

## Capacitation of mouse spermatozoa

### I. Correlation between the capacitation state and protein tyrosine phosphorylation

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#### SUMMARY

The molecular basis of mammalian sperm capacitation, defined functionally as those processes that confer on the sperm the acquisition of fertilization-competence either in vivo in the female reproductive tract or in vitro, is poorly understood. We demonstrate here that capacitation of caudal epididymal mouse sperm in vitro is accompanied by a time-dependent increase in the protein tyrosine phosphorylation of a subset of proteins of  $M_r$  40,000-120,000. Incubation of sperm in media devoid of bovine serum albumin,  $\text{CaCl}_2$  or  $\text{NaHCO}_3$ , components which individually are required for capacitation, prevent the sperm from undergoing 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. In each of these cases the protein tyrosine phosphorylation of the subset of capacitation-associated proteins does not occur. Protein tyrosine phosphorylation of these particular proteins, as well as sperm

capacitation, can be recovered in media devoid of each of these three constituents (bovine serum albumin,  $\text{CaCl}_2$  or  $\text{NaHCO}_3$ ) by adding back the appropriate component in a concentration-dependent manner. The requirement of  $\text{NaHCO}_3$  for these phosphorylations is not due to an alkalization of intracellular sperm pH or to an increase in media pH. Caput epididymal sperm, which lack the ability to undergo capacitation in vitro, do not display this capacitation-dependent subset of tyrosine phosphorylated proteins in complete media even after extended incubation periods, and do not fertilize metaphase II-arrested eggs in vitro. These data suggest that protein tyrosine phosphorylation in sperm may represent an important regulatory pathway that may ultimately modulate events associated with capacitation.

Key words: mouse, sperm, capacitation, fertilization, in vitro fertilization, tyrosine phosphorylation,  $\text{Ca}^{2+}$

#### INTRODUCTION

Unlike sperm of many lower species, mammalian sperm do not possess the ability to fertilize an egg immediately upon ejaculation, although they are motile and appear to be morphologically mature. In vivo, ejaculated sperm require a finite period of residence in the female reproductive tract to become fertilization-competent. This time-dependent acquisition of fertilization competence has been defined as 'capacitation' by both Chang (1951, 1955) and Austin (1951, 1952). Capacitation in vitro has also been accomplished in a number of different species using cauda epididymal and/or ejaculated sperm under a variety of different incubation conditions. Historically, capacitation was originally defined as the time interval of sperm incubation (either in vivo or in vitro) that is required to bring about this final functional maturation of the sperm (Chang, 1984). This loose definition takes into account all of the heretofore poorly understood biochemical

processes that ultimately regulate this event (reviewed by Yanagimachi, 1994). The definition of capacitation has also been modified over the years to include the acquisition of the ability of the acrosome-intact sperm to undergo the acrosome reaction in response to its interaction with the zona pellucida (ZP), the egg's extracellular matrix (Ward and Storey, 1984; Florman and Babcock, 1991; Kopf and Gerton, 1991).

Capacitation has been shown to be correlated with changes in sperm intracellular ion concentrations, plasma membrane fluidity, metabolism, and motility (Yanagimachi, 1994 and references therein). Although these changes have been known for many years to accompany the process of capacitation, the molecular basis underlying these events is poorly understood. Moreover, it is unclear which, if any, of these events is obligate for capacitation. It is clear that capacitation can be achieved in vitro in balanced salt solutions containing appropriate concentrations of electrolytes, metabolic energy

sources and serum albumin (as the primary protein source); this composition in many instances approximates that of the oviduct fluid (Yanagimachi, 1994). It appears that certain components of such media play an important role in promoting the capacitation process. Work in a variety of species has suggested that the presence of serum albumin (Go and Wolf, 1985; Langlais and Roberts, 1985),  $\text{Ca}^{2+}$  (Yanagimachi, 1982; Coronel and Lardy, 1987; Fraser, 1987; Ruknudin and Silver, 1990) and  $\text{NaHCO}_3$  (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Boatman and Robbins, 1991) are required for capacitation, although Fraser (1985) has suggested that the albumin requirement is for the acrosome reaction and not capacitation. Albumin is believed to be responsible for the removal of cholesterol from the sperm plasma membrane (Davis, 1976, 1980; Davis et al., 1979a,b; Go and Wolf, 1985; Langlais and Roberts, 1985; Suzuki and Yanagimachi, 1989), possibly accounting for the membrane fluidity changes that have been documented in many species during the capacitation process (Yanagimachi, 1994 and references therein). However, it must also be emphasized that since the strict endpoint of capacitation is the ability to fertilize an egg and that this property is dependent on many different sperm functions (i.e., normal motility, hyperactivated motility, ability to undergo an acrosome reaction) it is not clear which sperm function(s) are affected by these media constituents. For example, it is suggested that both  $\text{Ca}^{2+}$  and  $\text{NaHCO}_3$  are required for the induction of the acrosome reaction by the ZP (Lee and Storey, 1986; Kopf and Gerton, 1991; Yanagimachi, 1994), as well as hyperactivation (Neill and Olds-Clarke, 1987).

The transmembrane and intracellular signaling events regulating sperm capacitation are, likewise, poorly understood. As stated above some of these events may be coupled to changes in ionic movements within the sperm during this time. Changes in sperm cyclic nucleotide metabolism and protein phosphorylation have been implicated in a variety of sperm functions, including the initiation and maintenance of motility (Garbers and Kopf, 1980; Tash and Means, 1983; Lindemann and Kanous, 1989; Yanagimachi, 1994), induction of the acrosome reaction (Garbers and Kopf, 1980; Kopf and Gerton, 1991), and capacitation (Berger and Clegg, 1983; Stein and Fraser, 1984; Monks et al., 1986). Changes in tyrosine phosphorylation of specific sperm proteins have also been demonstrated to occur under conditions that support capacitation (Leyton and Saling, 1989; Duncan and Fraser, 1993), although a correlation or cause-and-effect relationship between these two parameters has not been examined.

We report here that incubation conditions conducive to capacitation of cauda epididymal mouse sperm *in vitro* promote the tyrosine phosphorylation of multiple sperm proteins. Moreover, bovine serum albumin (BSA),  $\text{Ca}^{2+}$  and  $\text{NaHCO}_3$  in the medium are absolutely required for these phosphorylations as well as capacitation, which we assessed by three independent methods. Caput sperm, which do not possess the ability to undergo capacitation and fertilize eggs, do not display the changes in protein tyrosine phosphorylation under incubation conditions normally conducive to the capacitation of cauda epididymal sperm. These data suggest that protein tyrosine phosphorylation may represent an important pathway that may ultimately regulate events associated with capacitation.

## MATERIALS AND METHODS

### 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). This medium was first prepared in the absence of  $\text{Ca}^{2+}$ , BSA and pyruvate, sterilized by passage through a 0.20  $\mu\text{m}$  filter (Nalgene) and frozen at  $-20^\circ\text{C}$  in aliquots for single use. Working 'complete' media were prepared by adding  $\text{Ca}^{2+}$  (1.7 mM), pyruvate (1 mM) and BSA (3 mg/ml), followed by gassing with 5%  $\text{CO}_2$ , 95% air to pH 7.3. HM medium was derived by replacing the 10 mM  $\text{NaHCO}_3$  with 10 mM NaCl, maintaining constant pH. In some experiments different concentrations of  $\text{NaHCO}_3$  were added back to medium HM, and the pH was maintained at 7.3 by incubation in closed Eppendorf tubes at  $37^\circ\text{C}$ . In some experiments,  $\text{Ca}^{2+}$ - and BSA-free HMB were used and the  $\text{Ca}^{2+}$  and BSA added back to various concentrations; in these media the pH was maintained at 7.3. When HM medium at different pHs were used, the pH was adjusted by the addition of NaOH.

### Preparation of sperm

Uncapacitated caudal epididymal sperm were collected from CD1 retired breeder males by placing one minced cauda epididymis in 0.5 ml of medium HM without  $\text{Ca}^{2+}$  or BSA. After 5 minutes, the sperm were washed in 10 ml of the same medium by centrifugation at 800 *g* for 10 minutes at room temperature. The sperm were resuspended to a final concentration of  $5\text{--}10 \times 10^6$  cells/ml and diluted ten times in the appropriate medium. After incubation for various periods of time, the sperm were concentrated by centrifugation at 5,000 *g* for 1 minute (room temperature), washed in 1 ml of phosphate buffered saline (PBS), resuspended in sample buffer (Laemmli, 1970) without mercaptoethanol and boiled for 5 minutes. After centrifuging at 5,000 *g* for 3 minutes, the supernatant was removed, boiled in the presence of 5% 2-mercaptoethanol for 5 minutes, and then subjected to SDS-PAGE as described below.

In some experiments, sperm were recovered from the caput epididymis, incubated in media that support capacitation and then examined for the presence of protein tyrosine phosphorylation by the methods described below.

### In vitro fertilization

*In vitro* fertilization of metaphase II-arrested eggs was performed as previously described (Moore et al., 1993), with the following modifications. Briefly, cumulus cell-enclosed metaphase II-arrested eggs were obtained from superovulated 6 week-old CF1 female mice 12 hours post hCG, and the cumulus cell mass was dispersed with hyaluronidase (0.05%) as previously described (Kurasawa et al., 1989). The collection medium was Waymouth medium (Gibco) supplemented with 5 mM  $\text{NaHCO}_3$ , pyruvate (100  $\mu\text{g}/\text{ml}$ ), gentamicin (10  $\mu\text{g}/\text{ml}$ ), fetal calf serum (FCS) (10%, Gibco) and 20 mM Hepes, pH 7.4 (Way/FCS). Sperm were obtained from (C57BL/6J $\times$ SJL/J)F<sub>1</sub> males (Jackson Laboratories). Cauda epididymal sperm from a single male were obtained by removing the epididymides and placing them in a 900  $\mu\text{l}$  drop of Whitten's medium (Whitten, 1971) supplemented with 0.1 mg/ml polyvinyl alcohol. The tissue was cut into sections and the sperm allowed to swim into the medium for 10-20 minutes. The resultant sperm suspension was then supplemented with fatty acid-poor BSA (ICN no. 103700) to the appropriate concentrations used for the particular experiments (see Results) and capacitated for 2 hours at a final concentration of  $20 \times 10^6/\text{ml}$ . Following capacitation, sperm were diluted with the same Whitten's medium to a final concentration of  $2.5 \times 10^5/\text{ml}$ . Eggs (10) and sperm ( $2.5 \times 10^3$ ) were mixed in 10  $\mu\text{l}$  drops of Whitten's medium containing the appropriate final concentration of BSA, and then cultured at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ , and 90%  $\text{N}_2$  for 2 hours. All *in vitro* fertilizations were performed approximately 14 hours post hCG. Eggs were

removed from the drop, washed six times in CZB medium (Chatot et al., 1989) to remove any loosely attached sperm adhering to the ZP, and then cultured in CZB medium. Fertilization was evaluated by the emission of the second polar body and the formation of both the male and female pronuclei, using phase contrast optics.

**Preparation and solubilization of mouse zonae pellucidae**

ZP were prepared from homogenized ovaries of virgin female 22-day old outbred CD-1 mice (Charles River) as described by Ward et al. (1992). The ZP, which were stored at -80°C in aliquots in a buffer containing 25 mM triethanolamine, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1% polyvinylpyrrolidone, pH 7.8, were centrifuged and then resuspended in a buffer containing 20 mM Tris-HCl, 130 mM NaCl, pH 7.4 at a final concentration of 200 ZP/μl. The ZP were then solubilized at 60°C for 1h, centrifuged at 15,000 g for 2 minutes at 24°C to remove particulate insoluble material and the supernatant incubated immediately with capacitated sperm for 30 minutes at a final concentration of 5 ZP/μl.

**Chlortetracycline assay**

In some experiments, sperm incubated under various conditions that either do or do not support capacitation were assessed for the capacitated state and the ability to undergo the ZP-induced acrosome reaction following incubation of the sperm with 5 ZP/μl for 30 minutes. Chlortetracycline (CTC) fluorescence assays to assess sperm capacitation and the ZP-induced acrosome reaction were carried out as described previously (Ward and Storey, 1984; Lee and Storey, 1985). It should be noted that the appearance of the CTC pattern 'B' was demonstrated by Ward and Storey (1984) to represent sperm that have undergone capacitation and are now capable of undergoing a ZP-induced acrosome reaction. Acrosomal status was also assessed using the monoclonal antibody HS-19 (Florman et al., 1984), using the protocols of Kligman et al. (1991), and similar results were observed.

**SDS-PAGE and immunoblotting**

SDS-PAGE (Laemmli, 1970) was performed in 10% gels. Electrophoretic transfer of proteins to Immobilon P in all experiments were 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; UBI) and enhanced chemiluminescence detection using an ECL kit (Amersham Corp.) according to the manufacturer's instructions. In some experiments the antiphosphotyrosine antibody was first absorbed with 10 mM O-phosphotyrosine for 30 minutes with constant rotation at 24°C prior to use for immunoblotting.

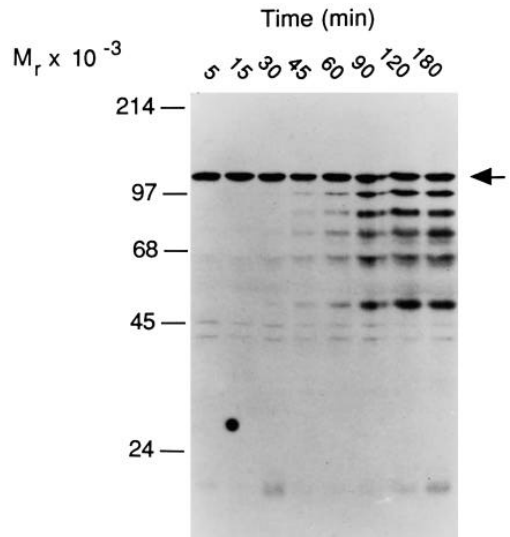
**Statistical methods**

Statistical comparison between the treatment groups was always performed against the control lacking the addition of the respective media constituent using a one-way analysis of variance (ANOVA). The acrosome reaction data were analyzed using a one-way ANOVA by comparing the inducible component of the acrosome reaction [e.g., the ZP-induced acrosome reaction following subtraction of the spontaneous acrosome reaction (AR<sub>ZP</sub> - AR<sub>spont.</sub>)].

**RESULTS**

**Time-dependent changes in protein tyrosine phosphorylation under conditions conducive to capacitation**

When epididymal sperm were incubated for various periods of time under conditions conducive to capacitation there was a

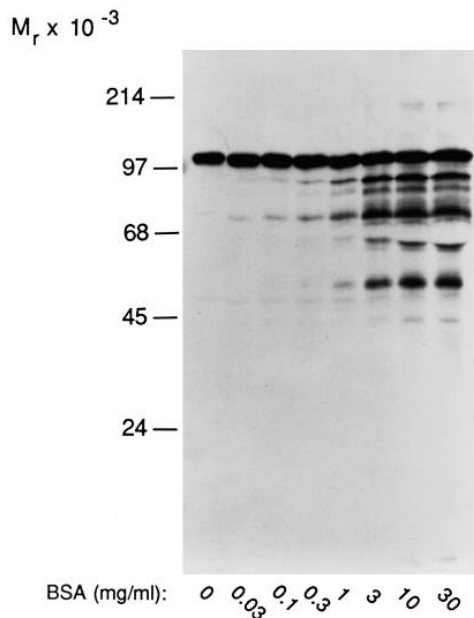


**Fig. 1.** Time course of protein tyrosine phosphorylation in caudal epididymal mouse sperm under incubation conditions that support capacitation. Sperm were incubated under conditions conducive to capacitation in complete medium (HMB) for the times indicated at the top of the figure. At those times an aliquot of the sperm suspension was removed ( $5 \times 10^5$  cells), centrifuged, washed with PBS, and the whole sperm extract then 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 (*α*-pY). This experiment was performed at least 5 times with similar results. Shown, is a representative experiment. The arrow on the right-hand side of the gel denotes the position of p95/116 ( $M_r=116,000$ ), the major phosphotyrosine-containing protein that has been identified as a hexokinase with unique properties (Kalab et al., 1994). Numbers on the left-hand side of this gel and in subsequent figures represent the position of the relative molecular mass standards ( $\times 10^{-3}$ ).

time-dependent increase in the phosphorylation on tyrosine residues of a group of proteins of  $M_r$  40,000-120,000 (Fig. 1). Tyrosine phosphorylation appeared to be maximal at about 90 minutes of incubation, a time during which capacitation was complete under these incubation conditions, as assessed by the ability of the sperm to undergo the ZP-induced acrosome reaction (Ward and Storey, 1984; Florman and Babcock, 1991) and to fertilize ZP-intact eggs *in vitro* (Wolf and Inoue, 1976; Yanagimachi, 1994 and references therein). These proteins were specifically phosphorylated on tyrosine residues since the immunoreactivity of the proteins observed with the anti-phosphotyrosine antibody (*α*-pY) was completely abolished when the antibody was first absorbed with O-phosphotyrosine (data not shown). It should be noted that the degree of tyrosine phosphorylation of the p95/116 hexokinase (denoted by the arrow in Fig. 1), which is the major phosphotyrosine-containing protein in mouse sperm membranes (Kalab et al., 1994), did not appear to change during the incubation period.

**Serum albumin requirement for protein tyrosine phosphorylation, capacitation, and *in vitro* fertilization**

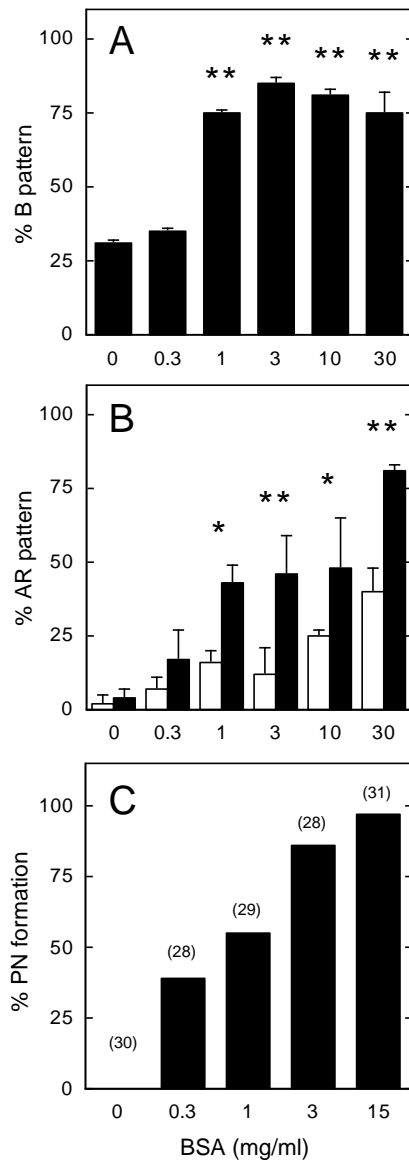
Since the presence of serum albumin in the culture medium has been demonstrated to be required for capacitation in a variety



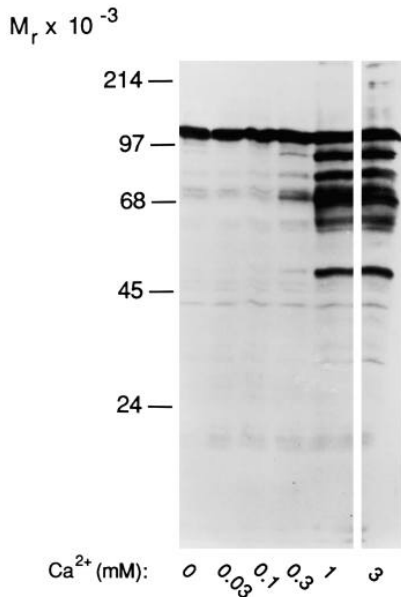
**Fig. 2.** Effects of bovine serum albumin concentration present in the capacitation media on the appearance of phosphotyrosine-containing proteins in caudal epididymal mouse sperm under incubation conditions that support capacitation. Sperm were incubated for a period of 90 minutes in HMB medium containing no added BSA or in media containing increasing concentrations of BSA (noted on the bottom of the figure). Sperm extracts were prepared as described in Fig. 1 and probed with a pY. This experiment was performed at least 5 times with similar results. Shown, is a representative experiment.

of species including the mouse (Go and Wolf, 1985; Langlais and Roberts, 1985; Yanagimachi, 1994), we examined the effects of BSA concentration in the media on the time-dependent changes in sperm protein tyrosine phosphorylation that were observed in Fig. 1. As shown in Fig. 2, when sperm were incubated in medium devoid of BSA for 90 minutes, the time normally necessary to observe maximal protein tyrosine phosphorylation of sperm incubated in the presence of complete medium (Fig. 1), protein tyrosine phosphorylation was restricted to the p95/116 hexokinase. In contrast, sperm incubated in media containing increasing concentrations of BSA displayed progressively increasing degrees of protein tyrosine phosphorylation, with the maximal degree of phosphorylation occurring at 3 mg/ml BSA (Fig. 2). The concentration dependence of the BSA requirement for protein tyrosine phosphorylation was clearly correlated with the capacitation state of the sperm, since increasing BSA concentrations resulted in an increase in the appearance of the B-pattern of CTC fluorescence (Fig. 3A), the competence of the sperm to undergo acrosomal exocytosis in response to solubilized ZP (Fig. 3B), and the ability to fertilize ZP-intact eggs in vitro (Fig. 3C).<sup>1</sup> All of these parameters can be taken as functional endpoints of the capacitated state (Ward and Storey, 1984;

<sup>1</sup>It should be noted that (C57BL/6J×SJL/J)F1 (in place of CD-1) males and the medium used for in vitro fertilization (Waymouth's medium) were different from that used in the protein tyrosine phosphorylation studies (HMB medium) (see Materials and Methods). However, when protein tyrosine phosphorylation was monitored in the sperm used for in vitro fertilization [(C57BL/6J×SJL/J)F1 males] under the same in vitro fertilization conditions, similar results to those seen with the sperm from CD-1 mice were obtained.



**Fig. 3.** Effects of bovine serum albumin concentration present in the capacitation media on the percentage of caudal epididymal mouse sperm displaying chlortetracycline fluorescence pattern 'B', the spontaneous and zona pellucida-induced acrosome reaction (chlortetracycline pattern 'AR') and pronucleus formation of metaphase II-arrested eggs inseminated in vitro. Sperm were incubated for a period of 90 minutes in HMB medium containing no added BSA or in media containing increasing concentrations of BSA (noted on the bottom of the figure) by the procedures outlined in Materials and Methods. (A) Sperm were assessed for the percentage of CTC pattern 'B', indicative of the capacitated state (Ward and Storey, 1984). 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.,  $n=3$ . (C) Sperm capacitated for 90 minutes at the various BSA concentrations were then added to metaphase II-arrested eggs and the percentage pronucleus formation (PN) assessed as described in Materials and Methods. Numbers in parentheses represent the numbers of eggs assessed in the different treatment groups. Statistical analysis was performed as described in Materials and Methods; \* $P<0.025$  and \*\* $P<0.005$ .



**Fig. 4.** Effects of calcium concentration present in the capacitation media on the appearance of phosphotyrosine-containing proteins in caudal epididymal mouse sperm under incubation conditions that support capacitation. Sperm were incubated for a period of 90 minutes in HMB medium containing no added CaCl<sub>2</sub> or in media containing increasing concentrations of CaCl<sub>2</sub> (noted on the bottom of the figure). Sperm extracts were prepared as described in Fig. 1 and probed with *a* pY. This experiment was performed at least 3 times with similar results. Shown, is a representative experiment.

Yanagimachi, 1994). It is interesting to note that the concentration of BSA required to support maximal protein tyrosine phosphorylation (3 mg/ml) is in the same range that supports functional capacitation, as assessed by the three parameters shown in Fig. 3.

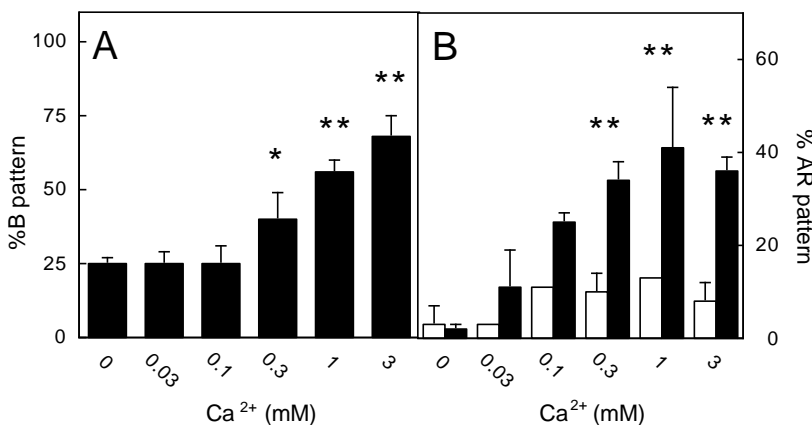
**Calcium requirement for protein tyrosine phosphorylation and capacitation**

The requirement for Ca<sup>2+</sup> in the sperm culture medium for capacitation and the ZP-induced acrosome reaction has been demonstrated in a variety of species (Yanagimachi, 1974,

1982, 1994; Didion and Graves, 1989), including the mouse (Storey and Kopf, 1991; Bailey and Storey, 1994). The relationship between protein tyrosine phosphorylation in mouse sperm and the capacitated state was further analyzed by incubating sperm in HMB medium containing 3 mg/ml BSA and various concentrations of added Ca<sup>2+</sup>. It should be noted that the normal Ca<sup>2+</sup> concentration of HMB medium is 1.7 mM. When cauda epididymal sperm were isolated in HMB medium devoid of added Ca<sup>2+</sup> and incubated for a period of 90 minutes it was demonstrated that, with the exception of the p95/116 hexokinase, the subset of phosphotyrosine containing proteins normally present during this time (Fig. 1) were absent (Fig. 4). However, incubation of sperm in media containing increasing concentrations of Ca<sup>2+</sup> for the 90 minute time period resulted in the appearance of these phosphotyrosine-containing proteins; their expression appeared maximal at 1 mM added Ca<sup>2+</sup> (Fig. 4). This Ca<sup>2+</sup> requirement for the appearance of the phosphotyrosine-containing proteins was correlated with a similar requirement for the appearance of the CTC B pattern (Fig. 5A). As a result of the appearance of the CTC B pattern, the sperm were then able to undergo acrosomal exocytosis in the presence of solubilized ZP (Fig. 5B). Unfortunately, an additional criterion of capacitation – fertilization of metaphase II-arrested eggs – could not be assessed since many steps in sperm-egg interaction are Ca<sup>2+</sup>-dependent processes (Storey and Kopf, 1991).

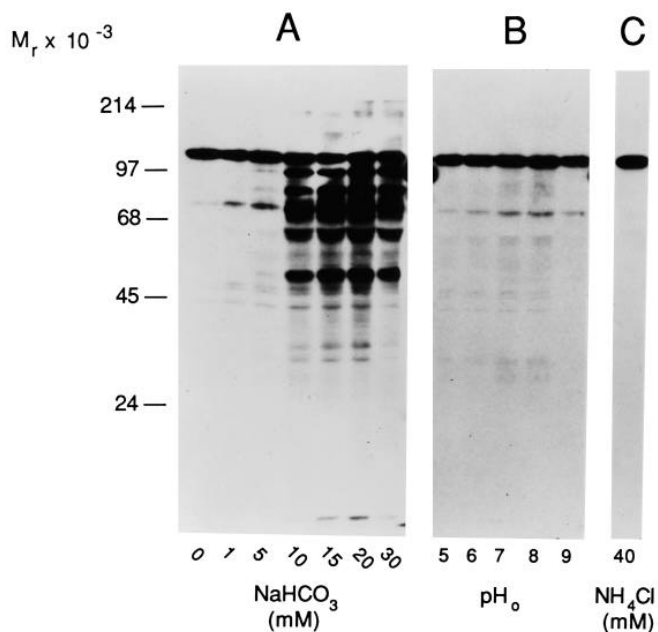
**Sodium bicarbonate requirement for protein tyrosine phosphorylation and capacitation**

Work by a number of different laboratories has demonstrated the importance of NaHCO<sub>3</sub> in the capacitation medium for the successful completion of this maturational event (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Boatman and Robbins, 1991). In order to determine the effect of NaHCO<sub>3</sub> on protein tyrosine phosphorylation, sperm were collected and incubated for 90 minutes in medium containing Ca<sup>2+</sup> and BSA, and with various concentrations of NaHCO<sub>3</sub>. As shown in Fig. 6A, sperm incubated in the absence of NaHCO<sub>3</sub> did not display the subset of phosphotyrosine-containing proteins that is normally present under conditions that lead to capacitation. Increasing concentrations of NaHCO<sub>3</sub> resulted in the appearance of these tyrosine phosphorylated proteins, with



**Fig. 5.** Effects of calcium concentration present in the capacitation media on the percentage of caudal epididymal sperm displaying chlortetracycline fluorescence pattern ‘B’ and the percentage of sperm undergoing the spontaneous and zona pellucida-induced acrosome reaction (chlortetracycline pattern ‘AR’). Sperm were incubated for a period of 90 minutes in HMB medium containing no added CaCl<sub>2</sub> or in media containing increasing concentrations of CaCl<sub>2</sub> (noted on the bottom of the figure). (A) Sperm were assessed for the percentage of CTC pattern ‘B’, indicative of the capacitated state (Ward and Storey, 1984). Data represent the mean ± s.e.m., n=4. (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=4. Statistical analysis was performed as described in Materials and Methods; \*P<0.025 and \*\*P<0.01.



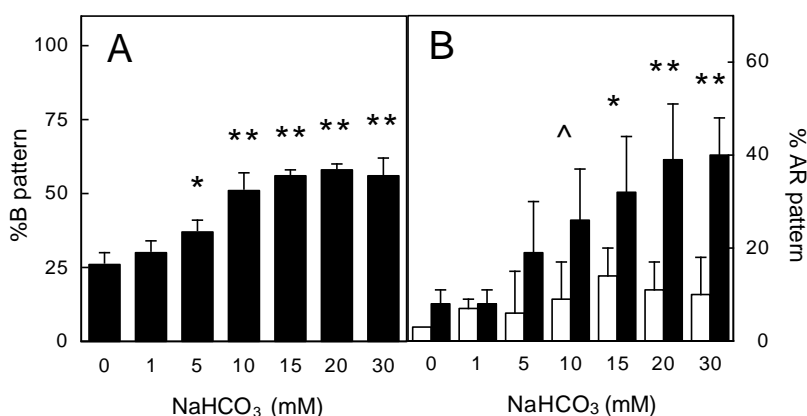
**Fig. 6.** Effects of  $\text{NaHCO}_3$  concentration present in the capacitation media, extracellular pH, and the permeant base,  $\text{NH}_4\text{Cl}$ , on the appearance of phosphotyrosine-containing proteins in caudal epididymal mouse sperm under incubation conditions that support capacitation. (A) Sperm were incubated for a period of 90 minutes in HM medium containing no added  $\text{NaHCO}_3$  or in media containing increasing concentrations of  $\text{NaHCO}_3$  (noted on the bottom of the figure). The pH was maintained throughout this period by incubation of the sperm in closed Eppendorf microcentrifuge tubes. Sperm extracts were prepared as described in Fig. 1 and probed with  $\alpha\text{P}_Y$ . (B) Sperm were incubated for a period of 90 minutes in HM medium containing no added  $\text{NaHCO}_3$  with the final pH of the media adjusted to the values noted on the bottom of the figure. (C) Sperm were incubated for a period of 90 minutes in HM medium, pH 7.3 containing no added  $\text{NaHCO}_3$  but in the presence of 40 mM  $\text{NH}_4\text{Cl}$ , a permeant base which has been demonstrated to alkalinize intracellular pH (Schackmann and Boon Chock, 1986). All of these experiments were performed at least 3 times with similar results. Shown, is a series of representative experiments.

maximal expression at 10–15 mM  $\text{NaHCO}_3$ , which is the normal concentration of  $\text{NaHCO}_3$  present in these media (Lee

and Storey, 1986). Again, the extent of protein tyrosine phosphorylation of the p95/116 hexokinase was completely independent of the  $\text{NaHCO}_3$  concentration. The ability of  $\text{NaHCO}_3$  to stimulate protein tyrosine phosphorylation was not due to an effect on the extracellular pH since media lacking  $\text{NaHCO}_3$  and buffered to various pH's (pH 5–9) did not mimic the effect of  $\text{NaHCO}_3$  on protein tyrosine phosphorylation (Fig. 6B). Moreover, alkalinization of intracellular pH with  $\text{NH}_4\text{Cl}$  (Schackmann and Boon Chock, 1986) did not mimic the  $\text{NaHCO}_3$  effect (Fig. 6C). Consistent with the results obtained by varying the BSA and  $\text{Ca}^{2+}$  concentrations of the media, the appearance of the CTC 'B' pattern, indicative of the capacitated state (Fig. 7A), and the ability of the sperm to undergo acrosomal exocytosis in response to solubilized ZP, a functional endpoint of capacitation (Fig. 7B), were also completely dependent on the presence of  $\text{NaHCO}_3$  in the media. These results confirm the earlier results of Lee and Storey (1986) and Boatman and Robbins (1991) regarding the essential role of  $\text{NaHCO}_3$  in capacitation and the acrosome reaction.

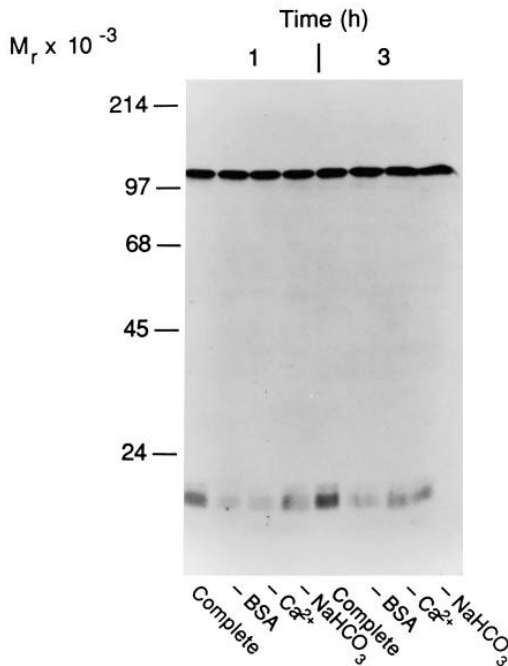
#### Caput epididymal sperm, which do not have the ability to undergo capacitation, do not display the time-dependent changes in protein tyrosine phosphorylation

All of the previous experiments utilized sperm that were recovered from the cauda epididymis. Such sperm have been demonstrated to undergo functional capacitation both in vitro and in vivo, as assessed by their ability to bind to the ZP/ZP3, undergo the ZP3-induced acrosome reaction, fertilize eggs and give rise to normal progeny (Fraser, 1993; Bleil, 1993). In contrast, sperm recovered from the caput epididymis of the mouse (Hoppe, 1975), as well as many other species (Yanagimachi, 1994 and references therein), do not have the ability to undergo capacitation and display full fertilizing capacity. In order to establish further the relationship between protein tyrosine phosphorylation and the capacitation state, sperm were recovered from the caput epididymis and then incubated in complete medium or media devoid of BSA,  $\text{Ca}^{2+}$  or  $\text{NaHCO}_3$ . As shown in Fig. 8, with the exception of the p95/116 hexokinase, protein tyrosine phosphorylation was not observed even under extended times of incubation (3 hours). Moreover, caput sperm were also demonstrated not to fertilize metaphase II-arrested eggs in vitro (data not shown).



**Fig. 7.** Effects of  $\text{NaHCO}_3$  concentration present in the capacitation media on the percentage of caudal epididymal sperm displaying chlortetracycline fluorescence pattern 'B' and the percentage of sperm undergoing the spontaneous and zona pellucida-induced acrosome reaction (chlortetracycline pattern 'AR'). Sperm were incubated for a period of 90 minutes in HM medium containing no added  $\text{NaHCO}_3$  or in media containing increasing concentrations of  $\text{NaHCO}_3$  (noted on the bottom of the figure). (A) Sperm were assessed for the percentage of CTC pattern 'B', indicative of the capacitated state (Ward and Storey, 1984). 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/ $\mu\text{l}$ ; closed bars). Data represent the mean  $\pm$  s.e.m.,  $n=5$ . Statistical analysis was performed as described in Materials and Methods;  $^{\wedge}P<0.1$ ,  $*P<0.05$  and  $**P<0.01$ .



**Fig. 8.** Effects of complete medium, or media devoid of BSA,  $\text{Ca}^{2+}$ , or  $\text{NaHCO}_3$  on the time-dependent appearance of the phosphotyrosine-containing proteins in caput epididymal mouse sperm under incubation conditions that support capacitation. Sperm were incubated in complete HMB medium (Complete), or media devoid of BSA (-BSA),  $\text{CaCl}_2$  ( $-\text{Ca}^{2+}$ ) or  $\text{NaHCO}_3$  ( $-\text{NaHCO}_3$ ) for either 1 or 3 hours. Sperm extracts were prepared at these times as described in Fig. 1 and probed with *a*Y. This experiment was performed at least 3 times with similar results. Shown, is a representative experiment.

**DISCUSSION**

Our results demonstrate that under a variety of different incubation conditions capacitation of caudal epididymal mouse sperm in vitro is correlated with the tyrosine phosphorylation of a subset of proteins of  $M_r$  40,000-120,000. These data suggest that protein tyrosine phosphorylation may ultimately be important in controlling events leading either to the capacitated state or to events that occur as a consequence of this final maturational process. It is of interest to note that the tyrosine phosphorylation of these proteins occurs in a time-dependent fashion in the absence of an apparent external stimulus. Nevertheless, this increase in phosphorylation is slow when compared to receptor-mediated changes in tyrosine phosphorylation that occur in cells that respond to growth factors or other ligands that function through cell surface receptors (Sefton and Campbell, 1991; Iwashita and Kobayashi, 1992). However, such observations are not inconsistent with what is known about the process of capacitation. For example, changes in sperm intracellular ion concentrations, metabolism and motility are known to accompany capacitation in a variety of species (Florman and Babcock, 1991; Yanagimachi, 1994 and references therein), and changes in such cellular parameters are normally regulated by extrinsic factors in somatic cells. Following sperm capacitation, such changes have also been demonstrated to be regulated by extracellular ligands (e.g., ZP,

progesterone) (Ward and Kopf, 1993). Although these changes occur in situ following residence in the female tract and could, therefore, be modulated by reproductive tract factors and/or environmental conditions (e.g. tract pH,  $\text{pO}_2$ ,  $\text{pCO}_2$ ), in vitro incubation of sperm in relatively simple defined media totally supports capacitation, thus suggesting that the initiation and completion of the capacitated state may be regulated by processes intrinsic to the sperm cell itself. This idea is further supported by the fact that capacitation does not appear to be species-specific or organ-specific [i.e., sperm can be capacitated in regions other than the female reproductive tract (Yanagimachi, 1994 and references therein)].

From the standpoint of understanding the fertilization process, it is of interest to study how mammalian sperm might intrinsically control events that lead to capacitation. One likely set of controlling factors related to this maturational process lies within the sperm plasma membrane. Epididymal sperm maturation has been shown to be associated with changes in sperm membrane lipid composition (Wolf and Vogelmayr, 1984; Nikolopoulou et al., 1985; Parks and Hammerstedt, 1985; Langlais and Roberts, 1985; Schlegel et al., 1986; Wolf et al., 1988), intramembraneous protein particle distribution (Olson, 1980; Suzuki, 1981) and an increase in membrane cholesterol (Suzuki, 1988; Seki et al., 1992). Such changes in membrane composition and structure might impart a stabilizing effect to the membrane of these cells. Of related interest is the fact that sperm obtained from regions distal to the corpus epididymis have, in many species, a greater fertilizing capacity than sperm obtained from the caput epididymis (Yanagimachi, 1994), related in part to their ability to undergo capacitation. The mixing of these capacitation-competent caudal epididymal sperm with the accessory seminal fluids prior to ejaculation might impart additional modulation of sperm membrane properties so that premature destabilization of the membrane does not occur during the extended residence time in the female reproductive tract. In this regard, it is of interest to note that in many species ejaculated sperm are more difficult to capacitate than epididymal sperm (Yanagimachi, 1994 and references therein), and this could be due in part to the coating of the sperm surface with proteins that inhibit processes normally associated with capacitation. Such proteins might include caltrin/seminal plasmin, an inhibitor of sperm  $\text{Ca}^{2+}$  uptake present in bovine seminal fluids (Rufo et al., 1982, 1984) and the poorly characterized 'decapacitation factor(s)' (Yanagimachi, 1994 and references therein).

Capacitation has also been shown to be accompanied by changes in membrane composition and structure, as assessed by changes in the distribution of a variety of surface proteins (Yanagimachi, 1994 and references therein), changes in the distribution of intramembraneous particles (Koehler and Gaddum-Rosse, 1975; Suzuki and Yanagimachi, 1989), and a reduction in the density of filipin-sterol complexes (Suzuki and Yanagimachi, 1989) indicative of a loss of cholesterol. Such changes could influence membrane fluidity (Wolf and Cardullo, 1991) which could theoretically increase the rate at which intrinsic pre-programmed membrane events are activated. The activation of such events might then account for the changes in ionic movements, metabolism and motility that accompany capacitation in many species. For example, Suarez et al. (1993) have demonstrated that intracellular  $\text{Ca}^{2+}$  levels increase during capacitation and hyperactivation of hamster sperm and that these changes oscillate with the flagellar beat

cycle of these sperm. It is believed that hyperactivated motility is tightly correlated with the capacitated state, although there are data in the mouse that support the notion that, under specific conditions, these two events can be uncoupled from one another (Neill and Olds-Clarke, 1987; Olds-Clarke, 1989). In any event, an extensive literature base dealing with the phenomenon of sperm capacitation suggests that changes in sperm membrane properties appear to be one of the key regulatory events that is needed for capacitation to occur.

Based on these aforementioned observations and the results presented in this report, it can be argued that those key components of culture media that are required to support capacitation also play an important obligatory role in bringing about the changes in protein tyrosine phosphorylation that are observed during capacitation. 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) and the consequent alteration in membrane fluidity and membrane destabilization, would help to accelerate those ionic changes that would ultimately result in the capacitated state (e.g., B pattern of CTC fluorescence; ability to undergo a ZP-induced acrosome reaction; ability to fertilize a ZP-intact egg) and would result in changes in protein tyrosine phosphorylation. It also appears that both external  $\text{Ca}^{2+}$  and  $\text{NaHCO}_3$  play a key role in those processes leading to protein tyrosine phosphorylation.

An argument could be made for the possibility that, under the experimental conditions utilized in these studies, these media constituents are important solely for maintaining the viability of the sperm, and not for signaling events leading to capacitation. Two observations argue against this possibility. First, incubating the sperm for a period of two hours in media devoid of such components, followed by the addition of the respective component back to the culture media, results in the ability of the sperm to display the protein tyrosine phosphorylations (Visconti, Connors and Kopf, unpublished observations). Second, as demonstrated in the accompanying paper (Visconti et al., 1995), the mechanism by which these components of the culture media regulate protein tyrosine phosphorylation appears to be through a protein kinase A-dependent pathway. Specifically, both protein tyrosine phosphorylation and capacitation can be rescued in sperm incubated in media devoid of any one of these three media components by the addition of cAMP agonists. Further experiments will be needed to understand the exact mechanism by which this regulation occurs and to determine the nature of the kinases and/or phosphatases involved. An understanding of the role of protein tyrosine phosphorylation in sperm capacitation will only be obtained once the protein substrates are localized and identified.

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