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Regulation of Protein Tyrosine Phosphorylation during Bovine Sperm Capacitation by a Cyclic Adenosine 3′,5′-Monophosphate-Dependent Pathway[1]

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ABSTRACT

Mammalian sperm capacitation, defined as an obligatory maturational process leading to the development of the fertilization-competent state, results from a poorly understood series of morphological and molecular events. We report here that ejaculated bovine sperm, incubated under conditions that support capacitation in vitro, display a reproducible pattern of protein tyrosine phosphorylations that are regulated by a cAMP-dependent pathway. The appearance of these tyrosine phosphorylated proteins correlated temporally with the time course of capacitation induced by heparin, and these phosphorylations displayed a similar heparin concentration dependence. Glucose, which inhibits capacitation, inhibited these protein tyrosine phosphorylations in media containing heparin. The biologically active cAMP analogues (dibutyryl cAMP [db-cAMP], 8-bromo cAMP, Sp-cAMPS) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) induced the same protein tyrosine phosphorylation patterns as seen with heparin. Moreover, these cAMP agonists could overcome the inhibition of the heparin-induced tyrosine phosphorylations by glucose. In contrast, Rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS), a protein kinase A (PK-A) antagonist, blocked the capacitation-associated increases in protein tyrosine phosphorylation. This cAMP regulation of the protein tyrosine phosphorylation pattern is mediated by PK-A since *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-dihydrochloride (H89), another inhibitor of PK-A, inhibited the heparin-induced protein tyrosine phosphorylation pattern in a concentration-dependent manner in either the absence or presence of db-cAMP, IBMX, and glucose. These data support a model for sperm capacitation that includes protein tyrosine phosphorylation as an important regulatory pathway, and a role for cAMP/PK-A in the regulation of this pathway leading to capacitation. These studies are the first to report a unique interrelationship between tyrosine kinase/phosphatase and cAMP signaling pathways at the level of PK-A in bovine sperm capacitation.

INTRODUCTION

Capacitation is defined as the obligatory maturational process(es) of sperm leading to the development of the fertilization-competent state, either in vivo in the female reproductive tract or under defined conditions in vitro [1]. This time-dependent acquisition of fertilization competence was originally defined as "capacitation" by both Chang [2] and Austin [3, 4]. Freshly ejaculated spermatozoa, which have undergone epididymal maturation, are motile but infertile until they undergo capacitation ([1] and references therein). In vivo, ejaculated sperm require a finite period of residence in the female reproductive tract to become fertilization competent. The definition of capacitation has been modified over the years to include acquisition of the ability of the acrosome-intact sperm to undergo the acrosome reaction (AR) in response to its interaction with the zona pellucida (ZP), the egg's extracellular matrix [5–7].

Capacitation has been shown to be correlated with changes in sperm intracellular ion concentrations, plasma membrane fluidity, metabolism, and motility ([1] and references therein; [8]). 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. 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 oviductal fluid [1]. It appears that certain components of such media play an important role in promoting the capacitation process.

Studies of bovine sperm capacitation have shown that in vitro capacitation can be accomplished in media containing heparin [9] or oviductal fluid, in which the capacitating factor is thought to be a heparin-like glycosaminoglycan (GAG) [10–13]. It has been postulated that GAGs may promote capacitation by binding to seminal plasma proteins that are adsorbed to the sperm plasma membrane, thereby changing the properties of the plasma membrane [14, 15]. Glucose inhibits heparin-induced capacitation by an undefined mechanism that could involve changes in sperm energy metabolism and intracellular pH [16–18].

The transmembrane and intracellular signaling events regulating sperm capacitation are poorly understood. As stated above, some of these events may be coupled to changes in ionic movements within the sperm. 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 [1, 19–21], induction of the AR [7, 19], and capacitation [22–25]. Changes in sperm cyclic nucleotide metabolism have been linked to capacitation in the bull [26, 27], pig [22], mouse (reviewed by [28]), and hamster [29]. Cyclic AMP also appears to be crucial for the capacitation of rhesus monkey and macaque sperm, both of which require incubation with the membrane-permeable cAMP analogue dibutyryl cAMP (db-cAMP) and the phosphodiesterase inhibitor, caffeine, before in vitro fertilization is possible [30, 31]. Changes in tyrosine phosphorylation of specific murine and human sperm proteins have also been demonstrated to

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occur under conditions that support capacitation [25, 32–35].

Previous studies in this laboratory have established a correlation between protein tyrosine phosphorylation and the capacitation of murine epididymal sperm, as confirmed by the competency of sperm to undergo the ZP-induced AR and in vitro fertilization [25, 34]. Moreover, both capacitation and protein tyrosine phosphorylation appear to be regulated by cAMP, since these two events can be stimulated or inhibited by cAMP analogues or protein kinase A (PK-A) inhibitors, respectively [25]. This cAMP regulation of protein tyrosine phosphorylation is at the level of PK-A [25], which represents a unique mode of signal transduction cross-talk. A recent study has similarly shown that protein tyrosine phosphorylation during human sperm capacitation is also regulated by a cAMP-dependent pathway [35].

In bovine sperm, it is postulated that the effects of heparin on capacitation are mediated by cAMP for the following reasons. First, heparin-induced capacitation can be blocked by the cAMP antagonist, Rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS) [27]. Second, inhibition of heparin-induced capacitation by Rp-cAMPS can be reversed by an active cAMP analogue, 8-bromo-cAMP [27]. Third, inhibition of heparin-induced capacitation by glucose can be reversed by 8-bromo-cAMP or the phosphodiesterase inhibitors, 3-isobutyl-1-methylxanthine (IBMX) or caffeine, in a concentration-dependent manner [26]. Fourth, sperm cAMP concentrations increase during heparin-induced capacitation [26]. Finally, inhibitors of heparin-induced capacitation, glucose and protamine, also suppress the increase in cAMP concentrations observed during capacitation [26]. Moreover, these same studies support a role for protein phosphorylation in capacitation, since bovine sperm could be capacitated in the presence of the protein phosphatase inhibitor, okadaic acid, in the absence of heparin [27]. Apparently, heparin stimulation and glucose inhibition of bovine sperm capacitation in vitro involve a cAMP/PK-A-dependent pathway, as well as protein phosphorylation, but the possible downstream effectors of such a pathway have not yet been examined [26, 27].

Based on the recent murine sperm capacitation studies [25, 34], as well as studies by others [1, 8, 35], a unifying model for sperm capacitation is emerging that involves plasma membrane changes leading to changes in ion fluxes, stimulation of adenylyl cyclase, an increase in cAMP concentrations, and activation of PK-A, which interacts with a sperm protein tyrosine kinase/phosphatase signaling pathway to regulate protein tyrosine phosphorylation and subsequent downstream events. The objectives of this study were threefold: 1) to examine the patterns of protein tyrosine phosphorylation in bovine sperm incubated under conditions that support capacitation, 2) to evaluate the effects of inhibitors and enhancers of the in vitro capacitation of bovine sperm on protein tyrosine phosphorylation, and 3) to assess the involvement of a cAMP-dependent pathway in the regulation of capacitation-associated protein tyrosine phosphorylation.

MATERIALS AND METHODS

Reagents

Heparin (from porcine intestinal mucosa, 176 USP U/mg), L-α-lysophosphatidylcholine (LC; type 1, from egg yolk), BSA (fraction V, catalog number A-4503), d-(+)-glucose, db-cAMP, 8-bromo cAMP, 8-bromo cGMP, 3-isobutyl-1-methylxanthine (IBMX), and the nuclear exclusion dye Hoechst 33258 (H258) were purchased from Sigma Chemical Company (St. Louis, MO). Fluoresceinated *Pisum sativum* agglutinin (fluorescein isothiocyanate [FITC]-PSA) was obtained from Vector Laboratories (Burlingame, CA). Sp-cAMPS and Rp-cAMPS were from Research Biochemicals International (Natick, MA). H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-dihydrochloride) was from LC Labs (Woburn, MA) and Calbiochem (La Jolla, CA). Anti-phosphotyrosine antibody (clone 4G10) was obtained from Upstate Biotechnology, Inc. (UBI, Lake Placid, NY). All other reagents were of the highest analytical grade.

Culture Media

Two basic media were used for all experiments: a modified Tyrode's bicarbonate-buffered medium (Sp TALP) and a modified Tyrode's Hepes-buffered medium (Sp TALPH), both as described by Parrish et al. [9]. These media were modified as follows. BSA was replaced by 1 mg/ml polyvinyl alcohol in Sp TALPH to reduce its ability to capacitate sperm [34], and 0.4 mM EDTA was added to all media. Both media were first prepared in the absence of Ca^{2+}, BSA, and pyruvate and frozen at −20°C in aliquots for single use. Working "complete" Sp TALPH medium was prepared by adding Ca^{2+} (2 mM), pyruvate (1 mM), $NaHCO_3$ (10 mM), polyvinyl alcohol (1 mg/ml), and gentamycin (50 μg/ml). Working complete Sp TALP medium was prepared by adding Ca^{2+} (2 mM), pyruvate (1 mM), $NaHCO_3$ (25 mM), and BSA (6 mg/ml). For some experiments, these media were prepared with the addition of glucose (5 mM). The pH of Sp TALPH was adjusted to 7.40–7.45 at 22–24°C; the pH of Sp TALP was adjusted to 7.4 after equilibration for 1 h at 39°C in an atmosphere of 5% CO_2 in air; and both media were sterilized by passage through a 0.20-μm filter (Nalgene, Fisher, Pittsburgh, PA).

Preparation of Sperm

Ejaculated bovine sperm (*Bos taurus*, Holstein), collected by artificial vagina, were generously provided by Atlantic Breeders' Cooperative (Lancaster, PA) and the Hofmann Center at New Bolton Center, University of Pennsylvania School of Veterinary Medicine (Kennett Square, PA). The sperm were immediately assessed for motility by light microscopy and then, at 10–20 min after collection, were split and diluted 1:5 into Sp TALPH with and without 5 mM glucose. These samples were then subjected to two washes by centrifugation at 375 × *g* for 10 min in Sp TALPH with and without 5 mM glucose to remove seminal plasma, as previously described [9]. Sperm were kept at 22–24°C in Sp TALPH after the washes for transport to the laboratory (1–2 h) and then transferred to Sp TALP, with or without 5 mM glucose, after a final centrifugation to remove Sp TALPH media. The concentration of sperm in the Sp TALP solution was determined by hemocytometer and adjusted to 50 × 10^6 cells/ml with Sp TALP, with or without 5 mM glucose, before the capacitation experiments were initiated.

For capacitation, 100-μl aliquots of the sperm suspension (5 × 10^6 cells), as well as the appropriate test reagents (1–3 μl), were incubated in uncapped, 1.5-ml polypropylene microcentrifuge tubes at 39°C in a Thermolyne 37900 (Fisher Scientific) culture incubator flushed continually with 5% CO_2 in air at high humidity as previously described [9]. Additions to the sperm suspension were made immediately before initiation of the incubation period. After the incubation period, for variable times between 0 and

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7 h, or at a fixed time of 4 h, the sperm were concentrated by centrifugation at 10 000 × g for 3 min at room temperature and then washed in 1 ml PBS containing 0.2 mM Na_2VO_3 at room temperature; the sperm pellet was then resuspended in sample buffer [36] without mercaptoethanol and boiled for 5 min. After centrifuging at 10 000 × g for 3 min, the supernatant was removed, boiled in the presence of 5% 2-mercaptoethanol for 5 min, and then subjected to SDS-PAGE as described below.

For all experiments, sperm from at least three different mature Holstein bulls, previously shown to be fertile, were assayed to control for individual variation.

Induction of Acrosome Reactions and Evaluation of Acrosomal Status

Sperm were incubated as described above at a concentration of 50 × 10^6 cells/ml and in a volume of 200 μl. At the conclusion of the 4-h incubation, the sample was divided for the assessment of protein tyrosine phosphorylation, uninduced ARs, and LC-induced ARs. Division of the sample was as follows. Two aliquots of 50 μl were each added to 200 μl Sp TALP with or without 5 mM glucose at 39°C equilibrated with 5% CO_2 in air for a final concentration of 10 × 10^6 cells/ml. One of these 1:5 dilutions was stained with H258/[FITC]-PSA as described below; the other was first incubated for 15 min at 39°C in 5% CO_2 in air with 100 μg/ml LC dissolved in Sp TALPH on the day of the experiment, as described previously, for induction of the AR in capacitated sperm [9]. This sample was then immediately stained with H258/[FITC]-PSA. The remaining 100 μl of sperm suspension at 50 × 10^6 cells/ml was processed for SDS-PAGE and phosphotyrosine immunoblotting, as described in the previous section, to correlate the protein tyrosine phosphorylations with these endpoints of capacitation.

H258/[FITC]-PSA staining was performed as described previously for stallion sperm [37]. Briefly, to assess viability, sperm were incubated at a concentration of 10 × 10^6 cells/ml with 2 μg/ml of H258, a nuclear exclusion dye, for 5 min. This was followed by an 8-min incubation with 1% paraformaldehyde to fix sperm. After placement of 5 μl of this suspension onto polycarbonate filters (4.0-μm pore size; Poretics Corporation, Livermore, CA), sperm were permeabilized with 95% ethanol (−20°C), and acrosomal contents were labeled with [FITC]-PSA and scored as described previously [37]. All incubations were performed in the dark. A total of 200 sperm were assessed for each sample. Sperm with no fluorescence over the acrosomal region and sperm with a thin band of green fluorescence over the equatorial region of the sperm head were scored as acrosome-reacted sperm. In addition, sperm were scored as acrosome reacted when the smooth acrosomal border was replaced by a wrinkled border, since the acrosomal membrane appeared disrupted both under oil and (for those sperm mounted on slides without a membrane) when examined with phase contrast (data not shown).

SDS-PAGE and Immunoblotting

SDS-PAGE [36] was performed using 10% gels. Electrophoretic transfer of proteins to Immobilon P (Amersham Life Science, Arlington Heights, IL) in all experiments was carried out according to the method of Towbin et al. [38], at 70 V (constant) for 2 h at 4°C. Immunodetection of proteins transferred to Immobilon P was performed at room temperature as described previously [39] using a primary monoclonal antibody against phosphotyrosine (clone 4G10; UBI), a secondary goat anti-mouse horseradish peroxidase-conjugated IgG from Bio-Rad Laboratories (Hercules, CA), and enhanced chemiluminescence detection (ECL) using an Amersham ECL kit according to the manufacturer's instructions. As controls for the specificity of the antiphosphotyrosine antibody, duplicate blots were probed either with antibody that was first absorbed with 10 mM *o*-phosphotyrosine for 30 min with constant rotation at 24°C prior to use for immunoblotting, or with the primary antibody omitted.

TABLE 1. Assessment of bovine sperm capacitation by the LC-induced acrosome reaction.*

	Expt. 1	Expt. 2	Expt. 3	Expt. 2 + Glucose[a]	Expt. 3 + Glucose[a]	Expt. 4 + Glucose[a]
Pre[b]	6	6	N/A	3	25	3
+ LC[c]	N/A	N/A	27	N/A	25	4
No Add.[d]	16	17	15	12	21	12
+ LC[c]	22	39	18	38	50	25
Heparin[e]	18	11	32	19	41	14
+ LC[c]	33	49	40	19	37	22
cAMP[f]	13	21	27	19	29	11
+ LC[c]	41	64	41	31	40	19
Hep/cAMP[g]	28	11	24	13	39	18
+ LC[c]	37	38	40	34	48	35

* Values represent the percentage of AR sperm of 200 sperm counted per treatment.
[a] + Glucose indicates incubation with 5 mM glucose; see *Materials and Methods* for details.
[b] Pre, preincubation sample.
[c] Under all conditions, the second row (+LC) shows the percentage of ARs following a 15-min incubation with 100 μg/ml LC.
[d] No add., no additions to the Sp TALP incubation media.
[e] Heparin, incubated with 10 μg/ml heparin.
[f] cAMP, incubated with 1 mM db-cAMP plus 100 μM IBMX.
[g] Hep/cAMP, incubated with 10 μg/ml heparin and 1 mM db-cAMP plus 100 μM IBMX.

RESULTS

Assessment of Sperm Capacitation Conditions by Measurement of the Percentage of LC-Induced ARs

While the conditions used in this study have been well characterized and have been shown to support the capacitation of ejaculated bovine sperm [9, 40], capacitation was assessed under our incubation conditions for a small number of samples to ensure that sperm were prepared and incubated appropriately. The AR is considered to be the major endpoint marker for the completion of capacitation [6]. While the zona pellucida-induced AR is considered the most physiologic assessment of acrosomal responsiveness [6], in the bovine system the lysophosphatidylcholine (LC)-induced AR is considered an appropriate endpoint marker for capacitation and has been shown to correlate with the rate of in vitro fertilization, another indicator of capacitation [9]. The purpose of this study was to show that our media and incubation conditions support LC-mediated membrane fusion events that have been associated with capacitation, rather than to validate the LC assay or to correlate levels of protein tyrosine phosphorylation and capacitation.

In the current study, sperm samples that had been incubated for 4 h at 39°C in 5% CO_2 in air were assessed for uninduced ARs and LC-induced ARs as described in *Materials and Methods*. Table 1 displays the total percentage

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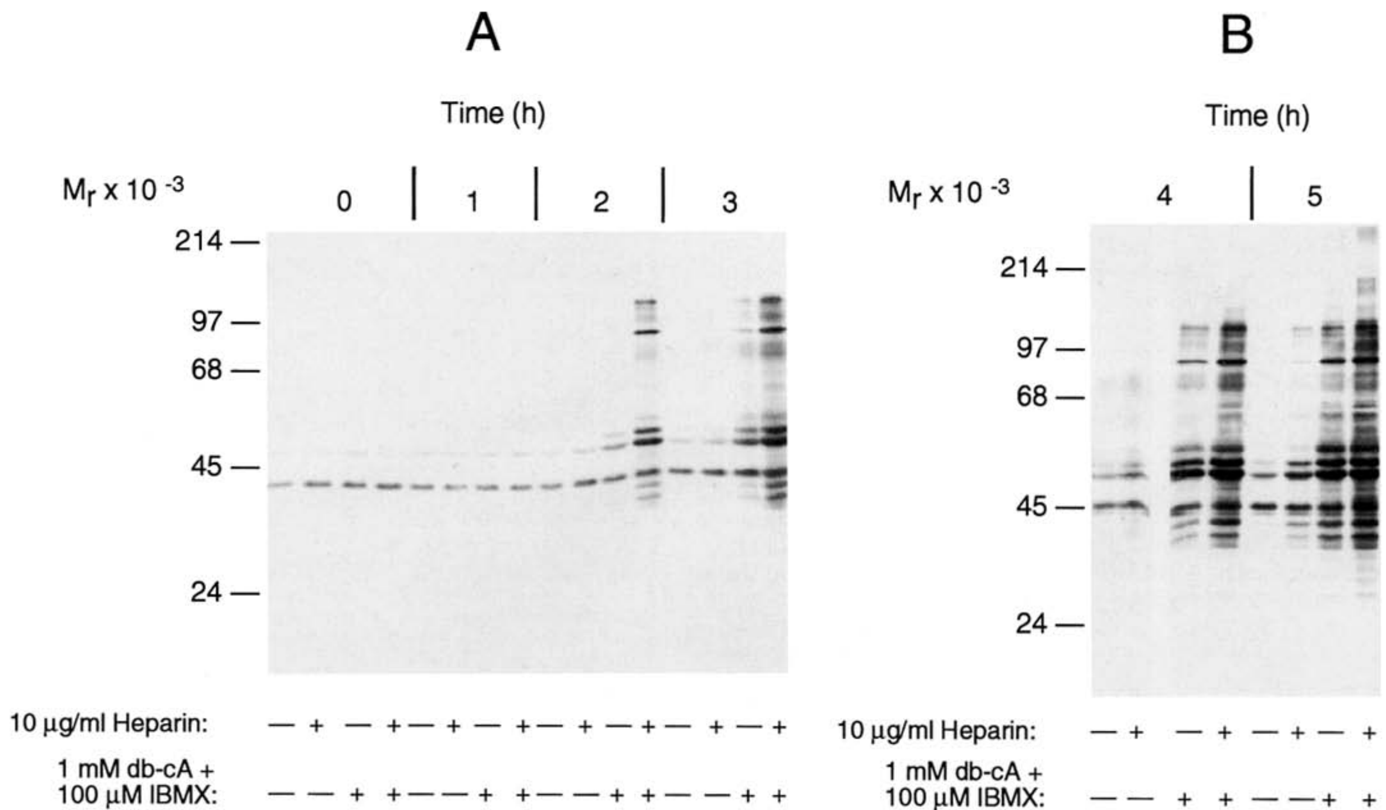

FIG. 1. Time course of protein tyrosine phosphorylation in ejaculated bovine sperm under conditions that support capacitation. Sperm were incubated under conditions conducive to capacitation in complete medium (Sp TALP) and the compounds as indicated. These agents were added to the sperm suspension at the start of the incubation period. At the times listed, sperm proteins were extracted and subjected to SDS-PAGE immunoblotting with a monoclonal antibody to phosphotyrosine (αPY) by the procedures outlined in *Materials and Methods*. Four conditions were tested at each time point. Both **A** and **B** are from a single experiment and were subjected to identical film exposure conditions. The space shown in **B** for the 4-h samples indicates that two different gels are shown, with the first two lanes under 4 h being from the same gel as **A**. This experiment was performed at least three times using sperm samples from three different bulls. A representative experiment is shown. Numbers on the left-hand side of the gels and in subsequent figures represent the positions of the relative molecular mass standards ($\times$ 10^{-3}).

of ARs detected in the presence and absence of 100 μg/ml LC under a variety of incubation conditions. ARs in the total sperm population, rather than only the live population, are shown because most of the AR sperm seen were dead (i.e., took up H258 stain; data not shown). Since increases in the dead/AR category were always accompanied by a decrease in the live/intact category, and since the LC-treated sample was from the same sperm sample as the untreated sample, it was assumed that the dead/AR sperm seen were the result of ARs in the live sperm population followed quickly by cell death. Under all conditions, 60–95% of sperm excluded the H258 stain before the addition of LC, and 42–92% excluded H258 stain after the addition of LC (data not shown).

While the individual responses are variable, LC consistently induced an increase in the percentage of acrosome reacted sperm under conditions supporting capacitation (Table 1). The increase in LC-induced ARs in the presence of heparin, db-cAMP, and IBMX is consistent with studies showing that heparin and cAMP agonists enhance the capacitation of bovine sperm [18, 26]. The presence of 5 mM glucose in the incubation media, which has previously been shown to inhibit heparin-induced capacitation [18], prevented the increase in LC-induced ARs in the heparin-treated sperm samples but, strangely, appeared to increase the rate of LC-induced ARs when no additives were present. The addition of cAMP agonists to the media containing glucose resulted in a return of LC-induced ARs; this was consistent with previous studies in which cAMP agonists were shown to bypass the inhibitory effects of glucose on capacitation [26]. The sperm therefore appear to be undergoing capacitation with use of our in vitro methods in the presence of enhancers and inhibitors of this maturational event.

Time-Dependent Changes in Protein Tyrosine Phosphorylation under Conditions That Support Capacitation

Since the in vitro capacitation of ejaculated bovine sperm in the presence of heparin has been shown to require a 4-h incubation [9, 18], and the appearance of tyrosine phosphorylation of mouse sperm proteins is temporally correlated with capacitation [34], changes in protein tyrosine phosphorylation of ejaculated bovine sperm over a time period of 0–7 h in Sp TALP medium were examined. As shown in Figure 1, when sperm were incubated for various periods of time between 0 and 5 h in media that support capacitation, there was a time-dependent increase in the tyrosine phosphorylation of a subset of proteins of M_r = 40 000–120 000. While two immunoreactive bands appeared under all conditions (discussed below), the remaining bands detected with the antiphosphotyrosine antibody appeared to be phosphorylated in a time-dependent manner. At the later time points, the increase in protein tyrosine phosphorylation was consistently enhanced by the addition

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of 10 μg/ml heparin and/or 1 mM db-cAMP plus 100 μM IBMX to the incubation media (Fig. 1B). This concentration of heparin has been shown to induce in vitro capacitation of bovine sperm [9, 12, 18, 40]. It should be noted that other cAMP agonists, 8-bromo-cAMP (500 μM) and IBMX (500 μM), have been shown to induce the capacitation of bovine sperm [26], and db-cAMP and IBMX supported the capacitation of bovine sperm in this study (Table 1). Moreover, these agents have a positive effect on both the capacitation and protein tyrosine phosphorylation of murine sperm [25].

Previous studies have shown that heparin-induced in vitro capacitation of bovine sperm starts to increase by 2 h, reaches a peak, and then plateaus at 4 h [9]. Consistent with these findings, extending the incubation time to 5 or 7 h resulted in little or no increase in the level of protein tyrosine phosphorylation (Fig. 1B; 7-h data not shown). In the experiment shown, the peak protein tyrosine phosphorylation response in the presence of heparin alone was seen at 5 h, but 4 h was routinely sufficient for the detection of a notable response to heparin (see Figs. 3 and 5–7). As shown in Figure 1A, db-cAMP and IBMX induced protein tyrosine phosphorylation at earlier time points (2 and 3 h) than heparin alone. Even in the presence of these cAMP agonists, maximum levels of protein tyrosine phosphorylation under those conditions were still not detected until 4 h. When combined, heparin, db-cAMP, and IBMX increased the intensity of protein tyrosine phosphorylation seen at all time points to a degree that appeared to be synergistic rather than merely additive. Again, in the presence of these three agents, protein tyrosine phosphorylation was detected at earlier time points (2 h), but the peak intensity with this treatment still occurred at 4 h.

As shown in Figure 2, nearly all of these proteins were specifically phosphorylated on tyrosine residues, since preadsorption of the anti-phosphotyrosine antibody with 10 mM *o*-phosphotyrosine abolished the signal on the blots, with the exception of a protein of M_r = 53 000 and a second, fainter band at M_r = 32 000 (see the arrows to the right of Fig. 2B). These two proteins were also observed with secondary antibody alone (data not shown). A specific immunoreactive protein of approximate M_r = 45 000 was detected under all experimental conditions, but its intensity was not affected by changes in capacitation conditions and it was therefore thought to represent a protein that is constitutively tyrosine phosphorylated (see all figures). Moreover, in order to assess whether these protein tyrosine phosphorylations occur in populations of dead cells, we examined protein tyrosine phosphorylation patterns in sperm that were frozen, then thawed, and observed to be nonmotile. In these populations, one band, just below the constitutively tyrosine phosphorylated band at M_r = 45 000, increased in intensity (data not shown). None of the other bands detected under capacitation-enhancing or capacitation-inhibiting conditions were detected in this population. These controls confirm that the protein tyrosine phosphorylations observed under conditions conducive to capacitation occurred only in viable cells.

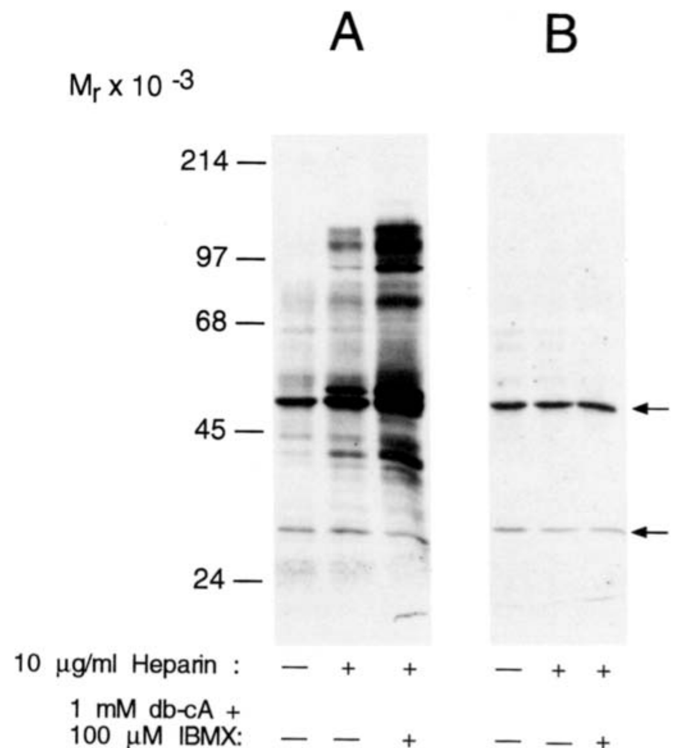

FIG. 2. The effects of heparin and db-cAMP plus IBMX on protein tyrosine phosphorylation in ejaculated bovine sperm under incubation conditions that support capacitation (**A**), and specificity of the antiphosphotyrosine antibody (**B**). Sperm were incubated for 4 h. **A**) Sperm extracts were prepared as described for Figure 1 and probed with the αPY antibody. **B**) Sperm were incubated under the same conditions as in **A**, except that the αPY antibody was first preabsorbed with 10 mM *o*-phosphotyrosine as described in *Materials and Methods*. The arrows to the right of the gel indicate the nonspecific bands detected under these conditions. This experiment was performed three times using sperm samples from three different bulls with similar results. A representative experiment is shown in which the exposure time to film was longer than in the other figures for the purpose of enhancing detection of nonspecific bands.

Effects of Heparin Concentration on Protein Tyrosine Phosphorylation under Conditions That Support Capacitation

In murine sperm, protein tyrosine phosphorylation is absolutely dependent on the presence of media components shown to be required for capacitation in vitro (i.e., Ca^{2+}, $NaHCO_3$, and BSA) [34]. Since heparin has been shown to support the in vitro capacitation of bovine sperm in a concentration-dependent manner, with a maximum effective concentration reported at 10 μg/ml [9], we examined the dependence of protein tyrosine phosphorylation on the heparin concentration in the media. Sperm were incubated in the absence or presence of various concentrations of heparin (0–20 μg/ml). As shown in Figure 3, the intensity of protein tyrosine phosphorylation detected after a 4-h incubation was positively correlated with the heparin concentration present in the media. As observed with capacitation [9], protein tyrosine phosphorylation in response to heparin was detectable between 1 and 5 μg/ml and was maximal at 5 μg/ml, with no further increase at 10 or 20 μg/ml (Fig. 3). Addition of db-cAMP plus IBMX to the media in the presence of 10 μg/ml heparin resulted in a greater degree of protein tyrosine phosphorylation than in the presence of heparin alone (Fig. 3).

Effects of Glucose on Heparin- and cAMP-Induced Protein Tyrosine Phosphorylation

Previous studies have shown that glucose has a strong inhibitory effect on heparin-induced capacitation of bovine sperm [18]. In order to determine whether or not glucose has a similar effect on protein tyrosine phosphorylation, the

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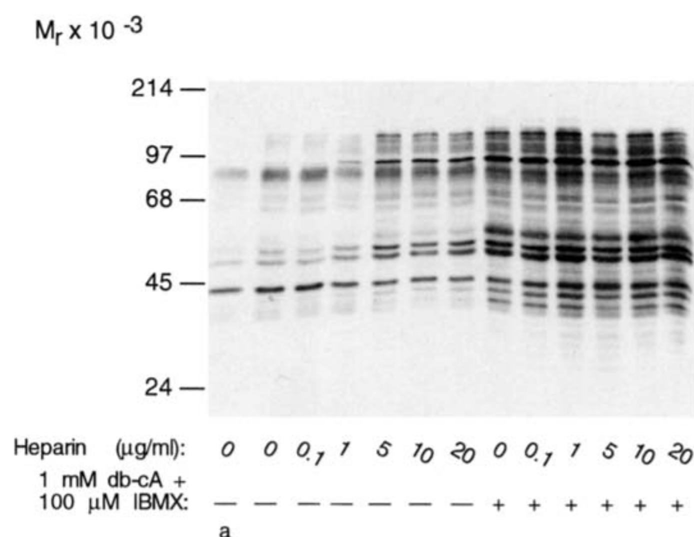

FIG. 3. Concentration-dependent effects of heparin on the appearance of phosphotyrosine-containing proteins in ejaculated bovine sperm under incubation conditions that support capacitation. Sperm were incubated for a period of 4 h in Sp TALP medium containing added compounds as shown. The lane marked "a" contains 5 mM glucose, which has been demonstrated to inhibit capacitation. Sperm extracts were prepared as described for Figure 1 and were probed with αPY antibodies. This experiment was performed at least three times using sperm samples from three different bulls with similar results. A representative experiment is shown.

effect of 5 mM glucose on the time and heparin dependence of protein tyrosine phosphorylation was examined in experiments performed in parallel to those shown in Figures 1 and 3. As shown in Figure 4, A and B, which are from experiments performed on the same sperm samples as those used in Figure 1, no increase in protein tyrosine phosphorylation was seen at any time point in either the absence or presence of 10 μg/ml heparin when 5 mM glucose was present in the incubation media. However, the addition of db-cAMP plus IBMX resulted in the appearance of increased protein tyrosine phosphorylation, although to a lower level of intensity than seen in the absence of glucose. Previous studies have shown that cAMP agonists can overcome the inhibitory effect of glucose on bovine sperm capacitation [26]. When cAMP agonists were present in the media, the time course of protein tyrosine phosphorylation was the same as for the experiments performed in the absence of glucose (Fig. 1). The increase in protein tyrosine phosphorylation appeared at 2 h (in the presence of db-cAMP plus IBMX), increased until 4 h, and then remained level (Fig. 4, A and B). Further increases were not observed after incubations of up to 7 h (data not shown). The most dramatic increase in protein tyrosine phosphorylation at 2–4 h was seen when the cAMP agonists and heparin were both present.

The ability of glucose to inhibit protein tyrosine phosphorylation in the presence of varying concentrations of heparin was examined. The presence of 5 mM glucose in the incubation media completely inhibited the increase in protein tyrosine phosphorylation at all concentrations of heparin tested after a 4-h incubation (Fig. 4C shows an experiment performed with the same sperm sample used in Fig. 3). Adding the cAMP agonists, db-cAMP and IBMX, to these incubation media overcame this glucose inhibition on heparin-induced protein tyrosine phosphorylation (Fig. 4C). It should be noted that a concentration-dependent increase in protein tyrosine phosphorylation was seen with increasing heparin concentrations in the presence of glucose, db-cAMP, and IBMX (Fig. 4C). When glucose was present, the heparin-induced protein tyrosine phosphorylation was abolished, and the cAMP agonist-induced response was decreased. In addition, the presence of both heparin and the cAMP agonists resulted in an apparently synergistic stimulation of protein tyrosine phosphorylation. However, even at the highest heparin concentration tested in the presence of cAMP agonists, the intensity of protein tyrosine phosphorylation was still less than that seen in the absence of glucose (see the lane labeled "a" in Fig. 4C for comparison to the results found in the absence of glucose).

Effects of dbcAMP and IBMX on the Glucose-Induced Inhibition of Protein Tyrosine Phosphorylation

Since capacitation studies (Table 1; [26]) and the current protein tyrosine phosphorylation studies indicated that cAMP agonists can overcome the inhibitory effects of glucose on heparin-mediated events, and since cAMP levels have been shown to increase during bovine sperm capacitation [26], we further characterized the involvement of cAMP as a possible regulator of protein tyrosine phosphorylation during capacitation. If db-cAMP and IBMX are mediating protein tyrosine phosphorylation by activating the cAMP pathway, the amount of protein tyrosine phosphorylation detected should correlate with the concentration of these cAMP agonists present in the media. As shown in Figure 5, this was found to be the case. When sperm were incubated in Sp TALP in the absence of glucose and heparin, the addition of db-cAMP plus IBMX resulted in a concentration-dependent increase in protein tyrosine phosphorylation (Fig. 5A). Over the concentrations tested, IBMX appeared to have a stronger effect on protein tyrosine phosphorylation than did db-cAMP. As shown in Figure 5B, addition of heparin (10 μg/ml) to incubation media containing the same concentrations of db-cAMP and IBMX as in Figure 5A resulted in a similar concentration dependence of protein tyrosine phosphorylation, but with an overall increased signal intensity. When glucose was added to the incubation media in the absence of heparin at the same cAMP agonist concentrations, no increase in protein tyrosine phosphorylation was seen at the lower concentrations (as in Fig. 5C, lane a) and only a small increase was seen at the highest concentrations (100 μM IBMX and 1 mM db-cAMP; data not shown). As shown in Figure 5C, detectable protein tyrosine phosphorylations were seen when heparin was added to the media, but the response could be observed only at the highest concentration of IBMX (100 μM) and was much weaker than the degree of protein tyrosine phosphorylation seen in the absence of glucose under otherwise identical conditions (compare to Fig. 5C, lane b). Therefore, IBMX and db-cAMP clearly displayed concentration-dependent effects on protein tyrosine phosphorylation that were enhanced by heparin and inhibited by glucose.

Specificity of cAMP in the Regulation of Protein Tyrosine Phosphorylation

While db-cAMP and IBMX enhanced protein tyrosine phosphorylation, and it seemed likely that these cAMP agonists were exerting their effects by stimulating the cAMP pathways and thereby activating PK-A, we confirmed that these effects were specific to cAMP. A panel of active and inactive cAMP analogues, as well as a cGMP analogue,

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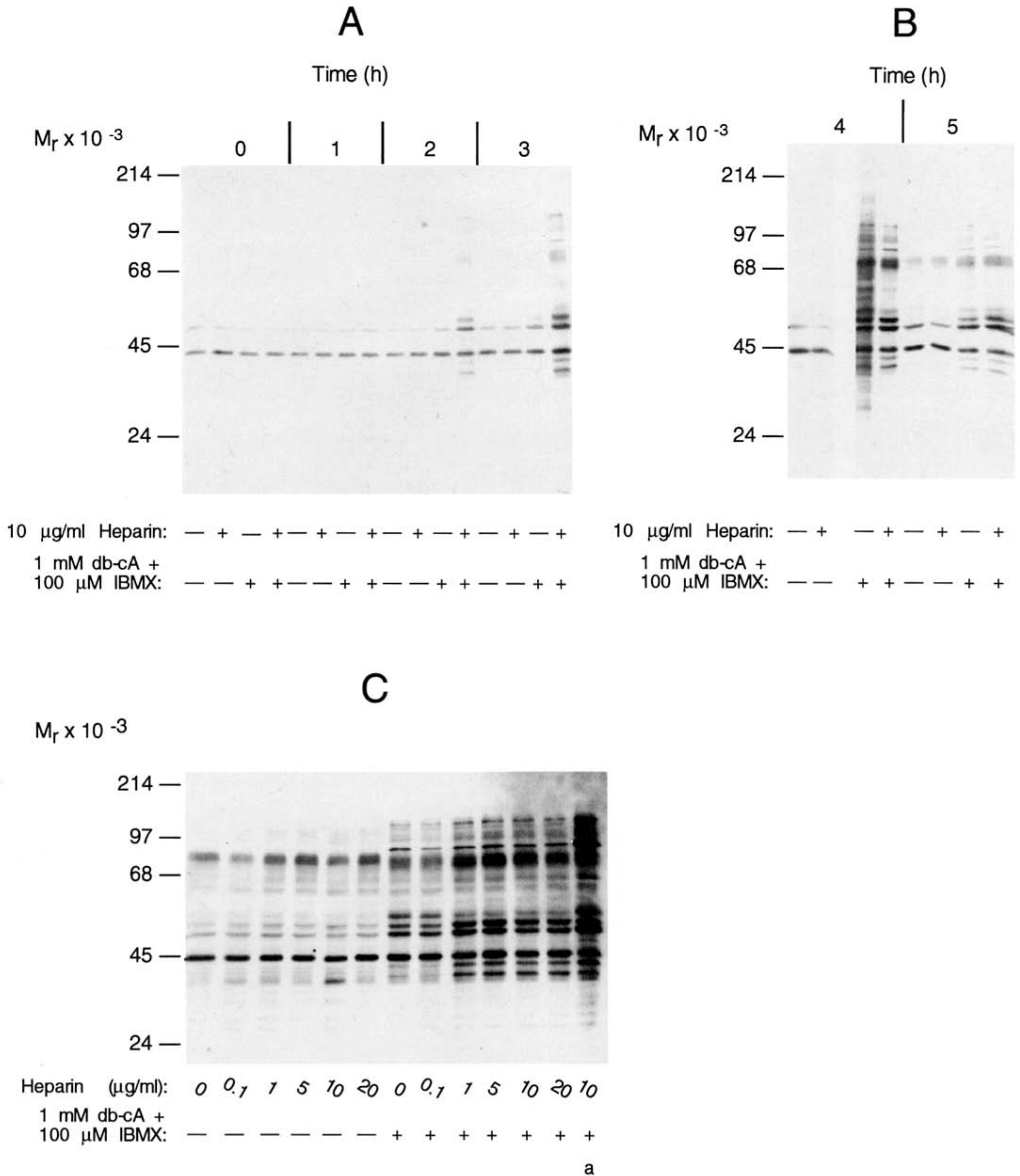

FIG. 4. Effects of glucose present in the capacitation media on the time-, heparin-, and cAMP analogue-dependent appearance of phosphotyrosine-containing proteins in ejaculated bovine sperm under incubation conditions that support capacitation. Sperm were incubated in Sp TALP containing 5 mM glucose and added compounds as indicated. **A** and **B**) Sperm extracts were prepared as described for Figure 1 and probed with αPY antibodies. Both **A** and **B** are from a single experiment performed on the same day and with the same sperm samples as used in Figure 1. Gels were subjected to similar ECL and film exposure conditions. The space shown in **B** for the 4-h samples indicates that two different gels are shown, with the first two lanes of **B** under 4 h being from the same gel as in **A**. **C**) The same sperm sample as in Figure 3 treated as indicated. For comparison, the lane marked "a" does not contain glucose. Sperm extracts were prepared as described for Figure 1 and probed with αPY antibodies. This experiment was performed at least three times using sperm samples from three different bulls with similar results. A representative experiment is shown.

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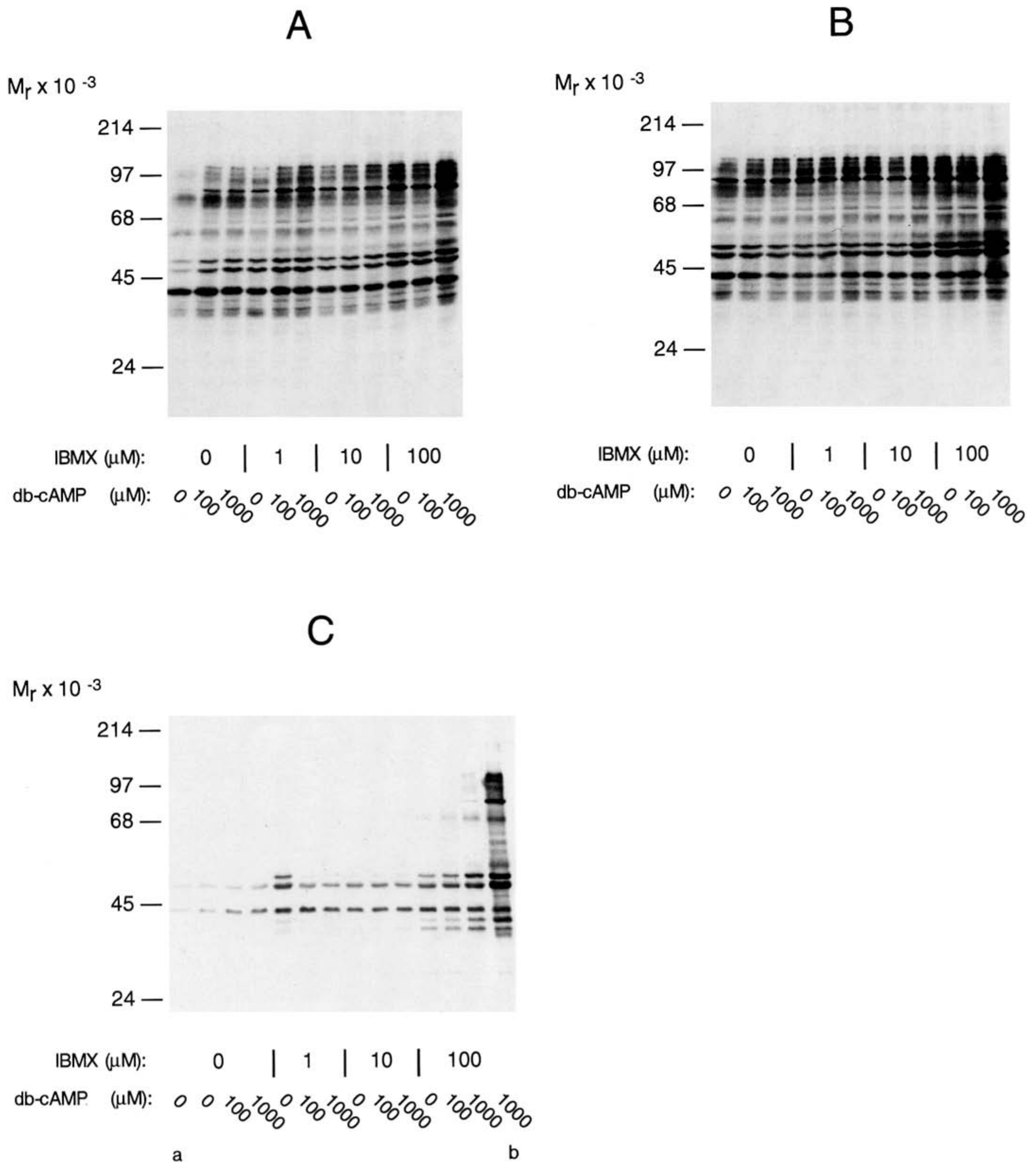

FIG. 5. Concentration-dependent effects of db-cAMP and IBMX, in the presence and absence of glucose or heparin, on the appearance of phosphotyrosine-containing proteins in ejaculated bull sperm incubated under conditions that support capacitation. **A**) Sperm were incubated for a 4-h period in Sp TALP, without added glucose or heparin, in the absence or presence of IBMX plus db-cAMP (db-cA) as indicated. Sperm extracts were prepared as described for Figure 1 and probed with αPY antibodies. **B**) Sperm were incubated for a 4-h period in Sp TALP without added glucose but containing 10 µg/ml heparin in either the absence or presence of IBMX plus db-cA as indicated. **C**) Sperm were incubated for a 4-h period in Sp TALP plus 5 mM glucose and 10 µg/ml heparin in either the absence or presence of IBMX and db-cA as indicated. The lane marked "a" denotes a sample that contains no heparin. The lane marked "b" is a duplicate of the last lane shown in **B** (no added glucose plus 10 µg/ml heparin). **A**, **B**, and **C** are from a single experiment and were subjected to identical ECL and film exposure conditions. This experiment was performed at least three times using sperm samples from three different bulls with similar results. A representative experiment is shown.

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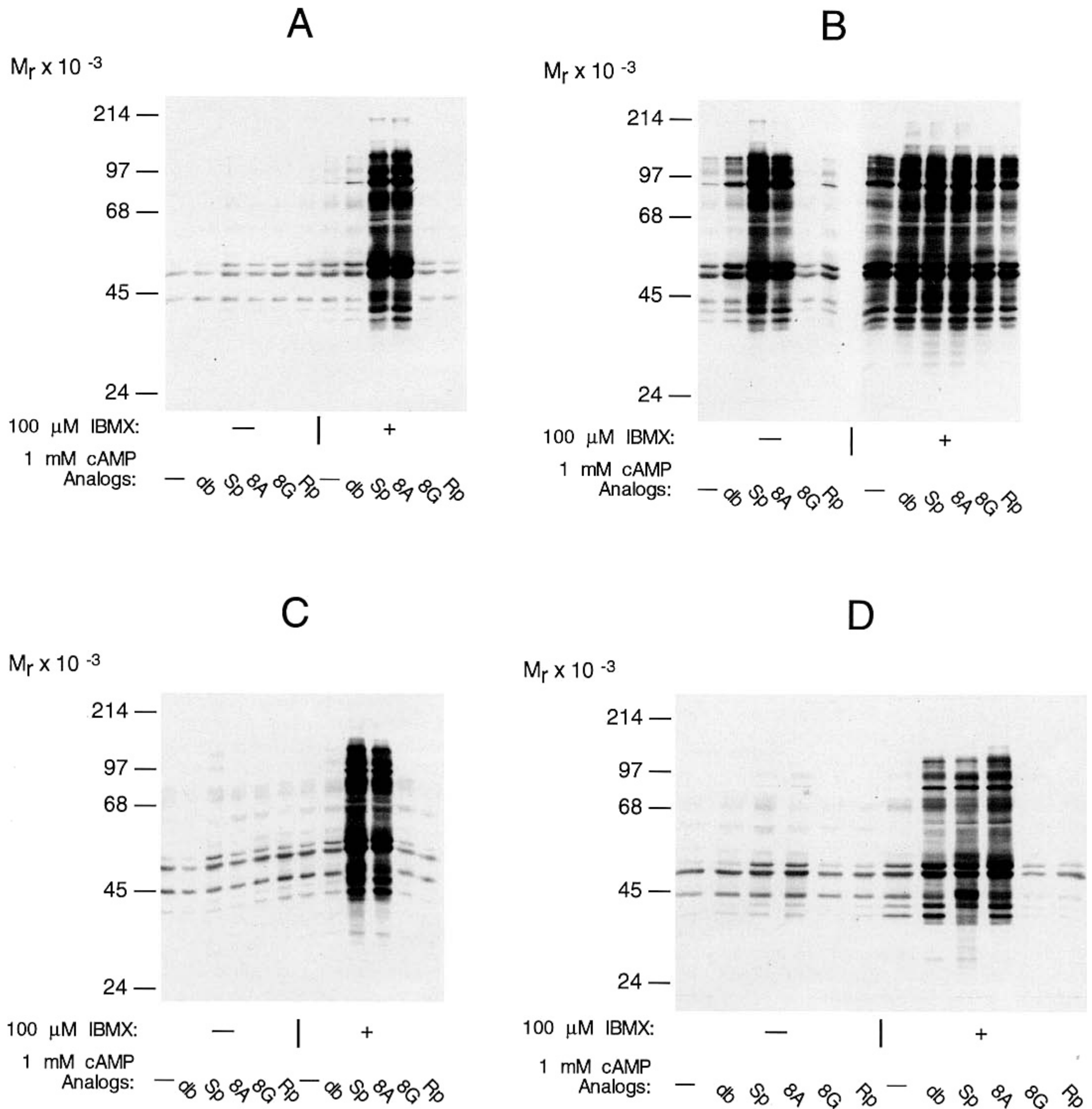

FIG. 6. Effects of cAMP analogues, a cGMP analogue, and IBMX on the appearance of phosphotyrosine-containing proteins in ejaculated bull sperm incubated in Sp TALP medium in either the presence or absence of glucose and heparin. Sperm were incubated for 4 h in Sp TALP media containing no glucose (**A**, **B**) or 5 mM glucose (**C**, **D**), in the absence of heparin (**A**, **C**) or in the presence of 10 µg/ml heparin (**B**, **D**), and in the presence or absence of 100 µM IBMX, as indicated below each gel. These media were also supplemented with 1 mM db-cAMP (db), 1 mM Sp-cAMPS (Sp), 1 mM 8-bromo-cAMP (8A), 1 mM 8-bromo-cGMP (8G), or 1 mM Rp-cAMPS (Rp) as indicated below the gels. Sperm extracts were prepared as described for Figure 1 and probed with αPY antibodies. All 4 gels show data from a single experiment. The space shown in **B** indicates that two different gels are shown. The left panel of **B** is from the same gel as **A**, and the right panel is from the same gel as **C**. This experiment was performed at least three times using sperm samples from three different bulls with similar results. A representative experiment is shown.

were tested for their abilities to affect protein tyrosine phosphorylation in the presence and absence of glucose, heparin, and IBMX (Fig. 6). The panel of cAMP agonists included the active, membrane-permeable cAMP analogues, db-cAMP, Sp-cAMPS, and 8-bromo cAMP; the cAMP competitive antagonist Rp-cAMPS; and the membrane-permeable active cGMP analogue, 8-bromo cGMP.

As shown in Figure 6A (first 6 lanes), none of the cAMP analogues induced a significant change in protein tyrosine phosphorylation over the baseline level in the absence of

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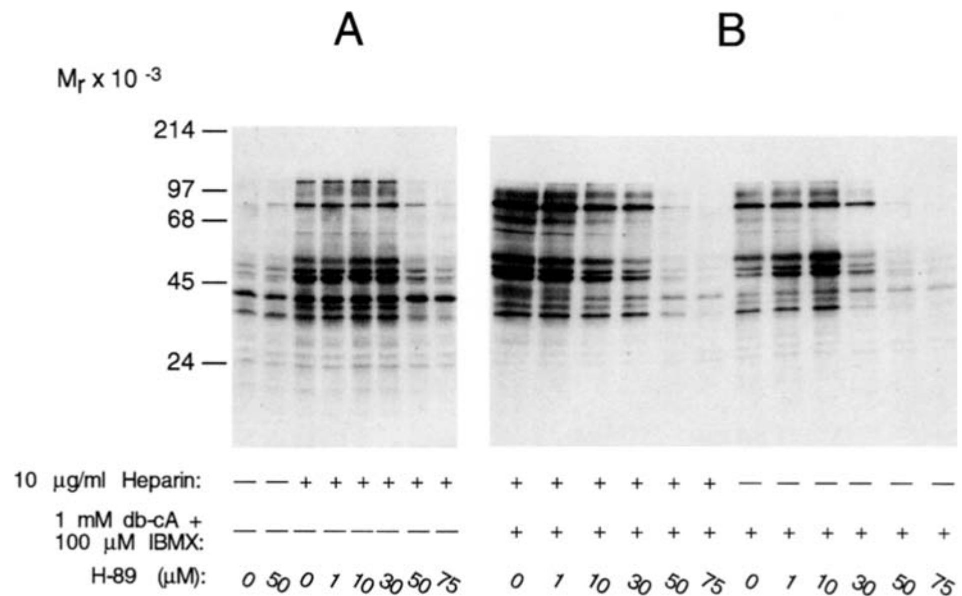

FIG. 7. Effects of the protein kinase A inhibitor, H-89, on the appearance of phosphotyrosine-containing proteins in ejaculated bovine sperm incubated under conditions that support capacitation. Sperm were incubated for a period of 4 h in Sp TALP medium containing added compounds as indicated. Sperm extracts were prepared as described for Figure 1 and probed with αPY antibodies. The data shown in **A** and **B** are from a single experiment. The lanes in **B** are wider than in **A** because this gel was run using a different-sized sample comb. This experiment was performed at least three times using sperm samples from three different bulls with similar results. A representative experiment is shown.

heparin and IBMX. While this was true for the experiment shown in Figure 6A, in other cases db-cAMP (see Fig. 5A, first 3 lanes), 8-bromo-cAMP, and Sp-cAMPS (data not shown for latter two) could promote protein tyrosine phosphorylation when present alone, although to a very low extent. In the presence of the phosphodiesterase inhibitor IBMX, but in the absence of heparin, an increase in protein tyrosine phosphorylation was observed when the active cAMP analogues were present (Fig. 6A). Under the same conditions, no increase in protein tyrosine phosphorylation was observed in the presence of 8-bromo cGMP or the inactive cAMP analogue, Rp-cAMPS (Fig. 6A). Also shown in Figure 6A (last six lanes), Sp-cAMPS, 8-bromo-cAMP, and to a lesser extent db-cAMP, in the presence of IBMX and absence of glucose and heparin, promoted protein tyrosine phosphorylation that resembled the pattern seen in the presence of heparin (compare to Fig. 6B).

As shown in Figure 6B, the addition of heparin to media containing the same panel of cAMP analogues as in Figure 6A yielded a similar pattern of protein tyrosine phosphorylation, but the level detected was more intense. In the presence of heparin and absence of IBMX, db-cAMP, Sp-cAMPS, and 8-bromo cAMP induced protein tyrosine phosphorylation, while the addition of 8-bromo cGMP and Rp-cAMPS resulted in little or no increase in tyrosine phosphorylation over the level seen in the presence of heparin alone (Fig. 6B, first six lanes). Occasionally (Fig. 6B), a low level of protein tyrosine phosphorylation was seen upon addition of Rp-cAMPS, but in other experiments the levels of these phosphorylations were actually less than those seen in the presence of heparin alone (data not shown). Addition of IBMX to the incubation media in the presence of heparin and absence of glucose had the strongest influence in supporting protein tyrosine phosphorylation (Fig. 6B, last six lanes). Again, the bioactive cAMP analogues supported the most intense protein tyrosine phosphorylation, but intense phosphorylation was also detected in the presence of heparin alone, 8-bromo cGMP, and Rp-cAMPS, consistent with a high level of endogenous cAMP activation that might have outcompeted the Rp-cAMPS for access to PK-A.

In this set of experiments, the results seen in the presence of glucose are quite informative (Figs. 6, C and D). In the absence of heparin and presence of glucose (Fig. 6C), the effects of the various analogues were similar to the results observed when glucose was absent under otherwise identical conditions (compare to Fig. 6A). Again, cAMP analogues had little to no effect on protein tyrosine phosphorylation when added alone. It is interesting to note that previous studies have also indicated that cAMP agonists cannot induce capacitation of bovine sperm in the presence of glucose if heparin is absent [26]. IBMX enhanced the protein tyrosine phosphorylation response detected in the presence of dbcAMP, Sp-cAMPS, and 8-bromo-cAMP, but had no effect on incubations performed in the presence of 8-bromo cGMP or Rp-cAMPS (Fig. 6C). In the experiment shown, db-cAMP plus IBMX did not induce a notable increase in protein tyrosine phosphorylation in the presence of glucose, but a more intense response could be seen in other experiments (see Fig. 4, B and C; 0 heparin, for example).

In contrast to the experiments performed in the absence of glucose (Fig. 6B), the addition of heparin plus the cAMP analogues to sperm in the presence of glucose had almost no effect on protein tyrosine phosphorylation (Fig. 6D). The active analogues still only minimally affected protein tyrosine phosphorylation (see Fig. 6D, first 6 lanes; compare to Fig. 6C, first 6 lanes). The addition of IBMX plus heparin to sperm in the presence of glucose (Fig. 6D, last 6 lanes) supported a minimal increase in protein tyrosine phosphorylation when no other cAMP analogues were added, and supported moderate increases in protein tyrosine phosphorylation in the presence of the active cAMP analogues. Under the same conditions, the response to 8-bromo cGMP and Rp-cAMPS was still undetectable, in contrast to the results shown in Figure 6B in the absence of glucose. Overall, the addition of IBMX had the strongest effect on protein tyrosine phosphorylation when glucose was present. In addition, the inability of 8-bromo cGMP to exert any effect upon protein tyrosine phosphorylation under any of the eight conditions examined supports the hypothesis that protein tyrosine phosphorylation during bovine sperm capacitation is mediated by a pathway specifically stimulated by cAMP.

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Effects of H-89, an Inhibitor of the cAMP-Dependent Protein Kinase, on Protein Tyrosine Phosphorylation

Since the primary intracellular target for cAMP action is PK-A, we examined whether PK-A played a role in protein tyrosine phosphorylation under conditions supporting in vitro capacitation as had been found in the murine model [25]. Ejaculated sperm were incubated for 4 h in complete medium supporting capacitation in either the absence or presence of H-89, a highly selective and cell-permeable inhibitor of PK-A [41]. H-89 was tested for its effects in the presence and absence of 5 mM glucose, 10 μg/ml heparin, and 1 mM db-cAMP plus 100 μM IBMX. As shown in Figure 7, H-89 inhibited protein tyrosine phosphorylation of sperm proteins in a concentration-dependent manner with the most complete inhibition observed at 50 μM.

In the presence of glucose, H-89 did not affect the level of protein tyrosine phosphorylation observed when heparin was added (data not shown). This result was expected, since 5 mM glucose has already been shown to inhibit the protein tyrosine phosphorylation associated with added heparin (Fig. 4). When db-cAMP and IBMX were present in glucose-containing media, H-89 inhibited protein tyrosine phosphorylation in a concentration-dependent manner with maximal effects observed at 10–30 μM H-89 (data not shown). These data, along with the inhibitory effects of Rp-cAMPS (another PK-A antagonist), support the idea that the cAMP regulation of protein tyrosine phosphorylation occurs at the level of PK-A.

DISCUSSION

Our results demonstrate that incubation conditions supporting the in vitro capacitation of ejaculated bovine sperm are correlated with the tyrosine phosphorylation of a subset of proteins of M_r = 40 000–120 000. The ability of cAMP agonists to enhance, and cAMP antagonists to inhibit, both capacitation and the pattern of protein tyrosine phosphorylation ([26, 27, 35]; this report) supports our hypothesis that mammalian sperm capacitation is mediated by a signaling pathway that involves cross-talk between cAMP/PK-A and tyrosine kinase/phosphatase signaling pathways.

We believe that these changes in protein tyrosine phosphorylation are a consequence of changes associated with capacitation, rather than cell death or ARs, as stated in *Results*. Moreover, the LC assays, performed to confirm that sperm incubated under our conditions were capable of undergoing membrane fusion events associated with capacitation, revealed variable rates of spontaneous ARs in the absence of LC (Table 1) but a consistent increase in protein tyrosine phosphorylation (Figs. 1–7). This variability observed in the rates of LC-induced ARs under our conditions, in comparison to our immunoblot results (Figs. 1–7), underscores the notion that the LC assay measures the ability of sperm membranes to fuse and undergo the AR [9]; but protein tyrosine phosphorylation might actually be a more consistent indicator of intracellular changes preceding and/or associated with capacitation. Further studies are planned to examine the connection between protein tyrosine phosphorylation and endpoints of capacitation such as the rates of in vitro fertilization and ZP-induced ARs.

Although the role of heparin as an enhancer of the in vitro capacitation of bovine sperm has been extensively studied, knowledge of its connection to downstream signaling pathways that may regulate this maturational event is relatively new. Our results show that heparin supports protein tyrosine phosphorylation at the same concentrations (Fig. 3) and over the same time course (Fig. 1) as has been shown with in vitro capacitation [9]. Furthermore, the ability of cAMP agonists to mimic and enhance the same pattern of protein tyrosine phosphorylation as observed with heparin (Figs. 1–3) is consistent with previous studies implicating a role for cAMP signaling in heparin-dependent capacitation [26, 27]. The ability of these cAMP agonists to substitute for heparin and to overcome the inhibition of heparin-induced protein tyrosine phosphorylation (Figs. 4–6) and capacitation (Table 1; [26]) by glucose suggests that cAMP functions as a second messenger acting downstream from the action of these particular media constituents. While the means by which heparin could activate a cAMP-dependent pathway remain to be elucidated, such a pathway could involve heparin-induced (or heparin-assisted) changes in intracellular ion levels that would then lead to the activation of intracellular signal transduction pathways. For example, it has been proposed that heparin may cause an increase in intracellular Ca^{2+} concentrations, as measured during capacitation, leading to activation of adenylyl cyclase [26]. Unlike somatic cell adenylyl cyclases, the sperm enzyme is not activated by coupling to the stimulatory G protein, G_s [28, 42], although it is clearly regulated by egg products [43, 44]. Mammalian sperm adenylyl cyclase can be regulated directly/indirectly by Ca^{2+} [24, 45–48], calmodulin [47, 49–51], and $NaHCO_3$ [25, 46, 52–54].

If little is known about the mechanism of heparin-induced capacitation, even less is understood concerning the inhibitory effects of glucose on this process. Glucose or glycolytic substrates block heparin- or oviduct fluid-induced capacitation [13, 18]. The inhibitory effect of glucose on heparin-induced capacitation appears to act upstream of cAMP or targets of cAMP (PK-A; protein substrates), since it can be reversed in a concentration-dependent manner by 8-bromo-cAMP, IBMX, or caffeine [17, 26]. The present results also indicate that the inhibitory effect of glucose on protein tyrosine phosphorylation is upstream of cAMP since the addition of cAMP agonists reverses the effect (Figs. 4–6). The steps in the pathway between glucose, cAMP-dependent events, and downstream effects on protein tyrosine phosphorylation and capacitation are, however, unknown. Glucose is thought to block the capacitation-associated increases in sperm Ca^{2+} uptake and increase in intracellular pH (pH_i) [17, 18]. Glucose does not interfere with [^{3}H]heparin binding to sperm [26], and appears to require glycolysis to have its inhibitory effect [18]. It has been suggested that glucose inhibits a capacitation-associated rise in pH_i [18]. It is postulated that heparin causes increases in the intracellular second messengers cAMP, pH, and Ca^{2+} independently [27] and that the glycolytic metabolites of glucose have inhibitory effects on these three second messengers that can be bypassed by cAMP agonists or phosphodiesterase inhibitors [26].

If, in fact, glucose does exert its inhibitory effect by altering pH_i and/or $[Ca^{2+}]_i$, such changes in ionic movements could ultimately affect the activities of the sperm adenylyl cyclases, protein kinases, phosphatases, and/or phosphodiesterases [21, 55]. Our current study supports this hypothesis since IBMX, a phosphodiesterase inhibitor, appears to have the most significant effect on reversing the inhibition of protein tyrosine phosphorylation by glucose, suggesting that the presence of endogenous cAMP and its rate of degradation by phosphodiesterase may be critical to the regulation of protein tyrosine phosphorylation during capacitation (Figs. 5 and 6). This may imply that glucose, either directly or indirectly, is up-regulating phosphodies-

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terase activity, thereby reducing the pool of available cAMP for activation of PK-A. It is possible, therefore, that the rate of destruction of cAMP by phosphodiesterase may be a more sensitive regulatory point than its rate of production by the adenylyl cyclase. There is certainly precedence for this during the process of epididymal sperm maturation [56]. We are currently examining the effects of stimulators and inhibitors of capacitation on the activities of sperm adenylyl cyclases, phosphodiesterases, and PK-A and hope to further clarify this issue in the future.

While the mechanisms by which glucose and heparin affect capacitation and protein tyrosine phosphorylation await further clarification, our data suggest that PK-A is a central mediator acting downstream from these agents and upstream of tyrosine kinase/phosphatase signaling pathways. The ability of two inhibitors of PK-A, one functioning as a cAMP antagonist at the level of the regulatory subunit of PK-A (Rp-cAMPS) and the other being a competitor for ATP binding to the catalytic subunit of PK-A (H-89), to inhibit protein tyrosine phosphorylation strengthens this conclusion (Figs. 6 and 7). It is interesting to note that the concentration of H-89 required to achieve the complete inhibition of protein tyrosine phosphorylation in bovine sperm (50 μM; Fig. 7) is higher than the 10 μM needed to inhibit the capacitation-associated increases in protein tyrosine phosphorylation in murine sperm [25]. The reasons for these differences are not clear, but they may reflect species differences in H-89 permeability and/or endogenous ATP concentrations, both of which could influence results based on in vitro or in vivo assays. The published K_i of H-89 for PK-A is 0.048 μM in in vitro assays. This K_i is 500- to 3000-fold lower than that for other serine/threonine kinases [41]. However, when H-89 was used for in vivo studies, 20–30 μM was required to inhibit PK-A and specific cAMP-mediated physiologic events; but this higher level still did not inhibit other serine/threonine kinases [41]. The disparity between the in vitro and in vivo inhibitory concentrations of this inhibitor in this case was also thought to be due to the cellular permeability of H-89 and the presence of high concentrations of ATP under the conditions studied [41]. In future studies, we plan to further characterize the role of PK-A in capacitation and protein tyrosine phosphorylation by examining the effect of H-89 on capacitation as measured by in vitro fertilization.

Our current and previous results, as well as the recent study of human sperm by Leclerc et al. [35], support the existence of a novel signaling pathway for mammalian sperm capacitation that includes cross-talk at the level of PK-A between cAMP and tyrosine kinase/phosphoprotein phosphatase signaling pathways [25, 34, 35]. To the best of our knowledge, these studies in mammalian sperm provide the first example of an up-regulation of protein tyrosine phosphorylation mediated by cAMP/PK-A. Evidence exists for the down-regulation of receptor tyrosine kinase activity by cAMP [57, 58], but up-regulation has thus far not been demonstrated. Protein tyrosine phosphatase activity has been demonstrated to be both inhibited [59, 60] and stimulated [61] by cAMP/PK-A, effects that could ultimately modulate the levels of protein tyrosine phosphorylation.

The only other example of cross-talk between cAMP/PK-A and tyrosine kinase/phosphoprotein phosphatase pathways that may be regulated at the level of PK-A is in trout sperm [62, 63]. In this species, a protein whose phosphorylation increases rapidly during activation of motility in concert with an increase in cAMP was found to be phosphorylated on tyrosine residues [62]. It was also shown that this tyrosine phosphorylation occurs in a cAMP-dependent fashion in an in vitro assay [63]. Moreover, in the invertebrate *Ciona*, sperm motility, which is known to be stimulated by cAMP, can be diminished by compounds that can inhibit tyrosine kinases [64]. Examination of the effects of specific tyrosine kinase inhibitors on protein tyrosine phosphorylation and capacitation await further characterization of the tyrosine kinase(s) involved in this process. This is due to concern over the specificity of many tyrosine kinase inhibitors and apparent effects of these inhibitors on unrelated events [65].

The level at which cAMP/PK-A functions to regulate the steady-state levels of protein tyrosine phosphorylation in bovine sperm under conditions conducive to capacitation (i.e., tyrosine kinases, phosphoprotein phosphatases), as well as the identification of the substrates undergoing tyrosine phosphorylation, will be the subject of intense scrutiny in our laboratory for the foreseeable future. It is likely that the identity of the tyrosine phosphorylated substrates will be informative with regard to functional roles in exerting the downstream effects of capacitation, or that they will be shown to be involved in regulating signaling events leading to capacitation. Investigations into the mechanism by which cAMP/PK-A regulates tyrosine kinases/phosphoprotein phosphatases might yield new information about alternative modes of cross-talk between these two signaling pathways.

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