# Tissue-specific PKA inhibition using a chemical genetic approach and its application to studies on sperm capacitation

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**Studies on cAMP signaling and protein kinase A (PKA) function *in vivo* are limited by the lack of highly specific inhibitors that can be used in primary cell culture and whole animals. Previously we reported that a mutation in the ATP binding pocket of a catalytic subunit (C$\alpha$) of PKA confers sensitivity to the pyrazolo[3,4-*d*]pyrimidine inhibitor, 1NM-PP1. We have now engineered the mouse *Pkraca* gene such that after Cre-mediated recombination *in vivo*, the C$\alpha$M120A mutant protein is expressed and the wild-type C$\alpha$ is turned off. We demonstrate the utility of this approach by examining the requirement for PKA activity during capacitation of sperm from mice that express C$\alpha$M120A mutant protein. For C$\alpha$M120A sperm, 10 $\mu$M of 1NM-PP1 prevented PKA-dependent phosphorylation and the activation of motility that are both rapidly (<90 s) evoked by the $HCO_3^-$ anion. A continuous (90 min) inhibition with 10 $\mu$M of 1NM-PP1 prevented the protein tyrosine phosphorylation of late-stage capacitation. Delayed application of 1NM-PP1 demonstrated that PKA activity was required for at least the initial 30 min of capacitation to produce subsequent protein tyrosine phosphorylation. Acute application of 1NM-PP1 rapidly slowed the accelerated beat of activated motility but did not affect the established waveform asymmetry of hyperactivated sperm. Our results demonstrate that PKA in C$\alpha$M120A mutant sperm is rapidly and reversibly inhibited by 1NM-PP1 and that this blockade has selective and time-dependent effects on multiple aspects of capacitation. The conditional C$\alpha$M120A-expressing mouse lines will be valuable tools for studying PKA function *in vivo*.**

cAMP | mouse genetics | protein phosphorylation | male fertility | hyperactivation

Mammalian sperm undergo a series of maturational changes in the female reproductive tract and this process, termed capacitation, is essential for subsequent fertilization of the egg (1). Although multiple signal transduction pathways are involved in capacitation, a central role for cAMP as a regulatory mediator is underscored by the male-infertility phenotypes of mice that carry targeted disruptions of the sperm-specific C$\alpha$2 catalytic subunit of protein kinase A (PKA) or of the atypical $HCO_3^-$-stimulated sperm adenylyl cyclase, SACY (formerly known as sAC). Studies of the mutant sperm that lack C$\alpha$2 (2) or SACY (3–5) have provided definitive evidence that neither protein is required for the initiation of motility or for maintenance of a slow basal flagellar beat. In contrast, both C$\alpha$2 and SACY are required for the acceleration of the flagellar beat that characterizes the rapid activation of motility by the $HCO_3^-$ anion, and for $HCO_3^-$ to rapidly facilitate evoked entry of $Ca^{2+}$. The capacitation sequence that prepares sperm for fertilization includes two additional components that also are $HCO_3^-$ dependent but delayed in onset. These delayed increases in protein tyrosine phosphorylation and hyperactivation of motility do not occur in the C$\alpha$2 or SACY null sperm. However, it has remained unclear whether cAMP and PKA are required continuously for these changes to develop or whether the initial activation of SACY and PKA sets in motion events that culminate in tyrosine phosphorylation and sperm hyperactivation without a continuing role for PKA.

Acute application of pharmacological inhibitors of PKA provides an approach to study the temporal requirements for PKA activity, but the specificity of the available competitive inhibitors of ATP binding is incomplete (6–8). Chemical genetics provides an alternative and powerful method for monospecific kinase inhibition in which the target kinase is replaced by an analog-sensitive variant whose enlarged ATP binding pocket accepts bulky inhibitors (9, 10). Our past work established that an M120A point mutation in the C$\alpha$ subunit of PKA confers sensitivity to inhibition by derivatives of pyrazolo[3,4-*d*]pyrimidine (11). In JEG-3 cells transfected with C$\alpha$M120A, the $IC_{50}$ for 1NM-PP1 was $\approx$150 nM. The mutant C$\alpha$M120A also has been stably expressed in *Escherichia coli* to provide a tool to identify direct targets of PKA *in vitro* (12). We have now created a "knockin" mouse that can be switched from expressing a wild-type C$\alpha$ to the mutant C$\alpha$M120A by the action of Cre recombinase. This mouse allows a chemical genetic analysis of the role for the C$\alpha$ subunit of PKA signaling in any cell type that can be targeted by a transgenic Cre recombinase. Although the 1NM-PP1 inhibitor has high specificity for the genetically mutated kinase, this approach also allows us to control for off-target effects of the inhibitor by testing it on wild-type mouse cells.

The mutant mice generated in this study provide a novel approach to *in vivo* studies of signal transduction involving the PKA pathway. Crossing the C$\alpha$LoxM120A line of mice with any cell-type-specific Cre recombinase transgenic converts the C$\alpha$ isoform in that tissue from a wild-type protein to the analog-sensitive C$\alpha$M120A. The analog-sensitive mutant kinase will then be inhibited specifically by administration of 1NM-PP1 to the whole animal. In the present study we have used a Cre recombinase transgenic to activate the mutation in all mouse tissues and then used isolated sperm to examine the requirement for PKA activity during the process of capacitation. Our results with analog-sensitive sperm demonstrate that PKA activity must be maintained for >30 min to initiate late-stage tyrosine phosphorylation. However, inhibition of PKA after capacitation has progressed does not rapidly reverse the established protein tyrosine phosphorylation and the flagellar waveform asymmetry of hyperactivation.

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The authors declare no conflict of interest.

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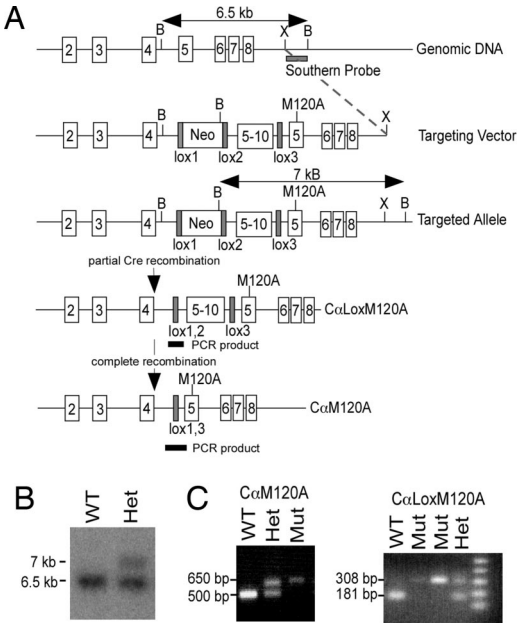


**Fig. 1.** Knockin of the CαM120A mutation. (*A*) Schematic diagram of the targeting vector that specified the CαM120A mutation in exon 5 of the *Prkaca* (Cα) gene. A NEO cassette was flanked by two loxP sites and a downstream minigene containing the wild-type form of exons 5–10. The NEO cassette was removed by partial recombination using EIIa-Cre transgenic animals to give CαLoxM120A mice. The wild-type minigene, also flanked by a downstream loxP site, was removed by complete Cre-mediated excision after mating with EIIa-Cre transgenic mice. Excision of NEO and the minigene by Cre caused expression of the CαM120A mutation. (*B*) Southern blot analysis of BamHI-digested genomic tail DNA using an external DNA probe identified correctly targeted ES cell clones that were used for microinjection. The mutant allele yielded a 7.0-kb band and the wild-type allele generated a 6.5-kb band. (*C*) PCR analysis of genomic DNA from the offspring of mice that carried the targeted allele and also an EIIa-Cre recombinase transgene. CαM120A is the product of complete recombination and was genotyped with a PCR product across the lox1,3 site. CαLoxM120A is the product of partial recombination between lox1 and lox2 and was genotyped by a PCR across the lox1,2 site.

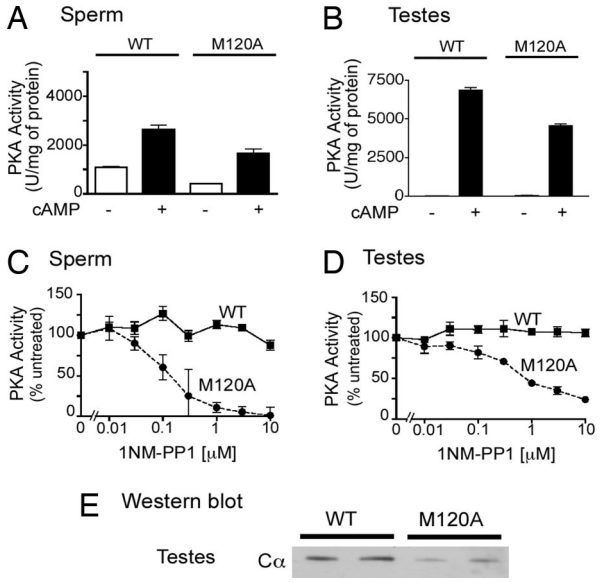


**Fig. 2.** 1NM-PP1 inhibits the mutant CαM120A form of PKA in testis and sperm. PKA activity in homogenates of sperm (*A* and *C*) and testis (*B* and *D*) from wild-type (WT) and mutant (M120A) mice assayed in the presence or absence of 25 μM of cAMP. In *C* and *D* assays also contained the indicated concentrations of 1NM-PP1. Averaged values (± SEM) from triplicate assays are shown. PKA activity is expressed as units (pmoles of phosphate incorporated per min) per mg of protein. (*E*) Western blot analysis for Cα and RIIα proteins in extracts from the testes of WT or M120A homozygotes. Twenty micrograms of protein were loaded per lane.

## Results

**Generation of CαM120A and CαLoxM120A Mice.** To express the modified Cα gene, we generated a targeting vector that contained the neomycin phosphotransferase gene (NEO), a Cα minigene (exons 5–10), and a mutant form of exon 5 with the CαM120A mutation (Fig. 1*A*). Note that both NEO and the wild-type minigene were located upstream of the CαM120A mutation and flanked with loxP sites. By this design the wild-type gene was expressed before and the CαM120A mutation was expressed only after Cre recombinase removed the NEO cassette and the wild-type minigene. Correctly targeted ES cell clones were identified by Southern blot analysis using a genomic DNA probe located outside of the targeting vector (Fig. 1*B*). Targeted ES cells were injected into wild-type blastocysts to produce chimeric mice that carried the nonrecombined targeted allele in the germ line. Chimeric males were mated with wild-type females to produce heterozygote founders carrying the targeted allele. These heterozygote animals, also identified by Southern blot, were mated with EIIa-Cre transgenic animals (13) to generate mice that carried the fully recombined CαM120A allele as identified by PCR analysis specific for recombination between lox1 and lox3 or the partially recombined CαLoxM120A allele identified by PCR specific for recombination between lox1 and lox2 (Fig. 1*C*). All of our studies on sperm were performed with mice expressing the fully recombined CαM120A allele in all tissues.

**Phenotype of CαM120A Mice.** At the age of weaning, homozygous CαM120A mutant mice were present in normal Mendelian ratios indicating that this mutation does not cause lethality. Adult male and female CαM120A mutants were fertile, viable, and exhibited no gross anatomical abnormalities. The body weight of CαM120A males was normal at 10 and 15 weeks of age. The wet weight of CαM120A testes was normal relative to testes of wild-type littermates, and CαM120A epididymides released normal numbers of "swim-out" sperm (data not shown).

**1NM-PP1 Inhibition of Mutant PKA in Testis, Sperm, and Other Tissues.** We examined basal and cAMP-stimulated PKA activity and its sensitivity to 1NM-PP1 in homogenates from wild-type and homozygous CαM120A sperm (Fig. 2*A* and *C*) and testis (Fig. 2 *B* and *D*). The total cAMP-stimulated PKA activity was reduced by ~30–40% in the CαM120A testis and sperm. A similar decrease in total PKA activity was observed in other tissues such as heart, kidney, and skeletal muscle expressing the CαM120A allele (data not shown). This reduction of PKA activity in the CαM120A-expressing tissues might be the result of decreased Cα protein or reduced enzymatic activity of the Cα mutant. To distinguish between these two possibilities, we performed Western blot analysis of testis extracts. The results (Fig. 2*E*) indicate that testis from CαM120A mutant mice contained reduced amounts of Cα protein compared to wild-type testis, suggesting that the lower PKA activity observed in CαM120A mutants is the result of a decrease in the level of the mutant protein. The ATP binding-site mutation that we created lowers the affinity of Cα for ATP (12), and it is likely that diminished ATP binding interferes with assembly of type I PKA holoenzyme and the consequent stabilization of the Cα subunit. However, we cannot rule out changes in the level of mutant Cα mRNA. We also examined the PKA activity in CαLoxM120A homozygotes that can only express Cα from the inserted minigene. PKA activity in these animals was also reduced by 30–40% in heart, kidney, and

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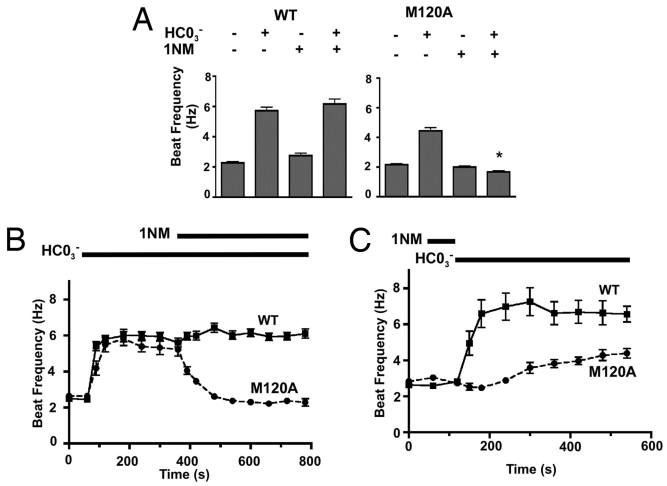


**Fig. 3.** Bicarbonate-evoked activation of sperm motility. (*A*) Averaged flagellar beat frequency was determined for wild-type (*Left*) and M120A (*Right*) sperm that were bathed in HS medium that lacked or contained 10 $\mu$M of 1NM-PP1 (1NM). Individual sperm were examined before and then again after 1 min of perfusion with the same media fortified with 15 mM of $HCO_3^-$ ($n$ = 19–39 cells. At least 9 cells were examined from each animal in 2–3 independent experiments.). *, $P < 0.05$ (M120A, $HCO_3^-$ vs. $HCO_3^-$ + 1NM-PP1). *B* and *C* compare averaged beat frequencies of wild-type (■) and M120A (●) sperm. In *B*, sperm were bathed in HS medium and sequentially perfused with HS medium containing 15 mM of $HCO_3^-$ for 5 min, then with HS containing both 10 $\mu$M of 1NM-PP1 and 15 mM of $HCO_3^-$ for 7 min ($n$ = 19–49 cells. At least 9 cells were examined from each animal in 2–3 independent experiments.). In *C*, individual sperm were sequentially perfused with HS containing 10 $\mu$M of 1NM-PP1 for 1 min, then with HS containing 15 mM of $HCO_3^-$ ($n$ = 9–15 cells from one wild-type animal, 16–67 cells from two to four M120A mutant animals). Error bars for all experiments represent standard error of the mean.

skeletal muscle (data not shown). Because the C$\alpha$ protein synthesized in this case is wild type, the lower expression is likely the result of decreased mRNA levels originating from the minigene. We found no inhibition of PKA activity in the presence of 1 $\mu$M of 1NM-PP1 in the C$\alpha$LoxM120A tissues.

The PKA activity of homogenates of wild-type testis (Fig. 2*C*) and sperm (Fig. 2*D*) was not inhibited by 1NM-PP1 at concentrations of up to 10 $\mu$M. However, the engineered C$\alpha$M120A kinase of mutant testis and sperm was inhibited with an $IC_{50}$ ≈175 nM, similar to the sensitivity found for the mutant kinase expressed in cell culture (11). PKA activity of mutant sperm was completely inhibited by 10 $\mu$M of 1NM-PP1. However ≈20% of the PKA activity in whole testis extracts was insensitive to 10 $\mu$M of 1NM-PP1. This residual activity is consistent with the observed expression of the C$\beta$ isoform in the somatic cells of the testis (14) because this isoform would not be sensitive to 1NM-PP1. Mature sperm do not express detectable levels of C$\beta$ (2).

**Activation of Sperm Motility.** We previously demonstrated that PKA is required for the increase in flagellar beat frequency that is evoked by the bicarbonate anion (2, 15). When wild-type and C$\alpha$M120A sperm (Fig. 3*A*) were bathed in HS medium alone, the slow resting beat frequencies (on average ≈2 Hz) were indistinguishable. Reexamination of the same individual cells 1 min after addition of 15 mM of $NaHCO_3$ to the perfusing medium showed that mean beat frequencies increased two- to threefold. When the wild-type or C$\alpha$M120A sperm were bathed with HS medium that also contained 10 $\mu$M of 1NM-PP1, the resting beat frequencies remained stable. When the same cells were reexamined 1 min after the further addition of 15 mM of $NaHCO_3$, the continued presence of 1NM-PP1 did not blunt the increase in beat frequency for wild-type sperm, but completely prevented the increase in C$\alpha$M120A sperm (Fig. 3*A*). These results are consistent with past work, indicating that PKA is not required for sperm to initiate and maintain motility with a slow resting beat, but is required for the cAMP-mediated increase in beat frequency that produces activated motility (2, 15).

The onset of action of 1NM-PP1 was quite rapid as shown by the decrease in flagellar beat frequency observed in the presence of $HCO_3^-$ (Fig. 3*B*). Individual wild-type and C$\alpha$M120A sperm were perfused with $HCO_3^-$-fortified medium for 5 min to maximally accelerate the flagellar beat. When 1NM-PP1 was then added to the perfusing medium, the wild-type sperm remained fully activated, whereas C$\alpha$M120A sperm rapidly ($t_{1/2}$ ≈40 s) returned to the resting beat rate of 2.5 Hz. When $HCO_3^-$ was simply removed by perfusion with bicarbonate-free medium, activated C$\alpha$M120A sperm returned to a resting beat frequency much more slowly ($t_{1/2}$ ≈200 s, data not shown), similar to the loss of motility activation observed for wild-type sperm in past work (15). This slower response to $HCO_3^-$ removal compared with the rapid onset of 1NM-PP1 inhibition reflects the time required for $HCO_3^-$ to decrease in sperm, SACY activity to return to baseline, and the phosphodiesterase-dependent degradation of the elevated cAMP to take place.

Although cAMP analogs rescue the activated motility of SACY null sperm (5, 16), rescue of activated motility has not been feasible for C$\alpha$2 null sperm. We addressed this issue by application of a selective, transient inactivation of the PKA of C$\alpha$M120A sperm followed by drug washout. Fig. 3*C* shows that for C$\alpha$M120A sperm the accelerating action of $HCO_3^-$ was slowly restored after 1NM-PP1 was removed from the perfusing medium (Fig. 3*C*). The beat rate increased twofold after 5 min of recovery.

**Sperm Hyperactivation Is Resistant to PKA Inhibition.** One of the hallmarks of late-stage capacitation is the asymmetrical flagellar waveform of hyperactivated motility. To produce a symmetrical waveform the planar flagellar wave must bend equally to each side. Fig. 4*A* shows how the angle between the initial segment of the flagellum and tangent line at distance d along the flagellum (the tangent angle, $\Theta_d$) reports the curvature at that point (yellow dot on Fig. 4*A*). The time-averaged curvature (tangent angle) then reports flagellar waveform asymmetry (15, 17). The cAMP-mediated activation of motility by $HCO_3^-$ initially speeds the beat of both wild-type and C$\alpha$M120A sperm, but the flagellar waveform remains symmetrical (Fig. 4*B*). In contrast, prolonged capacitating incubations (90 min) produce a hyperactivated motility that is characterized by an asymmetric flagellar waveform. Treatment of C$\alpha$M120A sperm with 1NM-PP1 in early stages of capacitation rapidly attenuates the accelerated beat rate (Fig. 3*B*), and chronic treatment with 1NM-PP1 throughout capacitation greatly reduces the number of sperm that display flagellar waveform asymmetry while at the same time causing a large increase in the proportion of immotile cells (data not shown). However, when C$\alpha$M120A sperm were first hyperactivated by capacitating incubations in the absence of 1NM-PP1 and then examined after a 5-min perfusion with 10 $\mu$M of 1NM-PP1, the 1NM-PP1 did not affect the flagellar asymmetry of hyperactivated C$\alpha$M120A sperm (Fig. 4*C*).

**Tyrosine Phosphorylation Susceptibility to PKA Inhibition.** An increase in tyrosine phosphorylation of sperm proteins is generally recognized as a signature of late-stage capacitation (18, 19). However, the temporal requirements for PKA activity during capacitation are not well understood. We first demonstrate that 1NM-PP1 specifically inhibits PKA substrate phosphorylation in C$\alpha$M120A sperm. As shown in Fig. 5*A*, the addition of 10 $\mu$M of 1NM-PP1 to wild-type sperm did not affect the increase in PKA-dependent phosphorylation seen after 90 s of capacitation

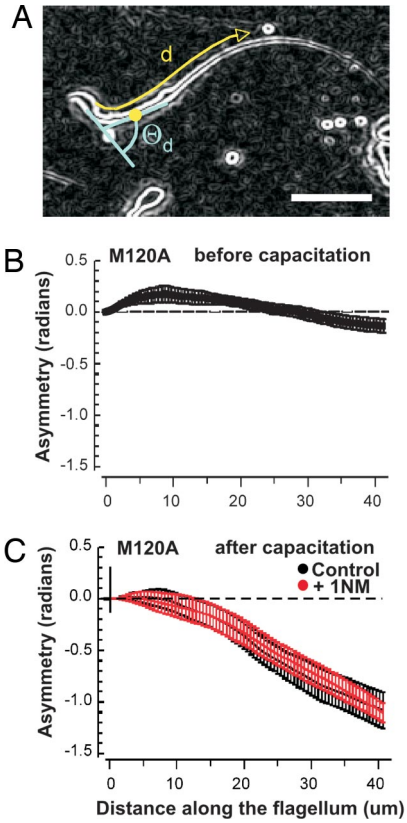


**Fig. 4.** PKA inhibition does not reverse the flagellar waveform asymmetry of hyperactivation. (*A*) Contrast-enhanced, stop-motion image of a sperm loosely tethered at the base of the head. The lines show d, the distance along the flagellum and $\Theta_d$, the tangent angle at point d (yellow dot). Scale bar, 25 $\mu$m. (*B* and *C*) Mean values of flagellar asymmetry (the time-averaged $\Theta_d$) obtained by waveform analysis of individual C$\alpha$M120A mutant sperm examined before *B* and after *C* capacitating incubation to produce hyperactivation. For *C* each cell was sampled twice, once after transfer form capacitating medium to medium HS alone (black squares and line), then again at the end of a 5-min perfusion with HS plus 10 $\mu$M of 1NM-PP1 (red circles and line) ($n$ = 11 cells from one wild-type animal, 23 cells from three M120A mutant animals).

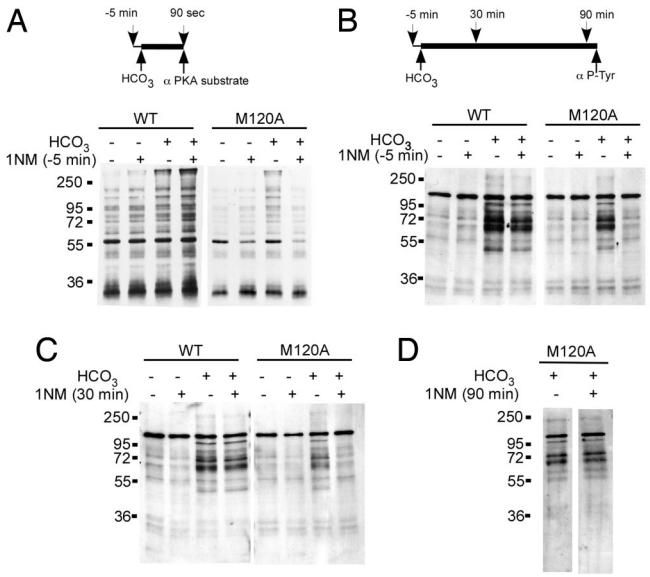


**Fig. 5.** Continuous PKA activity is required to initiate tyrosine phosphorylation during capacitating incubations. (*A*) Sperm from WT or M120A mutant mice were incubated at 37 °C in HS buffer containing 5 mg/ml of BSA. Thirty micromoles of 1NM-PP1 were added for a 5-min preincubation followed by addition of 15 mM of $HCO_3^-$ at 0 time where indicated. Sperm were harvested by centrifugation at 90 s and subjected to Western blot analysis using anti-phospho PKA substrate antibody. (*B*) Protein tyrosine phosphorylation was assessed by Western blot analysis of extracts from WT and M120A sperm after incubation for 90 min in HS plus BSA in the presence of continuous 30 $\mu$M of 1NM-PP1 and $HCO_3^-$ as indicated. (*C*) Same as *B* except that 1NM-PP1 was not added until 30 min after start of incubation. (*D*) Same as *C* except that 1NM-PP1 was added at 90 min and the sperm were harvested at 95 min for phosphotyrosine analysis on Western blots. Only the mutant M120A sperm samples are shown in *D*. The blots shown are representative of those obtained in three separate experiments.

by $HCO_3^-$ and calcium. However, PKA phosphorylation in mutant sperm was strongly inhibited by 1NM-PP1.

Fig. 5 *B–D* shows that protein tyrosine phosphorylation in sperm has a different temporal requirement for PKA activity. The ability of 1NM-PP1 to inhibit tyrosine phosphorylation was examined by first pretreating wild-type and mutant sperm for 5 minutes with 30 $\mu$M of 1NM-PP1 and then continuing the incubation for 90 min in capacitating medium that also contained 30 $\mu$M of 1NM-PP1 (Fig. 5*B*). Treatment with 1NM-PP1 had no effect on the induction of tyrosine phosphorylation detected in wild-type sperm after a 90 min incubation. However, continuous treatment of mutant sperm with 1NM-PP1 completely blocked the induction of tyrosine phosphorylation (Fig. 5*B*).

One important question regarding the role of PKA during capacitation is whether sustained PKA activity is required for capacitation to proceed normally. We addressed this question by treating mutant and wild-type sperm with the drug after allowing them to undergo capacitation for 30 min. Fig. 5*C* shows that the delayed application of 1NM-PP1 still blocked the normal induction of tyrosine phosphorylation. This result suggests that PKA activity must be sustained for capacitation to proceed normally. When 1NM-PP1 was added for just the final 5 min of incubation after tyrosine kinase substrates are already phosphorylated, the level of phosphotyrosine was not inhibited, suggesting the pathway is fully activated and continued PKA activation is not required (Fig. 5*D*). This result is consistent with the lack of effect of 1NM-PP1 on the asymmetric flagellar beat when the drug is added after the late stage of capacitation has already occurred. Although a 5-min inhibition of PKA did not appear to reduce tyrosine phosphorylation of substrates substantially, it is possible that longer periods of inhibition will cause a reversal of the tyrosine phosphorylation stimulated during capacitation.

## Discussion

Studies on the biological role of protein kinases in signal transduction have benefited greatly from the development of selective small-molecule inhibitors that target a single or more often a small subgroup of kinases from the >500 identified kinases in the mouse or human genome (20). The vast majority of these inhibitors act as competitive antagonists at the ATP binding site of the kinase. One major limitation of these inhibitors is that because of the high conservation of the ATP binding pocket structure in protein kinases, they usually cross-react with multiple off-target kinases, sometimes at very similar affinities to the targeted kinase (6–8). For example, H89, a frequently used PKA inhibitor, is also very efficient at inhibiting S6K1, MSK1, and ROCK-II. Another "specific" PKA inhibitor, KT5720, inhibited PDK1 and PHK (phosphorylase kinase) at much lower concentrations than those needed to inhibit PKA (8). A second limitation of the available small-molecule inhibitors is that they are much less useful *in vivo* because they cannot be directed to a specific cell type.

A chemical genetic approach has been developed that provides a novel tool for either inhibiting a specific kinase or

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detecting the direct substrates of a single kinase (21, 22). The targeted kinase is mutated at a specific residue in the ATP binding pocket (termed a "gatekeeper" residue) and this mutation enlarges the binding site sufficiently to allow entry and binding of either a novel small-molecule inhibitor or a modified ATP gamma phosphate donor. We have previously shown that the Cα subunit of PKA can be mutated at the gatekeeper Met-120 to either an Ala or Gly making the mutant kinase sensitive to inhibition by several PP1-based analogs including 1NM-PP1 without significantly interfering with normal kinase activity in the absence of the drug (11). We have now engineered a Met120Ala mutation into the Cα gene (*Prkaca*) of a mouse in such a way that the action of Cre recombinase will turn on the transcription of mutant CαM120A mRNA while turning off the transcription of wild-type Cα. As a first step in demonstrating the usefulness of this mouse line we used a germ line Cre recombinase to activate the mutation in all tissues and then isolated sperm from these animals to investigate the role of PKA in both early and late events of sperm capacitation.

Sperm capacitation is considered an obligatory maturational process that is required for sperm to fertilize the egg. Past work shows that bicarbonate activation of SACY, elevation of cAMP, and activation of PKA are essential initiating steps in this capacitation process. Mouse knockouts of either SACY (3–5) or PKA (2) fail to undergo bicarbonate-enhanced motility and never reach the late stages of capacitation characterized by tyrosine phosphorylation and asymmetric flagellar hyperactivation.

One question that has been difficult to address with available reagents is whether PKA activation is required only at the initial stage of capacitation or whether there is a continuing role for PKA activity during late stages of capacitation when tyrosine phosphorylation and sperm hyperactivation are occurring. We now show that acute inhibition of PKA with 1NM-PP1 during the late stages of capacitation does not diminish either the protein tyrosine phosphorylation or hyperactivated flagellar asymmetry of fully capacitated sperm. In contrast acute PKA inhibition prevents or reverses the $HCO_3^-$-induced increase in flagellar beat frequency during early stage capacitation. Application of 1NM-PP1 during the early stages of capacitation demonstrate that at least 30 min of chronic PKA activity is required to initiate secondary increases in tyrosine phosphorylation.

A major advantage of the chemical genetic approach compared with traditional PKA inhibitors is that the possible off-target effects of 1NM-PP1 can be revealed by examining the response of wild-type sperm to the drug. We found no effect of 1NM-PP1 treatment of wild-type sperm on any of the measures of sperm capacitation we have investigated, demonstrating that the effects on CαM120A mutant sperm are specific to PKA. A recent report on protein kinase inhibitors examined the specificity of PP1, 1NM-PP1, and other PP1 derivatives on a large panel of protein kinases and concluded that 1NM-PP1 did cause significant inhibition of purified Src, Src-related kinases, and several serine/threonine kinases including PKA. When wild-type PKA was assayed at 20 μM of ATP it was reported that 1NM-PP1 inhibited with an $IC_{50}$ of 0.5 μM (6). Our results using extracts from wild-type sperm and several other tissues have shown no direct effect of up to 30 μM of 1NM-PP1 on PKA activity, and treatment of intact wild-type sperm does not affect the PKA-dependent increase in motility. Because 1NM-PP1 and ATP are competing for the mutated binding site in Cα, it is possible that the use of 200 μM of ATP in our *in vitro* assay and the high physiological levels of ATP in sperm could account for the lack of inhibition we see. The possibility that 1NM-PP1 is inhibiting Src in sperm is also relevant because Src has been reported to be activated in sperm and responsible for the increase in tyrosine phosphorylation during late-stage capacitation (23). Because we see no decrease in tyrosine phosphorylation in wild-type sperm treated throughout capacitation with 30 μM of 1NM-PP1, we conclude that either this concentration is not sufficient to inhibit Src activity under capacitating conditions or Src activity is not required for tyrosine phosphorylation in sperm.

There are now several examples in which analog-sensitive mutations have been engineered directly into kinases in the mouse genome but one limitation of the previous studies is that the targeted kinases are expressed in many different tissues and it has not been possible to make the analog-sensitive kinase tissue specific. Mice with analog-sensitive knockin mutations in the neurotrophin receptors (TrkA, TrkB, or TrkC) were used to study the neurotrophin-Trk receptor signaling pathways during development of the nervous system *in vivo* (24) and the role of TrkB specifically in hippocampal mossy fiber/CA3 synaptic plasticity (25). Analog-sensitive mutation of *Jnk2* in the mouse germ line allowed the isolation of primary embryonic fibroblasts in which JNK2 could be specifically inhibited without affecting JNK1 activity (26). Transgenic expression of an analog-sensitive CaMKIIα in brain was used to investigate the effects of CaMKII overexpression on synaptic plasticity and memory formation (27, 28). The potential of this chemical genetic approach for *in vivo* studies would be even greater if the wild-type allele could be replaced by an analog-sensitive allele in a cell-type-specific fashion. By inserting a lox-flanked wild-type minigene allele into the Cα gene upstream of the analog-sensitive mutant exon we have created a mouse that expresses wild-type Cα protein until switched by Cre recombinase to the mutant allele, CαM120A. Many protein kinases including PKA are expressed in multiple cell types and regulate a variety of cell signaling events. This Cre-dependent expression of an analog-sensitive PKA allows investigators to take advantage of the many Cre recombinase-expressing mouse lines to study the role of PKA in specific cell types under physiological conditions.

## Materials and Methods

**Genetic Engineering of Mice Expressing the CαM120A Mutation.** A targeting vector containing three loxP sites was created to allow tissue-specific expression of a CαM120A mutation that confers sensitivity to inhibition by 1NM-PP1. The targeting vector also contains a neomycin phosphotransferase cassette (NEO) and a minigene containing exons 5–10 of the Cα cDNA followed by a poly(A) addition site from the human growth hormone gene (29). The presence of NEO allows positive selection of transfected ES cells in culture and the minigene was designed to allow expression of wild-type Cα *in vivo* in CαLoxM120A animals (Fig. 1*A*). Both insertions are flanked by loxP sites that allow them to be removed by Cre recombinase. A mutant form of exon 5 possessing the CαM120A point mutation is located downstream of NEO and the minigene. Mouse ES cells were transfected with targeting vector by electroporation and correctly targeted ES cells were identified by Southern blot and then microinjected into blastocysts to produce chimeras that were then bred to C57BL/6 mice to establish the colony. Mice expressing the targeted allele were mated with EIIa-Cre transgenic animals (13) to produce progeny mice expressing the fully (CαM120A) and partially (CαLoxM120A) recombined alleles. Genotyping for the CαM120A allele was performed using the PCR primer pair lox1F (5′-TCACATCCCACACGATTTT-3′) and the reverse primer, 5′-ACCAGGAGGATTGTGAGCCTAAGAC-3′. These primers yielded a 500-bp fragment from the wild-type allele and a 652-bp band from the mutant allele. The CαM120A males used in this study had been backcrossed onto a C57BL/6 background for at least three generations after the cross with EIIa-Cre transgenics to activate the CαM120A allele in all tissues. Genotyping for the CαLoxM120A was performed using lox1F (above) and the reverse primer: 5′GCAGGCATTGAAACCTCAGTG-3′, giving a 308-bp fragment for the CαLoxM120A allele and a 181-bp fragment for the wild-type allele.

**Sperm Preparation.** Mice were killed by $CO_2$ asphyxiation. The caudal epididymides and vasa deferentia were excised and cleaned in medium HS: 135 mM of NaCl, 5 mM of KCl, 1 mM of $MgCl_2$, 2 mM of $CaCl_2$, 1 mM of pyruvic acid, 20 mM of lactic acid, 5 mM of glucose, and 20 mM of Hepes, pH 7.4. Sperm were harvested by a 15-min swim out in medium HS supplemented with 5 mg of BSA/ml. Released sperm were sedimented then resuspended and stored in BSA-free HS medium. Sperm destined for Western blot analysis were washed

twice more by sedimentation and resuspension in BSA-free HS medium. Capacitating incubations (37 °C/5% $CO_2$/90 min) were in medium HS supplemented with 15 mM of $NaHCO_3$ and 5 mg of BSA/ml.

**In Vitro Analysis of PKA Activity and Western Blot Analysis.** Testis or sperm proteins were extracted in a buffer containing 1% Triton X-100, 20 mM of Tris pH 7.6, 0.1 mM of EDTA, 0.5 mM of EGTA, 5 mM of magnesium acetate, 250 mM of sucrose, and a protease-and-phosphatase-inhibitor mixture that contained 0.5 mM of 4-[2-aminoethyl]benzenesulfonyl fluoride, 0.15 nM of aprotinin, 1 $\mu$M of E-64, 1 $\mu$M of leupeptin, 25 $\mu$M of 4-bromotetramisole oxalate, 5 mM of cantharidin, 5 nM of microcystin-LR, 2 mM of imidazole, 1 mM of sodium orthovanadate, 1.2 mM of sodium molybdate, 1 mM of NaF, and 4 mM of sodium tartrate (EMD Biosciences). Testes were disrupted with a Polytron homogenizer followed by sonication, whereas sperm were disrupted by sonication alone. Particulate material was removed by centrifugation and the protein content of the supernatant determined by Bradford assay. PKA activity assays were performed in triplicate using the synthetic PKA substrate kemptide (30). The assay buffer contained 30 $\mu$M of kemptide, 0.2 mM of ATP, 10 mM of magnesium acetate, 20 mM of Tris-Cl (pH 7.6), 0.5 mM of IBMX, 10 mM of DTT, 5 mM of sodium fluoride, and 0.025 mCi/ml of $\gamma$-[$^{32}$P]-ATP. For Western blot analysis, sperm proteins were solubilized by boiling sedimented sperm for 5 min in a 1$\times$ Laemmli buffer containing 62.5 mM of Tris, pH 6.8, 2% SDS, 5% glycerol, 0.1% (wt/vol) bromophenol blue, 5% $\beta$-mercaptoethanol, and 10 mM of DTT. Proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes and analyzed by Western blot as previously described (2). Primary antibodies were obtained from the following sources and diluted as indicated: sc 909 anti-RII$\alpha$ (1:500) was from Santa Cruz Biotechnology; 4G10 anti-phosphotyrosine (1:1000) from Upstate Biotechnology; 9624 anti-phospho PKA substrate RRXS/T (1:5000) from Cell Signaling; and 306–1 anti-C$\alpha$ (1:5000) was a generous gift from Susan Taylor (University of California, San Diego).

**Analysis of Sperm Motility and Flagellar Waveform.** Motility and flagellar waveform were examined in individual sperm as previously described (2, 15). Briefly, stop-motion images were collected at 20- to 40-ms intervals for sperm loosely tethered to a glass surface at the head. A solenoid-controlled gravity-driven local perfusion device produced rapid changes in medium composition. Images were processed with MetaMorph (Universal Imaging) and waveform analysis used software written in the IgorPro (Wavemetrics) environment.

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