Bicarbonate/CO_2 induces rapid activation of phospholipase A_2 and renders boar spermatozoa capable of undergoing acrosomal exocytosis in response to progesterone

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Abstract **We tested whether capacitation, a phenomenon that renders spermatozoa capable of undergoing acrosomal exocytosis, may be completed rapidly after a short exposure to bicarbonate/CO_2. We found that, in the presence of Ca^{2+}, a 10-min exposure of boar spermatozoa to bicarbonate led to a partial activation of phospholipase A_2, primed spermatozoa for a major subsequent activation of this enzyme upon stimulation with progesterone and furthermore rendered spermatozoa capable of undergoing exocytosis in response to this steroid. These results suggest that capacitation may be completed in a relatively short period of time and open up new possibilities for unravelling molecular mechanisms underlying this process.**

Key words: Phospholipase A_2; Capacitation; Acrosome reaction; Exocytosis; Progesterone

1. Introduction

Upon release from the male genital tract, mammalian spermatozoa are still not ready to engage in fertilization. They need to spend a period of time in the female tract, or be incubated under adequate in vitro conditions, in order to develop the capacity to interact with the oocyte. This phenomenon, which is known as sperm capacitation, and appears to be unique to mammals, was first recognized over 40 years ago [1,2] and is known to be essential for fertilization. However, despite considerable attention and efforts, information regarding the underlying molecular mechanisms has remained largely fragmentary or even hypothetical [3,4]. Nevertheless, a variety of changes in membrane lipids and proteins have been found to occur in parallel to the development of the capacitated state [4], and changes in ionic regulation also seem to play a role in this process [5].

It was once thought that capacitation served to regulate the onset of exocytosis of the sperm acrosomal granule, a process believed to take place spontaneously [6]. Evidence gathered during the last decade has indicated that exocytosis in spermatozoa is initiated in response to oocyte-derived stimuli, and this has prompted a departure from that view [7]. Currently, the significance of capacitation is believed to relate to a loss of the stable state developed during epididymal storage [7].

There are now two conflicting views regarding the kinetics and timing of capacitation. One view holds that capacitation is a slow, time-dependent process, whose duration is species-specific, and which develops while spermatozoa ascend along the female tract [4,8]. Central to this idea is the concept that underlying molecular changes in sperm membrane lipids and proteins may need a long time to develop. The alternative view holds that spermatozoa kept in the upper portions of the female tract in a quiescent state may undergo or complete capacitation rapidly in synchrony with the release of oocytes; completion of capacitation may thus be thought of as an ovulation-related event [9]. The idea that capacitation may not occur over a long time-span is particularly important in species such as humans and domesticated animals with a long interval between sperm deposition and fertilization; premature capacitation may render spermatozoa unstable and incapable of interacting with oocytes and thus participating in fertilization [9]. In agreement with this idea, it was found that fully capacitated spermatozoa cannot migrate from uterus to oviduct and hence that precocious capacitation may result in spermatozoa being incapable of further ascent and, as a result, unable to take part in fertilization [10]. Thus, capacitation may develop quickly, perhaps in response to changes in the environment of the female tract, slightly before or around the time of ovulation [9].

In this study we have attempted to obtain evidence that would shed light on this controversy. We have concentrated here on processes that may 'prepare' spermatozoa to undergo acrosomal exocytosis, the latter being considered a separate, irreversible process, initiated in response to a natural agonist. It has to be mentioned that capacitation also involves the development of hyperactivated motility, a peculiar type of motion that allows spermatozoa to swim in the viscous fluids of the oviduct, and aids in the penetration of the oocyte vestments; this issue, though, will not be addressed here.

We have tested the hypothesis that preparation for exocytosis may be completed rapidly. This was done by investigating whether exposure to bicarbonate, a component of in vitro fertilization media which destabilizes sperm membranes over a short period of time [11], appears to be essential for capacitation [12], and is present in high concentrations in oviductal fluid [13], results in rapid changes in sperm membrane lipids. We have also investigated whether a short exposure to bicarbonate modifies spermatozoa in such a way that they are rendered capable of undergoing exocytosis upon challenge with a natural agonist. We report here that a very short (10 min) exposure to bicarbonate elicits partial activation of phos-

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Abbreviations: PLA_2, phospholipase A_2; PC, phosphatidylcholine; PS, phosphatidylserine

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pholipase A_2 (PLA_2) and changes in sperm membrane lipids, primes spermatozoa for a further PLA_2 activation upon challenge with progesterone, and renders spermatozoa capable of undergoing exocytosis in response to this agonist. These results strongly suggest that capacitation may indeed be completed in a short period of time.

2. Materials and methods

2.1. Reagents

[1-^{14}C]Arachidonic acid (54 mCi/mmol; toluene solution), and [*methyl*-^{14}C]choline chloride (54 mCi/mmol) were obtained from Amersham International (Amersham, Bucks., UK). Chemicals were of analytical grade, and were purchased from Sigma or BDH (both of Poole, Dorset, UK). Ionophore A23187 was from Calbiochem (Nottingham, UK). Percoll was from Pharmacia (Milton Keynes, UK). Organic solvents were of reagent grade and were purchased from BDH. Neutral lipids and phospholipids used as standards were from Sigma. Polyphosphoinositide standards were kindly provided by Dr R.F. Irvine of The Babraham Institute.

2.2. Preparation, labelling and treatment of spermatozoa

The medium used for storage and labelling of boar spermatozoa was the Beltsville extender (BTS) [14]: 10 mM KCl, 20.4 mM trisodium citrate ($2H_2O$), 15 mM $NaHCO_3$, 3.36 mM EDTA (disodium salt), 205 mM glucose, and 50 μg kanamycin monosulfate/ml. Incubation in this medium results in no or very little loss of sperm viability (assessed by exclusion of propidium iodide [15]) or fertility [14] for up to 4 days.

The Tyrode's medium used for incubation of spermatozoa was a modification of that used previously [11,16] and consisted of 102 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 15 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 16.7 mM sodium lactate, 1 mM sodium pyruvate, 20 μg phenol red/ml, 50 μg kanamycin/ml, and 3 mg bovine serum albumin (fraction V)/ml. The pyruvate and the albumin were added on the day of use. When required, $CaCl_2$ (3 mM), or EGTA (1 mM) was added. Tyrode's medium without bicarbonate was prepared by omitting this component and by modifying the concentration of NaCl in order to maintain the osmolality at 300 mOsmol/kg [11]. When bicarbonate was present, the medium was maintained in equilibrium with 5% CO_2 in air, so that all media had a pH of 7.4 at 38°C.

Sperm-rich fractions of semen, collected from Large White boars, were allowed to cool slowly to room temperature (about 22–24°C) and then filtered through gauze. Spermatozoa were incubated in BTS ($\sim 1\times10^8$ cells/ml) at 25°C, in the presence of 0.1 μCi [^{14}C]arachidonic acid/ml for 24–48 h, or 2 μCi [*methyl*-^{14}C]choline chloride/ml for 72 h. During these labelling periods, viability of spermatozoa remained constant (85–95%) as estimated using propidium iodide [15]. Aliquots of 4×10^8 spermatozoa were washed through two layers of 35% (4 ml) and 70% (2 ml) Percoll-saline, as described previously [11]. After centrifugation, 0.5 ml of infranatant containing loosely pelleted spermatozoa was diluted in saline medium [17], and washed through sucrose medium [17] for 10 min at $300\times g_{max}$, followed by 10 min at $750\times g_{max}$ to remove the Percoll. At this stage, ≥80% viable cells were found.

In a series of experiments, samples of the washed sperm suspension were added to 0.8 ml of Tyrode's medium with or without bicarbonate, Ca^{2+} or EGTA (final concentration 0.5×10^8 cells/ml), and were incubated at 38°C for 10 min before lipid extraction. In another series, spermatozoa were incubated for 10 min in Tyrode's medium with or without bicarbonate and/or Ca^{2+}, under identical conditions, and were then treated with progesterone (15 μM) for 10 min before lipid extraction; in addition, spermatozoa in Tyrode's medium without bicarbonate, but with Ca^{2+}, were treated with the ionophore A23187 (0.5 μM) for 20 min before lipid extraction. In a final series, spermatozoa were similarly treated, and exocytosis of the acrosome monitored by phase contrast microscopy of glutaraldehyde-fixed samples [18]; cell viability was examined by staining with propidium iodide.

2.3. Lipid analyses

To measure changes in phospholipids, incubations were stopped with 10% (v/v) perchloric acid [19] and lipids were then extracted as described previously [20,21]. For experiments in which only quantitation of arachidonic acid was required, incubations were stopped by addition of chloroform/methanol (1:2 v/v) and lipids were then extracted according to Bligh and Dyer [22].

Lipids were separated by thin layer chromatography on silica-gel 60-coated glass plates (0.25 mm thickness) or plastic sheets (0.2 mm thickness) (E. Merck, Darmstadt, Germany). Phospholipids were separated on 10×10 cm plastic sheets pretreated by spraying with 1% (w/v) potassium oxalate, activated by heating at 110°C for 10 min, and developed in a two-dimensional thin layer chromatography system [17,19,23]. The plates were air-dried briefly, and the various spots were detected by autoradiography using Fuji RX film. Using the autoradiographs as templates, the individual spots were scraped off and the radioactivity in each determined by liquid scintillation counting.

Neutral lipids were separated by thin layer chromatography, developing 20×20 cm glass plates twice using the solvent *n*-hexane/diethylether/acetic acid (70:30:1 v/v), and were detected by staining with iodine vapors. Lipid spots were identified by comparison with authentic standards of arachidonic acid, 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoylglycerol run on the same plate, scraped off, and the radioactivity in each determined by liquid scintillation counting.

For quantitation of lysophosphatidylcholine (lysoPC), spermatozoa were labelled with [*methyl*-^{14}C]choline chloride, and phospholipids were separated using a one-dimensional thin layer chromatography system. Plates (20×20 cm) were pretreated by spraying with 1% (w/v) EGTA, pH 5.5, activated by heating at 110°C for 60 min, and developed in the solvent chloroform/methanol/water/acetic acid (65:50:5:2 v/v). Lipid spots were detected by iodine staining, identified by comparison with internal standards of phosphatidylcholine (PC), lysoPC and sphingomyelin, scraped off, and radioactivity in each determined by liquid scintillation counting.

2.4. Statistical analyses

Results are means ± S.E.M.; data were transformed and analyzed as described previously [19].

3. Results and discussion

To test whether bicarbonate induces rapid changes in the composition of sperm membranes, boar spermatozoa were prelabelled with [^{14}C]arachidonic acid, washed, and exposed to a Tyrode's medium with or without Ca^{2+} and/or bicarbonate/CO_2 (hereafter referred to as 'bicarbonate'). Results in Fig. 1A show that a short (10 min) exposure to Ca^{2+} alone resulted in release of arachidonic acid. In the absence of Ca^{2+}, exposure to bicarbonate also led to release of arachidonic acid. The combination of both bicarbonate and Ca^{2+} caused a further increase in the release of arachidonic acid (the effect was significantly higher than that seen with bicarbonate alone; it tended to be higher than Ca^{2+} alone but it did not reach significance – see below). The magnitude of the arachidonic acid release seen after these treatments was considerably lower than that seen after stimulation of cells with A23187 in the presence of Ca^{2+} [19].

Sperm phospholipids only have unsaturated fatty acids in position 2 of the glycerol backbone [24]; therefore, the release of arachidonic acid described above strongly suggests PLA_2 activation (cf. [19]). Additional evidence for PLA_2 activation comes from the observation that the increases in arachidonic acid induced by Ca^{2+} and/or bicarbonate were accompanied by decreases in phospholipids. Interestingly, exposure to Ca^{2+} was paralleled by a decrease in PC (Fig. 1B), whereas exposure to bicarbonate was accompanied by a decrease in phosphatidylserine (PS) (Fig. 1C). Inclusion of both bicarbonate and Ca^{2+} resulted in a decrease in both PC and PS. No clear changes were seen in phosphatidylethanolamine or phospha-

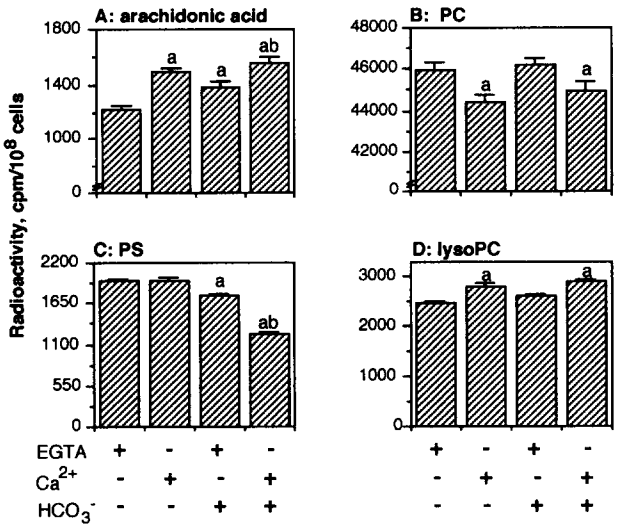

Fig. 1. Changes in lipids after exposure of boar spermatozoa to bicarbonate/CO_2 and/or Ca^{2+}. Spermatozoa were labelled with [^{14}C]arachidonic acid, washed and resuspended in media with or without bicarbonate, EGTA (1 mM) or Ca^{2+} (3 mM) for 10 min before lipid extraction and separation using two dimensional thin layer chromatography (see Section 2). Results are averages (±S.E.M.) of experiments carried out on three different occasions; a = different from medium with EGTA, $P<0.05$; b = different from medium with EGTA and bicarbonate/CO_2, $P<0.05$.

tidic acid (not shown). On the other hand, phosphatidylinositol was poorly labeled and no changes were seen. However, an increase in labelled lysoPC was also noticed after exposure to Ca^{2+} and/or bicarbonate (Fig. 1D), which suggests activation of a phospholipase A_1.

In order to obtain further evidence of PLA_2 activation following exposure to Ca^{2+} and/or bicarbonate, we used a different labelling protocol to examine changes in lysoPC, the other product of PLA_2. When cells were incubated with [*methyl*-^{14}C]choline a good labelling of the PC pool was obtained, and changes in lysoPC were unambiguous. Exposure to Ca^{2+} led to a clear generation of lysoPC, which was similar to that triggered by inclusion of bicarbonate (Fig. 2). Addition of both Ca^{2+} and bicarbonate resulted in higher levels of lysoPC. Generation of lysoPC thus confirms that activation of PLA_2 is taking place in response to these ions (cf. [19]).

The question that arises is how does exposure to Ca^{2+} and/or bicarbonate result in PLA_2 activation? Since activation of the enzyme is seen with inclusion of either Ca^{2+} or bicarbonate, and both show additive effects, such activation may not be entirely related to the destabilizing action of bicarbonate described earlier [11], mainly because in the latter study the effects of bicarbonate were noted regardless of the presence or absence of Ca^{2+}, unlike the results presented here. The findings we obtained with bicarbonate/Ca^{2+} are more reminiscent of the (Ca^{2+}, bicarbonate)-induced stimulation of adenylyl cyclase and generation of cAMP seen in spermatozoa from various species. Ca^{2+} alone was found to cause elevations of cAMP [25], and so was bicarbonate [26], and the effect was maximal with Ca^{2+} plus bicarbonate [26]; the ions appear to act directly on adenylyl cyclase [27–31]. The biological relevance of these findings relates to the fact that addition of exogenous permeable cAMP, or reagents that lead to increases in endogenous cAMP (such as caffeine), shorten the capacitation time [25,32–34]. Also, both an increase in adenylyl cyclase activity and a decrease in phosphodiesterase activity have been shown to accompany the development of capacitation in vitro [34]. Furthermore, exogenous permeable cAMP causes spermatozoa incubated in bicarbonate-deficient medium to display a chlortetracycline fluorescent pattern indicative of capacitation and to develop the ability to respond to zona pellucida with exocytosis [31]. In spite of these observations, no temporal link has been found between increases in, or addition of, cAMP during capacitation and ensuing acrosomal exocytosis [25,33]; this may be due to the fact that, in these latter studies, exocytosis was allowed to develop spontaneously.

To test further if the effects we observed with bicarbonate/Ca^{2+} may be linked to cAMP, we examined whether treatment with caffeine, a cyclic nucleotide phosphodiesterase inhibitor known to inhibit sperm phosphodiesterase [35] and to increase intracellular levels of cAMP [36], enhanced the release of arachidonic acid (as an indication of PLA_2 activity – see above). As shown in Fig. 3, Ca^{2+} or bicarbonate triggered release of arachidonic acid, in agreement with results obtained in the previous series of experiments. In the absence of Ca^{2+}, addition of caffeine resulted in levels of arachidonic acid that were higher than those seen in controls (with EGTA added), and similar to those observed after exposure to Ca^{2+} or bicarbonate. If Ca^{2+} was present, caffeine induced a considerable release of arachidonic acid (Fig. 3). Moreover, if caffeine and bicarbonate were added together in the presence of Ca^{2+}, the release of arachidonic acid was further increased (Fig. 3). Taken together, these results suggest that the effect of bicarbonate and Ca^{2+} could be related to a generation of cAMP. This raises the question of how cAMP would activate sperm PLA_2.

Unfortunately, it is not entirely clear which type of PLA_2 is present in spermatozoa, so we can only speculate about its regulation. The cytosolic PLA_2 that is translocated to the plasma membrane upon somatic cell activation, and that mediates arachidonic acid release for eicosanoid production, is a high molecular weight (85–100 kDa) enzyme, quite different from the low molecular weight extracellular types (16–20 kDa) which are apparently not involved in intracellular signalling [37,38]. Attempts to extract and purify PLA_2(s) from human spermatozoa have revealed a low molecular weight form akin to secreted PLA_2, based on its molecular weight of ~16 kDa, but apparently unique due to its N-terminal sequence [39]. However, the extraction protocol used in this latter study would have inactivated the high molecular weight form [37]. Therefore, the nature of the sperm PLA_2(s) that we characterized using various labelling protocols and that we found to be essential for acrosomal exocytosis [19] awaits clarification.

The cytosolic PLA_2 in somatic cells appears to be activated by two mechanisms: mitogen-activated protein (MAP) kinase-mediated phosphorylation, and translocation to the membrane after an intracellular Ca^{2+} rise; MAP kinase, in turn, seems to be regulated by protein kinase C [40,41]. Although protein kinase C does not appear to be involved in pathways regulating PLA_2 in spermatozoa ([42]; but see [43]), it is still possible that sperm PLA_2 is somehow phosphorylated. This is suggested by the fact that the enzyme remains activated after sperm stimulation and subsequent cell disruption, extraction and assay [44]. It is therefore very interesting that recent work has shown that MAP kinase could also be activated by cAMP

[45,46], a finding that introduces an attractive possibility to explain how sperm PLA_2 might be regulated. Alternatively, cAMP may act via protein tyrosine kinase [31]. We would therefore like to postulate that the activation of sperm PLA_2 resulting from exposure to bicarbonate/Ca^{2+} could be mediated by cAMP-induced phosphorylation.

In a final series of experiments, we examined whether the limited activation of PLA_2 promoted by bicarbonate/Ca^{2+} would render the cells capable of responding to a natural agonist and, thus, whether this activation may represent an event underlying capacitation. To test this idea, we exposed cells to bicarbonate/Ca^{2+} and then challenged them with progesterone. We predicted that if changes induced by bicarbonate/Ca^{2+} were meaningful, spermatozoa pre-exposed to these components and then challenged with progesterone might experience further activation of PLA_2 and, more importantly, would respond with exocytosis.

Exposure of spermatozoa to bicarbonate/Ca^{2+} for 10 min, and subsequent challenge with progesterone (15 μM) for 10 min, revealed a major release of arachidonic acid (Fig. 4A), which was considerably higher than the release of arachidonic acid seen when spermatozoa were exposed only to bicarbonate/Ca^{2+} for 20 min (control). The effect was clearly dependent on pre-exposure to bicarbonate, because progesterone treatment of cells not exposed to this anion resulted in no arachidonic acid release (Fig. 4A). As positive controls, we included results of spermatozoa in a bicarbonate-free medium stimulated with 0.5 μM A23187 for 20 min (Fig. 4A). It should be noted that although the concentration of progesterone we used may be regarded as high, and slightly above the levels present at the site of fertilization [47], our choice of progesterone concentration can be justified on the following grounds. First, we attempted to maximize the response of cells to progesterone challenge. Second, there is no indication that a high concentration of progesterone (up to 1 mM) affects cell

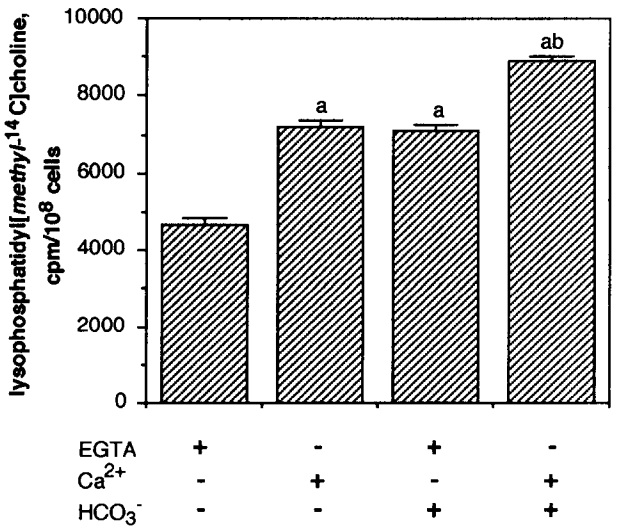

Fig. 2. Changes in lysophosphatidylcholine after exposure of boar spermatozoa to bicarbonate/CO_2 and/or Ca^{2+}. Spermatozoa were labelled with [*methyl*-^{14}C]choline, washed and resuspended in media with or without bicarbonate, EGTA (1 mM) or Ca^{2+} (3 mM) for 10 min before lipid extraction and separation (for details see Section 2). Results are averages (±S.E.M.) of experiments carried out on three different occasions; a = different from medium with EGTA, $P < 0.01$; b = different from medium with Ca^{2+} and from medium with EGTA and bicarbonate/CO_2, $P < 0.01$.

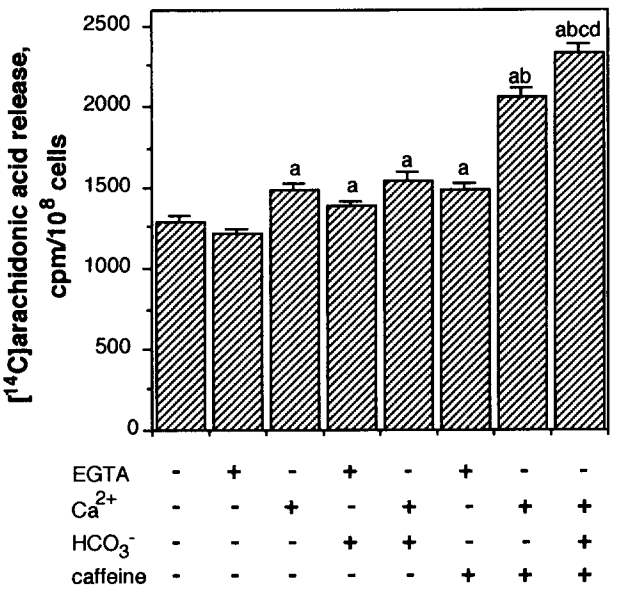

Fig. 3. Changes in arachidonic acid after exposure of boar spermatozoa to bicarbonate/CO_2, Ca^{2+} and/or caffeine. Spermatozoa were labelled with [^{14}C]arachidonic acid, washed and resuspended in media with or without bicarbonate, EGTA (1 mM), Ca^{2+} (3 mM) or caffeine (1 mM) for 10 min before lipid extraction and separation using a mono-dimensional thin layer chromatography system (see Section 2). Results are averages (±S.E.M.) of experiments carried out on three different occasions; a = different from medium with EGTA, $P \leq 0.01$; b = different from medium with caffeine alone, $P \leq 0.001$; c = different from medium with Ca^{2+} alone, $P < 0.001$; d = different from medium with Ca^{2+} and caffeine, $P = 0.03$.

viability [48]. Third, spermatozoa may, after all, be acutely exposed to a high concentration of progesterone on the cell surface [49].

In a parallel experiment, spermatozoa were subjected to similar treatments and were subsequently examined for occurrence of exocytosis. Exposure to bicarbonate/Ca^{2+} for 10 min, followed by progesterone for a further 10-min period, resulted in exocytosis (Fig. 4B). However, if spermatozoa were not exposed to bicarbonate, they did not undergo acrosomal exocytosis when they were treated with progesterone (Fig. 4B). Viability of the cells was ≥ 70%. As positive controls, spermatozoa treated with 0.5 μM A23187 showed that ~85% of cells underwent exocytosis by 30 min (not shown). Interestingly, the percentage of cells undergoing exocytosis after the sequence bicarbonate/Ca^{2+} → progesterone kept rising at 60 min, which suggests some heterogeneity in the initial response of spermatozoa to bicarbonate/Ca^{2+}. At both 30 min and 60 min, there was a higher percentage of spermatozoa without acrosomes when cells were exposed to bicarbonate/Ca^{2+}, as compared to figures seen when cells were not pre-exposed to bicarbonate and then challenged with progesterone. This is probably due to the destabilizing action of bicarbonate reported previously [11].

It could be argued that the effects described here could be due to the fact that spermatozoa were stored in BTS medium before exposure to bicarbonate, and that changes resembling capacitation may have taken place during the period of storage. However, this is unlikely because freshly collected (non-stored) spermatozoa were also able to undergo exocytosis in response to the sequence bicarbonate/Ca^{2+} → progesterone

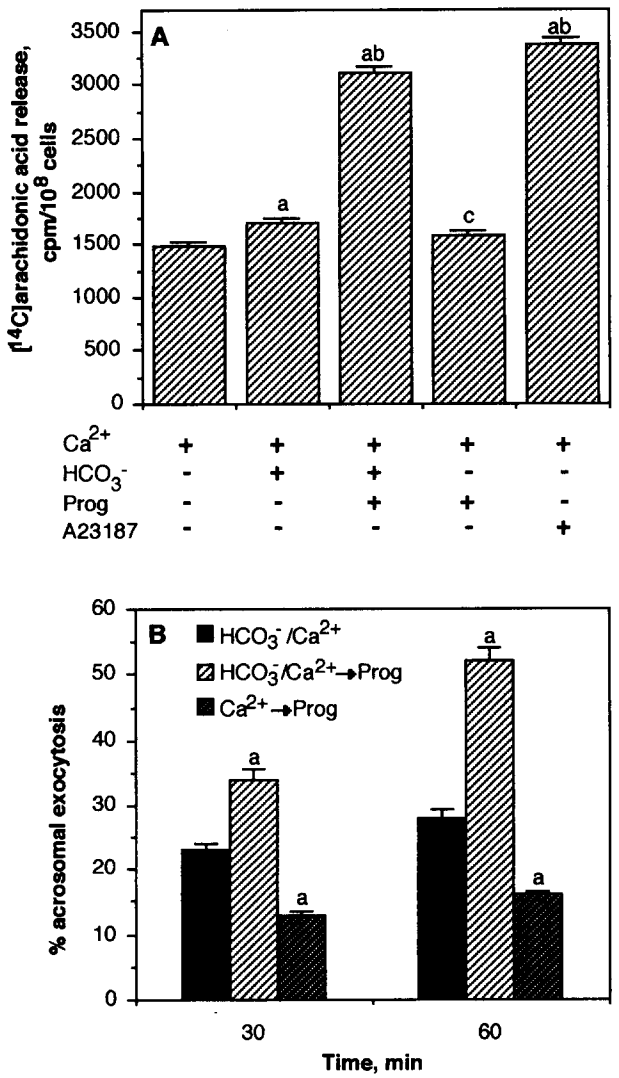

Fig. 4. Changes in arachidonic acid and occurrence of acrosomal exocytosis after exposure of boar spermatozoa to bicarbonate/CO_2 in the presence of Ca^{2+}, followed by treatment with progesterone (Prog). A: Spermatozoa were labelled with [^{14}C]arachidonic acid, washed and resuspended in media with Ca^{2+} (3 mM) and with or without bicarbonate for 10 min, and were then challenged with progesterone (15 μM) for 10 min before lipid extraction and separation using a mono-dimensional thin layer chromatography system. Spermatozoa in medium with Ca^{2+} were incubated for 20 min with or without bicarbonate to serve as controls. Spermatozoa in medium with Ca^{2+}, but without bicarbonate, were treated with 0.5 μM A23187 for 20 min as positive controls. Results are averages (±S.E.M.) of experiments carried out on five different occasions; a = different from Ca^{2+} alone, $P \leq 0.01$; b = different from bicarbonate/Ca^{2+}, $P < 0.001$; c = different from bicarbonate/Ca^{2+} → progesterone, $P < 0.001$. B: Unlabelled spermatozoa were suspended in medium containing Ca^{2+} and were incubated in the presence or the absence of bicarbonate for 10 min, and were then exposed to progesterone (15 μM). Spermatozoa in medium with Ca^{2+} were incubated with bicarbonate, but were not exposed to progesterone, and served as controls. At 30 and 60 min, sub-samples were taken and examined for the occurrence of acrosomal exocytosis. Results are averages (±S.E.M.) of experiments carried out on three different occasions; a = different from bicarbonate/Ca^{2+}, $P < 0.001$.

(not shown). Furthermore, it has recently been reported that the timing of oocyte penetration (i.e. an estimate of the time needed to complete capacitation) was not affected by storage of boar spermatozoa or by repeated washing [50].

In summary, our findings demonstrate that exposure to bicarbonate and Ca^{2+} results in activation of PLA_2, and that this treatment modifies spermatozoa in such a way that they become responsive to a subsequent challenge with a natural agonist; this, in turn, leads to a major activation of PLA_2 and exocytosis of the acrosome.

One final question to be raised is what, then, are the kinetics of sperm capacitation. The changes underlying the response to bicarbonate could take place in the upper portions of the female tract around the time of ovulation. Female oviductal fluids have a high concentration of bicarbonate (35 mM), which rises after ovulation (to 90 mM) [13], and therefore such fluids may trigger in vivo the responses described here. These changes could occur either when spermatozoa are kept 'repressed' in the lower isthmus (while attached to the oviductal epithelium), or soon after they start their journey towards the oocyte to achieve fertilization. The former hypothesis is more appealing. In fact, completion of capacitation may allow spermatozoa to detach and swim towards the oocyte.

The evidence presented here suggests that completion of capacitation could be a rapid process. Our results thus challenge the traditional view that regards preparation for exocytosis as a slow and long process, as inferred from in vivo and in vitro fertilization studies (see Section 1). Our results also support the idea that, since preparation for exocytosis may be induced and completed in a short time-span, at least in a significant sub-population of spermatozoa, it could occur in the oviduct not long before the sperm encounters the oocyte [9]. However, our findings do not necessarily imply that loss of so-called decapacitation factors or coating proteins [4] would take place at this stage, or that they are not an important step in the preparation for exocytosis. In vivo, this loss may occur earlier, during transport via the cervix, uterus, or utero-tubal junction, or perhaps early during the period of residence in the lower isthmus of the oviduct. But, in this context, it is important to bear in mind that spermatozoa that have lost these decapacitation factors or coating proteins (and by current definition would be 'partially capacitated') may be in an unstable state for several hours during residence in the lower oviductal isthmus. Perhaps a re-definition of capacitation, and a dissection and recognition of steps based on molecular evidence, would help to clarify this issue.

With regard to molecular mechanisms underlying capacitation, our findings suggest that regulation of PLA_2 activation may be an important part of this process. Activation of PLA_2 may lead to changes in membrane lipids which could, for instance, allow other lipids to move freely [51], or could modify the membrane for changes that would take place later during exocytosis. The study of mechanisms leading to the initial activation of PLA_2 during capacitation deserves further attention; in particular, how does the bicarbonate/Ca^{2+}-mediated activation of adenylyl cyclase, and generation of cAMP, relate to PLA_2 activation. Another phenomenon which is also believed to play an important role in capacitation is the loss of cholesterol from sperm membranes [52]. It is therefore very interesting that recent studies have shown that cAMP can stimulate cholesterol efflux [53], a fact which would reconcile previous observations ([52], and references therein) with our present findings.

On the other hand, a variety of protein changes are thought to underlie processes apparently attending capacitation [4]. It

would therefore be worth while examining which of these protein modifications take place in the bicarbonate/Ca^{2+} → progesterone system. Thus, this system represents a unique model to examine which changes are relevant in the sequence preceding exocytosis; treatment with bicarbonate/Ca^{2+} may be a very useful tool to attempt a more precise dissection of events based on molecular changes.

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