

## Capacitation of mouse spermatozoa

### II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway

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#### SUMMARY

In the accompanying report (Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P. and Kopf, G.S. (1995) *Development*, 121, 1129-1137) we demonstrated that the tyrosine phosphorylation of a subset of mouse sperm proteins of  $M_r$  40,000-120,000 was correlated with the capacitation state of the sperm. The mechanism by which protein tyrosine phosphorylation is regulated in sperm during this process is the subject of this report. Cauda epididymal sperm, when incubated in media devoid of  $\text{NaHCO}_3$ ,  $\text{CaCl}_2$  or bovine serum albumin do not display the capacitation-associated increases in protein tyrosine phosphorylation of this subset of proteins. This  $\text{NaHCO}_3$ ,  $\text{CaCl}_2$  or bovine serum albumin requirement for protein tyrosine phosphorylation can be completely overcome by the addition of biologically active, but not inactive, cAMP analogues. Addition of the active cAMP analogues to sperm incubated in media devoid of  $\text{NaHCO}_3$ ,  $\text{CaCl}_2$  or bovine serum albumin overcomes the inability of these media to support capacitation, as assessed by the ability of the cells to acquire the pattern B chlortetracycline fluorescence, to undergo the zona pellucida-induced acrosome reaction and, in some cases, to fertilize metaphase II-arrested eggs

in vitro. The effects of the cAMP analogues to enhance protein tyrosine phosphorylation and to promote capacitation appears to be at the level of the cAMP-dependent protein kinase (PKA), since two specific inhibitors of this enzyme (H-89 and Rp-cAMPS) block the capacitation-dependent increases in protein tyrosine phosphorylation in sperm incubated in media supporting capacitation. Capacitation, as assessed by the aforementioned endpoints, also appears to be inhibited by H-89 in a concentration-dependent manner. These results provide further evidence for the interrelationship between protein tyrosine phosphorylation and the appearance of the capacitated state in mouse sperm. They also demonstrate that both protein tyrosine phosphorylation and capacitation appear to be regulated by cAMP/PKA. Up-regulation of protein tyrosine phosphorylation by cAMP/PKA in sperm is, to our knowledge, the first demonstration of such an interrelationship between tyrosine kinase/phosphatase and PKA signaling pathways.

Key words: mouse, sperm, capacitation, fertilization, in vitro fertilization, tyrosine phosphorylation, cyclic AMP, protein kinase A

#### INTRODUCTION

Sperm capacitation is a poorly understood maturational process, which occurs in vivo in the female reproductive tract or can be accomplished in vitro in defined media, the endpoint of which confers upon the sperm the ability to undergo the acrosome reaction and to fertilize an egg (Yanagimachi, 1994). Capacitation has been shown to be correlated with changes in sperm plasma membrane fluidity, intracellular ion concentrations, metabolism, and motility (Yanagimachi, 1994 and references therein), but it is not clear how such changes are necessarily tied to the development of this fertilization-competent

state. Moreover, although these changes have been known for many years, the molecular basis underlying these events is poorly understood. It is clear from studies in somatic cells that many of these aforementioned cellular changes can be modulated by extrinsic factors that function intracellularly via receptors and second messengers. However, it appears that the initiation and completion of capacitation in mammalian sperm may be regulated by processes inherent to the sperm itself. The mechanism by which such an inherent regulation occurs is not clear, although it is likely that the controlling factor(s) lies with the sperm plasma membrane itself and that changes in the properties of the membrane might lead to a de-repression of

the preprogrammed cellular events that are ultimately necessary for the development of the capacitated state.

We have shown in the preceding report that the acquisition of the capacitated state in caudal epididymal mouse sperm, as assessed by the ability of the cells to acquire the pattern B chlortetracycline fluorescence, to undergo the ZP-induced acrosome reaction, and to fertilize metaphase II-arrested eggs in vitro, is accompanied by the protein tyrosine phosphorylation of a specific subset of sperm proteins (Visconti et al., 1995). The onset of both capacitation and the protein tyrosine phosphorylations are dependent on the presence of serum albumin,  $\text{Ca}^{2+}$  and  $\text{NaHCO}_3$  in the culture medium, three components that have been demonstrated to be essential for in vitro capacitation of sperm from a variety of different mammals (Yanagimachi, 1994 and references therein). Caput epididymal sperm, which do not possess the ability to undergo capacitation, do not display the protein tyrosine phosphorylations of this subset of proteins. We believe that protein tyrosine phosphorylation may represent an important pathway that may ultimately regulate events associated with capacitation.

The mechanism by which these protein tyrosine phosphorylations are regulated is the subject of this study. Of potential relevance to the requirement of extracellular  $\text{Ca}^{2+}$  and  $\text{NaHCO}_3$  for these protein tyrosine phosphorylations is the observation that these particular ions have been demonstrated to be involved in the regulation of mammalian sperm cAMP metabolism, and that changes in the concentration of this intracellular second messenger have been linked to both capacitation and the acrosome reaction. For example, it is known that  $\text{Ca}^{2+}$ -dependent elevations of guinea pig sperm cAMP concentrations are dependent on the presence of extracellular  $\text{NaHCO}_3$  and that the effects of  $\text{NaHCO}_3$  on this process do not appear related to changes in intracellular pH (Garbers et al., 1982). The effects of this cation and anion may be at the level of the adenylyl cyclase, since the mammalian sperm enzyme has been demonstrated to be regulated directly/indirectly by  $\text{Ca}^{2+}$  (Hyne and Garbers, 1979), calmodulin (Gross et al., 1987), and  $\text{NaHCO}_3$  (Garty and Salomon, 1987; Okamura et al., 1988; Visconti et al., 1990).  $\text{Ca}^{2+}$  and  $\text{NaHCO}_3$  also appear to be required for capacitation (Fraser, 1987; Neill and Olds-Clarke, 1987; Boatman and Robbins, 1991) and the induction of the acrosome reaction (Hyne, 1984; Lee and Storey, 1986; Kopf and Gerton, 1991 and references therein; Spira and Breitbart, 1992). Moreover, changes in the activity of adenylyl cyclase and cAMP have been postulated in some species to play a role in capacitation (Perreault and Rogers, 1982; Berger and Clegg, 1983; Boatman and Bavister, 1984; Stein and Fraser, 1984; Monks et al., 1986; Yanagimachi, 1994 and references therein).

The interrelationships between  $\text{NaHCO}_3$ ,  $\text{Ca}^{2+}$ , cAMP, protein tyrosine phosphorylation and capacitation in cauda epididymal mouse sperm was further examined in the present report. The capacitation-associated changes in protein tyrosine phosphorylation that are dependent on the presence of bovine serum albumin (BSA),  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  can be mimicked in the absence of each of these required media constituents by the addition of active, but not inactive, membrane-permeable cAMP analogues. Correlated with the stimulatory effects of these cAMP analogues on protein tyrosine phosphorylation is a stimulatory effect of these analogues on capacitation. The

stimulatory effects of cAMP on protein tyrosine phosphorylation and capacitation appear to be at the level of PKA.

## MATERIALS AND METHODS

### Reagents

Sp-cAMPS, Rp-cAMPS, 8-bromo cAMP and KN-62 were from Research Biochemicals International (Natick, MA). H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), staurosporine and chelerythrine were from LC Labs (Woburn, MA). Dibutyl cAMP and IBMX were purchased from Sigma. Anti-phosphotyrosine antibody (clone 4G10) was obtained from UBI (Lake Placid, NY). All other reagents were of the highest analytical grade.

### Culture media

The basic medium used throughout these studies for the preparation of sperm was a modified Krebs Ringer bicarbonate medium (HMB-Hepes buffered), as described by Lee and Storey (1986). The preparation of the media is as described in the accompanying report (Visconti et al., 1995).

### Preparation of sperm and in vitro fertilization

Uncapacitated caudal epididymal sperm were collected from CD1 retired breeder male mice by the methods described in the accompanying paper (Visconti et al., 1995). In vitro fertilization of metaphase II-arrested eggs was performed as previously described (Moore et al., 1993), with the modifications described in the accompanying report (Visconti et al., 1995).

### Preparation and solubilization of mouse zonae pellucidae

*Zonae pellucidae* were prepared from homogenized ovaries of virgin female 22-day old outbred CD-1 mice (Charles River) as described in Ward et al. (1992) and solubilized for all experiments by the procedures outlined in the previous report (Visconti et al., 1995).

### Chlortetracycline assay

This assay was used to assess both the capacitation state of the sperm and the percentage of acrosome reactions. The experimental design and use of these assays is described in Visconti et al. (1995).

### Preparation of sperm membranes and measurement of adenylyl cyclase activity

Caudal epididymal spermatozoa were recovered as described by Endo et al. (1987, 1988), and incubated at a concentration of  $1.3 \times 10^8$  cells/ml for 1 hour at  $37^\circ\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  in air in Hepes-buffered modified bicarbonate Krebs-Ringer medium (HMB) containing 20 mg/ml BSA. At the end of the incubation, the sperm cells were diluted twice in HMB+BSA and centrifuged for 20 minutes (150 g) at room temperature. The resultant pellet was resuspended in ice-cold homogenization buffer (50 mM Hepes, 1 mM EGTA, 10% DMSO, pH 7.5) and gently homogenized (10 strokes in a loose glass-teflon homogenizer) under conditions that have been shown to maintain the ZP-G protein coupling (Ward et al., 1992). The homogenate was subjected to a low speed centrifugation (1,000 g) and the resultant supernatant centrifuged for 1 hour (100,000 g;  $4^\circ\text{C}$ ) to pellet the membranes. The particulate crude membrane fraction was resuspended in the same homogenization buffer, aliquoted and kept frozen ( $-70^\circ\text{C}$ ). The adenylyl cyclase assays were performed on membrane preparations less than a week old. Generally, 250-350  $\mu\text{g}$  of membrane proteins were obtained using 20 males.

Adenylyl cyclase activity was measured by the conversion of [ $\alpha$ - $^{32}\text{P}$ ]ATP to [ $^{32}\text{P}$ ]cAMP (Salomon et al., 1974). The assay was carried out for 20 minutes at  $37^\circ\text{C}$  in the presence of 50 mM Hepes, 1.5 mM ATP, 15 mM  $\text{MgCl}_2$ , 10 mM KCl, 4 mM DTT, 1 mM IBMX, 16  $\mu\text{g}$  creatine kinase, 3.2 mM creatine phosphate and 2.5-3  $\mu\text{Ci}$  [ $\alpha$ -

$^{32}\text{P}$ ]ATP, pH 7.6 in a final volume of 30  $\mu\text{l}$ . The assay was started by the addition of 8  $\mu\text{l}$  of membrane suspension containing 0.5–1.2  $\mu\text{g}$  of protein, and was terminated by the addition of 25  $\mu\text{l}$  of the termination buffer (36.4 mM ATP, 10 mM cAMP, 1% SDS, and 3000 cpm [ $^3\text{H}$ ]cAMP) followed by heating in a boiling water bath for 5 minutes. The [ $^{32}\text{P}$ ]cAMP formed was purified following chromatography using Dowex and Alumina columns (Salomon et al., 1974) and the [ $^3\text{H}$ ]cAMP was used as an internal standard for the evaluation of recovery.

### SDS-PAGE and immunoblotting

SDS-PAGE (Laemmli, 1970) was performed in 10% gels. Electrophoretic transfer of proteins to Immobilon P in all experiments was carried out according to the method of Towbin et al. (1979) at 30 V (constant) for 6 hours at 4°C. Immunodetection of proteins on Immobilon P was performed at room temperature as described previously (Kalab et al., 1994) using a monoclonal antibody against phosphotyrosine (clone 4G10) and enhanced chemiluminescence detection using an ECL kit (Amersham Corp.) according to the manufacturer's instructions.

### Statistical methods

Statistical comparison between the treatment groups was always performed against the control lacking the addition of the respective media constituent using a Student's *t*-test. The acrosome reaction data were analyzed using a Student's *t*-test by comparing the inducible component of the acrosome reaction [e.g., the ZP-induced acrosome reaction following subtraction of the spontaneous acrosome reaction ( $\text{AR}_{\text{zp}} - \text{AR}_{\text{spont.}}$ )].

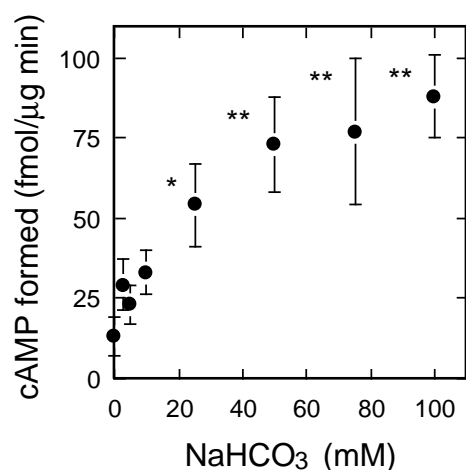
## RESULTS

### Effects of bicarbonate anion on mouse sperm adenylyl cyclase activity

We first examined whether the mouse sperm adenylyl cyclase could be stimulated by  $\text{HCO}_3^-$ . Membranes from capacitated mouse sperm were prepared under conditions that support ZP3- $\text{G}_i$  protein-coupled signal transduction (Ward et al., 1992) and assayed for adenylyl cyclase activity in the absence and presence of various concentrations of  $\text{NaHCO}_3$ . As shown in Fig. 1,  $\text{NaHCO}_3$  stimulated adenylyl cyclase activity in a concentration-dependent manner that was maximal at 50 mM. These stimulatory effects compare favorably with those effects seen by Okamura et al. (1985), Garty and Salomon (1987) and Visconti et al. (1990). The effect of  $\text{NaHCO}_3$  on mouse sperm adenylyl cyclase activity is due to the effect of  $\text{HCO}_3^-$ , since substituting the  $\text{NaHCO}_3$  with NaCl had no effect on the activity of the enzyme (data not shown). Moreover, the effect of  $\text{NaHCO}_3$  on enzyme activity did not result as a consequence of changes in assay pH, since adjusting the pH of the buffers used in the enzyme assay from pH 7–8 had no effect on enzyme activity (data not shown).

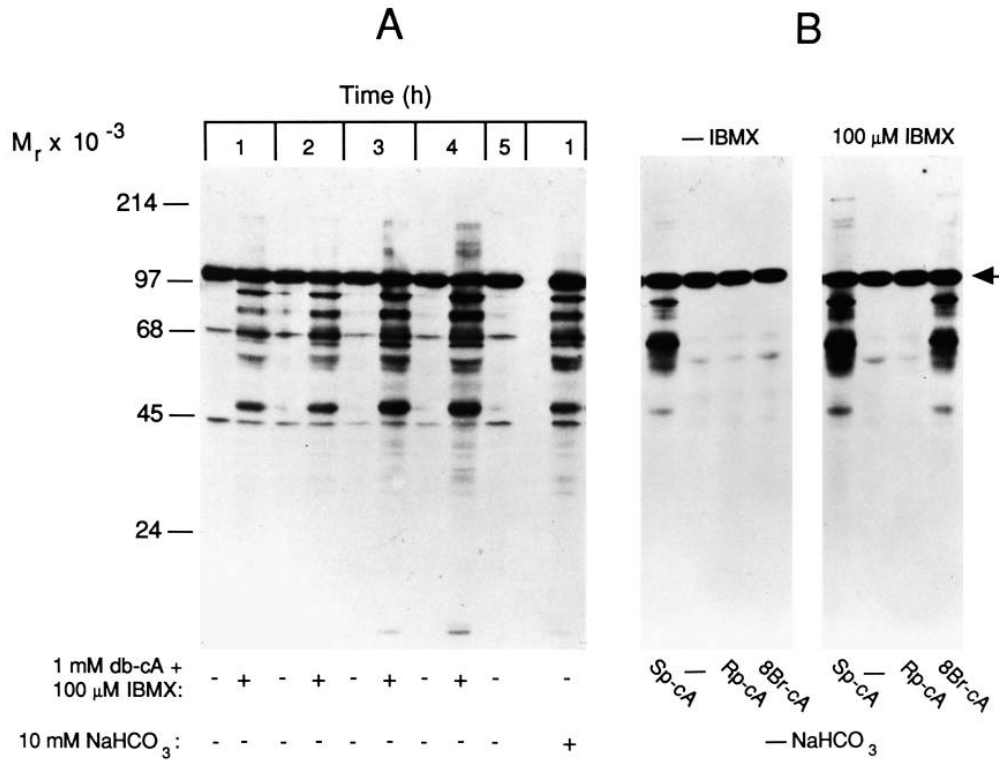
### Cyclic AMP analogues can substitute for bicarbonate anion in supporting both protein tyrosine phosphorylation and capacitation

As previously demonstrated, the presence of  $\text{NaHCO}_3$  in the medium is required for capacitation and the associated changes in protein tyrosine phosphorylation (Visconti et al., 1995, Figs 6, 7). Since  $\text{NaHCO}_3$  stimulated the mouse sperm adenylyl cyclase (Fig. 1), we determined whether the addition of membrane permeable cAMP analogues could substitute for



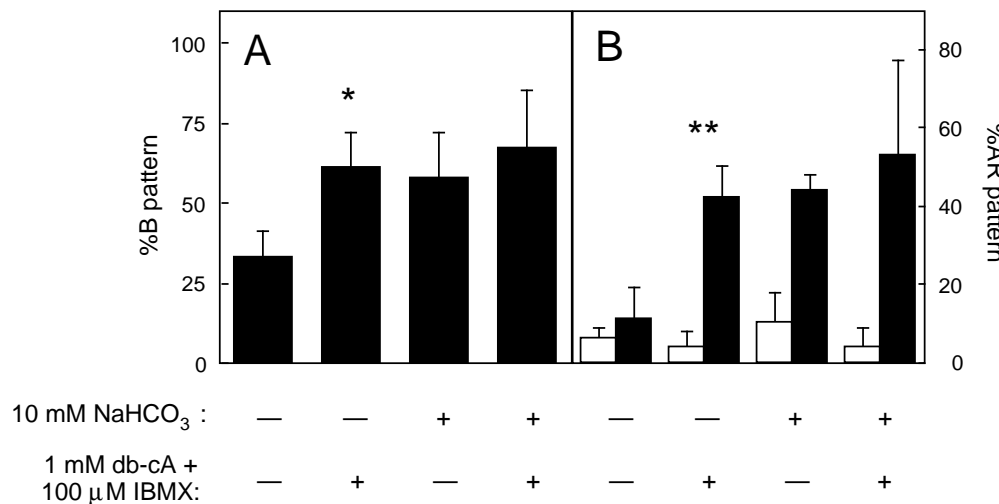
**Fig. 1.** Concentration-dependent effects of  $\text{NaHCO}_3$  on the adenylyl cyclase activity of membrane preparations of capacitated mouse sperm. Sperm membranes were prepared and assayed for adenylyl cyclase activity using  $\text{Mg}^{2+}$  as the divalent cation as described in Materials and Methods. The final concentration of  $\text{NaHCO}_3$  in the assay is shown on the abscissa. Data represent mean  $\pm$  s.e.m.;  $n=4$ . Statistical analysis was performed as described in Materials and Methods; \* $P<.05$ ; \*\* $P<.01$ .

$\text{NaHCO}_3$  in promoting both protein tyrosine phosphorylation and capacitation. Caudal epididymal sperm were incubated for different periods of time in complete medium or in medium devoid of  $\text{NaHCO}_3$  in either the absence or presence of 1 mM dibutyryl cAMP (db-cA) plus 100  $\mu\text{M}$  IBMX. When compared to sperm incubated in complete medium, sperm incubated in medium devoid of  $\text{NaHCO}_3$  did not display the capacitation-associated changes in protein tyrosine phosphorylation, even after incubation times up to 5 hours (Fig. 2A). However, when the  $\text{NaHCO}_3$ -free culture medium was supplemented with 1 mM db-cA plus 100  $\mu\text{M}$  IBMX, the protein tyrosine phosphorylation was observed and this was maximal by 3 hours of incubation (Fig. 2A). As described in the previous report (Visconti et al., 1995), the degree of tyrosine phosphorylation of the p95/116 hexokinase, which is the major phosphotyrosine-containing protein in mouse sperm membranes (Kalab et al., 1994), was not affected by the absence of  $\text{NaHCO}_3$  in the medium and its phosphorylation did not appear to be modulated by the addition of 1 mM db-cA plus 100  $\mu\text{M}$  IBMX (Fig. 2A). These effects on protein tyrosine phosphorylation were not limited to db-cA since 1 mM 8-bromo-cAMP (8Br-cA) and 1 mM Sp-cAMPS (Sp-cA) also supported protein tyrosine phosphorylation in the absence of  $\text{NaHCO}_3$  and in the presence of IBMX (Fig. 2B, right-hand panel). In contrast to the effects of these active cAMP analogues, addition of Rp-cAMPS (Rp-cA), which is a membrane-permeable cAMP analogue that binds to the regulatory subunit of PKA but fails to cause subunit dissociation and activation of PKA and thus antagonizes cAMP action (Van Haastert et al., 1984; Parker Botelho et al., 1988), did not support protein tyrosine phosphorylation in the absence of  $\text{NaHCO}_3$  (Fig. 2B, right-hand panel). In contrast to the effects seen when 8Br-cA was added to the sperm in the presence of IBMX, 8Br-cA added in the absence of IBMX failed to efficiently support protein tyrosine phosphorylation (Fig. 2B, left-



**Fig. 2.** Effects of cyclic AMP analogues and 3-isobutyl-1-methylxanthine (IBMX) on the appearance of phosphotyrosine-containing proteins in caudal epididymal mouse sperm incubated in media devoid of  $\text{NaHCO}_3$ . (A) Sperm were incubated for the periods of time indicated in the figure in HM medium (no added  $\text{NaHCO}_3$ ) or in the same medium containing 1 mM dibutyryl cAMP (db-cA) plus 100  $\mu\text{M}$  IBMX. At those times, an aliquot of the sperm suspension was removed ( $5 \times 10^5$  sperm), centrifuged and the whole sperm extract subjected to SDS-PAGE by the procedures outlined in Materials and Methods. The proteins were then transferred to Immobilon P and probed with a monoclonal antibody to phosphotyrosine (*a pY*). This experiment was performed at least three times with similar results. Shown is a representative experiment. (B) Sperm were incubated in  $\text{NaHCO}_3$ -depleted

medium (–), or in  $\text{NaHCO}_3$ -depleted medium containing 1 mM Sp-cAMPS (Sp-cA), Rp-cAMPS (Rp-cA) or 8 bromo-cAMP (8Br-cA) in either the absence (left-hand panel) or presence (right-hand panel) of 100  $\mu\text{M}$  IBMX. This experiment was performed at least three times with similar results; a representative experiment is shown. In both A and B, the major phosphotyrosine-containing protein that appears at  $M_r$  116,000 in all of the lanes is the p95/116 hexokinase. Numbers on the left-hand side of this gel and in subsequent figures represent the position of the relative molecular mass standards ( $\times 10^{-3}$ ).



**Fig. 3.** Effects of dibutyryl cAMP plus 3-isobutyl-1-methylxanthine (IBMX) on the percentage of caudal epididymal mouse sperm displaying chlortetracycline fluorescence pattern 'B' and the percentage of sperm undergoing the spontaneous and zona pellucida-induced acrosome reaction (chlortetracycline pattern 'AR') in media containing  $\text{NaHCO}_3$  or in media devoid of  $\text{NaHCO}_3$ . Sperm were incubated for a period of 90 minutes in  $\text{NaHCO}_3$ -free HMB,  $\text{NaHCO}_3$ -free HMB containing 10 mM  $\text{NaHCO}_3$ , or in these media containing 1 mM dibutyryl cAMP (db-cA) plus 100  $\mu\text{M}$  IBMX. (A) Sperm were

assessed for the percentage of CTC pattern 'B', indicative of the capacitated state. Data represent the mean  $\pm$  s.e.m.,  $n=3$ . (B) Sperm were assessed for the percentage of CTC pattern 'AR', representing cells undergoing acrosomal exocytosis, following a 30 minute incubation in buffer [open bars] or solubilized zonae pellucidae (5 ZP/ $\mu\text{l}$ ) [solid bars]. Data represent the mean  $\pm$  s.e.m.,  $n=3$ . Statistical analysis was performed as described in Materials and Methods; \* $P<0.05$  and \*\* $P<0.025$ .

hand panel). Such a differential effect was not observed when Sp-cA was used (Fig. 2B, compare left-hand and right-hand panels); this is probably due to its inability to be hydrolyzed by cyclic nucleotide phosphodiesterases. These effects appeared to be specific for cAMP analogues since dibutyryl

cGMP, in either the absence or presence of IBMX, had no effect on protein tyrosine phosphorylation (data not shown).

Sperm incubated in medium devoid of  $\text{NaHCO}_3$  also did not display the CTC fluorescence B pattern indicative of the capacitated state (Fig. 3A). The ZP-induced acrosome reaction was,

likewise, completely dependent on the presence of  $\text{NaHCO}_3$  in the medium (Fig. 3B). As was seen with protein tyrosine phosphorylation, the addition of 1 mM db-cA plus 100  $\mu\text{M}$  IBMX to medium devoid of  $\text{NaHCO}_3$  could substitute for  $\text{NaHCO}_3$  in supporting the ability of the sperm to display the CTC fluorescence B pattern and to undergo the ZP-induced acrosome reaction (Fig. 3A,B).

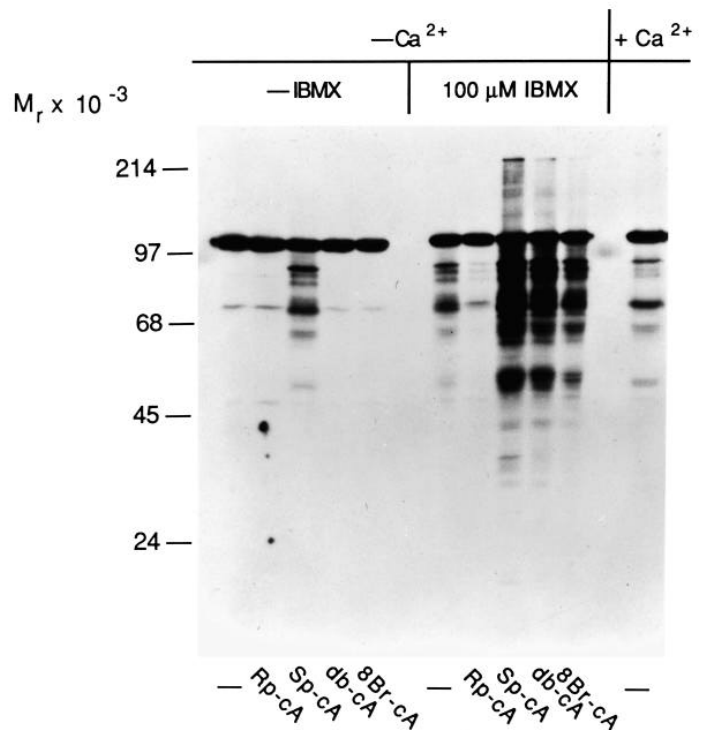
#### Cyclic AMP analogues can substitute for $\text{Ca}^{2+}$ in supporting both protein tyrosine phosphorylation and capacitation

When compared to complete medium, sperm incubated in medium devoid of added  $\text{Ca}^{2+}$  failed to display the capacitation-dependent changes in protein tyrosine phosphorylation (Fig. 4; Visconti et al., 1995). When sperm were incubated in  $\text{Ca}^{2+}$ -free medium containing various cAMP analogues plus IBMX for a period of 90 minutes, the capacitation-dependent changes in protein tyrosine phosphorylation were observed when biologically active (Sp-cA; db-cA; 8Br-cA), but not inactive (Rp-cA), cAMP analogues were used (Fig. 4, right-hand panel). It is of interest that IBMX alone supported protein tyrosine phosphorylation, but not to the degree of that observed in the presence of the active cAMP analogues (Fig. 4, right-hand panel). This is due, presumably, to its function as an inhibitor of cyclic nucleotide phosphodiesterase activity. IBMX alone also supported a greater degree of protein tyrosine phosphorylation but Rp-cA appeared to inhibit the IBMX-induced changes in protein tyrosine phosphorylation. This is presumably due to the fact that Rp-cA, in addition to its ability to bind to the regulatory subunit of PKA but not activate the enzyme, has been demonstrated to function as a cAMP antagonist (Van Haastart et al., 1984; Parker Botelho et al., 1988) and might therefore abrogate the effects of IBMX as a cyclic nucleotide phosphodiesterase inhibitor by competing with endogenous cAMP. In contrast to the effects seen in the presence of IBMX, in the absence of this phosphodiesterase inhibitor only Sp-cA appeared to support the appearance of the capacitation-dependent changes in protein tyrosine phosphorylation (Fig. 4, left-hand panel). This is presumably due to the fact that this analogue is very effective in activating PKA and is very poorly hydrolyzed by cyclic nucleotide phosphodiesterases. These data suggest that cAMP analogues can substitute for  $\text{Ca}^{2+}$  deficiency in supporting protein tyrosine phosphorylation.

Sperm incubated in medium devoid of  $\text{Ca}^{2+}$ , likewise, did not display the CTC fluorescence B pattern indicative of the capacitated state (Fig. 5A). The ZP-induced acrosome reaction was also completely dependent on the presence of  $\text{Ca}^{2+}$  in the medium (Fig. 5B). As was seen with protein tyrosine phosphorylation, the addition of 1 mM db-cA plus 100  $\mu\text{M}$  IBMX to medium devoid of  $\text{Ca}^{2+}$  could substitute for  $\text{Ca}^{2+}$  in supporting the ability of the sperm to display the CTC fluorescence B pattern (Fig. 5A). In contrast, these agents did not support the ability of the sperm to undergo a ZP-induced acrosome reaction (Fig. 5B), presumably due to the fact that extracellular  $\text{Ca}^{2+}$  is essential for this exocytotic event (Yanagimachi, 1994).

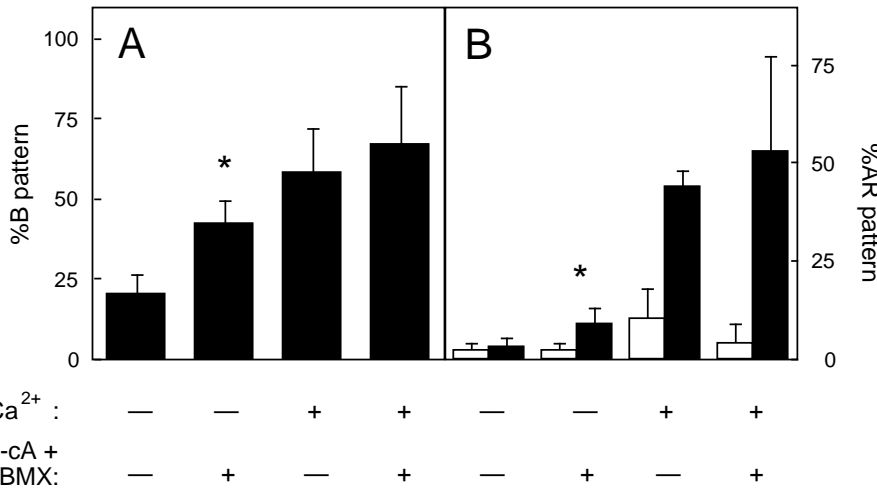
#### Cyclic AMP analogues can substitute for serum albumin in supporting both protein tyrosine phosphorylation and capacitation

Since serum albumin is required for both capacitation (Yanagimachi,



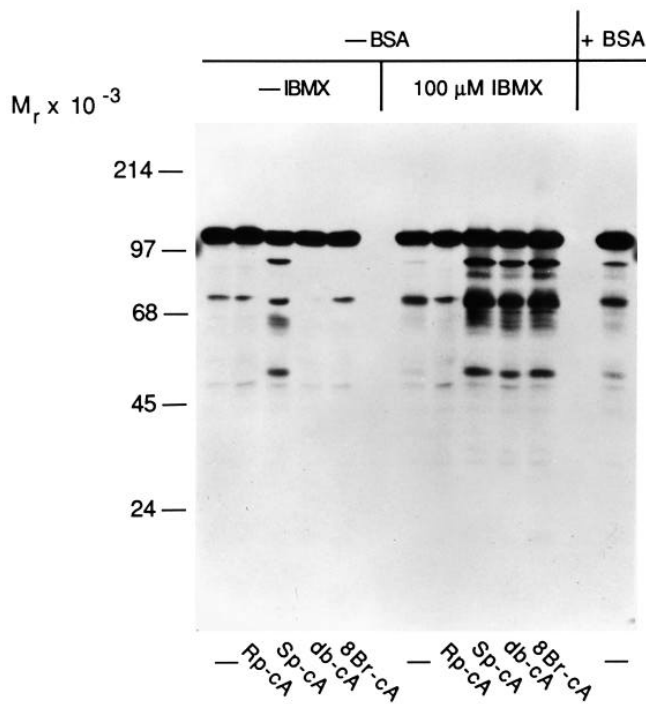
**Fig. 4.** Effects of cyclic AMP analogues and 3-isobutyl-1-methylxanthine (IBMX) on the appearance of phosphotyrosine-containing proteins in caudal epididymal mouse sperm incubated in media devoid of  $\text{CaCl}_2$ . Sperm were incubated for 90 minutes in HMB medium containing no added  $\text{CaCl}_2$  ( $\text{Ca}^{2+}$ -free HMB) in either the absence (left-hand panel) or presence (right-hand panel) of 100  $\mu\text{M}$  IBMX. These media were also supplemented with either no cAMP analogues (—) or with 1 mM Rp-cAMPS (Rp-cA), 1 mM Sp-cAMPS (Sp-cA), 1 mM dibutyl cAMP (db-cA) or 0.5 mM 8-bromo-cAMP (8Br-cA). Sperm were also incubated in the presence of complete HMB (extreme right lane marked  $+\text{Ca}^{2+}$ ). This experiment was performed at least three times with similar results. Shown, is a representative experiment.

imachi, 1994 and references therein) and the appearance of the phosphotyrosine-containing proteins in sperm (Visconti et al., 1995), we examined whether cAMP analogues could substitute for BSA in supporting both protein tyrosine phosphorylation and capacitation. Sperm incubated in medium devoid of added BSA failed to display the capacitation-dependent changes in protein tyrosine phosphorylation when compared to sperm incubated in medium containing this protein supplement (Fig. 6). When sperm were incubated in BSA-free medium containing various cAMP analogues plus IBMX for a period of 90 minutes, the capacitation-dependent changes in protein tyrosine phosphorylation were observed when biologically active (Sp-cA; db-cA; 8Br-cA), but not inactive (Rp-cA), cAMP analogues were used (Fig. 6, right-hand panel). In contrast to the protein tyrosine phosphorylation seen with IBMX alone in the absence of  $\text{Ca}^{2+}$  and presence of BSA (Fig. 4), IBMX added alone in  $\text{Ca}^{2+}$ -containing and BSA-free media did not effectively support protein tyrosine phosphorylation (Figs 2, 6). Although the reasons for these differences are not known it is probable that they may be due to the fact that sperm contain  $\text{Ca}^{2+}$ /calmodulin activated cyclic nucleotide phosphodiesterases and phosphoprotein phosphatases (Kopf and



percentage of CTC pattern 'B', indicative of the capacitated state. Data represent the mean  $\pm$  s.e.m.,  $n=3$ . (B) Sperm were assessed for the percentage of CTC pattern 'AR', representing cells undergoing acrosomal exocytosis, following a 30 minute incubation in buffer [open bars] or solubilized zonae pellucidae (5 ZP/ $\mu$ l) [closed bars]. Data represent the mean  $\pm$  s.e.m.,  $n=3$ . Statistical analysis was performed as described in Materials and Methods; \* $P<0.05$ .

**Fig. 5.** Effects of dibutyryl cAMP plus 3-isobutyl-1-methylxanthine (IBMX) on the percentage of caudal epididymal mouse sperm displaying chlortetracycline fluorescence pattern 'B' and the percentage of sperm undergoing the spontaneous and zona pellucida-induced acrosome reaction (chlortetracycline pattern 'AR') in medium containing CaCl<sub>2</sub> or in medium devoid of CaCl<sub>2</sub>. Sperm were incubated for a period of 90 minutes in Ca<sup>2+</sup>-free HMB, Ca<sup>2+</sup>-free HMB containing 1.7 mM CaCl<sub>2</sub>, or in these media containing 1 mM dibutyryl cAMP (db-cA) plus 100  $\mu$ M IBMX. (A) Sperm were assessed for the

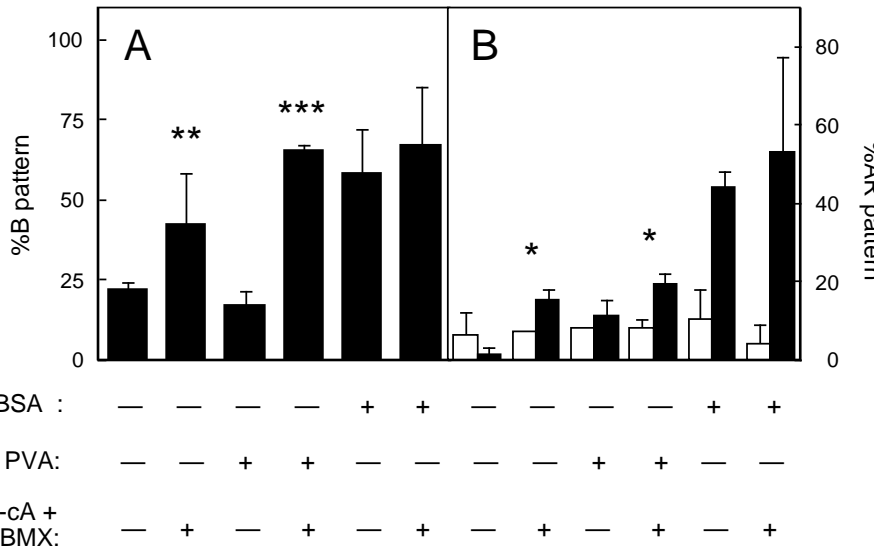


**Fig. 6.** Effects of cyclic AMP analogues and 3-isobutyl-1-methylxanthine (IBMX) on the appearance of phosphotyrosine-containing proteins in caudal epididymal mouse sperm incubated in medium devoid of bovine serum albumin. Sperm were incubated for 90 minutes in HMB medium containing no added bovine serum albumin (BSA-free HMB) in either the absence (left-hand panel) or presence (right-hand panel) of 100  $\mu$ M IBMX. These media were also supplemented with either no cAMP analogues (-) or with 1 mM Rp-cAMPS (Rp-cA), 1 mM Sp-cAMPS (Sp-cA), 1 mM dibutyryl cAMP (db-cA) or 0.5 mM 8 bromo-cAMP (8Br-cA). Sperm were also incubated in the presence of HMB containing 3 mg/ml BSA (extreme right lane marked +BSA). This experiment was performed at least five times with similar results. Shown, is a representative experiment.

Gerton, 1991; Tash et al., 1988) that may be more active in sperm incubated in the presence of Ca<sup>2+</sup>. In contrast to the effects seen in the presence of IBMX, in the absence of this phosphodiesterase inhibitor only Sp-cA appeared to support the appearance of the capacitation-dependent changes in protein tyrosine phosphorylation (Fig. 6, left-hand panel). These data suggest that cAMP analogues can substitute for BSA deficiency in supporting protein tyrosine phosphorylation.

Sperm incubated in medium devoid of BSA, likewise, did not display the CTC fluorescence B pattern indicative of the capacitated state (Fig. 7A). Capacitation was also not observed when polyvinyl alcohol (PVA) was substituted for BSA. It should be noted that protein tyrosine phosphorylation is also not supported when sperm are incubated in BSA-free medium supplemented with PVA (data not shown). The ZP-induced acrosome reaction was also completely dependent on the presence of BSA in the medium (Fig. 7B). The addition of 1 mM db-cA plus 100  $\mu$ M IBMX to the BSA-free medium, however, could substitute fully for BSA in supporting the ability of the sperm to display the CTC fluorescence B pattern (Fig. 7A) but not to undergo the ZP-induced acrosome reaction (Fig. 7B).

Although the effects of BSA depletion on capacitation using in vitro fertilization as an endpoint could not be assessed due to the fact that serum albumin is needed for the ZP-induced acrosome reaction (Fig. 7B), we did observe that sperm incubated under capacitation conditions in the absence of BSA but in the presence of 1 mM db-cA plus 100  $\mu$ M IBMX, and then incubated with ZP-intact eggs in the presence of BSA, displayed a higher percentage fertilization (79%; 32 eggs assessed) than sperm incubated in capacitation medium devoid of both BSA and db-cA/100  $\mu$ M IBMX (55%; 48 eggs assessed) (data not shown). These data are consistent with the notion that db-cA plus IBMX may function in the absence of BSA to accelerate the capacitation process and/or synchronize capacitation in a greater population of sperm (Fraser, 1981).



**Fig. 7.** Effects of dibutyryl cAMP plus 3-isobutyl-1-methylxanthine (IBMX) on the percentage of caudal epididymal mouse sperm displaying chlortetracycline fluorescence pattern 'B' and the percentage of sperm undergoing the spontaneous and zona pellucida-induced acrosome reaction (chlortetracycline pattern 'AR') in medium containing bovine serum albumin, or in medium devoid of bovine serum albumin in either the absence or presence of polyvinyl alcohol. Sperm were incubated for a period of 90 minutes in BSA-free HMB, BSA-free HMB containing 1 mg/ml polyvinyl alcohol (PVA), HMB containing 3 mg/ml BSA, or in these media containing 1 mM dibutyryl cAMP (db-cA) plus 100

μM IBMX. (A) Sperm were assessed for the percentage of CTC pattern 'B', indicative of the capacitated state. Data represent the mean ± s.e.m., n=3. (B) Sperm were assessed for the percentage of CTC pattern 'AR', representing cells undergoing acrosomal exocytosis, following a 30 minute incubation in buffer (open bars) or solubilized *zonae pellucidae* (5 ZP/μl; closed bars). Data represent the mean ± s.e.m., n=3. Statistical analysis was performed as described in Materials and Methods; \*P<0.1, \*\*P<0.025 and \*\*\*P<0.005.

**Protein tyrosine phosphorylation is accelerated by dibutyryl cAMP and IBMX in media that support capacitation**

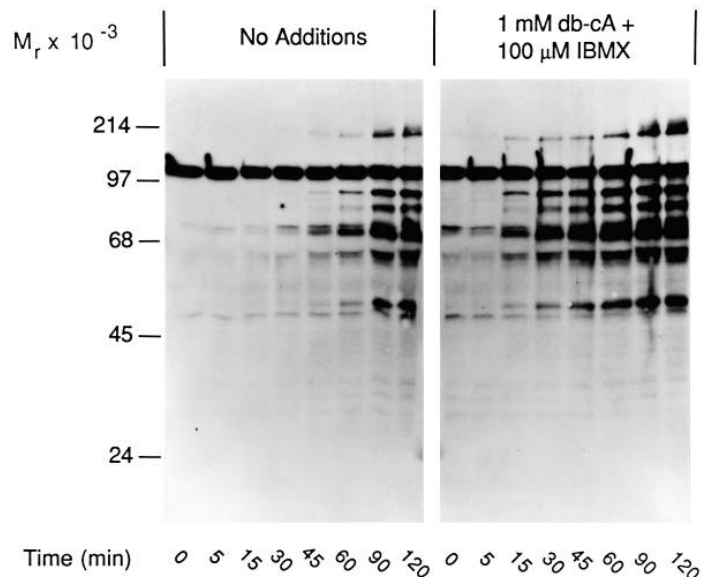
Since it has been demonstrated that dibutyryl cAMP accelerates capacitation in vitro in mouse sperm (Fraser, 1981), we examined whether the addition of db-cA plus IBMX influenced the time-dependent onset of protein tyrosine phosphorylation that occurs during the normal time course of capacitation in vitro. As shown in Fig. 8, when compared to controls, addition of 1 mM db-cA plus 100 μM IBMX to sperm in complete medium significantly reduced the time during which changes in protein tyrosine phosphorylation occurred.

**Protein tyrosine phosphorylation and capacitation appear to be regulated by cAMP-dependent protein kinase**

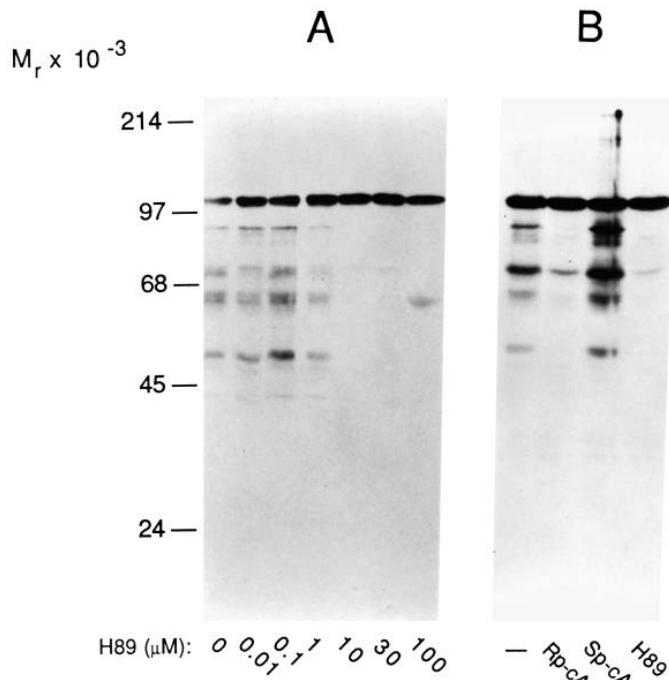
Since the primary intracellular target for cAMP action is the PKA, we examined whether PKA played a role in protein tyrosine phosphorylation and capacitation. Caudal epididymal sperm were incubated for 90 minutes in complete medium that supports capacitation in either the absence or presence of N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), a highly selective and cell permeable inhibitor of PKA (Chijiwa et al., 1990). As shown in Fig. 9A, H-89 inhibited protein tyrosine phosphorylation of sperm proteins in a concentration-dependent manner with maximal effects observed at 10 μM. In contrast, the tyrosine phosphorylation of the p95/116 hexokinase was unaffected by H-89 at all concentrations tested. In contrast to the effects of this PKA inhibitor on protein tyrosine phosphorylation, inhibitors of protein kinase C (1 μM staurosporine; 10 μM chelerythrine) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase (10 μM KN-62) had no effects on protein tyrosine phosphorylation that accompanied capacitation (data not shown).

Sperm incubated in complete medium for 90 minutes did not display protein tyrosine phosphorylation when incubated with

the cAMP antagonist Rp-cAMPS, whereas phosphorylation was observed in either the absence of a cAMP analogue or in the presence of the active cAMP analogue, Sp-cAMPS (Fig. 9B). The phosphorylation observed with Sp-cAMPS appeared to be higher than that observed in the absence of added cAMP analogue.

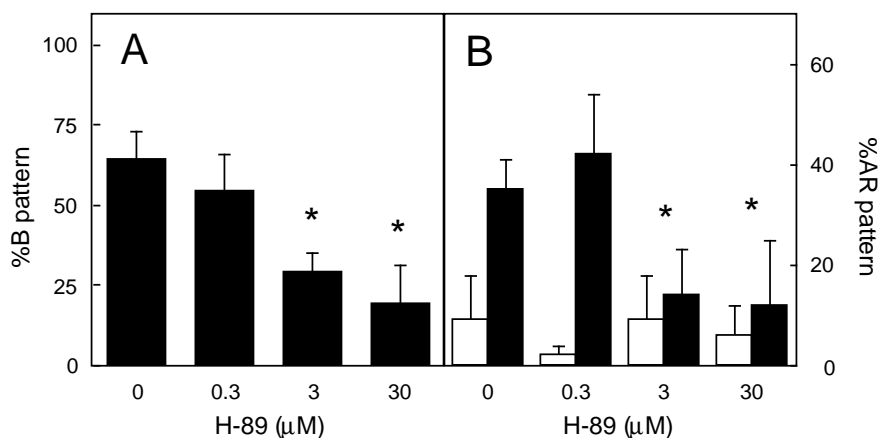


**Fig. 8.** Effects of dibutyryl cAMP plus IBMX on the time course of protein tyrosine phosphorylation in caudal epididymal mouse sperm under incubation conditions that support capacitation. Sperm were incubated under conditions conducive to capacitation in complete medium (HMB) for the times indicated at the bottom of the figure in either the absence (No Additions) or presence of 1 mM db-cA plus 100 μM IBMX. This experiment was performed two times with similar results. Shown, is a representative experiment.



**Fig. 9.** Effects of the protein kinase A inhibitor H-89, as well as Rp-cAMPS and Sp-cAMPS, on the appearance of phosphotyrosine-containing proteins in caudal epididymal mouse sperm incubated in complete media that support capacitation. (A) Sperm were incubated for 90 minutes in complete HMB medium in the absence or presence of increasing concentrations of H-89 and protein tyrosine phosphorylation assessed. (B) Sperm were incubated for 90 minutes in complete HMB medium in either the absence (–) or presence of 1 mM Rp-cAMPS (Rp-cA), 1 mM Sp-cAMPS (Sp-cA) or 30  $\mu$ M H-89, and protein tyrosine phosphorylation assessed. This experiment was performed at least three times with similar results. Shown, is a representative experiment.

H-89 was also observed to inhibit capacitation, as assessed by a number of criteria. The appearance of the CTC B pattern fluorescence was inhibited by H-89 in a concentration-dependent manner (Fig. 10A). This was accompanied by a loss in the ability of the sperm to undergo a ZP-induced



**Fig. 10.** Effects of the protein kinase A inhibitor H-89 on the percentage of caudal epididymal mouse sperm displaying chlortetracycline fluorescence pattern 'B' and the percentage of sperm undergoing the spontaneous and zona pellucida-induced acrosome reaction (chlortetracycline pattern 'AR') in complete media that support capacitation. Sperm were incubated for a period of 90 minutes in HMB containing 3 mg/ml BSA in either the absence or presence of various concentrations of H-89. (A) Sperm were assessed for the percentage of CTC pattern 'B', indicative of the capacitated state. Data represent the mean  $\pm$  s.e.m.,  $n=3$ . (B) Sperm were assessed for the percentage of CTC pattern 'AR', representing cells

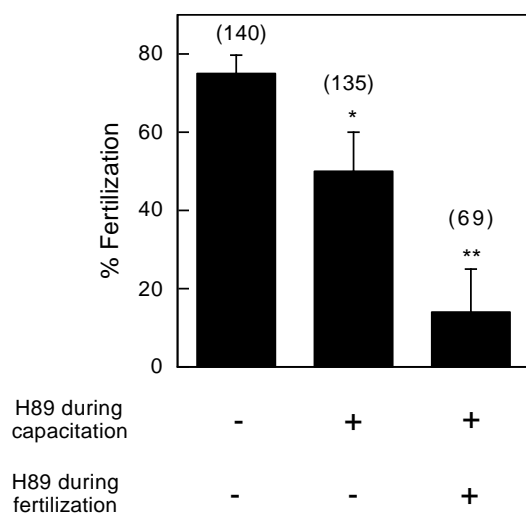
undergoing acrosomal exocytosis, following a 30 minute incubation in buffer (open bars) or solubilized zona pellucida (5 ZP/ $\mu$ l; solid bars). Data represent the mean  $\pm$  s.e.m.,  $n=3$ . Statistical analysis was performed as described in Materials and Methods; \* $P<0.005$ .

acrosome reaction (Fig. 10B). Moreover, if sperm were incubated under capacitating conditions in the presence of 30  $\mu$ M H-89, a concentration that inhibits tyrosine phosphorylation, the appearance of the CTC B pattern of fluorescence and the ZP-induced acrosome reaction (Figs 9, 10), in vitro fertilization of ZP-intact eggs is inhibited, as evidenced by a significant reduction in the formation of a pronucleus in eggs inseminated with H-89-treated sperm (Fig. 11, H-89 in capacitation media; H-89 in both capacitation and fertilization media). The effect on fertilization is especially pronounced when the H-89 concentration is held at 30  $\mu$ M by keeping the inhibitor in both the capacitation and fertilization media. The reduction in the inhibitory effect on fertilization observed when the H-89 is present only in the capacitation medium is probably due to the dilution of the H-89 at the time of in vitro insemination (from 30  $\mu$ M to 0.2  $\mu$ M). The lowering of the H-89 concentration during the 2 h insemination period might then permit capacitation to occur so that the sperm can bind to the ZP, undergo the acrosome reaction, penetrate and fertilize the egg. Consistent with this hypothesis is the fact that after dilution of the sperm to the same extent in an H89-free medium, the protein tyrosine phosphorylation increases steadily (data not shown). The inhibitory effects of H-89 on fertilization does not appear to be due to an inhibitory effect of H-89 on the egg itself, since insemination of ZP-free eggs in H-89-containing fertilization medium with sperm that have been capacitated in the absence of H-89 does not inhibit in vitro fertilization (data not shown).

## DISCUSSION

The data outlined in this report further support the interrelationship between protein tyrosine phosphorylation and the capacitation state of mouse sperm, since modulation of capacitation by the addition of cAMP analogues also modulates protein tyrosine phosphorylation. Moreover, inhibition of protein tyrosine phosphorylation and capacitation by two inhibitors of PKA (Rp-cAMPS; H89) that act on this enzyme by totally different mechanisms further support the linkage between these two events and strengthen the argument that PKA is required for the regulation of these two processes. The





**Fig. 11.** Effects of the protein kinase A inhibitor H-89 on in vitro fertilization of zona pellucida-intact and zona pellucida-free metaphase II-arrested eggs. Sperm were capacitated in either the absence (-) or presence (+) of 30  $\mu$ M H-89 for a period of 2 hours and then incubated with zona pellucida-intact eggs for 2 hours in fertilization medium containing no added H-89 (-) or 30  $\mu$ M H-89 (+), according to the procedures outlined in the Methods. When sperm were capacitated in the presence of H-89 and then diluted into fertilization medium devoid of H-89, the final H-89 concentration in the insemination droplet was 0.2  $\mu$ M. Following insemination and washing the eggs were then evaluated for the presence of a pronucleus. Numbers in parentheses above the bars indicate the numbers of eggs analyzed in the different treatment groups. The data represent the mean  $\pm$  s.e.m. of three individual experiments. Statistical analysis was performed as described in Materials and Methods; \* $P$ <0.01, \*\* $P$ <0.005.

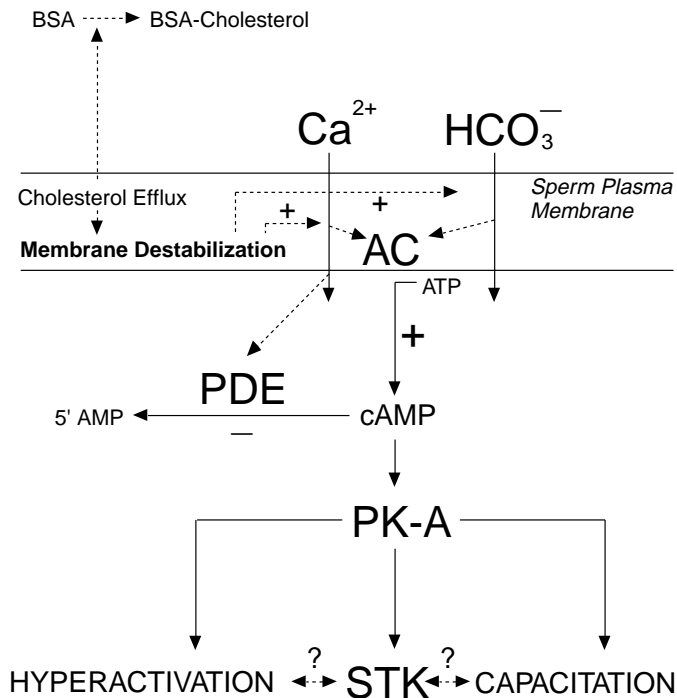
fact that biologically active cAMP analogues can overcome the deficiency in protein tyrosine phosphorylation and capacitation seen in media devoid of  $\text{NaHCO}_3$ ,  $\text{Ca}^{2+}$  or BSA suggests that this second messenger functions downstream from events stimulated by these media constituents. Although some cAMP analogues added alone could completely substitute for specific media components in supporting protein tyrosine phosphorylation, the effects of some of these analogues on protein tyrosine phosphorylation was augmented when the experiments were carried out in the presence of a cyclic nucleotide phosphodiesterase inhibitor such as IBMX (Figs 2, 4, 6). Such an effect is consistent with the idea that cAMP is up-regulating protein tyrosine phosphorylation and that inhibition of cAMP hydrolysis by IBMX augments these effects by elevating the endogenous sperm cAMP concentrations. It is interesting to note that these effects appear most pronounced in the experiments where  $\text{Ca}^{2+}$  is depleted (Fig. 4), a condition where  $\text{Ca}^{2+}$ /calmodulin activated cyclic nucleotide phosphodiesterases might not be expected to be as active. Such  $\text{Ca}^{2+}$ /calmodulin-stimulated cyclic nucleotide phosphodiesterases have been demonstrated to be present in sperm from a number of different species (Garbers and Kopf, 1980; Tash and Means, 1983; Wasco and Orr, 1984). In addition, the augmentation of protein tyrosine phosphorylation in IBMX-containing medium devoid of added  $\text{Ca}^{2+}$  could arise as a consequence of a reduction in the activity of  $\text{Ca}^{2+}$ -activated phosphoprotein

phosphatases, the activity of which is present in sperm (Tash et al., 1988).

Since both  $\text{Ca}^{2+}$  and  $\text{NaHCO}_3$  have been linked to capacitation and the regulation of mammalian sperm cAMP metabolism (Kopf and Gerton, 1991) and this intracellular second messenger has been implicated in both mammalian sperm capacitation and the acrosome reaction, we were also interested in determining whether these membrane permeable cAMP analogues could support capacitation in the absence of these ions. The results shown in Fig. 3 shows this to be the case in the experiments performed in media devoid of  $\text{NaHCO}_3$ . However, in the case of the  $\text{Ca}^{2+}$  depletion (and BSA depletion) experiments (Figs 5 and 7, respectively), the membrane permeable cAMP analogues do not completely restore the ZP-induced acrosome reaction under conditions where protein tyrosine phosphorylation appears to be completely restored (see Figs 4 and 6 respectively). This could be due to the fact that final stages of capacitation, presumably either independent of or downstream of the protein tyrosine phosphorylations, are not being completed, and/or  $\text{Ca}^{2+}$  and BSA are important components for the induction of the acrosome reaction (Fraser, 1985; Yanagimachi, 1994). Presently, it is impossible to distinguish experimentally between these two possibilities. In any event these results are consistent with a role for cAMP in the capacitation process, as concluded by other groups (Fraser, 1981; Perreault and Rogers, 1982; Berger and Clegg, 1983; Stein and Fraser, 1984; Monks et al., 1986; Fraser and Monks, 1990). The role of this intracellular messenger in capacitation is further supported by the inhibitory effects of H-89 on capacitation, as assessed by the appearance of the CTC pattern B, the ability of the sperm to undergo a ZP-induced acrosome reaction (Fig. 10), and the ability of the sperm to fertilize ZP-intact eggs (Fig. 11).

As discussed in the accompanying manuscript (Visconti et al., 1995), it is of interest that the sperm appears to intrinsically control events leading to capacitation and that the controlling factor(s) related to this maturational process may lie within the plasma membrane. Destabilization of the plasma membrane by changes in membrane fluidity could ultimately set in motion a series of intrinsic pre-programmed membrane and intracellular events that ultimately regulate those sperm functions that are normally associated with the capacitated state (e.g., changes in sperm motility; ability to undergo a ZP-induced acrosome reaction). Our data would not only support the idea that protein tyrosine phosphorylation might represent a key set of signaling events that are entrained as part of the onset of the capacitated state but that there appears to be a regulatory hierarchy that involves PKA.

Fig. 12 provides a working model for how such changes at the level of the sperm plasma membrane might engage a cAMP-dependent pathway that leads to protein tyrosine phosphorylation and the concomitant endpoints of capacitation. In this model, serum albumin, by virtue of its ability to serve as an extracellular sink for sperm membrane cholesterol efflux (Davis, 1976, 1980; Davis et al., 1979a,b; Go and Wolf, 1985; Langlais and Roberts, 1985), could lead to alterations in membrane fluidity with resultant changes in the permeability of the sperm to  $\text{Ca}^{2+}$  (Singh et al., 1978; Ruknudin and Silver, 1990) and/or  $\text{HCO}_3^-$  (Okamura et al., 1988). Changes in the permeability of the sperm towards these ions can then regulate intracellular cAMP concentrations through stimulatory effects



**Fig. 12.** Diagramme depicting the mechanism by which sperm surface changes that occur under conditions conducive to capacitation give rise to cAMP-dependent regulation of protein tyrosine phosphorylation and the capacitated state. Incubation of uncapacitated sperm under conditions conducive to capacitation gives rise to changes in the permeability of the sperm to  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  via a change in membrane fluidity or membrane destabilization. These changes in membrane properties are thought to occur by the loss of cholesterol from the membrane; this loss may be accelerated by the presence of serum albumins, which can bind cholesterol. As a consequence of the increase in  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  permeability, cAMP metabolism is altered. This could occur by the  $\text{Ca}^{2+}$  and/or  $\text{HCO}_3^-$ -induced activation of adenylyl cyclase (AC), resulting in an increase in intracellular cAMP.  $\text{Ca}^{2+}$  could also alter the hydrolysis of cAMP by stimulating the activity of a cyclic nucleotide phosphodiesterase(s) (PDE). The increase in cAMP then results in the activation of protein kinase A (PK-A) which leads to the activation of sperm tyrosine kinase(s) (STK) and/or inactivation of phosphoprotein phosphatases, the net result being an increase in protein tyrosine phosphorylation. As a consequence of an increase in protein tyrosine phosphorylation, events leading to capacitation and/or hyperactivation of motility are initiated. In this model capacitation and hyperactivation may or may not be coupled to one another. Arrows with dashed lines indicate hypothetical/uncharacterized pathways of regulation. Arrows with solid lines indicate characterized pathways of regulation observed in sperm. +, indicates positive regulation; -, indicates negative regulation.

on adenylyl cyclase. Adenylyl cyclase could be regulated either directly by  $\text{Ca}^{2+}$  (Hyne and Garbers, 1979) or via calmodulin (Kopf and Vacquier, 1984; Gross et al., 1987). Moreover, sperm cAMP concentrations could be additionally modulated by  $\text{Ca}^{2+}$  at the level of a  $\text{Ca}^{2+}$ /calmodulin-stimulated cyclic nucleotide phosphodiesterase. Adenylyl cyclase could, likewise, be regulated by  $\text{HCO}_3^-$ , as we have demonstrated in Fig. 1 and has been also demonstrated in sperm from other species (Garty and Salomon, 1987; Okamura et al., 1985;

Visconti et al., 1990). The resultant changes in cAMP would modulate the activities of PKA, of which there appears to be both Types I and II present in mouse sperm (Visconti and Kopf, unpublished observations), and such changes in enzyme activity would lead ultimately to changes in protein tyrosine phosphorylation.

The endpoints of capacitation that such tyrosine phosphorylations might regulate are also unknown. Changes in sperm motility appear to accompany capacitation process (Neill and Olds Clarke, 1987; Olds Clarke, 1990). Since it is known that cAMP plays an important regulatory role in the initiation of flagellar motility (Tash and Means, 1983) and in alterations of the flagellar curvature (Lindemann and Kanous, 1989; White and Aitken, 1989; Lindemann et al., 1991; Si and Okuno, 1993), it is possible that such cAMP-dependent changes in protein tyrosine phosphorylation might be related to these motility changes. For example, it has been demonstrated that the initiation of flagellar motility in the sperm of the rainbow trout is accompanied by the tyrosine phosphorylation of a 15,000  $M_r$  flagellar protein that occurs in a cAMP-dependent manner (Hayashi et al., 1987). This protein and the tyrosine kinase that regulates its phosphorylation have yet to be identified. It is also possible that changes in tyrosine kinase and/or phosphoprotein phosphatase activities and resultant protein tyrosine phosphorylations are involved in the regulation of ion channels (Siegelbaum, 1994 and references therein). It is interesting to note that the regulation of the voltage-gated cation channel in *Aplysia* bag neurons by tyrosine phosphorylation appears to be modulated in a cAMP-dependent manner (Wilson and Kaczmarek, 1993).

Perhaps the most intriguing observations that have resulted from these studies are that the protein tyrosine phosphorylation state in mouse sperm appears to be regulated by cAMP through the action of PKA. To the best of our knowledge, these observations provide the first example of an up-regulation of protein tyrosine phosphorylation by cAMP/PKA, and suggests that there is cross-talk at some level between PKA and tyrosine kinase/phosphoprotein phosphatase signaling pathways in the sperm. Evidence exists for the down regulation of receptor tyrosine kinase actions by cAMP (Wu et al., 1993; Cook and McCormick, 1993) but up-regulation has thus far not been demonstrated. Protein tyrosine phosphatase activity has been demonstrated to be both inhibited (Begum et al., 1992) and stimulated (Brautigan and Pinault, 1991) by cAMP, effects of which could ultimately modulate the levels of protein tyrosine phosphorylation. The level at which cAMP/PKA functions to regulate the steady state levels of protein tyrosine phosphorylation in mouse sperm under conditions conducive to capacitation (i.e., tyrosine kinases; phosphoprotein phosphatases) will be the subject of intense scrutiny in our laboratory for the foreseeable future. Subsequent investigations into the mechanism by which cAMP/protein kinase regulates those tyrosine kinases/phosphoprotein phosphatases might yield new information about alternative modes of cross-talk between these two signaling pathways.

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