatozoa mediated by T-type ( $\text{Ca}_v 3.x$ ) voltage-dependent  $\text{Ca}^{2+}$  channels. This is distinct from the acrosome reaction induced by zona pellucida or progesterone (Anderson et al, 2006).

This study was carried out to extend our understanding of how PPCM induces premature AL by examining signal transduction downstream from  $\text{Ca}^{2+}$  entry. In view of the

relatively low percentage of human spermatozoa that respond to stimuli of AL (typically, 30% to 35%; eg, Anderson et al, 1992; Revelli et al, 1999; Liu et al, 2008), a pharmacologic approach was taken, considering only spermatozoa with potential for AL in vitro as the dependent variable. Other approaches such as measurement of transduction pathway intermediates or enzymatic studies are less informative in this instance, because results would include activities derived from the bulk of spermatozoa that are nonresponsive, at least in vitro.

The results suggest that PPCM-induced AL (PAL) occurs by a nitric oxide (NO)–dependent process, through activation of endothelial NO synthase (eNOS), and downstream activation of soluble guanylate cyclase and type II cyclic guanosine monophosphate (cGMP)-dependent protein kinase (protein kinase G; cGK-II). NO donors also induce AL. However, signal transduction elements responsible for NO donor-induced AL and PAL are independent. The data also point to the importance of activity of the NO/cGMP/cGK pathway as a determinant of acrosomal status.

## Materials and Methods

### Reagents

Calcium ionophore A23187, 6-anilino-5,8-quinolinedione (LY83583), 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), S-methylthiocitrulline, S-methylisothiourea, N-(3-aminomethyl)benzylacetamide (1400W),  $\text{N}^G$ -nitro-L-arginine,  $\text{N}^G$ -nitro-L-arginine methyl ester (L-NAME), genistein, KT5720, KT5823, Rp-8-pCPT-cGMPS, Rp-8-Br-PET-cGMPS, DT-3 (cell-permeable cGK-I inhibitor), and human atrial natriuretic peptide (hANP), were products of EMD Biosciences, Inc (Calbiochem, La Jolla, California). Progesterone, dibutyryl cyclic adenosine monophosphate (dbcAMP), nitroprusside, and modified Griess reagent (catalog number G4410) for 1-step determination of nitrite were purchased from Sigma-Aldrich Corporation (St Louis, Missouri). Other reagents were of the highest quality commercially available.

### Synthesis of PPCM and Derivatives

PPCM is an oligomer with average molecular weight 1550 to 1860. It is synthesized by a proprietary method by reacting D,L-mandelic acid with sulfuric acid. The sodium salt is prepared by reacting the free acid with alcoholic NaOH, yielding an off-white powder.

The NO donor 3-nitrooxypropan-1-ol (nitrooxypropanol), a nitrate semier, is prepared by reacting silver nitrate with 3-bromo-1-propanol in acetonitrile. Silver nitrate (18.7 g, 110 mmol) was dissolved in acetonitrile (200 mL). 3-Bromo-1-propanol (18.9 g; 100 mmol), dissolved in 25 mL of acetonitrile, was added and protected from light. The reaction mixture was stirred at room temperature for about 96 hours; after 2 hours, a yellow precipitate ( $\text{AgBr}$ ) was formed. The reaction mixture was filtered through a cellite pad to remove

AgBr. The solvent was removed by rotary evaporation to yield a pale yellow to clear oil, which was dissolved in dichloromethane. This solution was extracted with saturated NaCl in water to remove residual silver as AgCl, and dried over anhydrous sodium sulfate. After solvent removal by rotary evaporation and distillation (50 mm Hg), nitrooxypropanol (colorless to pale yellow oil) was obtained in about 62% yield. Identity was confirmed with infrared (IR) spectroscopy and  $^{13}\text{C}$ - and  $^1\text{H}$ -nuclear magnetic resonance (NMR) spectroscopy. IR spectrum:  $3359\text{ cm}^{-1}$  (OH);  $2964$  and  $2896\text{ cm}^{-1}$  (CH);  $1626\text{ cm}^{-1}$  ( $\text{NO}_3$  asymmetric);  $1281\text{ cm}^{-1}$  ( $\text{NO}_3$  symmetric);  $871\text{ cm}^{-1}$  (N=O);  $759\text{ cm}^{-1}$  ( $\text{NO}_2$  bend).  $^{13}\text{C}$  NMR spectrum: 70.62 ppm (C1), 58.01 ppm (C2), 29.30 ppm (C3).  $^1\text{H}$  NMR spectrum (in  $\text{CDCl}_3$ ): 4.62 ppm (2H, H1), 3.78 ppm (2H, H3), 1.99 ppm (2H, H2), 1.70 ppm (1H, OH); numerical prefixes refer to number of protons, and suffixes refer to position on the structure. Nitrooxypropanol releases NO, measured by nitrite formation (Griess reaction). Nitrooxypropanol (0.1 mM) was reacted with 50 mM L-cysteine at  $37^\circ\text{C}$  for 24 hours. Maximum nitrite formation approaches  $58.3\text{ }\mu\text{M}$  (data not shown).

A PPCM derivative (NO donor-substituted poly-acidic polymer [NOSPPA]) was prepared, in which nitrooxypropanol was substoichiometrically esterified (approximately 23% substitution) to PPCM carboxyl groups. PPCM (3.19 g; 23.8 acid mEq) was dissolved in 500 mL dimethyl formamide (DMF) in a flask with attached drying tube and cooled to  $0^\circ\text{C}$  (ice bath). Excess 1,1'-carbonyldiimidazole (9.73 g; 60 mmol; coupling agent) was dissolved in 40 mL DMF and added to the stirred PPCM solution. After further addition of 30 mL DMF, the reaction was continued for 45 minutes with stirring at  $0^\circ\text{C}$ . Nitrooxypropanol (0.86 g; 7.1 mmol) in 5 mL dry DMF was added drop-wise over a 2-minute period. The reaction temperature was allowed to rise to ambient temperature. The reaction was continued for 15 hours with stirring at ambient temperature. The mixture was decanted into 1000 mL water and acidified to pH 1.6 with 6 N HCl. The precipitate was suction-filtered, washed with water (two 200-mL portions), and suction-filtered. The precipitate was lyophilized to yield 2.78 g of NOSPPA-23 (approximately 73% yield). Elemental analysis (calculated and observed): C, 66.4% and 66.0%; H, 4.5% and 4.8%; N, 1.9% and 2.0%. IR spectrum:  $1722\text{ cm}^{-1}$  (COO-R);  $1712\text{ cm}^{-1}$  (COOH);  $1629\text{ cm}^{-1}$  (asymmetric  $\text{NO}_2$  stretch);  $1280\text{ cm}^{-1}$  (symmetric  $\text{NO}_2$  stretch). NOSPPA-23 releases NO, measured with the Griess reaction. NOSPPA-23 (0.1 mg/mL; approx  $63\text{ }\mu\text{M}$ ) was reacted with 50 mM L-cysteine at  $37^\circ\text{C}$  for 24 hours; nitrite formation approached  $11.3\text{ }\mu\text{M}$  (data not shown).

### Human Subjects

In each experiment, fresh semen was collected from 2 or 3 out of a total of 9 healthy donors (age [ $\bar{x} \pm \text{SEM}$ ] =  $32 \pm 3.5$  years). Details regarding medical histories and inclusion criteria for these individuals have been presented (Anderson et al, 2006). Donors participated in this study with informed consent. The study was approved by and in compliance with the medical center institutional review board. Semen was of high quality, with average volume of  $4.8 \pm 0.68\text{ mL}$  and average sperm count of  $82$  (90% confidence limits =  $70.1$ –

$97.2) \times 10^6$  cells/mL. Initial fraction of motile sperm for all experiments was 70.2% (66.90% to 73.42%).

### Procedures

*Preparation of Spermatozoa and Induction of AL*—Within the context of this study, AL refers to the disruption of the sperm acrosome in response to a treatment or chemical entity. No inference is made as to whether this response is identical to a physiological acrosome reaction, during which the acrosome is also lost.

Within 90 minutes of collection, liquefied semen samples (2 or 3) were pooled after a cursory examination of sperm motility and count by light microscopy with a Neubauer hemacytometer (bright field,  $\times 400$ ). The pooled sample was divided for each assay, such that approximately 0.1 mL (total sperm number =  $5 \times 10^6$ ) was layered onto 1 mL of 11% (wt/vol) buffered Ficoll (containing 120 mM NaCl and 25 mM HEPES, pH 7.4), in plastic centrifuge tubes. Samples were centrifuged at  $15\,000 \times g$ -min ( $22^\circ\text{C}$  to  $24^\circ\text{C}$ ), and the supernatant was aspirated from the sperm pellet. The pellet was resuspended in modified Biggers, Whitten, and Whittingham (BWW; Biggers et al, 1971) medium (less albumin), and the suspension was recentrifuged at  $1000 \times g$ -min ( $22^\circ\text{C}$  to  $24^\circ\text{C}$ ). The supernatant was aspirated, and the pellet was resuspended in 1 mL BWW medium. Details of sperm preparation have been described (Anderson et al, 1992, 1994).

After equilibration of washed spermatozoa for 10 minutes at  $37^\circ\text{C}$ , transduction pathway modulators were added, as indicated. After an additional 10 minutes at  $37^\circ\text{C}$ , AL was induced, either by addition of stimulus (PPCM, dbcAMP, hANP, or progesterone) or by adding  $\text{CaCl}_2$ , as indicated. Fifteen minutes after AL induction, spermatozoa were fixed with buffered glutaraldehyde and stained with rose bengal and Bismarck brown for acrosome visualization (Anderson et al, 1992, 2006). Approximately 450 cells were scored per slide; typical cell counts in a given experiment ranged from approximately 400 to 700. From 3 to 4 replicates were measured in each experiment. The total number of replicates for each condition are presented in "Results." Data are expressed as averages, with 90% confidence limits, of percentage of AL induced by a maximally stimulating concentration of A23187 (Anderson et al, 1992). The percentage of spermatozoa lacking acrosomes after treatment with A23187 under these conditions is 30.7% (90% confidence limits = 30.5% to 31.0%;  $n = 30$ ).

In some instances, stock solutions of modulators (ie, KT5720, KT5823, progesterone, genistein, ODQ) were prepared in dimethyl sulfoxide (DMSO). The DMSO concentration was not greater than 1.5% (vol/vol). Equivalent concentrations of DMSO were added to the appropriate controls. DMSO is without effect on sperm motility and acrosomal status under these conditions.

*Estimation of NO Production*—NO production by spermatozoa was estimated with a modification of the method of Revelli et al (1999). The method determines stimulus-induced production of nitrite, measured spectrophotometrically with the Griess reaction.

Approximately  $30 \times 10^6$  spermatozoa were suspended in arginine-supplemented (2 mM) BWW medium. Incubations with different additions were carried out for 1 hour at 37°C, after which cells were disrupted with 0.2% Triton X-100, followed by 1 freeze-thaw cycle (−80°C to 37°C). Particulates were sedimented at  $80\,000 \times g$ -min).

Equal volumes of supernatant and modified Griess reagent were combined and reacted in the dark for 10 minutes before reading the absorbance at 540 nm. Nitrite formation (pmol nitrite/ $10^6$  cells) was quantified with a nitrite standard linear curve ( $r^2 = 0.9997$ ), at 5 concentrations ranging from 0.25 to 5.0  $\mu$ M, and is expressed as  $\bar{x} \pm$  SEM.

### Data Collection and Analysis

Frequency (%) data were subjected to arcsine transformation before analysis (Sokal and Rohlf, 1981b). Values are presented as average percentage of maximal AL, with 90% confidence limits. Analysis of variance and the Newman-Keuls multiple range test were used to identify differences among treatment groups within individual experiments. Where appropriate, a 2-tailed unpaired *t* test was used to determine the significance of differences between treated samples and their respective controls. Differences among treatment groups were considered significant at  $P < .05$ . Differences were not considered significant at  $P > .1$ .

### Determination of Independence of Actions of 2 AL Stimuli

In some instances, inhibitors of signal transduction pathways induced AL when added alone. To determine the effect of these agents on stimulus-induced AL, data were examined for interactive effects.

Interactive effects of 2 agents on a normally distributed variable can be evaluated with 2-way analysis of variance, looking for deviations from additive effects. However, this cannot be applied to frequency data, because of constraints placed on the possible range of values (0% to 100%). This is clearly seen if 2 agents, each of which produces 60% inhibition of some biological activity, are added in combination. The expected outcome is somewhat less than 100% inhibition, assuming that the 2 agents acted independently. In this instance the predicted effect would be 84% inhibition, unless interactive effects alter the outcome.

AL data are binary. With binary data, the probability of an event not occurring in the presence of 2 independent agents is equal to the product of the probabilities of that event not occurring in the presence of each agent alone. If agent A produces AL in 90% of the spermatozoa, and agent B causes the same outcome, but by an independent mechanism, the expected frequency of AL when both A and B are added in combination to the cells is  $(1 - [1 - 0.9][1 - 0.9]) \times 100 = (1 - 0.01)(100) = 99\%$ . A detailed discussion of this subject is available (Piegorisch and Margolin, 1989).

Interactive effects were evaluated by comparing the observed with the predicted responses to combined treatment with inhibitor (or second stimulus) and stimulus, based on the response to the 2 agents, each added alone. The interactive effect was evaluated with the log likelihood ratio test (G test) of independence (Sokal and Rohlf, 1981a). When the second stimulus was a pathway inhibitor, the extent to which that

agent inhibited the response to the primary stimulus was estimated with the following equation:

$$\% \text{ inhibition} = ([A - B]/[A - C]) \times 100$$

where A = predicted combination response, assuming no interaction; B = observed combination response; and C = observed response to inhibitor alone.

## Results

### PPCM Activates an Endothelial-like Isoform of NO Synthase

All NO synthase (NOS) inhibitors except for S-methylthiocitrulline promote AL when added alone, in the presence of added extracellular  $\text{Ca}^{2+}$ ; 1400W has a small stimulatory effect (Figure 1). The most effective NOS inhibitors as AL stimuli are the relatively nonselective  $\text{N}^G$ -nitro-L-arginine and L-NAME.

$\text{N}^G$ -nitro-L-arginine inhibits PAL from the predicted response of PPCM and  $\text{N}^G$ -nitro-L-arginine added in combination. S-methyl-isothiourea, selective for inducible NOS (also known as iNOS or NOS-2) and eNOS (NOS-3; Szabo et al, 1994), and L-NAME (nonselective; partially selective for eNOS; Vaupel et al, 1995; Pozzoli et al, 2001) inhibit PAL to a similar extent.

Neither S-methylthiocitrulline, selective for the neuronal isoform, nNOS (NOS-1; Furfine et al, 1994), nor 1400W, selective for iNOS (Garvey et al, 1997), inhibits PAL (Figure 1).

### PAL Requires Soluble Guanylate Cyclase

LY83583 (50 nM), an inhibitor of soluble and receptor-linked guanylate cyclases (Anand-Srivastava and Trachte, 1993) inhibits PAL (Figure 2). Similar to the iNOS and eNOS inhibitors, LY83583 induces AL in the presence of added  $\text{Ca}^{2+}$ . LY83583 is without effect on AL when  $\text{Ca}^{2+}$  is not added (2.2% [0.82% to 4.53%]). PAL is nearly 90% inhibited by LY83583.

LY83583 also inhibits by over 90% the AL induced by 0.1 nM hANP (Figure 2). hANP exerts its actions through activation of receptor-linked guanylate cyclase (Anand-Srivastava and Trachte, 1993).

ODQ (100 nM), a selective soluble guanylate cyclase inhibitor (Garthwaite et al, 1995), inhibits PAL, but not hANP-induced ( $t = 0.54$ ,  $df = 6$ ,  $P > .1$ ) AL (Figure 2). In the presence of added  $\text{Ca}^{2+}$ , ODQ alone exerts a small stimulatory effect on AL. ODQ has no effect on AL in the absence of added  $\text{Ca}^{2+}$  (0.5% [−0.27% to 3.70%]).

When reactions are carried out with added  $\text{Ca}^{2+}$ , responses to 0.25  $\mu$ g/mL PPCM and 0.1 nM hANP are 50% (49.0% to 51.4%;  $n = 18$ ) and 59% (56.4% to 61.3%;  $n = 4$ ) maximal AL, respectively. The predicted response to combined addition of these agents is 79.5%,

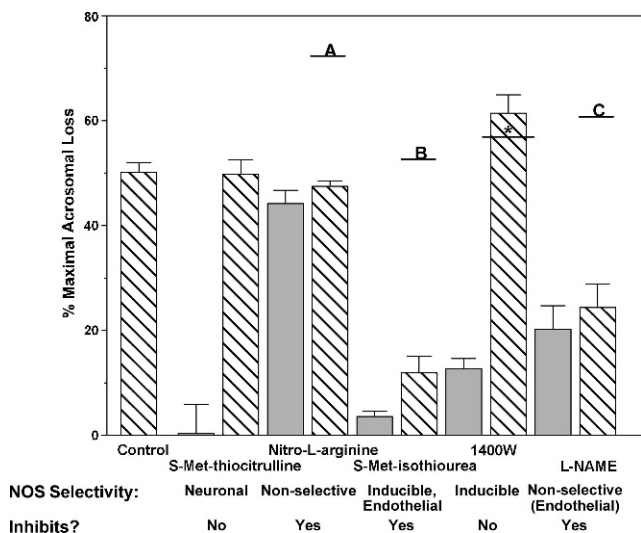


Figure 1. Previously designated sulfuric acid-modified mandelic acid (PPCM)-induced acrosomal loss requires endothelial nitric oxide synthase (NOS). Hatched bars represent samples that contain 0.25 µg/mL PPCM. Shaded bars represent samples without PPCM. Error bars represent 90% confidence limits ( $n = 4$ ). Horizontal bars represent the predicted response to PPCM and inhibitor added in combination, assuming independent actions. Sperm suspensions were incubated with either 1) buffer alone (control); 2) 0.5 µM S-methylthiocitrulline (selective against nNOS); 3) 5 µM  $N^G$ -nitro-L-arginine (nonselective NOS inhibitor); 4) 10 µM S-methylisothiurea (selective against eNOS and iNOS); 5) 2 µM 1400W (selective against iNOS); or 6) 10 µM  $N^G$ -nitro-L-arginine methyl ester (L-NAME; partially selective against eNOS). After 10 minutes, reactions were initiated with 1.3 mM  $Ca^{2+}$ . Fifteen minutes thereafter, spermatozoa were fixed and stained for acrosome visualization. <sup>A</sup> Observed increase attributable to PPCM over the observed response to  $N^G$ -nitro-L-arginine alone (44%), when PPCM and  $N^G$ -nitro-L-arginine are added in combination, is 91% inhibited from the predicted increase (28.1%), assuming independent actions of these agents. There is an antagonistic interactive effect of these agents ( $G = 26.1$ ;  $P < .001$ ). <sup>B</sup> Observed increase attributable to PPCM (8.3%) over the observed response to S-methylisothiurea alone (3.6%), when PPCM and S-methylisothiurea are added in combination, is 83% inhibited from the predicted increase, assuming independent actions of PPCM and S-methylisothiurea. There is an interactive (antagonistic) effect of these agents ( $G = 30.1$ ,  $P < .001$ ). <sup>C</sup> Observed increase attributable to PPCM (4.3%) over the observed response to L-NAME alone (20.2%), when PPCM and L-NAME are added in combination, is 89% inhibited from the predicted increase (40.6%), assuming independent actions of PPCM and L-NAME. There is an interactive (antagonistic) effect of these agents ( $G = 31.1$ ,  $P < .001$ ). \* Observed increase attributable to PPCM (49%) over the observed response to 1400W alone (13%), when PPCM and 1400W are added in combination, is not inhibited from the predicted increase (44.5%), assuming independent actions of PPCM and 1400W. There is no interactive effect of these agents ( $G = 0.68$ ,  $P > .1$ ).

if they act independently. The observed response to PPCM and hANP added in combination is 79% (76.7% to 81.7%;  $n = 4$ ).

#### PAL Is Antagonized by cGK Inhibition

The selective cGK inhibitor KT5823 (Nakanishi, 1989) completely inhibits PAL. The AL response to combined

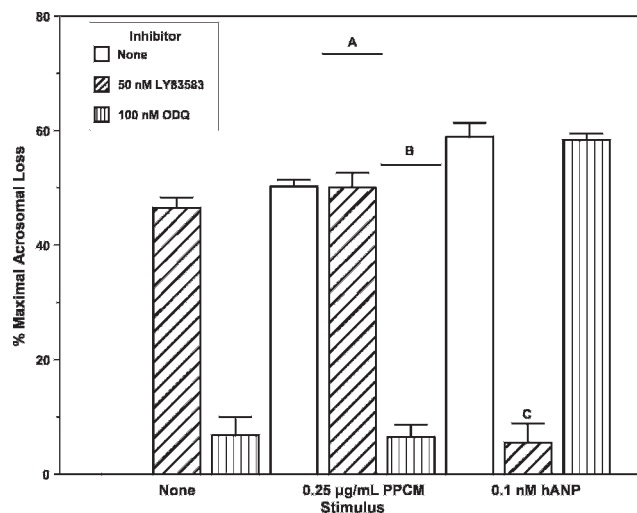


Figure 2. Previously designated sulfuric acid-modified mandelic acid (PPCM)-induced acrosomal loss requires soluble guanylate cyclase. Cross-hatched bars represent samples that contain 50 nM of the nonselective guanylate cyclase inhibitor LY83583. Vertical hatched bars represent samples that contain 100 nM of the selective soluble guanylate cyclase inhibitor ODQ. Error bars are 90% confidence limits ( $n = 8$  for LY83583 alone;  $n = 18$  for PPCM alone;  $n = 4$  for all other conditions). Horizontal bars represent the predicted response to stimulus and inhibitor added in combination, assuming independent actions. Sperm suspensions were incubated with either buffer alone (control), LY83583, or ODQ for 10 minutes. Reactions were initiated with 0.25 µg/mL PPCM and 1.3 mM  $Ca^{2+}$  (final concentrations) in combination (indicated as "PPCM"), or with 1.3 mM  $Ca^{2+}$  alone (indicated as "None"), or with 0.1 nM human atrial natriuretic peptide (hANP). Fifteen minutes thereafter, spermatozoa were fixed and stained for acrosome visualization. <sup>A</sup> Observed increase attributable to PPCM (4%) over the observed response to LY83583 alone (46%), when PPCM and LY83583 are added in combination, is 87% inhibited from the predicted increase (26.9%), assuming independent actions of PPCM and LY83583. There is an antagonistic interactive effect of these agents ( $G = 23.0$ ;  $P < .001$ ). <sup>B</sup> There is no increase because of PPCM over the observed response to ODQ (6.8%), when PPCM and ODQ are added in combination. <sup>C</sup> Value is inhibited compared with that observed in the presence of hANP alone ( $t = 21.4$ ,  $df = 6$ ,  $P < .001$ ).

addition of 2 µM KT5823 and 0.25 µg/mL PPCM represents a highly antagonistic interaction (Table 1).

#### PPCM Promotes NO Formation

PPCM increases sperm production of NO, as measured by an increase in nitrite formation. Nitrite formation is increased by 57% as compared with control reactions containing no additions (Table 2). This response is inhibited by approximately 88% by 10 µM  $N^G$ -nitro-L-arginine.  $N^G$ -nitro-L-arginine reduces baseline nitrite by approximately 22%; variability of the data precludes a significant difference (Table 2). NO production in response to PPCM is much lower than that in response to the NO donor SNAP, although both agents produce comparable (50% to 80%) AL.

Table 1. Effect of cGK inhibitors on AL induced by PPCM and SNAP<sup>a</sup>

Inhibitor	% Maximal AL (90% Confidence Limits)					
	Inhibitor Alone			PPCM		
	Ca <sup>2+</sup> Added	No Ca <sup>2+</sup> Added		0.25 µg/mL	0.5 µg/mL	0.4 mM SNAP
None	—	—	—	50 (49.0 to 51.4) n = 18	79 (77.5 to 80.9) n = 12	77 (75.4 to 78.6) n = 12 <sup>b</sup>
2.0 µM KT5823 <sup>c</sup>	58 (56.9 to 59.8) n = 24	1.9 (0.3 to 4.8) n = 10	—	59 (58.0 to 59.8) n = 4 <sup>d</sup>	—	75 (69.9 to 79.0) n = 4 <sup>b,e</sup>
5.0 µM Rp-8-pCPT-cGMPS <sup>f</sup>	33 (29.4 to 37.6) n = 8	4.1 (1.1 to 8.7) n = 8	—	—	46 (41.5 to 50.2) n = 4 <sup>d</sup>	62 (57.2 to 66.8) n = 4 <sup>d,g</sup>
0.35 µM Rp-8-Br-PET-cGMPS <sup>h</sup>	21 (18.6 to 23.3) n = 6	3.5 (2.4 to 4.8) n = 6	—	—	78 (74.3 to 82.7) n = 4	73 (66.5 to 79.5) n = 4 <sup>b,e</sup>
0.25 µM DT-3 (cell permeable cGK I-α inhibitor) <sup>i</sup>	0.1 (0.1 to 0.8) n = 4	0.9 (0.0 to 4.2) n = 8	—	—	82 (80.4 to 82.6) n = 4	72 (69.7 to 74.6) n = 4 <sup>e</sup>

Abbreviations: AL, acrosomal loss; cGMP, cyclic guanosine monophosphate; PPCM, previously designated sulfuric acid-modified mandelic acid.

<sup>a</sup> Reactions were carried out in the presence of the indicated inhibitor for 10 minutes prior to adding either PPCM or SNAP. AL was measured as described in footnote a to Table 3. Reactions with PPCM contained added Ca<sup>2+</sup> (1.3 mM). No Ca<sup>2+</sup> was added to reactions that contained SNAP.

<sup>b,e,g</sup> Values with different superscript designations are different ( $P < .01$ , Newman-Keuls multiple range test).

<sup>c</sup> An antagonistic interactive effect exists between PPCM and KT5823 ( $G = 207$ ,  $df = 1$ ,  $P < .001$ ). The observed response is completely inhibited as compared with the predicted response to combined addition of PPCM and KT5823, assuming independent actions of these agents.

<sup>d</sup> Inhibits.

<sup>f</sup> An antagonistic interactive effect exists between PPCM and Rp-8-pCPT-cGMPS ( $G = 79$ ,  $df = 1$ ,  $P < .001$ ). The observed response is 76% inhibited as compared with the predicted response (86%) to combined addition of PPCM and Rp-8-pCPT-cGMPS, assuming independent actions of these agents. An antagonistic interactive effect exists between SNAP and Rp-8-pCPT-cGMPS ( $G = 16.8$ ,  $df = 1$ ,  $P < .001$ ). The observed response is 23% inhibited as compared with the predicted response to combined addition of SNAP and Rp-8-pCPT-cGMPS, assuming independent actions of these agents.

<sup>h</sup> The observed response to combined addition of Rp-8-Br-PET-cGMPS and PPCM is not different from that predicted (83.5%), assuming independent actions of these 2 agents ( $G = 2.46$ ,  $df = 1$ ,  $P > .1$ ).

<sup>i</sup> Cell-permeable cGK I-α inhibitor (DT-3) has no effect on AL induced by either PPCM or SNAP (with or without Ca<sup>2+</sup>). Two-way analysis of variance shows no interactive (inhibitory) effect between stimulus and the presence/absence of DT-3 ( $F [3,72] = 1.43$ ,  $P > .1$ ).

Table 2. Nitrite formation promoted by PPCM is inhibited by the nitric oxide synthase inhibitor N<sup>G</sup>-nitro-L-arginine

Addition	[Nitrite], pmol/10 <sup>6</sup> Spermatozoa
None	29 ± 4.7 <sup>b,c</sup>
10 μM nitroarginine	23 ± 2.6 <sup>b</sup>
2 μg/mL PPCM	46 ± 5.6 <sup>d</sup>
PPCM + nitroarginine	31 ± 2.4 <sup>c</sup>
0.4 mM SNAP	391 ± 10.3 <sup>f</sup>

Abbreviation: PPCM, previously designated sulfuric acid-modified mandelic acid.

<sup>a</sup> Spermatozoa (30 × 10<sup>6</sup>) were incubated in Biggers, Whitten, and Whittingham (BWW, Biggers et al, 1971) medium supplemented with 2 mM L-arginine and the indicated additions at 37°C. After 1 hour, cells were lysed and sedimented; nitrite concentration was determined, as described in "Materials and Methods." Values are  $\bar{x} \pm \text{SEM}$  (n = 4).

<sup>b-f</sup> Values with different superscripts differ ( $P < .025$ , Newman-Keuls multiple range test).

*AL by PPCM and NO Donors Occurs by Different Pathways*

Unlike PAL (Anderson et al, 2006), SNAL does not require added Ca<sup>2+</sup>. SNAP (0.4 mM) induces, in the presence and absence of added Ca<sup>2+</sup>, 81% (79.6% to 83.1%; n = 8) and 79% (76.9% to 80.4%; n = 16) maximal loss, respectively ( $t = 1.70$ ,  $P > .1$ ).

As expected, SNAL is sensitive to the soluble guanylate cyclase inhibitor ODQ. However, inhibition

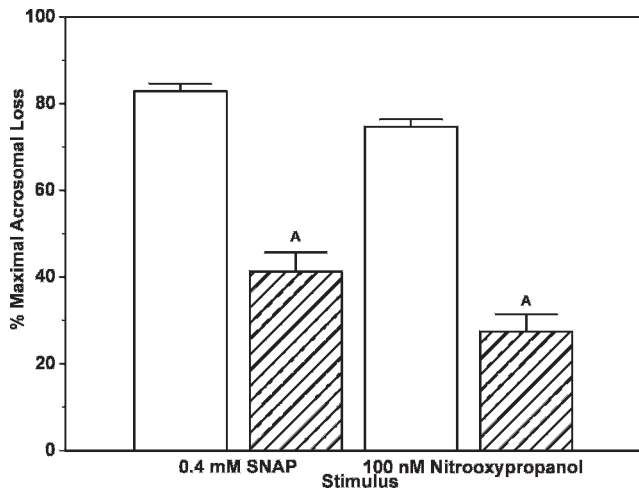


Figure 3. Involvement of soluble guanylate cyclase in acrosomal loss (AL) induced by nitric oxide (NO) donors. Cross-hatched bars represent samples that contain 100 nM of the selective soluble guanylate cyclase inhibitor ODQ. Error bars are 90% confidence limits (n = 16 for SNAP alone; n = 4 for all other conditions). Sperm suspensions were incubated for 10 minutes in Biggers, Whitten, and Whittingham (BWW, Biggers et al, 1971) medium to which Ca<sup>2+</sup> was not added, either in the presence or absence of ODQ. At this time reactions were initiated with either 0.4 mM SNAP or 100 nM nitrooxypropanol, as indicated. Fifteen minutes thereafter, spermatozoa were fixed and stained for acrosome visualization. <sup>A</sup> Value is inhibited relative to respective control. For SNAP,  $t = 14.8$  (df = 18;  $P < .001$ ); for nitrooxypropanol,  $t = 24.0$  (df = 6,  $P < .001$ ).

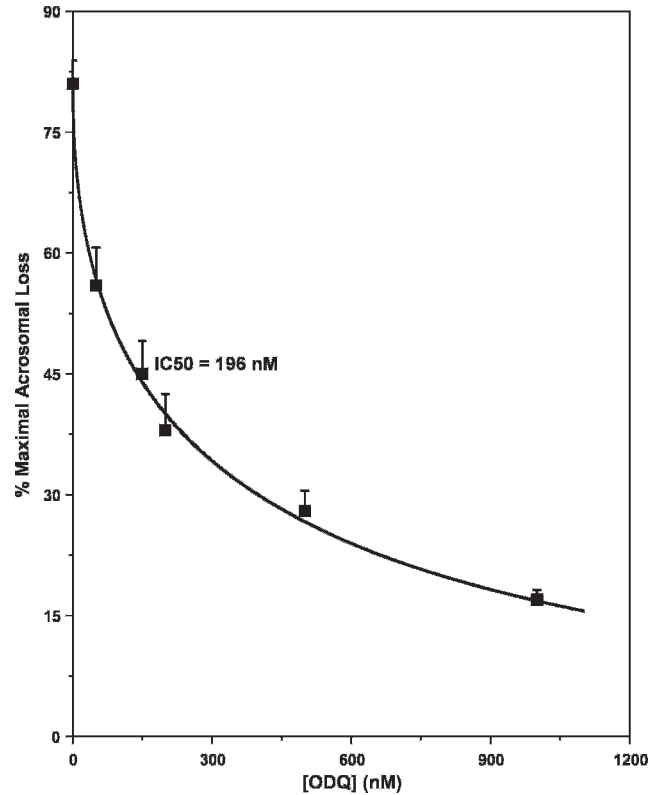


Figure 4. SNAP-induced acrosomal loss (SNAL) is less sensitive to inhibition by the soluble guanylate cyclase inhibitor ODQ than that induced by previously designated sulfuric acid-modified mandelic acid (PPCM). Error bars are 90% confidence limits. Washed spermatozoa were incubated with different concentrations of ODQ, as indicated. After 10 minutes at 37°C, 0.4 mM SNAP was added. Fifteen minutes after addition of stimulus, spermatozoa were fixed and stained for acrosome visualization. Data were fit to a curve ( $r^2 = 0.997$ ) described by the equation  $Y = a + bX^{0.5}$ , where  $a = 4.3900849$  and  $b = -0.049520827$ . This curve was used to estimate the IC<sub>50</sub> of ODQ (196 nM) as an inhibitor of SNAL.

is incomplete (approximately 50%) at 100 nM ODQ (Figure 3), a concentration sufficient to completely block PAL (Figure 2). Similar incomplete inhibition by ODQ occurs in AL induced by the nitrate ester NO donor nitrooxypropanol (63% inhibition at 100 nM ODQ). Even at 10-fold higher concentration, inhibition of SNAL approaches only 80% (Figure 4).

Unexpectedly, SNAL is unaffected by the cGK inhibitor KT5823 when added at a concentration (2 μM) that completely inhibits PAL. Apparent lack of cGK involvement is also demonstrated for several other NO donors, independent of mechanism of NO release (Table 3).

Effects of several other cGK inhibitors on PAL and SNAL were examined. PAL is inhibited by KT5823 (selective for cGK-I and cGK-II; Mishra et al, 2001; Tischkau et al, 2004) and by Rp-8-pCPT-cGMPS (inhibits cGK-I and cGK-II; Butt et al, 1994; Gambaryan

Table 3. No effect of KT5823 on acrosomal loss induced by NO donors<sup>a</sup>

	% Maximal AL (90% Confidence Limits)	
	No KT5823	2 $\mu$ M KT5823 <sup>b</sup>
NO donor		
0.4 mM SNAP	76 (72.0 to 81.1)	74 (70.0 to 79.0)
22 $\mu$ M nitroprusside	74 (69.7 to 79.0)	74 (69.3 to 78.7)
1 $\mu$ M SIN-1	47 (41.5 to 52.9)	46 (41.8 to 51.3)
100 nM 4-phenyl-3-furoxan carbonitrile	58 (55.1 to 61.8)	58 (53.8 to 62.7)

Abbreviations: AL, acrosomal loss; NO, nitric oxide.

<sup>a</sup> Sperm suspensions were incubated with or without 2  $\mu$ M KT5823 (cGK inhibitor) in the absence of added  $Ca^{2+}$ . After 10 minutes, reactions were initiated with NO donor, as indicated. After 15 minutes, spermatozoa were fixed and stained for acrosome visualization (n = 4).

<sup>b</sup> KT5823 has no effect on AL induced by NO donors. KT5823 alone has no effect on AL under the conditions of these experiments (ie, no added  $Ca^{2+}$ );  $\bar{x}$  maximal loss = 2 (0.3 to 4.8)%, n = 10.

et al, 1996; also inhibits protein kinase A (PKA) at concentrations effective against cGK; Smolenski et al, 1998). However, PAL is unaffected by either Rp-8-Br-PET-cGMPS (inhibits cGK-I $\alpha$  and cGK-I $\beta$ ; Schwede et al, 2000; also inhibits cGMP-specific phosphodiesterase type 5 [PDE-5]; Butt et al, 1995) or DT-3 (selective for cGK-I $\alpha$ ; Peluso and Pappalardo, 2004). Of the inhibitors examined, only Rp-8-pCPT-cGMPS weakly inhibits SNAL (Table 1).

The cGK inhibitors, KT5823 and Rp-8-pCPT-cGMPS (nonselective for cGK isotype), induce AL when added alone in the presence of added  $Ca^{2+}$ . Under similar conditions, somewhat lower (approximately 21% maximal loss) AL is induced by the cGK-I inhibitor Rp-8-Br-PET-cGMPS; however, DT-3, selective for cGK-I $\alpha$ , is without effect (Table 1). All inhibitors are without effect on AL by themselves in the absence of added  $Ca^{2+}$ .

The ability of cGK inhibitors to induce  $Ca^{2+}$ -dependent AL is similar to that seen for NOS and guanylate cyclase inhibitors (Figures 1 and 2), suggesting that the resting or basal state NO/cGMP/cGK

transduction pathway may be a determinant of acrosomal status, possibly through control of  $Ca^{2+}$  entry. AL induced by N<sup>G</sup>-nitro-L-arginine (inhibits NOS), LY83583 (inhibits guanylate cyclase), and KT5823 (inhibits cGK) are inhibited by the  $Ca^{2+}$  channel blocker nifedipine (Table 4). Nifedipine alone has a negligible effect on AL.

The PKA inhibitor KT5720 (Nakanishi, 1989) inhibits SNAL, but similarly to inhibition by ODQ, inhibition is incomplete. The concentration of KT5720 used is sufficient to completely block AL induced by dbcAMP (Figure 5). KT5720 is ineffective against PAL. SNAL was examined in the presence of KT5720 and ODQ, added alone or in combination, to determine possible interaction between the cAMP and cGMP pathways. There is no interaction between these inhibitors (Figure 5).

The protein tyrosine kinase (PTK) inhibitor genistein (25  $\mu$ g/mL; 93  $\mu$ M) partially inhibits SNAL (39%). In contrast, genistein is without effect on PAL. This concentration of genistein inhibits by over 92% the AL induced by progesterone (Figure 6), known to act via protein tyrosine phosphorylation (Rathi et al, 2003).

Although SNAL is inhibited by either KT5720 or genistein, genistein fails to inhibit AL induced by dbcAMP (Figure 6), suggesting involvement of cAMP and protein tyrosine phosphorylation through separate pathways. Inhibitor sensitivities of PAL and SNAL are summarized in Table 5.

PPCM at 0.25  $\mu$ g/mL and 0.25 mM SNAP induce 54% (50.6% to 58.7%) and 56% (54.4% to 58.8%) maximal AL, respectively. Observed response to combined addition of PPCM and SNAP is 82% (79.4% to 84.5%), reflecting no interactive effect of PPCM and SNAP (G test of independence = 0.345, df = 1,  $P > .1$ ).

The above results suggest that AL mediated by NO produced by 2 different types of stimuli (PPCM and NO donors) occurs by independent mechanisms. Covalent attachment of an NO donor to PPCM may result in a derivative with enhanced biological activity.

Table 4. Acrosomal loss induced by NO/cGMP/cGK pathway inhibitors is mediated by voltage-dependent  $Ca^{2+}$  channels<sup>a</sup>

Inhibitor	Target Enzyme	% Maximal AL (90% Confidence Limits)	
		No Nifedipine	10 $\mu$ M Nifedipine
N <sup>G</sup> -nitro-L-arginine	NOS (nonselective)	46 (41.9 to 50.4)	7.6 (3.3 to 13.7) <sup>b</sup>
LY83583	Guanylate cyclase (nonselective)	44 (42.5 to 46.5)	14 (10.2 to 19.2) <sup>b</sup>
KT5823	cGK (nonselective)	58 (57.1 to 59.5)	19.1 (14.2 to 24.5) <sup>b</sup>

Abbreviations: AL, acrosomal loss; cGK, cyclic guanosine monophosphate-dependent protein kinase; cGMP, cyclic guanosine monophosphate; NO, nitric oxide; NOS, NO synthase.

<sup>a</sup> Sperm suspensions were incubated with the indicated inhibitor in the presence or absence of 10  $\mu$ M nifedipine (voltage-dependent  $Ca^{2+}$  channel blocker). After 10 minutes, reactions were initiated with 1.3 mM  $Ca^{2+}$ . Fifteen minutes thereafter, spermatozoa were fixed and stained for acrosome visualization (n = 4). Nifedipine alone produces a negligible response (3.7% [1.3% to 7.4%] maximal AL; n = 8).

<sup>b</sup> Values are different from their respective controls ( $t > 9.6$  in each instance, df = 6,  $P < .001$ ).

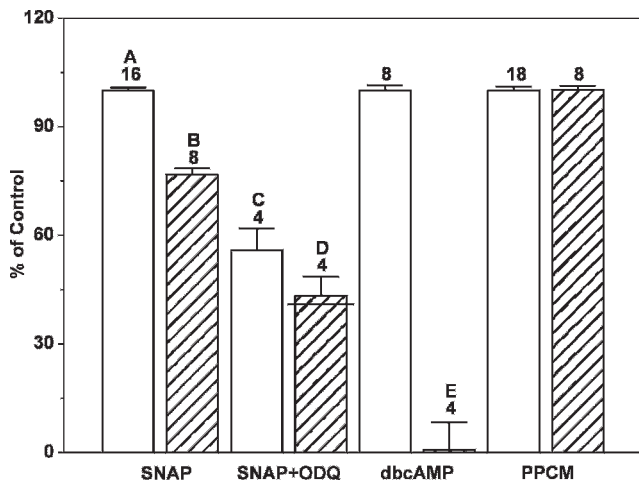


Figure 5. ODQ and KT5720 inhibit SNAP-induced acrosomal loss by independent, noninteracting pathways. Values are presented as average percentage of respective control reactions. Error bars are 90% confidence limits. Numerical values above each bar represent the sample size. Hatched bars represent reactions carried out in the presence of 1  $\mu$ M KT5720 (protein kinase A inhibitor). Reactions with previously designated sulfuric acid-modified mandelic acid (PPCM) were carried out in medium to which 1.3 mM  $Ca^{2+}$  was added.  $Ca^{2+}$  was excluded from all other reactions. The following concentrations of agents were added, where indicated: SNAP, 0.4 mM; ODQ, 0.15  $\mu$ M; dibutyl cyclic adenosine monophosphate (dbcAMP), 20  $\mu$ M; PPCM, 0.25  $\mu$ g/mL. KT5720 and/or ODQ were added 10 minutes prior to addition of stimulus. Fifteen minutes thereafter, sperm were fixed and stained, and acrosomal status was visualized. Stimulus and percentage maximal acrosomal loss (AL) for the control reactions are as follows: SNAP, 79% (76.9% to 80.4%), n = 16; dbcAMP, 59% (57.3% to 60.9%), n = 8; PPCM, 50% (49.0% to 51.4%), n = 18. Neither ODQ (3.3% [1.41% to 5.84%] maximal loss; n = 10) nor KT5720 (0.3% [0.06% to 1.94%]; n = 12) induces AL in the absence of added  $Ca^{2+}$ . KT5720 is without effect on AL in the presence of added  $Ca^{2+}$  (0.7% [0.00% to 3.02%], n = 8). The horizontal line passes through the bar representing the response to SNAP, ODQ, and KT5720 at the predicted percentage of control (59.0% inhibition), assuming no interactive effect between ODQ and KT5720. The difference between predicted and observed outcomes is not significant ( $G = 0.07$ ,  $df = 1$ ,  $P > .1$ ). <sup>A-D</sup> Values with different letter designations are different ( $P < .0025$ , Newman-Keuls multiple range test). <sup>E</sup> Value is different from its respective control ( $t = 12.4$ ,  $df = 10$ ,  $P < .001$ ).

To test this hypothesis, the NO donor nitrooxypropanol was covalently coupled to PPCM (see "Materials and Methods") to form a derivative in which approximately 23% of available carboxyl groups are substituted with the NO donor by esterification (NOSPPA-23).

NOSPPA-23 induces AL in the absence of extracellular  $Ca^{2+}$  (Figure 7). The response to 0.02  $\mu$ g/mL NOSPPA-23 in the absence of added  $Ca^{2+}$  (47%) is nearly 7-fold higher than the predicted response (6.8%) to an equivalent amount of the NO donor (36 nM nitrooxypropanol) from which NOSPPA-23 was derived (based on dose-response of nitrooxypropanol-induced AL; Figure 8, panel B). AL induced by nitrooxypropanol is independent of added  $Ca^{2+}$  (data not shown); PPCM is without effect in the absence of added  $Ca^{2+}$  (Anderson et al, 2006).

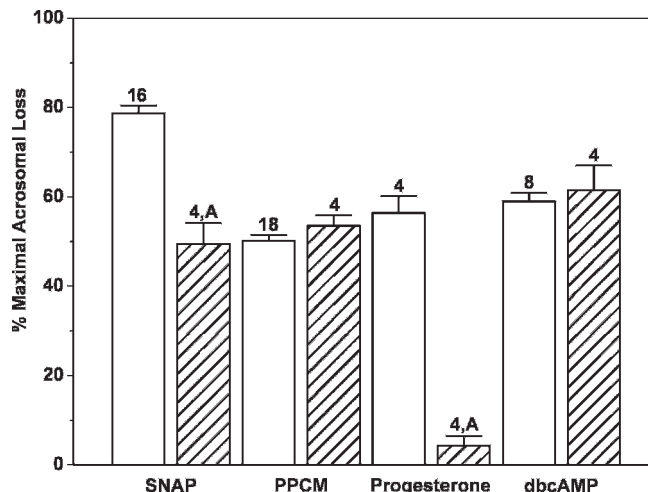


Figure 6. Genistein inhibits acrosomal loss induced by SNAP, but not by previously designated sulfuric acid-modified mandelic acid (PPCM). Error bars are 90% confidence limits. Numerical values above each bar represent the sample size. Hatched bars represent reactions carried out in the presence of 93  $\mu$ M genistein (protein tyrosine kinase inhibitor). Reactions with PPCM and progesterone were carried out in medium to which  $Ca^{2+}$  (1.3 mM) was added.  $Ca^{2+}$  was excluded from reactions that contained SNAP or dibutyl cyclic adenosine monophosphate (dbcAMP). The following concentrations of agents were added, as indicated: SNAP, 0.4 mM; PPCM, 0.25  $\mu$ g/mL; progesterone, 5 ng/mL (16 nM); dbcAMP, 20  $\mu$ M. Genistein was added 10 minutes prior to addition of stimulus. Fifteen minutes after addition of stimulus, sperm were fixed and stained, and acrosomal status was visualized. When added alone, genistein does not induce AL (with added  $Ca^{2+}$ : 0.4% [-0.13% to 2.53%] maximal loss, n = 8; without added  $Ca^{2+}$ : 0.4% [0.02% to 1.75%], n = 12). <sup>A</sup> Value is different from its respective control. For SNAP,  $t = 11.6$ ,  $df = 18$ ; for progesterone,  $t = 24.2$ ,  $df = 6$  ( $P < .001$ ).

The response to the same concentration of NOSPPA-23 in the presence of added  $Ca^{2+}$  is over 8-fold greater than that predicted (10.9%) from combined addition of the equivalent concentrations of PPCM (19 ng/mL) and nitrooxypropanol, assuming independent mechanisms of action (Figure 7). The increase in AL in the presence of NOSPPA-23 over that seen with the equivalent concentration of NO donor alone is over 21-fold higher than the predicted increase over the NO donor-induced AL because of the addition of PPCM. The  $ED_{50}$  of NOSPPA-23 in the presence of  $Ca^{2+}$  (8 ng/mL, or approximately 4.8 nM; Figure 8, panel A) is more than 30 times less than the  $ED_{50}$  for PPCM, on the basis of mass. On a molar basis, NOSPPA-23 is approximately 35-fold more active than PPCM. The  $ED_{50}$  of NOSPPA-23 in the absence of added  $Ca^{2+}$  (27.9 ng/mL) is equivalent to 40 nM NO donor, based on the nitrogen content (2.0%) of this material. This is approximately 3-fold less than the  $ED_{50}$  of the parent NO donor, nitrooxypropanol (120 nM; Figure 8, panel B).

In no instance is average sperm motility reduced more than 5% (after addition of either 50 nM LY83583 or 0.5  $\mu$ M S-methyl-L-thiocitrulline) from control values

Table 5. Different transduction pathways are responsible for SNAP- and PPCM-induced AL<sup>a</sup>

Stimulus	Inhibitor	% Maximal AL (90% Confidence Limits)
0.4 mM SNAP (no added Ca <sup>2+</sup> )	None	77 (75.8 to 79.0) <sup>b</sup>
	1.0 μM KT5720	59 (56.9 to 61.1) <sup>c</sup>
	93 μM genistein	50 (44.8 to 54.2) <sup>d</sup>
	0.2 μM ODQ	38 (33.9 to 42.7) <sup>e</sup>
	2 μM KT5823	75 (69.9 to 79.0) <sup>b</sup>
0.25 μg/mL PPCM (with 1.28 mM Ca <sup>2+</sup> )	None	50 (49.0 to 51.4) <sup>f</sup>
	1.0 μM KT5720	50 (47.7 to 52.9) <sup>f</sup>
	93 μM genistein	52 (49.6 to 54.5) <sup>f</sup>
	0.2 μM ODQ	6.5 (4.7 to 8.6) <sup>g</sup>
	2 μM KT5823	59 (58.0 to 59.8) <sup>h</sup>

Abbreviations: AL, acrosomal loss; PPCM, previously designated sulfuric acid-modified mandelic acid.

<sup>a</sup> In reactions with SNAP, inhibitor was incubated with spermatozoa for 10 minutes prior to initiating reactions with stimulus. In reactions with PPCM, inhibitor and stimulus were incubated with spermatozoa for 10 minutes prior to initiating reactions with CaCl<sub>2</sub>. After 15 minutes, spermatozoa were fixed and stained for acrosome visualization. All inhibitors are without effect on AL in the absence of added Ca<sup>2+</sup>. KT5720 and genistein are without effect in the presence of Ca<sup>2+</sup> (1.6% [−0.3% to 9.1%], n = 4, and 0.4% [−0.1% to 2.6%], n = 8, respectively).

<sup>b–e</sup> Values with different letter designations are different ( $P < .0025$ , Newman-Keuls multiple range test).

<sup>f</sup> Values do not differ ( $P > .1$ , Newman-Keuls multiple range test).

<sup>g</sup> AL induced by ODQ in the presence of added Ca<sup>2+</sup> is 6.8% (4.2% to 10.0%; n = 8); an antagonistic interactive effect exists between PPCM and ODQ ( $G = 39$ ,  $df = 1$ ,  $P < .001$ ).

<sup>h</sup> AL induced by KT5823 in the presence of added Ca<sup>2+</sup> is 59% (57.7% to 60.7%; n = 20); an antagonistic interactive effect exists between PPCM and KT5823 ( $G = 207$ ,  $df = 1$ ,  $P < .001$ ).

(no additions) as a result of treatment. Motility after addition of transduction pathway inhibitors ranges from 95% to 108% of control. Average motility loss caused by 0.25 μg/mL PPCM (concentration required to produce 50% maximal AL) is 0.1% (−0.8% to 1%). The fraction of motile spermatozoa increases in response to either 20 μM dbcAMP or 16 nM progesterone (by 24% and 35%, respectively;  $P < .05$ ). No relation exists between AL and the change in percentage of motile spermatozoa (Kendall's rank order correlation coefficient  $\tau = -0.156$ , n = 10,  $P > .1$ ). The likelihood of changes in AL being secondary to decreased cell viability is therefore minimal.

## Discussion

The objective of this study was to determine intracellular events responsible for PAL that are downstream from Ca<sup>2+</sup> entry. We have used a pharmacologic approach toward that end. This, as any approach, is subject to limitations and misinterpretation. Data interpretation can be confounded by any of the following: 1) the

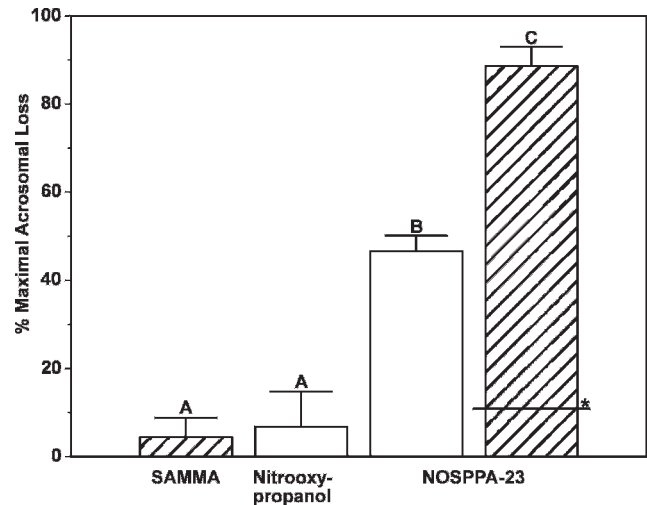


Figure 7. Synergistic response to nitric oxide donor-substituted poly-acidic polymer (NOSPPA)-23 compared with parent compounds. Error bars are 90% confidence limits. Either nitrooxypropanol (36.4 nM), previously designated sulfuric acid-modified mandelic acid (PPCM) (19 ng/mL), or NOSPPA-23 (20 ng/mL) was added to washed spermatozoa, with (hatched bars) or without (open bars) added Ca<sup>2+</sup>. After 15 minutes, sperm were fixed and stained, and acrosomal status was visualized. Concentrations of PPCM and nitrooxypropanol are equivalent to the concentrations of PPCM and nitric oxide-donor moieties present in NOSPPA-23. <sup>A–C</sup> Values with different letter designations are different ( $P < .01$ , Newman-Keuls multiple range test). \* The horizontal bar represents the predicted response to NOSPPA-23 (10.9% maximal loss), based on equivalent concentrations of the parent compounds, PPCM and nitric oxide-donor moieties present in NOSPPA-23. A significant synergistic interaction is observed ( $G = 191$ ,  $df = 1$ ,  $P < .001$ ).

selected inhibitor(s) lack specificity or selectivity; 2) the inhibitor is selective, but fails to readily enter intact cells; or 3) the inhibitor is selective, but it is not used at pharmacologically relevant concentrations, thus reducing its selectivity. Whenever possible, we have minimized these shortcomings by 1) using modulators selective for intended targets; 2) using multiple modulators of a single target; 3) verifying the efficacy of selected inhibitors through the use of alternative stimuli with known mechanisms of action; and 4) using concentrations of modulators that are consistent with actions observed in cell-free systems.

Our examination of possible involvement of signal transduction pathways in PAL was based in part on putative mechanism(s) of the mammalian acrosome reaction. Reviews on the subject have considered several post-Ca<sup>2+</sup> entry pathways and second messengers. These include, among others, increased protein tyrosine phosphorylation, increased cAMP levels, activation of PKA, increased adenylate cyclase activity and activation of L-type voltage-dependent calcium channels (Breitbart and Spungin, 1997; Benoff, 1998; Guraya, 2000). cGMP (Anderson et al, 1995; Kobori et al, 2000) and NO (Kameshwari et al, 2003) may also participate. Our

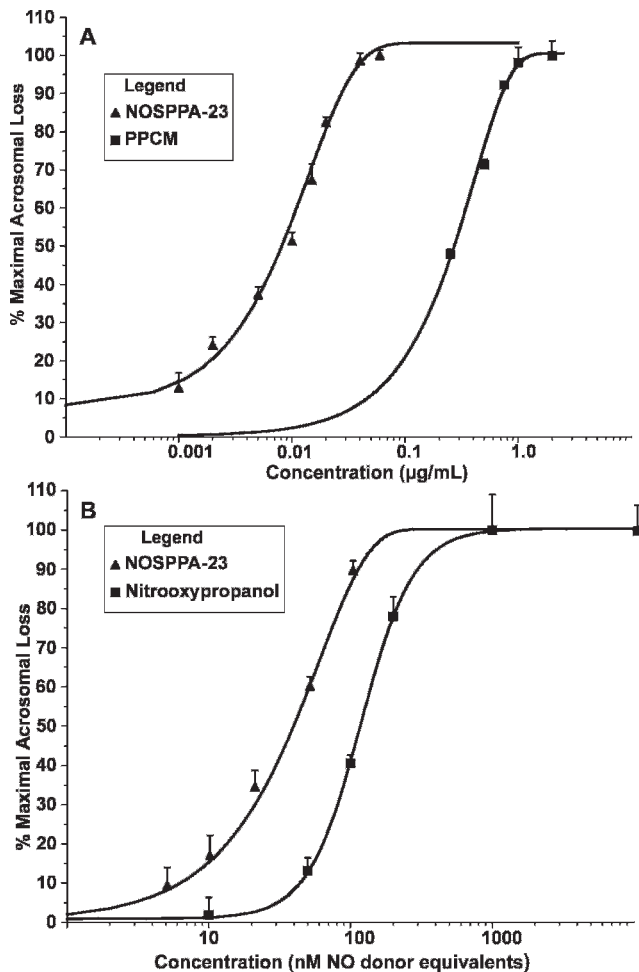


Figure 8. Acrosomal loss (AL) in response to nitric oxide donor-substituted poly-acidic polymer (NOSPPA)-23 compared with that induced by parent compounds, previously designated sulfuric acid-modified mandelic acid (PPCM) and nitrooxypropanol. (A) Either NOSPPA-23 or PPCM was added at the indicated concentrations to washed spermatozoa with added  $\text{Ca}^{2+}$ . After 15 minutes, sperm were fixed and stained, and acrosomal status was visualized. Error bars are 90% confidence limits. Curves were fit to the data with TableCurve 2D curve-fitting software, and were used to estimate concentration of test agent required to produce 50% maximal AL ( $\text{ED}_{50}$ ). The  $\text{ED}_{50}$  of PPCM in  $\text{Ca}^{2+}$ -sufficient medium is 250 ng/mL (approximately 167 nM); the  $\text{ED}_{50}$  of NOSPPA-23 in  $\text{Ca}^{2+}$ -sufficient medium is 8.1 ng/mL (approximately 4.8 nM). (B) Either NOSPPA-23 or nitrooxypropanol was added at the indicated concentrations to washed spermatozoa that contained no added  $\text{Ca}^{2+}$ . AL was determined and presented as in (A). Concentrations are expressed as nM nitric oxide (NO) donor equivalents of each compound. The  $\text{ED}_{50}$  of NO donor equivalent in NOSPPA-23 is 38.7 nM (27 ng/mL NOSPPA-23). The  $\text{ED}_{50}$  of the parent NO donor nitrooxypropanol is 120 nM.

objective was to determine whether PAL is mediated by pathways common to those mediating the physiological acrosome reaction.

#### *eNOS Is a Transduction Element Leading to PAL*

PAL requires NOS activity. Based on the activities of several NOS inhibitors (Figure 1), we conclude that

PAL is mediated by NO produced by eNOS, or an eNOS-like isoform of NOS.

NO is produced by different isoforms of NOS (Knowles and Moncada, 1994). eNOS and nNOS have been identified in spermatozoa by immunohistochemistry (O'Bryan et al, 1998) and Western blotting (Revelli et al, 1999). iNOS may also be present in spermatozoa (Balercia et al, 2004). Involvement of eNOS in PAL is consistent with its localization to the postacrosomal region and equatorial segment (O'Bryan et al, 1998).

The nonselective NOS inhibitor,  $\text{N}^{\text{G}}$ -nitro-L-arginine, is the most effective at inducing AL when added alone in the presence of added  $\text{Ca}^{2+}$ . No effect is observed when  $\text{Ca}^{2+}$  is not added (data not shown). The nNOS inhibitor S-methylthiocitrulline is without effect on PAL or on AL when added alone, either with or without added  $\text{Ca}^{2+}$  (Figure 1), suggesting that nNOS does not contribute substantially either to PAL or to resting NO levels.

#### *Soluble Guanylate Cyclase Is a Transduction Element Leading to PAL*

PAL is inhibited by guanylate cyclase inhibitors LY83583 and ODQ (Figure 2). LY83583 inhibits soluble and particulate (receptor-linked) guanylate cyclases (Mulsch et al, 1988) and inhibits hANP-induced AL (Anderson et al, 1994). hANP exerts its effect through the particulate enzyme (Anand-Srivastava and Trachte, 1993). The soluble guanylate cyclase inhibitor ODQ (Garthwaite et al, 1995) has no effect on hANP-induced AL (Figure 2). From the inhibitor data, PPCM could be acting through particulate, as well as soluble, guanylate cyclase. Particulate enzyme involvement would be supported by antagonism between PPCM and hANP as AL stimuli. However, hANP and PPCM induce AL by independent mechanisms ("Results, PAL Requires Soluble Guanylate Cyclase"). Therefore, PAL is likely mediated by activation of only soluble guanylate cyclase.

#### *cGK-II Is a Transduction Element Leading to PAL*

There are 2 isoforms of cGK, cGK-I and cGK-II. cGK-I exists in 2 isoforms (cGKI- $\alpha$  and - $\beta$ ; Smolenski et al, 1998). Failure of inhibitors that are selective for isoforms of cGK-I to inhibit PAL (Table 1) argues against involvement of the I isoform. Spermatozoa from cGK-I-deficient knockout mice are fertile and can undergo "spontaneous" acrosome reaction (Hedlund et al, 2000), suggesting that cGK-I plays little, if any, role in this aspect of sperm function. AL is induced by NO that is likely produced/released at or near the plasma membrane in response to PPCM. This favors involvement of the membrane-bound (Ruth, 1999; Hofmann et al, 2004) cGK-II for NO production responsible for PAL.

### *Resting Level of cGMP as a Determinant of Acrosomal Status*

This study extends our early work on induction of AL by agents that lower resting cGMP levels. LY83583, by itself, promotes AL in a  $\text{Ca}^{2+}$ -dependent manner (Anderson et al, 1995). Our observation with LY83583 has been extended to include induction of AL by compounds that inhibit different elements of the NO/cGMP/cGK pathway. Induction of  $\text{Ca}^{2+}$ -dependent AL by these agents is inhibited by the L-type ( $\text{Ca}_v$  1.x)  $\text{Ca}^{2+}$  channel blocker nifedipine (Bean, 1989; Table 4). These findings support our earlier contention that basal cGMP levels exert an inhibitory effect on calcium channel activity in spermatozoa, as they do in other tissue (Keef et al, 2001), and exert control over acrosomal status.

Interestingly, the cGK-I inhibitor cGMPSRp-8-Br-PET-cGMPS, added alone, is a stimulus of AL in the presence of  $\text{Ca}^{2+}$ . Reasons for this effect are not clear. Our data with other cGK-I inhibitors, and immunologic work by others (Willipinski-Stapelfeldt et al, 2004), suggest that cGK-I is not active in human spermatozoa. cGMPSRp-8-Br-PET-cGMPS is also an inhibitor of cGMP-specific PDE-5 (Butt et al, 1995). AL induced by this inhibitor may be caused by an increase in cGMP, secondary to inhibition of PDE-5.

Biphasic activity of cGMP such as seen in this study (ie, induction of AL by either an increase or a decrease in cGMP) has been observed in other cells. Elevated cGMP directly activates  $\text{Ca}^{2+}$  channels (Kaupp, 1991), but inhibits  $\text{Ca}^{2+}$  channels via cKG activation (Hofmann et al, 1992).

### *Importance of NO in Spermatozoal Function*

NO exerts several effects on spermatozoa, including induction of acrosome reaction (Kameshwari et al, 2003), increased cGMP (Revelli et al, 2001) and cAMP (Herrero et al, 2000), and increased protein tyrosine phosphorylation (Kameshwari et al, 2003). Excessive NO production may cause premature AL, leading to infertility (Herrero and Gagnon, 2001). However, spermatozoa have resting levels of NO that may contribute to cGMP levels in noncapacitated spermatozoa (Revelli et al, 2002) and thus preserve an intact acrosome.

### *PAL and AL Induced by NO Donors Occur by Different Pathways*

PAL, although mediated by NO, differs from AL induced by NO donors (eg, SNAP). AL induced by SNAP or other NO donors is not inhibited by cGK inhibitors (Tables 1 and 3), regardless of the mechanism of NO release. SIN-1 releases NO spontaneously by an oxygen-dependent mechanism (Feelisch et al, 1989); 4-phenyl-3-furoxan carbonitrile release of NO is thiol-

activated and NO release by nitroprusside depends on redox activation (Ferioli et al, 1995); release of NO from SNAP is spontaneous (Noack and Feelisch, 1991), but is also dependent on other factors, including cell membrane components (Kowaluk and Fung, 1990).

PAL, but not SNAL, depends on added  $\text{Ca}^{2+}$  ("Results;" Anderson et al, 2006). This difference is not unexpected, assuming that  $\text{Ca}^{2+}$  entry promoted by PPCM occurs upstream from NO formation. SNAL, but not PAL, is sensitive to PKA inhibition by KT5720 (Table 5, Figure 5). Both PAL and SNAL are inhibited by ODQ (Figure 2; Table 5), strongly suggesting that cGMP mediates AL induced by both agents.

The most common effectors of cGMP are 1) type 3 phosphodiesterase (PDE-3) inhibition, resulting in increased cAMP levels/PKA activation (Marletta, 2003); 2) cGK activation; and 3) opening of cyclic nucleotide-gated cation channels (Felix, 2005), through which  $\text{Ca}^{2+}$  can pass. None of these possibilities are completely consistent with the available data regarding SNAL.

First, inhibition of SNAL by ODQ and inhibition of SNAL by KT5720 appear to be independent (Figure 5). If cGMP were acting through PKA activation (secondary to increased cAMP levels or to crossover activation of PKA by cGMP; Keef et al, 2001), then the effect of PKA inhibition would be influenced by the extent to which cGMP was produced (affected by guanylate cyclase inhibitors, such as ODQ). An interactive effect between ODQ and KT5720 should exist if PKA were being activated by cGMP under these conditions. This does not occur.

Second, SNAL is essentially unaffected by cGK inhibitors; it is unaffected by the cGK inhibitor KT5823 and only approximately 20% inhibited by Rp-8-pCPT-cGMPS. However, Rp-8-pCPT-cGMPS also inhibits PKA at concentrations that inhibit cGK (Smolenski et al, 1998). Inhibition by this inhibitor is similar to inhibition by the PKA inhibitor KT5720 (24%; Table 1; Figure 5), suggesting that its effect may be attributable to PKA inhibition.

Third, SNAL does not require addition of  $\text{Ca}^{2+}$ . This minimizes the possibility that SNAP is acting through gated  $\text{Ca}^{2+}$  channels. Based on inhibition by ODQ, approximately 80% of AL induced by SNAP (and possibly other NO donors) is dependent on cGMP, which likely acts on an effector other than cGK, gated  $\text{Ca}^{2+}$  channels, or PDE-3.

### *PKA Is a Transduction Element Leading to SNAL*

The inhibitor response profile of SNAL is similar to that observed in other tissues treated with NO donors. Vila-Petroff et al (1999) reported an increase in adenylate cyclase activity in myocytes in response to SNAP, and

showed that inhibition of SNAP-induced contractile response by ODQ and the PKA inhibitor Rp-8CPT-cAMPS are independent, thus separating increased cGMP levels from PKA activation. A direct effect of NO on adenylyl cyclase is suggested.

Results from combined addition of KT5720 and ODQ (Figure 5) suggest that these agents inhibit SNAL independently. This finding, together with less than complete inhibition of SNAL by ODQ, is consistent with other work suggesting that NO may have a direct effect on adenylyl cyclase independent of increased cGMP (Vila-Petroff et al, 1999; Herrero and Gagnon, 2001).

KT5720 inhibits SNAL by only approximately 24% at a concentration that is nearly 20-fold higher than its  $K_i$  (Kase et al, 1987) for PKA (a concentration that remains selective for PKA; see Nakanishi, 1989). This concentration completely inhibits AL induced by dbcAMP (Figure 5). Higher inhibition of SNAL would be expected if all activity caused by cGMP were mediated by PKA activation. Similarly, not all SNAL can be explained by changes in cGMP. ODQ inhibits SNAL by only 80% at a concentration approximately 10-fold greater than its  $K_i$  for guanylate cyclase. NO and cGMP produced in the same cell may exert actions through activation of separate transduction pathways (Hofmann et al, 2006).

#### *Protein Tyrosine Phosphorylation Is a Transduction Element Leading to SNAL but not to PAL*

Tyrosine phosphorylation is increased by NO donors (Herrero and Gagnon, 2001). This is consistent with our observation that genistein, a PTK inhibitor (Hidaka and Kobayashi, 1992), inhibits SNAL. However, PAL is unaffected by genistein (Figure 6; Table 5). This might be explained by high concentrations (2  $\mu$ M) of cGMP required to increase tyrosine phosphorylation in human spermatozoa (Willipinski-Stapelfeldt et al, 2004). NO (and by inference, cGMP) produced by SNAP is much higher than that produced by PPCM (Table 2).

#### *Pathway Leading to NO-Induced AL May Depend on Location and Concentration of NO*

The pathway activated in response to NO is likely determined by the concentration and intracellular location of NO. NO production in response to PPCM is relatively low (Table 2) and likely occurs at or near the site of initial interaction of PPCM with the plasma membrane. NO production is mediated by eNOS (Figure 1), a particulate or membrane form of the enzyme. In contrast, NO produced from SNAP occurs at much higher levels (Table 2). NO release from SNAP likely occurs in a more diffuse fashion, allowing for the opportunity to interact with a wider array of intracel-

lular effector elements. However, relatively localized release of NO from NOSPPA-23 resulting from a vector-mediated delivery could explain the greatly increased efficacy of this compound as a stimulus of AL.

#### *Mechanism-Based Design of Compound as a More Efficacious Stimulus of AL*

Discovery of better second-generation compounds can be approached through structural modifications of the parent compound, based on its intracellular mechanism(s) of action. The proposed intracellular mechanism of PAL formed the basis for the design and synthesis of NOSPPA-23.

We propose that the PPCM moiety of NOSPPA-23 acts as a vector for the targeted delivery of NO. As part of the delivery system, the sperm-selective properties of the vector are enhanced. NOSPPA-23 was predicted to: 1) induce AL in the presence or absence of  $Ca^{2+}$ , because of the  $Ca^{2+}$ -independent actions of the NO donor; 2) induce AL in the absence of  $Ca^{2+}$  at a lower concentration of NO donor equivalents than required for NO donor alone, because the source of NO is directed to the surface of the spermatozoon by the PPCM moiety; and 3) be synergistic compared with either NO donor or PPCM alone in the presence of  $Ca^{2+}$ , because of different, but related, mechanisms by which the parent compounds induce AL (ie, an interactive effect is predicted).

Data presented in Figures 7 and 8 substantiate these predictions. NOSPPA-23, a prototype of an intramolecular combinatorial approach to an improved sperm-active compound, induces premature AL in either the presence or absence of added extracellular  $Ca^{2+}$ . The response, either in the presence or absence of  $Ca^{2+}$ , is synergistic to the predicted response to the NO donor and PPCM added in combination. The  $ED_{50}$  of NOSPPA-23 as a stimulus of AL in the presence of added extracellular  $Ca^{2+}$  (Figure 8, panel A) is substantially less than that of the NO donor from which it is derived, and 35-fold less than that of PPCM.

NO has known antimicrobial activity (Fang, 1997). Preliminary studies with NOSPPA-23 as an agent against HIV-1 and HSV-2 (Anderson, unpublished) suggest that covalent attachment of an NO donor to PPCM represents a viable approach to producing a contraceptive microbicide with improved biological activity.

The present study clearly shows synergistic activity of NOSPPA-23 regarding its ability to induce premature AL. NOSPPA-23 is only a single prototype of this new class of compounds, and may not represent the optimum vector (represented here by PPCM), NO donor (nitrooxypropanol, in this instance), or degree

of substitution (where possible; 23% in the present study). Further work with this vector-assisted system for targeted microbicide enhancement is highly warranted, in which these variables are considered, and the range of in vitro and in vivo biological activities is examined.

## References

- Alliance for Microbicide Development. Continuing coverage of closure of cellulose sulfate trials. *News Dig Alliance Microbicide Dev.* 2007; 8(5):2–5.
- Anand-Srivastava MB, Trachte GJ. Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmacol Rev.* 1993;45:455–497.
- 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;13:398–408.
- Anderson RA, Feathergill KA, Drisdell 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.
- Anderson RA, Feathergill KA, Rawlins RG, Mack SR, Zaneveld LJD. Atrial natriuretic peptide: a chemoattractant of human spermatozoa by a guanylate cyclase-dependent pathway. *Mol Reprod Dev.* 1995;40:371–378.
- Anderson RA, Feathergill KA, Waller DP, Zaneveld LJD. SAMMA induces premature human acrosomal loss by Ca<sup>2+</sup> signaling dysregulation. *J Androl.* 2006;27:568–577.
- Balercia G, Moretti S, Vignini A, Magagnini M, Mantero F, Boscaro M, Ricciardo-Lamonica G, Mazzanti L. Role of nitric oxide concentrations on human sperm motility. *J Androl.* 2004;25:245–249.
- BBC News. Merck abandons HIV vaccine trials. <http://news.bbc.co.uk/1/hi/health/7007734.stm>. Published September 21, 2007. Accessed January 22, 2008.
- Bean BP. Classes of calcium channels in vertebrate cells. *Ann Rev Physiol.* 1989;51:367–384.
- Benoff S. Modelling human sperm-egg interactions in vitro: signal transduction pathways regulating the acrosome reaction. *Mol Hum Reprod.* 1998;4:453–471.
- 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, CA: WH Freeman;1971:86–116.
- Breitbart H, Spungin B. The biochemistry of the acrosome reaction. *Mol Hum Reprod.* 1997;3:195–202.
- Butt E, Eigenthaler M, Genieser HG. (Rp)-8-pCPT-cGMPS, a novel cGMP-dependent protein kinase inhibitor. *Eur J Pharmacol.* 1994; 269:265–268.
- Butt E, Pohler D, Genieser HG, Huggins JP, Bucher B. Inhibition of cyclic GMP-dependent protein kinase-mediated effects by (Rp)-8-bromo-PET-cyclic GMPS. *Brit J Pharmacol.* 1995;116:3110–3116.
- Chang TL, Teleshova N, Rapista A, Paluch M, Anderson RA, Waller DP, Zaneveld LJD, Granelli-Piperno A, Klotman ME. SAMMA, a mandelic acid condensation polymer, inhibits dendritic cell-mediated HIV transmission. *FEBS Lett.* 2007;581:4596–4602.
- Cheshenko N, Keller MJ, MasCasullo V, Jarvis GA, Cheng H, John M, Li J-H, Hogarty K, Anderson RA, Waller DP, Zaneveld LJD, Proffy AT, Klotman ME, Herold BC. Candidate topical microbicides bind herpes simplex virus glycoprotein B and prevent viral entry and cell-to-cell spread. *Antimicrob Agents Chemother.* 2004;48:2025–2036.
- D’Cruz OJ, Uckun FM. Clinical development of microbicides for the prevention of HIV infection. *Curr Pharm Des.* 2004;10:315–336.
- European AIDS Treatment Group. Cellegy announces results of data monitoring committee review of Savvy Nigeria phase 3 HIV prevention trial. <http://www.eatg.org/news/newsitem.php?id=210>. Published August 28, 2006. Accessed January 22, 2008.
- Fang FC. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest.* 1997;99:2818–2825.
- Feelisch M, Ostrowski J, Noack E. On the mechanism of NO release from sydnonimines. *J Cardiovasc Pharmacol.* 1989;14(suppl 11): S13–S22.
- Felix R. Molecular physiology and pathology of Ca<sup>2+</sup>-conducting channels in the plasma membrane of mammalian sperm. *Reproduction.* 2005;129:251–262.
- Feroli R, Folco GC, Ferretti C, Gasco AM, Medana C, Fruttero R, Civelli M, Gasco A. A new class of furoxan derivatives as NO donors: mechanism of action and biological activity. *J Pharmacol.* 1995;114:816–820.
- Furfine ES, Harmon MF, Paith JE, Knowles RG, Salter M, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA, Garvey EP. Potent and selective inhibition of human nitric oxide synthases. *J Biol Chem.* 1994;269:26677–26683.
- Gambaryan S, Wagner C, Smolenski A, Walter U, Poller W, Haase W, Kurtz A, Lohmann SM. Endogenous or overexpressed cGMP-dependent protein kinases inhibit cAMP-dependent renin release from rat isolated perfused kidney, microdissected glomeruli, and isolated juxtaglomerular cells. *Proc Natl Acad Sci U S A.* 1996;95: 9003–9008.
- Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol Pharmacol.* 1995;48:184–188.
- Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJR, Knowles RG. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J Biol Chem.* 1997;272:4959–4963.
- Guraya SS. Cellular and molecular biology of capacitation and acrosome reaction in spermatozoa. *Int Rev Cytol.* 2000;199:1–64.
- Hedlund P, Aszodi A, Pfeifer A, Alm P, Hofmann F, Ahmad M, Fassler R, Andersson K-E. Erectile dysfunction in cyclic GMP-dependent kinase I-deficient mice. *Proc Natl Acad Sci U S A.* 2000; 97:2349–2354.
- Henshaw SK. Unintended pregnancy in the United States. *Fam Plann Perspect.* 1998;30:24–29.
- Herrero MB, Chatterjee S, Lefievre L, De Lamirande E, Gagnon C. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. *Free Rad Biol Med.* 2000;29: 522–536.
- Herrero MB, Gagnon C. Nitric oxide: a novel mediator of sperm function. *J Androl.* 2001;22:349–356.
- Hidaka H, Kobayashi R. Pharmacology of protein kinase inhibitors. *Ann Rev Pharmacol Toxicol.* 1992;32:377–397.
- Hofmann F, Ammendola A, Schlossmann J. Rising behind NO: cGMP-dependent protein kinases. *J Cell Sci.* 2004;113:1671–1676.
- Hofmann F, Dostmann W, Keilbach A, Landgraf W, Ruth P. Structure and physiological role of cGMP-dependent protein kinase. *Biochim Biophys Acta.* 1992;1135:51–60.
- Hofmann F, Feil R, Kleppisch T, Schlossmann J. Function of cGMP-dependent protein kinases as revealed by gene deletion. *Physiol Rev.* 2006;86:1–23.
- Kameshwari DB, Siva AB, Shivaji S. Inhibition of in vitro capacitation of hamster spermatozoa by nitric oxide synthase inhibitors. *Cell Mol Biol.* 2003;49:421–428.

- Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun*. 1987;142:436-440.
- Kaupp UB. The cyclic nucleotide-gated channels of vertebrate photoreceptors and olfactory epithelium. *Trends Neurosci*. 1991;14:150-157.
- Keef KD, Hume JR, Zhong J. Regulation of cardiac and smooth muscle Ca(2+) channels (Ca(V)1.2a,b) by protein kinases. *Am J Physiol Cell Physiol*. 2001;281:C1743-C1756.
- Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J*. 1994;298:249-258.
- Kobori H, Miyazaki S, Kuwabara Y. Characterization of intracellular Ca<sup>2+</sup> increase in response to progesterone and cyclic nucleotides in mouse spermatozoa. *Biol Reprod*. 2000;63:113-120.
- Kowaluk EA, Fung H-L. Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by S-nitrosothiols. *J Pharmacol Exp Ther*. 1990;255:1256-1264.
- Lirri E. Doctors speak out on failed AIDS trials. Monitor Publications, Ltd. <http://allafrica.com/stories/200708180112.html>. Published August 17, 2007. Accessed January 22, 2008.
- Liu R-Z, Na W-L, Zhang H-G, Lin Z-Y, Xue B-G, Xu Z-G. Assessment of released acrosin activity as a measurement of the sperm acrosome reaction. *Asian J Androl*. 2008;10:236-242.
- Mantell JE, Myer L, Carballo-Dieguez A, Stein Z, Ramjee G, Morar NS, Harrison PF. Microbicide acceptability research: current approaches and future directions. *Soc Sci Med*. 2005;60:319-330.
- Marletta MA. Cellular signaling with nitric oxide. In: *Calbiochem Signal Transduction Technical Resource, 2003-2004*. San Diego, CA: EMD Biosciences; 2003:579-581.
- Mishra M, Wagner MB, Wang Y, Joyner RW, Kumar R. Expression of cGMP-dependent protein kinase in human atrium. *J Mol Cell Cardiol*. 2001;33:1467-1476.
- Mulsh A, Busse R, Liebau S, Forstermann U. LY 83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. *J Pharmacol Exp Ther*. 1988;247:283-288.
- Nakanishi S. K-252 derivatives—K252a, K252b, KT5720, KT5962, KT5823. *Seitaino-Kagaku*. 1989;40:364-365.
- Noack E, Feelisch M. Molecular mechanisms of nitrovasodilator bioactivation. *Basic Res Cardiol*. 1991;86(suppl 2):37-50.
- O'Bryan MK, Zini A, Cheng CY, Schlegel PN. Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. *Fertil Steril*. 1998;70:1143-1147.
- Panchaud C, Singh S, Feivelson D, Darroch JE. Sexually transmitted diseases among adolescents in developed countries. *Fam Plann Perspect*. 2000;32:24-32.
- Peluso JJ, Pappalardo A. Progesterone regulates granulosa cell viability through a protein kinase G-dependent mechanism that may involve 14-3-3 $\mu$ . *Biol Reprod*. 2004;71:1870-1878.
- Piegorsch WW, Margolin BH. Quantitative methods for assessing a synergistic or potentiated genotoxic response. *Mutat Res*. 1989;216:1-8.
- Pozzoli G, Tringali G, Dello Russo C, Vairano M, Preziosi P, Navarra P. HIV-1 Gp120 protein modulates corticotropin releasing factor synthesis and release via the stimulation of its mRNA from the rat hypothalamus in vitro: involvement of inducible nitric oxide synthase. *J Neuroimmunol*. 2001;118:268-276.
- Rathi R, Colenbrander B, Stout TAE, Bevers MM, Gadella BM. Progesterone induces acrosome reaction in stallion spermatozoa via a protein tyrosine kinase dependent pathway. *Mol Reprod Dev*. 2003;120-128.
- Revelli A, Costamagna C, Moffa F, Aldieri E, Ochetti S, Bosia A, Massobrio M, Lindblom B, Ghigo D. Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. *Biol Reprod*. 2001;64:1708-1712.
- Revelli A, Ghigo D, Moffa F, Massobrio M, Tur-Kaspa H. Guanylate cyclase activity and sperm function. *Endocr Rev*. 2002;23:484-494.
- Revelli A, Soldati G, Costamagna C, Pellerey O, Aldieri E, Massobrio M, Bosia A, Ghigo D. Follicular fluid proteins stimulate nitric oxide (NO) synthesis in human sperm: a possible role for NO in acrosomal reaction. *J Cell Physiol*. 1999;178:85-92.
- Roberts JD, Caserio MC. Acid-catalyzed alcohol dehydration; reactions involving the C-O bond of alcohols. In: *Basic Principles of Organic Chemistry*. New York, NY: WA Benjamin Inc; 1964a:391-400.
- Roberts JD, Caserio MC. Electrophilic substitution. In: *Basic Principles of Organic Chemistry*. New York, NY: WA Benjamin Inc; 1964b:797-799.
- Ruth P. Cyclic GMP-dependent protein kinases: understanding in vivo functions by gene targeting. *Pharmacol Ther*. 1999;82:355-372.
- Schwede F, Maronde E, Genieser H-G, Jastorff B. Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharmacol Ther*. 2000;87:199-226.
- Smolenski A, Burkhardt M, Eigenthaler M, Butt E, Gambaryan S, Lohmann SM, Walter U. Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects. *Naunyn-Schmiedeberg's Arch Pharmacol*. 1998;358:134-139.
- Sokal R, Rohlf FJ. Analysis of frequencies. In: *Biometry*. 2nd ed. San Francisco, CA: WH Freeman; 1981a:691-778.
- Sokal R, Rohlf FJ. Assumptions of analysis of variance. In: *Biometry*. 2nd ed. San Francisco, CA: WH Freeman; 1981b:400-453.
- Szabo C, Southan GJ, Thiemeermann C. Beneficial effects and improved survival in rodent models of septic shock with S-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. *Pharmacology*. 1994;91:12472-12476.
- Tischkau SA, Mitchell JW, Barnes JA, Gillette MU. Protein kinase G type II is required for night-to-day progression of the mammalian circadian clock. *Neuron*. 2004;43:539-549.
- Trussell J. The cost of unintended pregnancy in the United States. *Contraception*. 2007;75:168-170.
- UNAIDS. AIDS epidemic update: December 2007. Geneva, Switzerland: UNAIDS; 2007. UNAIDS/07.27E/JC1322E.
- Vaupel DB, Kimes AS, London ED. Nitric oxide synthase inhibitors. Preclinical studies of potential use for treatment of opioid withdrawal. *Neuropsychopharmacology*. 1995;13:315-322.
- Vila-Petroff MG, Younes A, Egan J, Lakatta EG, Sollott SJ. Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ Res*. 1999;84:1020-1031.
- Ward M, Yu B, Wyatt V, Griffith J, Craft T, Neurath AR, Strick N, Li Y-Y, Wertz DL, Pojman JA, Lowe AB. Anti-HIV-1 activity of poly(mandelic acid) derivatives. *Biomacromolecules*. 2008;8:3308-3316.
- Whitesell JK, Pojman JA. Homochiral and heterochiral polyesters: polymers derived from mandelic acid. *Chem Mater*. 1990;2:248-254.
- Willipinski-Stapelfeldt B, Lubstedt J, Stelter S, Vogt K, Mukhopadhyay AK, Muller D. Comparative analysis between cyclic GMP and cyclic AMP signalling in human sperm. *Mol Hum Reprod*. 2004;10:543-552.
- 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.
- Zaneveld LJD, Anderson RA, Diao XH, Young PR, Waller DP, Garg S, Chany CJ, Kim DSHL, inventors; Rush Presbyterian, St Luke's Medical Center, assignee. Method for preventing sexually transmitted diseases. US patent 5,932,619. August 3, 1999.