Bicarbonate/CO₂ Is Not Required for Zona Pellucida- or Progesterone-Induced Acrosomal Exocytosis of Mouse Spermatozoa But Is Essential for Capacitation

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ABSTRACT

We investigated whether bicarbonate/CO₂ is required for capacitation or for acrosomal exocytosis triggered with zona pellucida or progesterone. Mouse spermatozoa, incubated for 90 min in a modified Tyrode's medium with bicarbonate and equilibrated with 5% CO₂ in air, were washed in medium without bicarbonate but with Hepes, resuspended in media with or without bicarbonate, and then stimulated with 1 zona pellucida/μl or 15 μM progesterone. Spermatozoa were able to undergo exocytosis, regardless of the medium in which they were resuspended, as ascertained via the chlorotetracycline assay. If, however, spermatozoa were first incubated in a medium in which bicarbonate was replaced by Hepes and were then washed and resuspended in various media, they were unable to undergo exocytosis in response to zona pellucida or progesterone even when resuspended in bicarbonate-containing medium. This indicated that spermatozoa were not capacitated. Furthermore, the proportion of cells exhibiting the "B" pattern (characteristic of capacitated cells) after incubation in medium without bicarbonate was lower than that in cells incubated with the anion and the appropriate gas phase. Extended incubation in medium without bicarbonate (up to 3 h) did not increase the proportion of cells that exhibited the "B" pattern. These results demonstrate that bicarbonate is not required for acrosomal exocytosis but that it is essential for capacitation, exerting roles beyond its action as pH buffer.

INTRODUCTION

Upon release from the male genital tract at ejaculation, mammalian spermatozoa are still not ready to fertilize oocytes; they must spend a period of time in the female tract, or be incubated under appropriate in vitro conditions, before they can engage in fertilization [1]. During the period of residence in the uterus and oviducts, a series of poorly defined changes, collectively known as "capacitation," takes place. Acquisition of the capacitated state enables spermatozoa to interact with the oocyte(s) and undergo another essential process in the series leading to fertilization, i.e., exocytosis of the acrosomal granule in response to oocyte-associated stimuli (the acrosome reaction) [1-3]. Acrosomal exocytosis involves the release or exposure of enzymes contained in the acrosomal granule, which in turn allows spermatozoa to penetrate the oocyte vestments and eventually fuse with the oolemma [1].

Our understanding of the molecular sequence of events underlying capacitation and acrosomal exocytosis is still largely fragmentary, although a considerable amount of information has accumulated in recent years [3-6]. Many of the studies carried out so far have tried to unravel which ions are required for these processes and thus infer which mechanisms might be regulating ion fluxes [5, 7-9]. In early studies, ions were omitted from in vitro fertilization assays in attempts to investigate this issue. However, this approach has not been adequate to probe the question of the stage at which a particular ion is required. In more recent studies, ions have been omitted at different stages of in vitro sperm incubation, and this has allowed for some inferences regarding the involvement of some ions in capacitation and/or the acrosome reaction. For instance, the use of this experimental approach has revealed that Ca²⁺ has fundamental roles at different steps leading up to and during sperm-oocyte interaction [10, 11]. Nevertheless, the approach has not been satisfactory when used to try to unravel unmistakably the role of other ions such as bicarbonate.

Thus, there is still some controversy regarding the role of bicarbonate/CO₂ during capacitation and the acrosome reaction. Although there are some good indications that bicarbonate/CO₂ may have a role early in capacitation, rather than during the acrosome reaction [12, 13], some studies have suggested that these components are essential for exocytosis [14]. However, none of these studies have dissected events in enough detail to allow for firm conclusions. The importance of whether bicarbonate/CO₂ is essential for any of these processes relates not only to fundamental aspects of ion regulation and the ion's potential roles; it relates also to practical implications with respect to the loss of CO₂ from the small volumes used for gamete manipulation and the resultant rise in pH, which is a constant problem in in vitro fertilization and work involving gametes [14]. In particular, studies of the biochemical mechanisms underlying capacitation or the acrosome reaction are hampered by the need to manipulate gametes in an environment containing CO₂.
The present study was therefore undertaken to examine whether bicarbonate/CO$_2$ (hereafter referred to as bicarbonate) is required for acrosomal exocytosis when spermatozoa are stimulated with two natural agonists, progesterone or zona pellucida, and whether bicarbonate is needed to sustain prior changes during capacitation. The results presented here indicate that bicarbonate is essential for the acquisition of capacitation but not for the process of exocytosis.

**MATERIALS AND METHODS**

*Materials*

All chemicals were of analytical grade and were purchased from Sigma or BDH (both of Poole, Dorset, UK). Water for all aqueous solutions was purified by reverse osmosis and then "polished" by circulation through mixed-bed ion-exchange resin and activated charcoal (Labro/Spectrum system; Elga Ltd., High Wycombe, Bucks, UK). BSA (fraction V), progesterone, and chlortetracycline (CTC) were obtained from Sigma or BDH (both of Poole, Dorset, UK). Hepes (type III), and 0.5 mg hyaluronidase (type IV) were placed in a Dounce homogenizer and subjected to 20 strokes of the pestle. The homogenate (~2 ml) was gently loaded on top of a two-step gradient of 1.5 ml of ice-cold HB buffer (15 mM NaCl, 25 mM triethanolamine, 1 mM MgCl$_2$, 1 mM CaCl$_2$) with 0.2% (w/v) polyvinylalcohol (PVA), 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) sodium deoxycholate, 0.5 mg DNAse (type III), and 0.5 mg hyaluronidase (type IV) were placed in a Dounce homogenizer and subjected to 20 strokes of the pestle. The homogenate (~2 ml) was gently loaded on top of a two-step gradient of 1.5 ml of 10% Percoll/HB with 0.2% (w/v) PVA and 1.5 ml of 20% Percoll/HB-PVA in seal-cap plastic tubes. Percoll solutions were prepared according to Vincent and Nadeau [19]. The tubes were centrifuged at 800 x g for 10 min. Zonae were isolated by examining samples of various layers under a phase-contrast microscope. The band containing the majority of zonae was located in the interphase between the homogenate and the 10% Percoll/buffer. The typical yield of this isolation protocol was 250–300 zonae/ovary. Zonae were stored in HB buffer with 0.2% (w/v) PVA at -80°C. On the day of the experiment, they were thawed, the concentration was adjusted to 100 zonae/µl, and they were then solubilized by incubation at 60°C for 1 h. The preparation was centrifuged at 13,000 rpm for 1 min to remove particulate debris, and the supernatant was used for experiments.

*Media*

The standard medium used in this study was a modified Tyrode's solution [15] containing 25 mM NaHCO$_3$ and supplemented with 5.56 mM glucose and 4 mg BSA/ml on the day of use; this medium is referred to as mT-B25 (Table 1). Variants of this medium were prepared by reducing the concentration of NaHCO$_3$ to 15 mM (mT-B15), reducing NaHCO$_3$ to 15 mM and including 20 mM Hepes (mT-BH), or by omitting NaHCO$_3$ entirely and including 20 mM Hepes (mT-H) (Table 1). The concentration of NaCl was modified for each medium to give a final osmolality of 295 mOsmol/kg. When NaHCO$_3$ was present, that medium was maintained in equilibrium with 5% CO$_2$ in air. When Hepes was present, the pH was adjusted with NaOH. All media had a pH of 7.4 at 37°C. The medium mT-BH was also used under air.

**Isolation and Preparation of Zona Pellucida**

Female mice (22–24 days old) of the TO strain (Harlan Olac Ltd., Bicester, UK; or B&K Universal Ltd., Hull, UK) were killed with CO$_2$ and the ovaries removed. Zonae pellucidae were isolated as described previously [16–18], with some modifications. Briefly, ovaries of 50–75 mice in 2 ml of ice-cold HB buffer (15 mM NaCl, 25 mM triethanolamine, 1 mM MgCl$_2$, 1 mM CaCl$_2$) with 0.2% (w/v) polyvinylalcohol (PVA), 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) sodium deoxycholate, 0.5 mg DNAse (type III), and 0.5 mg hyaluronidase (type IV) were placed in a Dounce homogenizer and subjected to 20 strokes of the pestle. The homogenate (~2 ml) was gently loaded on top of a two-step gradient of 1.5 ml of 10% Percoll/HB with 0.2% (w/v) PVA and 1.5 ml of 20% Percoll/HB-PVA in seal-cap plastic tubes. Percoll solutions were prepared according to Vincent and Nadeau [19]. The tubes were centrifuged at 800 x g for 10 min. Zonae were isolated by examining samples of various layers under a phase-contrast microscope. The band containing the majority of zonae was located in the interphase between the homogenate and the 10% Percoll/buffer. The typical yield of this isolation protocol was 250–300 zonae/ovary. Zonae were stored in HB buffer with 0.2% (w/v) PVA at -80°C. On the day of the experiment, they were thawed, the concentration was adjusted to 100 zonae/µl, and they were then solubilized by incubation at 60°C for 1 h. The preparation was centrifuged at 13,000 rpm for 1 min to remove particulate debris, and the supernatant was used for experiments.

**Collection and Preparation of Spermatozoa**

Male mice of the outbred TO strain weighing more than 30 g (12–16 wk old) were used. Animals were killed with CO$_2$ and spermatozoa were obtained from caudae epididymides and vasa deferentia. Spermatozoa were placed in

| TABLE 1. Composition of modified Tyrode’s (mT) media used in this study. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Component       | mT-B25 (mM)     | mT-B15 (mM)     | mT-BH (mM)      | mT-H (mM)       |
| NaCl            | 124.54          | 134.39          | 116.89          | 131.89          |
| KCl             | 2.68            | 2.68            | 2.68            | 2.68            |
| CaCl$_2$        | 1.80            | 1.80            | 1.80            | 1.80            |
| MgCl$_2$·6H$_2$O| 0.49            | 0.49            | 0.49            | 0.49            |
| NaHCO$_3$       | 25.00           | 15.00           | 15.00           | -               |
| NaH$_2$PO$_4$·2H$_2$O | 0.36          | 0.36            | 0.36            | 0.36            |
| Hepes           | -               | -               | 20.00           | 20.00           |
| Glucose         | 5.56            | 5.56            | 5.56            | 5.56            |
| Bovine serum albumin | 4 mg/ml        | 4 mg/ml         | 4 mg/ml         | 4 mg/ml         |
| Phenol red      | 5 µg/ml         | 5 µg/ml         | 5 µg/ml         | 5 µg/ml         |
| Kanamycin       | 50 µg/ml        | 50 µg/ml        | 50 µg/ml        | 50 µg/ml        |
| pH adjusted with NaOH | no            | no              | yes             | yes             |
| Gas phase       | 5%CO$_2$/air    | 5%CO$_2$/air    | 5%CO$_2$/air or air | air             |

Measured osmolality of complete media: 285 mOsm/kg. pH = 7.4 at 37°C after equilibration with the appropriate gas phase.
Evaluation of Acrosomal Status by CTC Staining

Experimental Design

Spermatozoa diluted in different media (final concentration 3–6 × 10⁶ cells/ml; motility ≥ 70%) were dispersed in a microdrop of 198 μl under oil. Then 2 μl of progesterone stock solution in dimethylsulfoxide (DMSO; final progesterone concentration: 15 μM), zona pellucida (final concentration: 1 zona/μl), or DMSO (final concentration 1%) was added; this concentration of DMSO did not affect viability or acrosomal integrity. Spermatozoa were incubated for 15 min at 37°C under the appropriate atmosphere; then samples were taken and processed for CTC staining.

Evaluation of Acrosomal Status by CTC Staining

Spermatozoa were stained with CTC as previously described [21, 22]. The stain solution was prepared by dissolving CTC-HCl at a concentration of 250 μM in TN buffer (20 mM Tris, 130 mM NaCl) with 5 mM cysteine (pH 7.8). The solution was wrapped with foil and kept on ice until use; fresh CTC stock was prepared daily. Before use, aliquots of 20 μl in 1 ml of sperm suspension) were layered over two-step cushions of 1 ml of 35% Percoll/mT-H and 1 ml of 70% Percoll/mT-H, which had been pre-warmed to 37°C, and were then centrifuged for 20 min at 600 × g. The Percoll/mT-H solutions were prepared so that they were iso-osmotic [19, 20]. After centrifugation, the supernatant layers were aspirated off to leave in each tube about 0.3 ml of the 70% Percoll/mT-H, which had been pre-warmed to 37°C, and were then centrifuged again at 600 × g for 10 min. After centrifugation the supernatant was removed and spermatozoa were diluted in media to be tested.

Results are means ± SE. Significance of results was examined by transforming percentages of acrosome reactions [arcsin √(x+100)] and analyzing them by Student’s t-test or the Mann-Whitney U test. Values of p < 0.05 were regarded as statistically significant.

RESULTS

Spermatozoa were incubated in a modified Tyrode’s medium containing 25 mM NaHCO₃ (mT-B25) for 90 min under 5% CO₂ in air. Sperm cells were then washed through a two-step Percoll gradient in a medium without bicarbonate but with Heps (mT-H); this was followed by dilution of the resulting infranatant in mT-H and a second centrifugation. The rationale for this washing protocol was that handling and washing of spermatozoa in a bicarbonate-containing medium under air would result in loss of CO₂ and lack of control of extracellular pH during the procedure. After washings, ≥ 70% of spermatozoa showed vigorous motility; about 10–20% exhibited the “F” pattern; 65–70%, the “B” pattern characteristic of capacitated sperm; and about 15–20%, the “AR” pattern. The latter corresponded either to spontaneous reactions or to dead cells.

In an initial series of experiments, we examined whether bicarbonate/CO₂ was required for the occurrence of mouse acrosomal exocytosis. Spermatozoa incubated in mT-B25 and washed as described above were resuspended in different media and were challenged with progesterone (15 μM) or zona pellucida (1 zona/μl) for 15 min. We reasoned that if bicarbonate was necessary for the acrosomal reaction, exocytosis would not occur in medium lacking this anion. Mouse spermatozoa resuspended in bicarbonate-containing medium (mT-B25) underwent exocytosis in response to challenge with either progesterone or zona pellucida (Fig. 1). Reduction of the bicarbonate concentration to 15 mM (mT-B15) did not reduce the ability of spermatozoa to respond to the agonist challenge. The absence of bicarbonate (medium mT-H; gas phase: air) did not impair the ability of spermatozoa to undergo exocytosis in response to either progesterone or zona pellucida, demonstrating that the acrosome reaction does not require the presence of this anion. The presence of Heps did not alter the response to these agonists, because the percentage of acrosomal exocytosis in spermatozoa suspended in media containing both bicarbonate and Heps (mT-BH), and incubated under 5% CO₂ or under air, was not different from the values seen when cells were suspended in media without Heps.

In a second series of experiments, the effect of bicarbonate during capacitation was examined. Spermatozoa were suspended in a medium containing bicarbonate (mT-B25)
or lacking this anion (mT-H) and were incubated in the appropriate gas atmosphere. After a 90-min incubation, spermatozoa were washed through a two-step Percoll/mT-H gradient, resuspended in mT-H, and centrifuged again. After the second centrifugation, spermatozoa initially incubated in mT-B25 were resuspended in the same medium to serve as control, whereas spermatozoa incubated initially in mT-H were resuspended in mT-B25, mT-B15, mT-BH, or mT-H. All samples were then challenged with progesterone (15 μM) or zona pellucida (1 zona/μl) for 15 min. As can be seen in Figure 2, spermatozoa initially incubated in mT-B25 and resuspended in the same medium responded to challenge with the agonists and experienced acrosomal exocytosis. On the other hand, spermatozoa incubated initially in mT-H and then resuspended in various media did not undergo acrosomal exocytosis even if they were resuspended in the bicarbonate-containing medium (mT-B25). Motility of spermatozoa was not different between treatments.

As evidenced by analysis of the proportion of cells exhibiting the "F" or the "B" pattern after 90-min incubation of spermatozoa in the absence or presence of bicarbonate, washing, and resuspension in various media, a lower proportion of cells underwent the "F" to "B" transition in the absence of the anion (Table 2).

In a third and final series of experiments, we examined the occurrence of spontaneous acrosome reactions when spermatozoa were incubated for 90 min in medium with or without bicarbonate, washed through Percoll/mT-H as described above, and resuspended in medium with or without bicarbonate (i.e., spermatozoa were resuspended in the same medium used for the initial incubation) with the incubation extended for a total of 3 h. The logic behind this experiment was that given enough time, spermatozoa in medium without bicarbonate might eventually become capacitated. When this experimental protocol was used, spermatozoa showed an increase in the percentage of cells exhibiting the "AR" pattern over time regardless of the medium in which they were incubated (Fig. 3); no attempt was made to distinguish between spontaneous exocytosis and cell death. There was a considerable proportion of cells that underwent the transition from the "F" to the "B" pattern in medium containing bicarbonate (mT-B25); this proportion increased as incubation time progressed. Interestingly, when spermatozoa were incubated in medium lacking bicarbonate (mT-H), the "F" to "B" transition did take place in some cells and these eventually went on to undergo spontaneous acrosome reactions. Nevertheless, a high proportion of cells retained the "F" pattern even after 3 h of incubation.

### TABLE 2. Effect of bicarbonate on the capacitation of mouse spermatozoa.

<table>
<thead>
<tr>
<th>Media</th>
<th>% pattern &quot;F&quot;</th>
<th>% pattern &quot;B&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>mT-B25 → mT-B25</td>
<td>26.5 ± 0.9a</td>
<td>54.0 ± 4.0a</td>
</tr>
<tr>
<td>mT-H → mT-H</td>
<td>54.3 ± 2.7a</td>
<td>22.7 ± 2.2b</td>
</tr>
<tr>
<td>mT-H → mT-B25</td>
<td>50.0 ± 4.6a</td>
<td>32.7 ± 2.7b</td>
</tr>
</tbody>
</table>

Spermatozoa were incubated in bicarbonate-containing medium (mT-B25) or in medium without bicarbonate but with Hapes (mT-H) for 90 min under the appropriate gas phase. They were then washed and resuspended in mT-B25 or mT-H for an additional period of 15 min and then stained with CTC. Results are averages ± SE of three experiments. Different superscripts within columns indicate significant differences: p < 0.01.
DISCUSSION

The results of this study clearly demonstrate that bicarbonate is not required for exocytosis when capacitated mouse spermatozoa are stimulated with natural agonists but that bicarbonate is essential for the acquisition of the capacitated state.

Spermatozoa incubated for 90 min in a bicarbonate-containing modified Tyrode's medium (mT-B25), and then washed through a Percoll gradient prepared with medium containing Hepes but lacking bicarbonate (in fact, with no added bicarbonate; Percoll/mT-H), underwent exocytosis when they were resuspended in bicarbonate-containing medium (mT-B25) and challenged with zona pellucida or progesterone. These results were not different from those obtained when spermatozoa were incubated continuously in bicarbonate-containing medium (i.e., no wash), stimulated with zona pellucida or progesterone, and examined after a similar total period of time (Q.X. Shi and E.R.S. Roldan, unpublished data); this indicates that washing cells through a Percoll gradient prepared with medium lacking bicarbonate did not affect spermatozoa. These results also demonstrate that mouse spermatozoa are able to undergo exocytosis in response to progesterone, as previously reported for spermatozoa from other species [2, 23, 24]. It could be argued that the concentration of progesterone we used (15 μM) is high—above the levels thought to be present at the site of fertilization [23]. However, this was the concentration of progesterone that in preliminary trials resulted in maximal stimulation (Q.X. Shi and E.R.S. Roldan, unpublished data); moreover, there is no indication that a high progesterone concentration affects cell viability [25]. In addition, it is possible that spermatozoa may, in fact, be exposed to a high concentration of progesterone on their cell surface when they interact with the oocyte vestments [26].

When mouse spermatozoa were capacitated in a bicarbonate-containing medium (mT-B25), washed, resuspended in a bicarbonate-free medium (mT-H), and challenged with natural agonists, acrosomal exocytosis took place at a rate similar to that seen in cells incubated in the presence of bicarbonate (Fig. 1). This is consistent with the observations of Boatman and Robbins [13], who found that once hamster spermatozoa were presumably capacitated, a reduction in the concentration of bicarbonate did not impair the ability of cells to penetrate zona-intact oocytes. It was deduced from this work that bicarbonate was not required for zona pellucida-induced exocytosis [13], but two

FIG. 3. Time course of changes in CTC patterns of mouse spermatozoa incubated in the presence (solid triangles, solid squares, solid circles) or the absence (open triangles, open squares, open circles) of bicarbonate. A) Changes in pattern "F," characteristic of uncapacitated cells. B) Changes in pattern "B," characteristic of capacitated, acrosome-intact cells. C) Changes in pattern "AR," characteristic of acrosome-reacted cells. Results are averages ± SE of three experiments.
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crucial aspects were not investigated: first, whether spermatozoa would undergo exocytosis in the absence of bicarbonate and, second, what was the proportion of cells undergoing exocytosis in bicarbonate-containing and bicarbonate-free media after interaction with zona pellucida—two aspects that we did explore in our study. Yoshimatsu and Yanagimachi [27] quantified the number of hamster spermatozoa that underwent acrosomal exocytosis on the surface of intact zona pellucida when cells were suspended in bicarbonate-free medium. They suggested that sperm cells capacitated in bicarbonate-containing medium do not undergo exocytosis in response to zona stimulation when transferred to medium lacking bicarbonate. However, in their study the total number of spermatozoa with detached + modified acrosomal caps did not differ when groups with and without bicarbonate during exposure to zona pellucida were compared [27]. On the other hand, Lee and Storey [14] concluded that bicarbonate was required for exocytosis in mouse spermatozoa because acrosome reactions did not take place spontaneously, or on intact zona pellucida, if spermatozoa were suspended in bicarbonate-free medium. The reason behind the discrepancy between the work of Lee and Storey [14] and that by us and others is not clear at the present time.

Interestingly, work carried out by Thomas and Meizel [28, 29] has revealed that various cell responses take place if human spermatozoa are stimulated with progesterone in medium lacking bicarbonate. Although the purpose of those studies was not to investigate bicarbonate requirements, the results are noteworthy and highly relevant in the context of the work presented and discussed here. A fraction of human follicular fluid (G-75FR), whose stimulatory ability was later shown to relate to its progesterone content [23, 30], was able to stimulate exocytosis of human spermatozoa suspended in Hepes-containing, bicarbonate-free medium; the proportion of cells undergoing exocytosis in bicarbonate-free medium was not different from that seen when spermatozoa were suspended in bicarbonate-containing medium [28]. Moreover, G-75FR and progesterone triggered Ca\(^{2+}\) influx [28, 29] in human spermatozoa suspended in medium lacking bicarbonate; and G-75FR was able to induce hydrolysis of the polyphosphoinositides, and a concomitant generation of inositol phosphates, under similar conditions [29].

Lastly, it is also worth mentioning that in a variety of somatic cells, responses including exocytosis can be stimulated in the absence of bicarbonate (e.g., [31–35]), so it is not surprising that acrosomal exocytosis takes place in the absence of this anion. Other sperm processes may, however, require the presence of bicarbonate.

Our results indicate that mouse spermatozoa need bicarbonate to undergo capacitation. Spermatozoa incubated in a Hepes-containing medium without bicarbonate were not able to undergo exocytosis when they were challenged with progesterone or zona pellucida, even after resuspen-
sion in a medium with bicarbonate (Fig. 2). By definition, spermatozoa that do not exhibit exocytosis in response to a challenge with natural agonists are not capacitated [1]. When spermatozoa were examined after CTC staining, some spermatozoa (about 20%) incubated in the absence of bicarbonate for 90 min exhibited the "B" staining pattern (Table 2) regarded as characteristic of capacitated spermatozoa [21]. Likewise, when spermatozoa were incubated in media without bicarbonate and exocytosis was allowed to develop spontaneously, some cells with the "B" pattern were seen along with a concomitant decrease in cells exhibiting the "F" pattern (Fig. 3). Although these results suggest that a few cells can undergo capacitation and spontaneous exocytosis in the absence of bicarbonate, they also indicate that the majority of cells cannot and, therefore, that bicarbonate is required during capacitation.

Our observation that mouse sperm require bicarbonate for capacitation is in agreement with results obtained by other groups using mouse or hamster spermatozoa [12–14]. It should be pointed out that Lee and Storey [14] and Boatman and Robbins [13] also found that a proportion of cells managed to undergo capacitation in a bicarbonate-free medium, apparently at a slower pace [14], in contrast to the total absence of capacitation noted by Neill and Olds-Clarke [12]. Interestingly, a lack of adequate capacitation may be the reason behind the low proportion of guinea pig spermatozoa undergoing exocytosis in bicarbonate-free media, under a variety of conditions, in the study by Bhattacharyya and Yanagimachi [36], although this issue was not addressed directly.

It is interesting to speculate about the reasons why bicarbonate might be needed for capacitation. It has been well documented that exposure of spermatozoa to bicarbonate results in a rapid stimulation of adenyl cyclase and generation of cAMP [37–40], indicating that bicarbonate exerts important actions beyond its role as pH buffer. That cAMP may underlie processes taking place during capacitation is illustrated by findings that exogenous permeable cAMP, or inhibition of cAMP catabolism, shortens capacitation time [41, 42]. Furthermore, the development of capacitation in vitro is accompanied by an increase in adenyl cyclase activity and a decrease in phosphodiesterase activity [43]. Recently it has been shown that bicarbonate triggers a rapid increase in membrane fluidity (looser packing) in the outer leaflet of the sperm plasma membrane [44] as well as a much slower destabilization of the sperm plasma membrane [20] and changes in the sperm surface [45]. However, the relevance of these changes for the development and completion of capacitation is still unknown. In any case, a potential role for bicarbonate in capacitation is possible because of the high concentrations of this anion found in oviductal fluids [46]. It is feasible, then, that spermatozoa residing in the lower sections of the oviduct (upper isthmus) may be exposed to high levels of bicarbonate and as a result undergo or complete capacitation. There-
fore, the mechanisms underlying bicarbonate action deserve further investigation.

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