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The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane

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SUMMARY

A flow cytometric procedure was used to follow the effect of bicarbonate, a key inducer of sperm capacitation in vitro, on the transbilayer behavior of C6NBD-phospholipids in the plasma membrane of living acrosome-intact boar spermatozoa under physiological conditions. In the absence of bicarbonate, 97% of C6NBD-phosphatidylserine and 78% of C6NBD-phosphatidylethanolamine was rapidly translocated from the outer leaflet to the inner, whereas relatively little C6NBD-phosphatidylcholine and C6NBD-sphingomyelin was translocated (15% and 5%, respectively). Inclusion of 15 mM bicarbonate/5%CO_2 markedly slowed down the rates of translocation of the aminophospholipids without altering their final distribution, whereas it increased the proportions of C6NBD-phosphatidylcholine and C6NBD-sphingomyelin translocated (30% and 20%, respectively). Bicarbonate activated very markedly the outward translocation of all four phospholipid classes. The changes in C6NBD-phospholipid behavior were accompanied by increased membrane lipid disorder as detected by merocyanine 540, and also by increased potential for phospholipase catabolism of the C6NBD-phospholipid probes. All three changes were mediated via a cAMP-dependent protein phosphorylation pathway. We suspect that the changes result from an activation of the non-specific bidirectional translocase ('scramblase'). They have important implications with respect to sperm fertilizing function.

Key words: Phospholipid asymmetry, Bicarbonate, Membrane fluidity, Protein kinase A, Sperm capacitation, Phospholipase

INTRODUCTION

To carry out its fertilizing role, the mammalian spermatozoon must express several key functions amongst which is the exocytosis of its acrosome (the so-called 'acrosome reaction') as a response to contact with the extracellular egg coat (the zona pellucida). As ejaculated, the mature spermatozoon is incapable of this response, and indeed of the expression of other fertilizing functions: a priming process is first required, termed 'capacitation'. In vivo, capacitation takes place in the female reproductive tract, but it can be induced in vitro by incubation of the spermatozoon in special media that have been developed empirically to support in vitro fertilization. The component of these media particularly responsible for inducing capacitation appears to be bicarbonate/CO_2. (For recent reviews, see Yanagimachi, 1994; Harrison, 1996.)

Studies over the years have identified a range of changes in sperm that take place during incubation under capacitating conditions. The large majority appear to constitute or reflect changes in the architecture of the sperm plasma membrane and/or cell surface. This is hardly surprising, given the importance of membrane fusion events in fertilization, viz. the sperm' exocytotic acrosome reaction and actual fusion between the sperm and the egg. It seems clear that capacitation is a process that somehow renders the sperm plasma membrane less stable, i.e. more fusible (see Harrison, 1996).

Changes in plasma membrane architecture and function are generally considered to concern principally the protein and glycoprotein membrane components. However, recently, increasing attention has been paid to the lipid components. In all mammalian cell types studied, including spermatozoa (Müller et al., 1994; Nolan et al., 1995; Gadella et al., 1999), the two leaflets of the plasma membrane bilayer differ in phospholipid composition. The aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are concentrated in the inner leaflet and the choline phospholipids sphingomyelin (SM) and phosphatidylcholine (PC) in the outer leaflet. In somatic cell types, it appears that

this asymmetry is established and maintained by the action of several translocating enzymes with differing phospholipid specificities (for review, see Bevers et al., 1998). Aminophospholipid translocase (also known as 'flippase), is responsible for transfer of PS and PE from the outer to the inner lipid leaflet; a second enzyme transfers phospholipids from inner to outer leaflet ('floppase'); a third acts as a bi-directional carrier with little specificity ('scramblase'), simply moving all four phospholipid species in both directions (inward and outward) across the membrane lipid bilayer and thereby reducing phospholipid asymmetry. Such reduction in phospholipid asymmetry ('scrambling') is believed to play a role both in cell adhesion (Schlegel et al., 1985; Verhoven et al., 1992) and in membrane fusion processes (Lucy, 1993; Bailey and Cullis, 1994), including exocytosis (Bogdanov et al., 1993, Müller et al., 1996). Given the increasing importance being accorded generally to phospholipid distribution within the plasma membrane as a factor in membrane function, investigation of such distribution in the spermatozoon during capacitation is of obvious interest.

In a previous study (Gadella et al., 1999), we established a reliable protocol for assessing by flow cytometry the transmembrane distribution and behavior of C6NBD-labeled phospholipid analogues in living sperm cells. Particular attention is paid to ensure (i) that incorporation of the phospholipids analogs from carrier vesicles into the sperm is via monomeric transfer rather than by fusion implying that initial incorporation only takes place into the outer lipid leaflet of the plasma membrane; (ii) that catabolism of C6NBD-phospholipids by endogenous phospholipases present in sperm cells is blocked, so that only the behavior of the parent phospholipid is being detected; (iii) that deteriorated cells are distinguishable as a separate subpopulation, so that they can be eliminated from the analyses.

Using this protocol as the basis of our approach, we have now analyzed the asymmetric behavior of C6NBD-phospholipids in boar spermatozoa during incubation under in vitro capacitating conditions, focussing particularly on the effect of bicarbonate. Because capacitated sperm tend to undergo a spontaneous acrosome reaction a change that would itself distort C6NBD-phospholipid labeling, we have improved our methodology so as to monitor in parallel acrosomal integrity and therefore focus our analyses on the acrosome-intact live sperm. Having noted the ability of bicarbonate to induce changes in phospholipid asymmetry, we have investigated the signal transduction mechanisms linking bicarbonate with such changes. Finally, we have assessed the relationship between phospholipid asymmetric behavior and membrane lipid disorder (as detected by merocyanine stainability), since Harrison et al. (1996) have demonstrated that bicarbonate induces a rapid increase in plasma membrane lipid disorder in boar sperm.

MATERIALS AND METHODS

Materials

R-phycoerythrin peanut agglutinin (PNA-RPE) conjugate was prepared from peanut agglutinin (Sigma Chemie, Zwijndrecht, Netherlands) and R-phycoerythrin pyridylsulfide (Molecular Probes Europe, Leiden, Netherlands), according to the specific instructions accompanying the Molecular Probes R-PE protein conjugation kit. C6NBD-phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA); propidium iodide and headgroup-labeled phosphatidylethanolamines were obtained from Molecular Probes. Sources of other reagents were as given by Gadella et al. (1999).

Sperm cell preparation

Sperm-rich fractions of semen were collected from Large White boars (presumed fertile) kept at the Babraham Institute, Cambridge (United Kingdom) or from highly fertile Dutch Landrace boars kept at the Collaborative Artificial Insemination Center Bunnik (Netherlands). After collection, the semen samples were diluted and stored in Beltsville Thawing Solution as described previously (Gadella et al., 1999).

To prepare spermatozoa for experimentation, an aliquot of about 5×10^8 cells was isolated by centrifugation through a two-step discontinuous gradient of 35% and 70 % isotonic Percoll® (Harrison et al., 1993; Gadella et al., 1999). The washed spermatozoa were diluted to a suitable concentration ($5\text{-}50\times10^6$ cells/ml, depending on experimental requirements) in a Hepes-buffered Tyrode's medium (HBT: see below).

Incubation media

The investigations centered on sperm behavior during incubation in one of two media. (i) The 'control' medium HBT (120 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5 mM glucose, 21.7 mM lactate, 1 mM pyruvate, 20 mM Hepes, and 100 μg kanamycin; 300 mOsm/kg, pH 7.4) and (ii) the 'capacitating' medium HBT-Bic, i.e. HBT containing 15 mM $NaHCO_3$ in equilibrium with 5% CO_2 in humidified atmosphere (the bicarbonate replaced a molar equivalent of NaCl so that osmolality was maintained). HBT, though relatively physiological, does not induce capacitative changes whereas HBT-Bic induces capacitative changes (Harrison et al., 1993, 1996; Suzuki et al., 1994; Harkema et al., 1998). Effectors and inhibitors were included in these media as described in the relevant Results sections.

Although BSA is required for full capacitation (e.g. Andrews and Bavister, 1989), this protein was not included in any of the incubation systems in which C6NBD-phospholipid labeling was performed (BSA, with its high affinity for C6NBD-phospholipids, interferes with the labeling process: see Gadella et al., 1999).

Unless otherwise stipulated, all media were routinely supplemented with 5 mM PMSF to block phospholipase catabolism of C6NBD-phospholipids (see Gadella et al., 1999). Moreover, 10 minutes prior to labeling with C6NBD-phospholipids (see below), 2.5 μg/ml propidium iodide (PI) and 10 μg/ml PNA-RPE were added to the incubating sperm suspensions; PI enabled subsequent distinction of live (unstained) from deteriorated (stained) cells (Harrison et al., 1993; Gadella et al., 1999) while PNA-RPE enabled distinction of acrosome-intact (unstained) from acrosome-reacted (stained cells) (see Gadella et al., 1999). (In experiments in which merocyanine was used to examine lipid disorder, 25 nM Yo-Pro 1 was substituted for PI and 10 μg/ml PNA-F was substituted for PNA-RPE; both were added to the media at the start of incubation.)

Labeling with C6NBD-phospholipids

Spermatozoa were labeled with C6NBD-phospholipids by means of incubation with small unilamellar donor vesicles, prepared and quality-tested as described previously (Gadella et al., 1999). Labeling was initiated by adding 10 μl of a suspension of donor vesicles containing the desired C6NBD-phospholipid to 2.99 ml aliquants of the incubated sperm suspensions (5×10^6 sperm cells/ml) at 38°C. Final concentration of C6NBD-phospholipid label was 0.3 μM, approx. 3% of endogenous sperm phospholipid. Sperm suspensions in media containing bicarbonate were maintained in equilibrium with 5% CO_2.

Analysis of inward translocation

Spermatozoa were incubated in the experimental media for 2 hours at

38°C after which they were mixed with suitable C6NBD-phospholipid vesicle preparations as described above. At predetermined intervals, 100 μl samples of the sperm/vesicle suspensions were subjected to flow cytometric analysis using a FACScalibur flow cytometer equipped with a 100 mW argon laser (Becton Dickinson, San Mateo, CA).

The cells were maintained at or close to 38°C as they passed through the instrument; the sheath fluid (HBT) was kept at 38°C and passed continuously through the flow-cell both during and between measurements, with the result that the temperature of liquid exiting from the flow-cell assembly was approx. 36.5°C (the instrument was pre-equilibrated for 1 hour before analyses were begun). During the analysis, non-sperm events (i.e. donor C6NBD-phospholipid vesicles and indeterminate debris) were gated out on the basis of their forward and sideways scatter profiles (see Gadella et al., 1999). Excited at 488 nm, NBD fluorescence was detected using a 530/30 nm band-pass filter (FL-1) while PI and RPE fluorescence were detected together using a 620 nm long-pass filter (FL-3); all fluorescence detectors were in log mode, and data were collected for 10,000 'events'.

Two analyses were carried out in rapid succession. The first, performed directly as above, analyzed total NBD fluorescence. Immediately afterwards, a further aliquot of 100 μl from the same sperm/vesicle suspension was mixed with 1 μl of 1 M sodium dithionite (freshly dissolved in 1 M Tris, pH 10), and data from a further 10,000 events collected. The concept of this procedure was that dithionite treatment instantly destroyed the NBD fluorescence in the plasma membrane outer leaflet of intact cells whereas the internalized NBD fluorescence remained protected. Comparison of the two analyses enabled estimation of the proportion of total C6NBD-phospholipid that had been translocated across the plasma membrane lipid bilayer (Gadella et al., 1999). By prestaining the sperm suspensions with PI and PNA-RPE, the plasma membrane bilayer distributions of NBD could be quantified on the live acrosome-intact (i.e. propidium iodide and PNA-RPE negative) subpopulation only.

The quantitative estimations were carried out according to Gadella et al. (1999). Briefly, from two-dimensional dot-plots of the fluorescence data, computer-generated boundaries were set so as to include the FL1 fluorescence limits of the live acrosome-intact spermatozoa (see Fig. 2); calculations were based on the statistics of the sperm populations detected within these boundaries. The mean initial FL1 value (i.e. prior to dithionite addition) represented the total amount of NBD fluorescence incorporated into the sperm cells (i.e. 100%). The dithionite-resistant NBD fluorescence was expressed as a proportion of the initial FL1 value and represented the percentage of label that had been translocated to the interior of the cells. The mean FL1 value of cells that had been treated with dithionite in the presence of 1% (w/v) Triton X-100 (i.e. where all NBD-fluorescence was destroyed) represented the base line (0%).

Analysis of outward translocation

Sperm cells were incubated in HBT or HBT-Bic for 2 hours at 38°C after which they were labeled for 10 minutes with C6NBD-phospholipids; in this case, 29.9 ml of sperm suspension (5×10^6 cells per ml) were mixed with 100 μl of vesicle suspension. Dithionite was then added (10 μl per 3 ml of sperm/vesicle suspension) in order to destroy the outer leaflet NBD fluorescence. The sperm cells were immediately sedimented through a Percoll® gradient as above (to remove the non-incorporated C6NBD-phospholipids as well as dithionite), and were then resuspended in either HBT or HBT-Bic as appropriate, both supplemented with 5 mg/ml delipidated BSA (preparation according to Gadella et al., 1999). Incubation was continued at 38°C (the cells in HBT-Bic were maintained in equilibrium with 5% CO_2), and at intervals samples were subjected to flow cytometric analysis as described above. At each time point, a single analysis of total sperm-associated NBD fluorescence in the live acrosome-intact subpopulation was made, in order to estimate the relative amount of C6NBD-phospholipid label that remained resistant to BSA back-extraction

The concept of this procedure was that C6NBD-phospholipids that resisted dithionite reduction were within the sperm, most likely in the inner leaflet of the plasma membrane bilayer. If these phospholipids were subsequently translocated to the outer leaflet, they would be extracted from the sperm by the BSA, and would no longer be detectable in the flow cytometer, whence the total sperm-associated NBD fluorescence would be seen to have decreased.

Analysis of C6NBD-phospholipid catabolism

Sperm suspensions (50×10^6 cells/ml) were suspended in HBT at 38°C either in the presence or the absence of 5 mM PMSF (Gadella et al., 1999). Donor C6NBD-phospholipid-bearing vesicles were then added (30 μl per ml, final concentration 3 μM C6NBD-phospholipid) and labeling continued for 10 minutes at 38°C. The sperm were immediately washed through a Percoll® gradient as described above to remove the donor vesicles, resuspended to their original concentration in various experimental medium, and incubated at 38°C for 1 hour. The suspensions were then treated with chloroform and methanol to extract total lipids. After separation on HPTLC, the C6NBD-conjugated lipids were detected by UV illumination, scraped off and quantified by fluorimetry; details of the procedure are described by Gadella et al. (1999).

Determination of membrane lipid disorder

Spermatozoa were subjected to incubation under various conditions (media were supplemented with 25 nM Yo-Pro and 10 μg/ml PNA-F to enable detection of the live acrosome-intact cells). At predetermined times, aliquots of the suspensions were stained briefly with 2.7 μM merocyanine 540 according to Harrison et al. (1996) and subjected to flow cytometric analysis. Settings were as described above, save that FL1 in combination with a 530/30 nm band-pass filter was used to detect both Yo-Pro and PNA-F fluorescence while FL-3 in combination with a 620 nm long-pass filter was used to detect merocyanine fluorescence. Analysis of the data was carried out essentially according to Harrison et al. (1996).

RESULTS

Exclusion of acrosome-reacted cells from analyses

Acrosome-reacted cells are able to incorporate C6NBD-phospholipids into the exposed acrosomal membranes; moreover, C6NBD-phospholipids in the outer leaflet of such membranes are susceptible to dithionite reduction. We therefore stained our suspensions routinely with PI and PNA-RPE to detect simultaneously both overall plasma membrane integrity (cells unstained by PI) and acrosomal integrity (cells unstained by PNA: as applied to spermatozoa, PNA specifically binds only to exposed acrosomal components – see Gadella et al., 1999). When viewed by fluorescence microscopy, completely intact cells were labeled homogeneously with C6NBD-phospholipids after incubations in HBT-Bic (Fig. 1A). This pattern was the same as that seen in cells incubated in HBT (Gadella et al., 1999). Sperm cells that were live (plasma membrane-intact) but acrosome-reacted showed a higher intensity of C6NBD-phospholipid labeling in the apical region of the head but did not show higher labeling intensity in the mid-piece (Fig. 1B). Plasma membrane damaged cells showed more intense labeling in the mid-piece region due to C6NBD-PC incorporation into the mitochondrial membranes (Fig. 1C). When the acrosomal cap was still present, the apical region of the head (anterior acrosomal

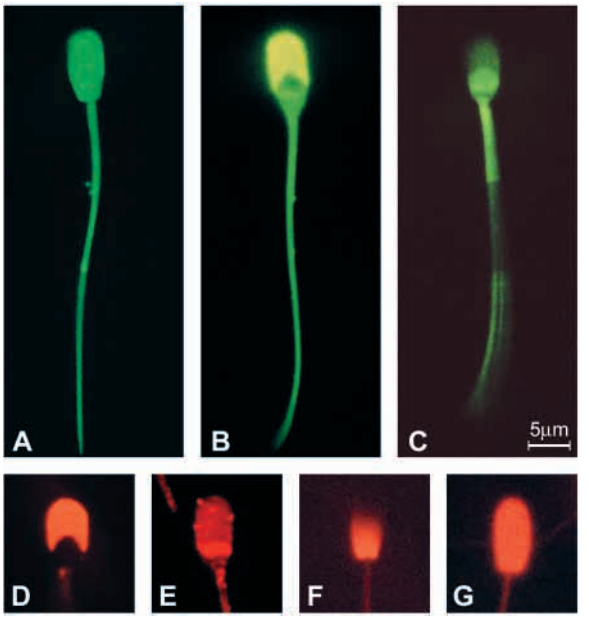

Fig. 1. Fluorescent labeling patterns of C6NBD-phospholipids in boar spermatozoa: differences between acrosome-intact, acrosome-reacted and deteriorated cells. After incubation for 2 hours in HBT-Bic, spermatozoa were labeled for 10 minutes with C6NBD-PC and then washed through Percoll® to remove the donor C6NBD-PC-bearing vesicles. The epifluorescence images were obtained using excitation via a 470/30 nm BP filter; emission was detected via a 510 nm dichroic mirror, using a 530/30 nm BP filter for NBD and a 620 LP filter for PI and PNA-RPE. To minimize bleaching of NBD, preparations were illuminated with a reduced level of excitation light. (A) C6NBD fluorescence: fully intact sperm cell (unstained by both PNA-RPE and PI – these latter were added to the sperm preparation 10 minutes before labeling with C6NBD-PC). Note homogeneous fluorescence over the entire sperm surface. (B) C6NBD fluorescence: live acrosome-reacted sperm cell (i.e. negative for PI but positive for PNA-RPE). Note enhanced acrosomal labeling due to the incorporation of C6NBD-PC into the acrosomal membranes; no enhancement of labeling of mid-piece. (C) C6NBD fluorescence: deteriorated acrosome-less sperm cell (PNA-RPE staining pattern similar to that in E). Note enhanced labeling by C6NBD-PC of both mid-piece and posterior region of head. (D) PNA-RPE fluorescence: live acrosome-reacted sperm cell (unstained by PI). Note homogeneous staining over the anterior acrosomal region (C6NBD-PC labeling was similar to that shown in B). (E) PNA-RPE fluorescence: deteriorated (i.e. PI-positive) sperm cell that had lost its acrosomal cap. Note homogeneous intense staining of equatorial acrosomal region (C6NBD-PC labeling was similar to that shown in C). Overall red head color is due to PI staining; the punctate spots of red fluorescence seen over the anterior acrosomal region are due to adhering donor vesicles, which showed a tendency to stick to the exposed acrosomal surface (the vesicles contained both C6NBD-PC and *N*Rh-PE, so as to enable routine testing of their donor efficacy, and their red fluorescence was due to fluorescence resonance energy transfer – for explanation, see Gadella et al., 1999). (F) PI-fluorescence: deteriorated sperm cell with graded staining of head, most intense at posterior end. (For the purposes of obtaining the illustration, this particular preparation did not receive PNA-RPE as an acrosomal stain). (G) PI-fluorescence: deteriorated sperm cell with homogeneous PI staining over entire head. (Preparation untreated with PNA-RPE.) Note that all PI-positive cells showed intense labeling of the mid-piece by C6NBD-PC as in C; in such cells, acrosomal labeling by C6NBD-PC varied between the pattern extremes shown in B and C.

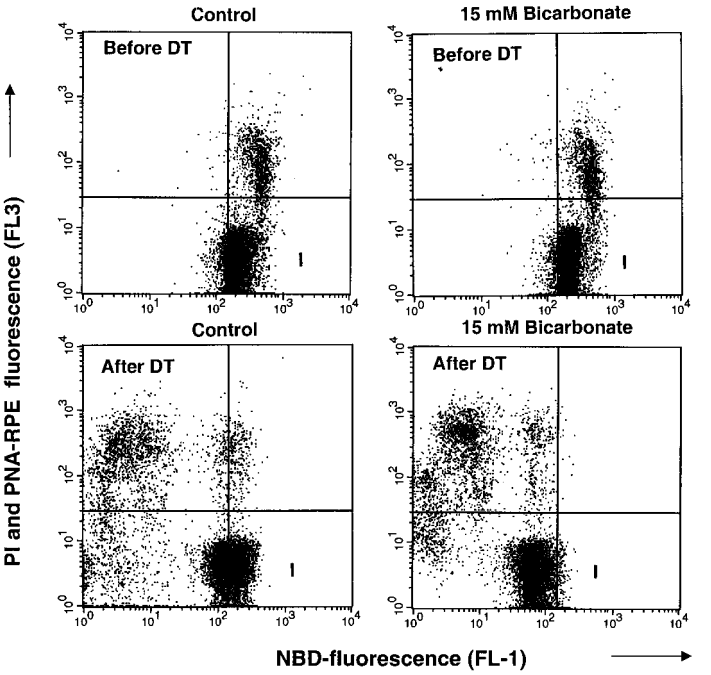

Fig. 2. Bicarbonate-induced changes in the asymmetric distribution of C6NBD-PS in boar spermatozoa: two-dimensional flow cytometric dot-plots. Spermatozoa were incubated for 2 hours in either HBT or HBT-Bic, after which they were labeled with C6NBD-PS for 10 minutes in the presence of PNA-RPE and PI, and then analyzed by flow cytometry. The left-hand panels show the C6NBD-PS labeling of 'control' cells incubated in HBT while the right-hand panels show C6NBD-PS staining of cells incubated in HBT containing 15 mM bicarbonate (HBT-Bic). The upper pair of panels shows total C6NBD-PS fluorescence, whereas the lower pair of panels show dithionite-resistant C6NBD-PS fluorescence. The horizontal line in each panel indicates the 'cut-off' value of FL3 above which cells were considered 'positive' for PNA-RPE and/or PI fluorescence and were therefore acrosome-reacted and/or deteriorated (c.f. patterns B-G of Fig. 1); these cells were excluded from further analyses. The asymmetric distribution of C6NBD-PS was determined for all cells that were 'negative' with respect to FL3 (i.e. the live acrosome-intact subpopulation, indicated with I, c.f. pattern A of Fig. 1), by comparing the C6NBD fluorescence before and after dithionite treatment (Gadella et al., 1999). The vertical line is used as a reference in the four dot-plots to show the loss of C6NBD-PS fluorescence in the 'I' subpopulation caused by dithionite and the increase in this loss that resulted from incubation in HBT-Bic as compared with incubation in HBT.

region) was also more intensely labeled (similarly to Fig. 1B), whereas when the acrosomal cap had been lost intense labeling was seen instead in the equatorial segment of the head (see Fig. 1C). Acrosome-reacted sperm cells were labeled in the acrosomal region with PNA-RPE. Reacted cells that were live (i.e. PI-negative) generally showed more-or-less uniform intense labeling over the anterior region of the acrosome (Fig. 1D) whereas deteriorated (PI-positive) cells tended to show intense labeling over the equatorial region of the acrosome (Fig. 1E). Sperm cells with damaged plasma membranes stained with PI as depicted in Fig. 1F and G.

Only the subpopulation of cells unstained by both PNA-RPE and PI, therefore both acrosome-intact and plasma membrane-intact was used for analyzing C6NBD-phospholipid

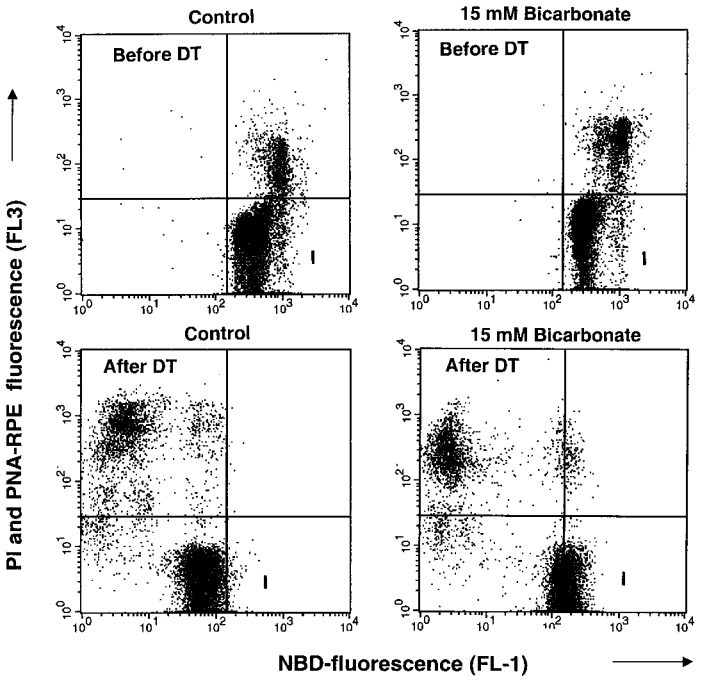

Fig. 3. Bicarbonate-induced changes in the asymmetric distribution of C6NBD-PC in boar spermatozoa: two-dimensional flow cytometric dot-plots. See Fig. 2 for a full explanation of the figure. Following 2 hours incubation in either HBT or HBT-Bic, spermatozoa were labeled with C6NBD-PC for 60 minutes. Note that incubation in HBT-Bic caused a reduction in the loss of C6NBD-PC fluorescence after dithionite treatment.

translocation and asymmetry; this subpopulation (illustrated in Fig. 1A) is indicated as I ('intact') in Figs 2 and 3.

Effect of bicarbonate on transbilayer movement of C6NBD-phospholipids

As compared with incubation in HBT, incubation in HBT containing 15 mM bicarbonate (HBT-Bic) altered considerably the inward movements of C6NBD-phospholipids (Figs 2-4). Inward translocation of C6NBD-PS was strongly inhibited by HBT-Bic, although after 60 minutes labeling steady-state distributions of C6NBD-PS had reached values comparable to those in control HBT incubations (Fig. 4A). Inward translocation of C6NBD-PE was also inhibited in HBT-Bic (Fig. 4B): only 50% of incorporated C6NBD-PE had translocated across the plasma membrane after 60 minutes in HBT-Bic as compared to 78% in HBT. In contrast, HBT-Bic stimulated the inward translocation of C6NBD-PC (Fig. 4C) and C6NBD-SM (Fig. 4D): in HBT-Bic, 30% C6NBD-PC and 20% C6NBD-SM had been translocated across the plasma membrane after 1 hour labeling whereas in HBT the respective values were only 15% and 5%.

The effect of bicarbonate on the outward movement of C6NBD-phospholipids was also monitored. As the experimental approach depended on the ability of BSA to extract C6NBD-lipids from the outer leaflet of the plasma membrane, inclusion of PMSF in the media to block metabolism of C6NBD-phospholipids (Gadella et al., 1999) was of particular importance so as to ensure that BSA extraction of NBD fluorescence represented only removal of

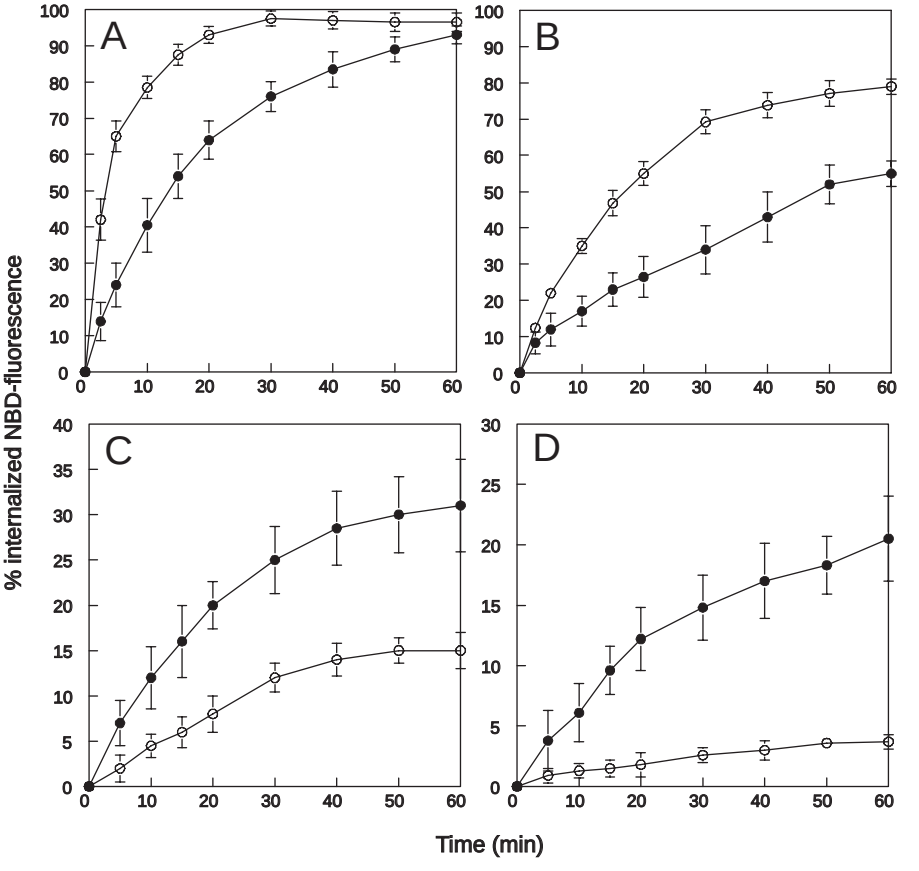

Fig. 4. Bicarbonate-induced changes in inward transbilayer movements of C6NBD-phospholipids in living, acrosome-intact boar spermatozoa. Spermatozoa were incubated for 2 hours either in HBT (open symbols) or in HBT-Bic (filled symbols), before labeling with C6NBD-phospholipids was initiated in the presence of PNA-RPE and PI. The fraction of label that had been internalized by the live acrosome-intact sperm subpopulation was estimated at intervals using flow cytometry in combination with dithionite treatment, as defined in Figs 1-3. Values are means ± s.d. from 8 independent experiments. (A) C6NBD-PS; (B) C6NBD-PE; (C) C6NBD-PC; (D) C6NBD-SM.

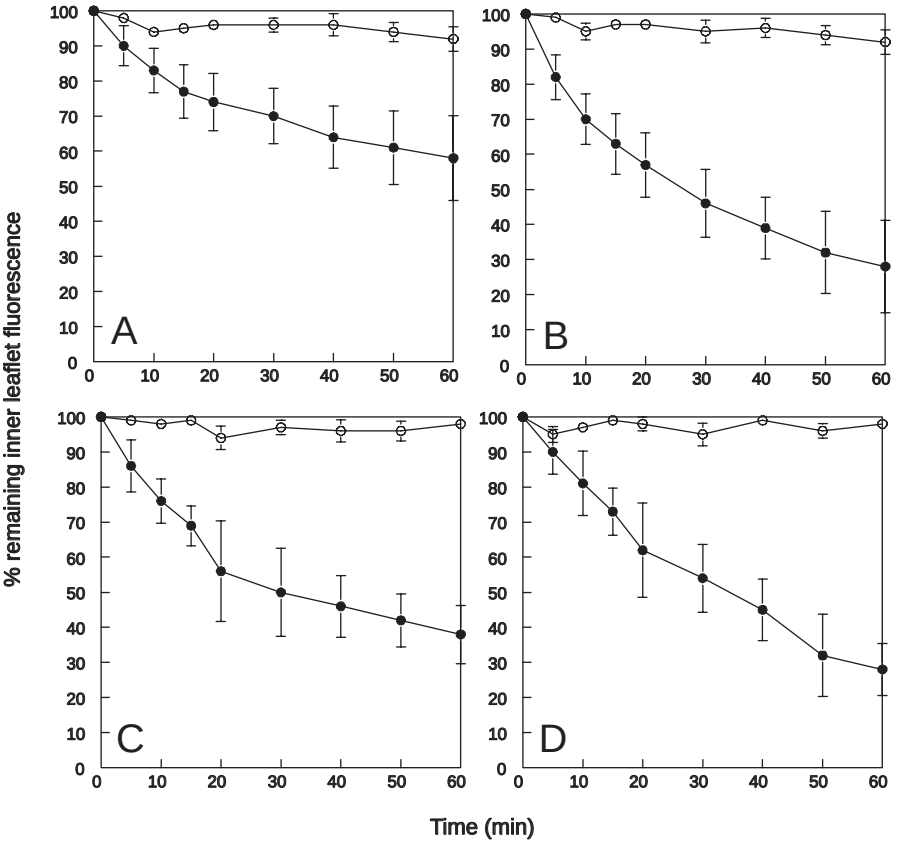

Fig. 5. Bicarbonate-induced changes in outward transbilayer movements of C6NBD-phospholipids in living, acrosome-intact boar spermatozoa. Spermatozoa were incubated for 2 hours either in HBT (open symbols) or in HBT-Bic (filled symbols). They were then labeled with the chosen C6NBD-phospholipid for 10 minutes in the presence of PNA-RPE and PI, after which the outer-leaflet fluorescence was destroyed by addition of dithionite. The cells were immediately washed through a Percoll® gradient, resuspended, and incubated with 5 mg/ml BSA in HBT or HBT-Bic as appropriate. To follow outward movements of the internalized phospholipid, at intervals samples of the sperm suspension were analyzed by flow cytometry in order to estimate the fraction of NBD fluorescence in the living acrosome-intact sperm subpopulation (as defined in Figs. 1-3) that resisted BSA back-extraction. For details, see Materials and Methods. (A) C6NBD-PS, (B) C6NBD-PE, (C) C6NBD-PC, (D) C6NBD-SM. Data, expressed as percentages of the original amount of internalized label, are means ± s.d. from 4 independent experiments.

intact C6NBD-phospholipids. (Preliminary experiments indicated that PMSF blocked C6NBD-phospholipid catabolism in HBT-Bic-treated cells in the same way as in control cells incubated in HBT.) Fig. 5 shows that during incubation in HBT (control conditions) very little outward movement of any of the C6NBD-phospholipids was detectable whereas, during incubation in HBT-Bic, considerable outward movement of all four C6NBD-phospholipid classes was observed.

Effect of bicarbonate concentration

To test the effect of bicarbonate concentration on transbilayer movement of C6NBD-phospholipids, spermatozoa were incubated in HBT, which had been supplemented with different dilutions of 0.3 M $NaHCO_3$ saturated with 100% CO_2. This stock bicarbonate solution contained the same proportion of CO_2 in relation to bicarbonate as our standard additive of 15 mM bicarbonate/5% CO_2, thus all such dilutions resulted in the same pH of 7.4 at 38°C (Harrison et al., 1996). During incubation, the CO_2 concentration in the gas phase was maintained at the correct level relative to the bicarbonate concentation, and pH values were routinely checked.

Bicarbonate-induced changes of inward translocation rates were found to be concentration-dependent (Fig. 6); little or no effect was seen below 2 mM while responses were beginning to maximize at 16 mM (the highest practical bicarbonate concentration that could be tested in this way).

Effect of calcium

Millimolar levels of external Ca^{2+} enhance greatly the lateral migration of a plasma membrane glycolipid component during in vitro capacitation (Gadella et al., 1994, 1995). However, addition of 2 mM $CaCl_2$ to HBS-Bic did not modify the inward translocation and distribution of C6NBD-phospholipids (data not presented). Forward- and side-scatter data from the flow cytometric analyses indicated that Ca^{2+} supplementation enhanced the sperm's tendency to agglutinate in HBT-Bic (see Harrison et al., 1996); moreover, in back-exchange experiments (i.e. in the presence of BSA as well as bicarbonate), added Ca^{2+} caused significant cell deterioration.

Role of cAMP-dependent protein phosphorylation in the control of asymmetric distribution of C6NBD-phospholipids

In spermatozoa of boar and other species, bicarbonate stimulates adenylyl cyclase to raise intracellular levels of cAMP; this latter acts as an important second messenger to enhance sperm fertilizing functions (Okamura et al., 1985; Tajima et al., 1987; Visconti et al., 1995b). Cyclic AMP's second messenger role is generally exerted through its activation of protein kinase A (PKA) to phosphorylate specific cellular proteins and thereby alter their molecular function. Accordingly, we investigated the effect of various modulators of cAMP-dependent phosphorylation on C6NBD-phospholipid translocation and distribution (Table 1). Incubation of sperm cells in HBT with cyclic-nucleotide phosphodiesterase (PDE) inhibitors (caffeine, theophylline, IBMX or papaverine) resulted in changes in C6NBD-phospholipid behavior similar to those observed for HBT-Bic treated cells. The concentrations of PDE inhibitors used (500 μM for caffeine,

Table 1. Modulation of transbilayer distribution of C6NBD-phospholipids and merocyanine staining in boar spermatozoa by signalling effectors

Treatment	Final concn (μM)	C6NBD-PS*	C6NBD-PE*	C6NBD-PC‡	C6NBD-SM‡	MC540§
HBT		78.8±2.5	40.2±1.9	12.7±1.5	5.5±0.7	3.4±1.4
+Caffeine	500	49.3±7.2	21.7±4.3	29.4±9.6	15.4±4.9	25.4±5.4
Theophylline	500	45.6±6.9	18.0±4.1	32.6±7.3	16.3±3.6	21.6±3.2
IBMX	500	40.8±5.4	16.9±3.8	37.5±4.6	21.7±3.1	35.2±6.8
Papaverine	100	37.2±3.8	14.8±2.9	38.1±4.1	22.6±2.0	49.0±8.5
Bt_2-cAMP	100	47.3±7.9	18.8±4.6	33.5±5.0	18.3±4.3	32.7±5.3
8Br-cAMP	100	46.7±8.2	16.4±5.2	30.7±5.4	15.6±3.9	38.7±3.9
cpt-cAMP	100	49.5±8.6	21.3±5.0	36.4±5.3	20.0±4.0	29.8±4.1
8Br-cGMP	100	79.9±2.8	42.0±2.1	14.8±1.7	5.3±0.5	5.3±2.0
Forskolin¶	10	77.9±2.4	41.3±1.9	17.6±2.6	7.2±0.8	5.5±1.9
Forskolin¶	100	67.3±4.6	34.6±5.0	18.9±3.4	8.0±0.9	14.1±3.8
Okadaic acid¶	0.1	47.5±5.8	19.7±4.9	32.8±5.3	27.1±4.6	45.5±9.1
Calyculin¶	0.1	45.2±7.2	17.6±4.1	28.7±5.2	20.1±5.3	38.2±7.4
HBT-Bic		41.3±5.8	18.7±4.1	32.0±5.7	20.5±4.5	35.2±7.2
+H89	50	80.1±3.4	43.1±4.6	12.7±2.3	5.0±1.1	5.4±1.7
Rp-cAMPS	1000	81.2±4.3	40.9±3.8	13.2±2.0	4.7±0.6	4.9±1.2

Spermatozoa were incubated at 38°C for 2 h in either HBT or HBT-Bic containing the effectors indicated, after which C6NBD-phospholipid labelling was initiated. Inward translocation of the phospholipids was determined in the live acrosome-intact sub-population as indicated in Materials and Methods and Fig. 4. In parallel, spermatozoa were incubated in the same media for 30 minutes before merocyanine staining was estimated in the live acrosome-intact subpopulation (see Fig. 8). Values are means ± s.d. from 8 independent experiments.

*Percent of total C6NBD-PS and C6NBD-PE label internalized after 10 minutes labelling.

‡Percent of total C6NBD-PC and C6NBD-SM label internalized after 60 minutes labelling.

§Percent highly merocyanine-staining cells.

¶In combination with 50 μM IBMX. This concentration of IBMX in HBT had no effect on its own (data not presented).

theophylline and IBMX and 100 μM for papaverine) caused cellular cAMP levels to rise from 7.3±2.1 to 20.1±4.7 pmol/100 million cells (n=8), increases comparable to those resulting from bicarbonate stimulation (Harrison, 1997; Harrison and Miller, 2000). Changes in C6NBD-phospholipid behavior were likewise seen when HBT was supplemented with Bt_2-cAMP, 8Br-cAMP or cpt-cAMP. The latter was particularly potent (as might be expected from its higher membrane permeability and resistance to PDE destruction: see Sandberg et al., 1992). Effects appeared specific for cAMP analogs because 8Br-cGMP did not induce changes.

Incubation of sperm in the presence of forskolin, an established activator of somatic cell adenylyl cyclase (Seamon et al., 1981), resulted in only relatively minor changes in transbilayer behavior of the C6NBD-phospholipids (see Table 1), even in the presence of low levels of the PDE inhibitor IBMX (50 μM). However, this result was not unexpected since numerous studies have indicated that forskolin has only a minor ability to activate sperm adenylyl cyclase (see Harrison and Miller, 2000, and refs therein).

Mechanisms enhanced by protein phosphorylation are controled simultaneously by protein dephosphorylation as a means of maintaining a balanced level of the active phosphorylated form of the protein target. We were able to induce changes in C6NBD-phospholipid behavior similar to those induced by bicarbonate when we included in the HBT

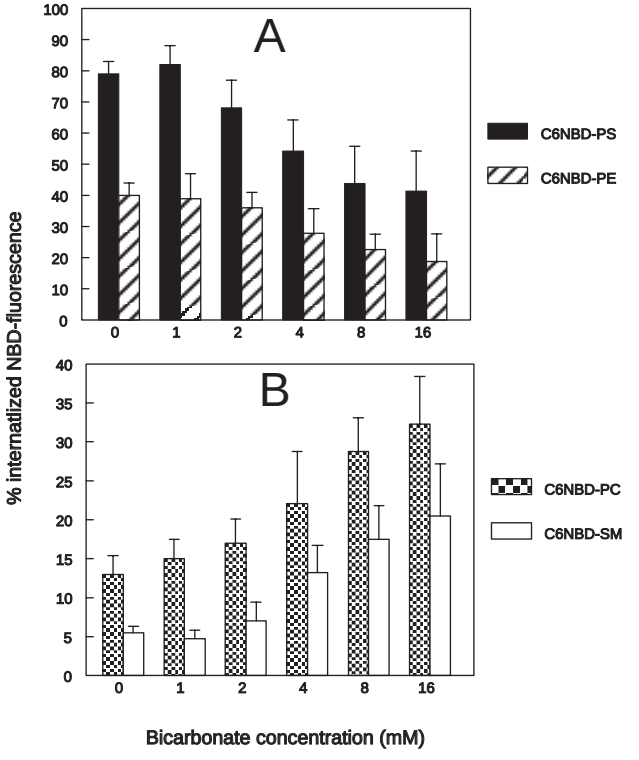

Fig. 6. Concentration dependency of bicarbonate-induced alterations in inward transbilayer movements of C6NBD-phospholipids. Spermatozoa were incubated for 2 hours in HBT supplemented with various concentrations of bicarbonate/CO_2 (achieved by adding suitable quantities of 0.3 M $NaHCO_3$ that had been saturated with 100% CO_2: see Results section). C6NBD-phospholipid-bearing donor vesicles were then added, and the inward movement of C6NBD-phospholipids was followed by flow cytometric analysis of the live acrosome-intact subpopulation (c.f. Figs 2-4). During all incubations, CO_2 concentration in the gas phase was maintained proportionate to the final bicarbonate concentration. (A) Amounts of translocated C6NBD-PS and C6NBD-PE were determined after 10 minutes labeling. (B) Amounts of translocated C6NBD-PC and C6NBD-SM were determined after 60 minutes labeling. Values are means ± s.d. from 4 independent experiments.

Table 2. Enhancement of C6NBD-phospholipid catabolism in boar spermatozoa by signalling effectors

Treatment	Final concn (μM)	C6NBD-metabolite:	C6NBD-PS		C6NBD-PE		C6NBD-PC		C6NBD-SM
			FA	DAG	FA	DAG	FA	DAG	Cer
HBT			(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)
+Caffeine	500		1.2	1.9	1.1	1.6	1.4	2.7	3.8
IBMX	500		1.8	2.7	1.5	2.1	2.1	4.2	6.3
Bt_2-cAMP	100		1.3	2.1	1.2	1.7	1.8	2.8	4.5
cpt-cAMP	100		1.7	2.0	1.4	1.6	1.9	2.7	4.9
8Br-cGMP	100		0.9	1.2	1.0	1.1	1.0	1.1	1.2
Forskolin*	10		1.1	1.1	1.0	1.1	1.1	1.2	1.4
Forskolin*	100		1.1	1.2	1.0	1.3	1.2	1.4	1.7
Okadaic acid*	0.1		2.0	3.1	1.9	2.7	2.5	4.0	9.7
Calyculin*	0.1		2.1	2.6	1.8	2.4	2.2	3.6	7.1
HBT-Bic			1.6	2.4	1.4	1.9	2.0	3.3	5.3
+H89	50		0.9	1.0	1.0	1.0	1.0	1.0	1.0
Rp-cAMPS	1000		0.9	1.2	1.0	1.1	1.0	1.0	1.2

Spermatozoa were labelled with the indicated C6NBD-phospholipid for 10 minutes, after which they were washed through Percoll® to remove unincorporated label. The cells were then further incubated in either HBT or HBT-Bic containing 2 mM $CaCl_2$ as well as the effectors indicated; PMSF was absent. After 1 hour, lipid extracts were prepared and subjected to HPTLC. The individual fluorescent spots of the C6NBD-metabolites (C6NBD-FA, C6NBD-DAG and C6NBD-Cer) as well as the parent phospholipids (see Fig. 7) were scraped off and the amounts of C6NBD fluorescence quantified. Data are expressed as the amount of specified metabolites relative to the control values (i.e. after similar incubation in HBT alone), and are means from three independent experiments.

*In combination with 50 μM IBMX. This concentration of IBMX in HBT had no effect on its own (data not presented).

incubating medium either of the protein phosphatase inhibitors okadaic acid or calyculin, provided a low level (50 μM) of IBMX was also included (Table 1). (This latter concentration of IBMX was unable on its own to exert any effect on C6NBD-phospholipid behavior: data not presented.)

The above results suggested that bicarbonate was exerting its effect on C6NBD-phospholipid behavior by increasing intracellular levels of cAMP and thereby stimulating PKA to raise protein phosphorylation states. To test this hypothesis further, we incubated sperm in HBT-Bic in the presence of PKA inhibitors H89 (Chijiwa et al., 1990) or Rp-cAMPS (De Wit et al., 1984); both compounds indeed blocked the effect of bicarbonate (Table 1).

Bicarbonate enhancement of C6NBD-phospholipid catabolism

In the experiments described above, PMSF was included in all media to prevent digestion of C6NBD-phospholipids by endogenous phospholipases, and thus ensure that changes in NBD location were true reflections of changes in the location of the intact phospholipids (Gadella et al., 1999). When PMSF was omitted from the incubation media, however, it was possible to observe the effect of bicarbonate and other signaling modulators on the catabolism of sperm-incorporated C6NBD-phospholipids. Sperm were labeled with individual C6NBD-phospholipids for 10 minutes and then incubated for 1 hour in various modifications of PMSF-free HBT and HBT-Bic (Fig. 7; Table 2). Catabolism of all 4 classes of C6NBD-phospholipid was increased in the presence of bicarbonate (Fig. 7). Formation of C6NBD-FA and C6NBD-DAG probably reflected the activities of phospholipases A_2 and C respectively, whereas formation of C6NBD-Cer was likely due to sphingomyelinase activity. Bicarbonate treatment resulted in mild stimulations (1.4- to 3.3-fold) of phospholipase A_2 and C (most pronounced effects were found for C6NBD-PC: see Table 2), whereas stimulation of sphingomyelinase was markedly higher (5.5-fold: see Table 2). Cyclic AMP analogs, PDE inhibitors, and protein phosphatase inhibitors stimulated C6NBD-phospholipid catabolism similarly

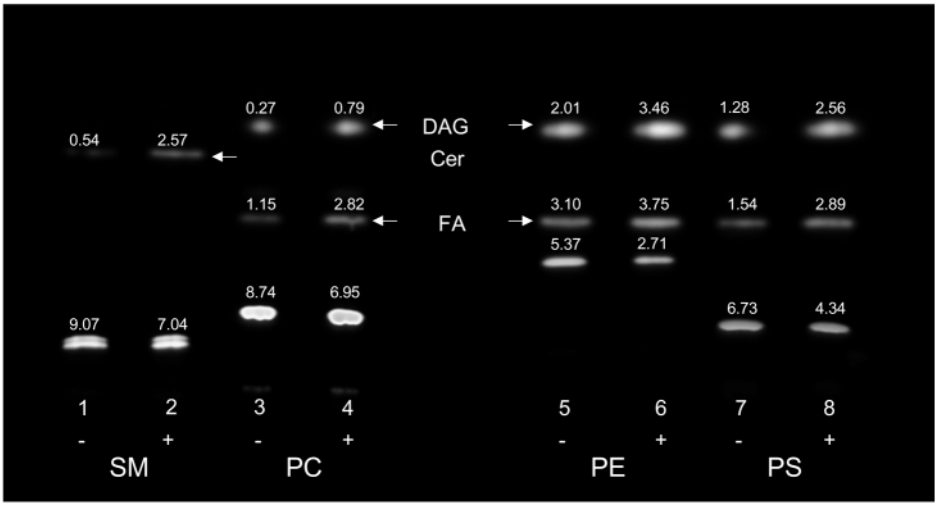

Fig. 7. Effect of bicarbonate on C6NBD-phospholipid catabolism in boar spermatozoa. Spermatozoa in HBT in the absence of PMSF were labeled with different C6NBD-phospholipids for 10 minutes (without any preincubation), after which time the non-incorporated label was removed by sedimenting the spermatozoa through Percoll®. The labeled cells were then incubated in either HBT (lanes 1, 3, 5 and 7) or in HBT-Bic (lanes 2, 4, 6 and 8); both media were supplemented with 2 mM $CaCl_2$ but neither contained PMSF. After 1 hour, the sperm lipids were extracted and separated by HPTLC. Sperm labeled with: C6NBD-SM, lanes 1 and 2; C6NBD-PC, lanes 3 and 4; C6NBD-PE, lanes 5 and 6; C6NBD-PS lanes 7 and 8. Arrows indicate the running position of reference lipids C6NBD-FA (FA), C6NBD-Cer (Cer) and C6NBD-DAG (DAG). The figure associated with each spot indicates its absolute molar content of C6NBD; 10 nmole of C6NBD-lipid were applied to each lane.

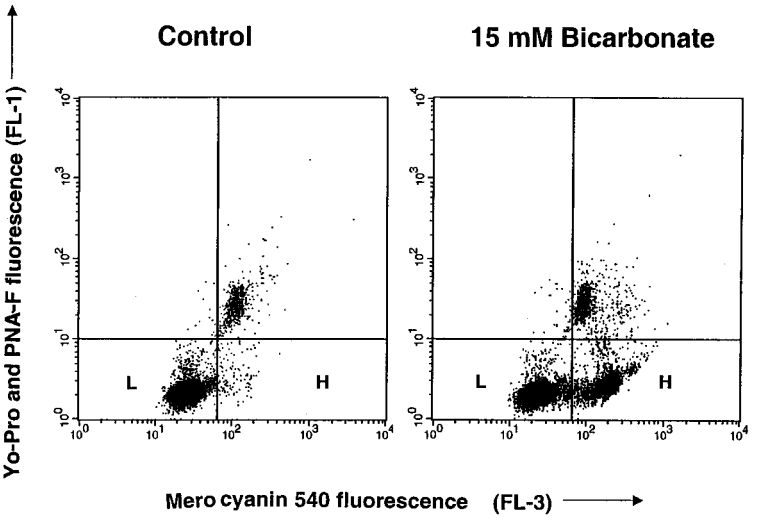

Fig. 8. Bicarbonate-induced changes in merocyanine fluorescence intensity of boar spermatozoa. Spermatozoa were incubated for 30 minutes in HBT (A) or HBT-Bic (B) in the presence of Yo-Pro 1 and PNA-F. Samples were then mixed briefly with merocyanine before being analyzed by flow cytometry (details of methodology given in Materials and Methods). The horizontal line in each panel indicates the 'cut-off' value of FL1 above which cells were considered 'positive' for PNA-F and/or Yo-Pro fluorescence and were therefore acrosome-reacted and/or deteriorated (c.f. patterns B-G of Fig. 1); these cells were excluded from further analyses. The merocyanine fluorescence (FL3) was determined for all spermatozoa that were 'negative' with respect to FL1 (i.e. the live acrosome-intact subpopulation). The subpopulation of cells with low merocyanine fluorescence is indicated with L while the subpopulation of sperm cells with high merocyanine fluorescence is indicated with H.

to bicarbonate (Table 2). Their catabolic effects correlated approximately with their effects on C6NBD-phospholipid distribution; notably forskolin and the cGMP analog had little or no effect. On the contrary, PKA inhibitors that blocked HBT-Bic effects on C6NBD-phospholipid asymmetry behavior (Table 1) also blocked the stimulatory effect of HBT-Bic on phospholipases (Table 2). In the presence of PMSF, HBT-Bic incubation resulted in the production of only trace amounts of C6NBD-labeled catabolites (<5% of the amounts detected in the absence of PMSF: data not presented). It should be noted that all the observations on C6NBD-phospholipid metabolism were done on complete sperm suspensions. One could argue that the observed changes were due to altered metabolism in deteriorating cells or acrosome-reacting cells (intracellular labeling in presence of non-surface phospholipases). However, instead of increased phospholipase activities dramatic decreases of C6NBD-phospholipid breakdown was observed in Triton X-100 permeabilized cell samples (data not shown). This indicates that we observed activation of phospholipases in capacitating cells rather than the activity of intracellular phospholipases in deteriorating cells.

Relationship between changes in transbilayer movement of C6NBD-phospholipids and merocyanine-detectable changes in membrane lipid disordering

The lipophilic fluorescent dye merocyanine 540 is able to detect plasma membrane changes that have been interpreted as increases in lipid disorder (e.g. McEvoy et al., 1988; Aussel et al., 1993; Mower et al., 1994). Recent studies (Harrison et al., 1996; Harrison and Miller, 2000) have shown that both bicarbonate and signaling modulators related to PKA-driven protein phosphorylation enhance markedly the merocyanine fluorescence intensity of boar spermatozoa. We therefore tested whether the bicarbonate-induced changes in transbilayer behavior of C6NBD-phospholipids corresponded with changes in merocyanine fluorescence intensity. Bicarbonate induced an increase in the live acrosome-intact subpopulation showing high merocyanine staining (Fig. 8); the change was essentially dependent on bicarbonate but not on BSA or calcium (Fig. 9),

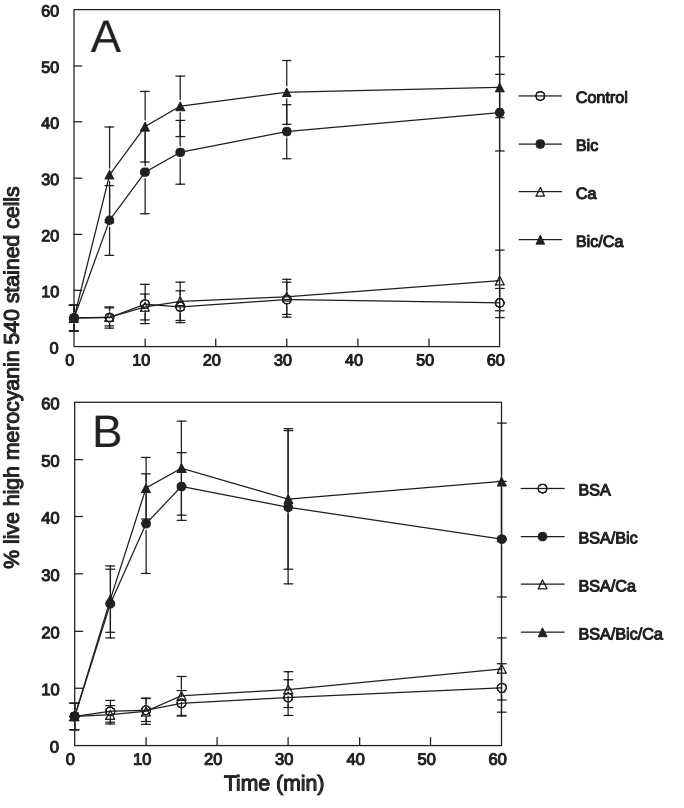

Fig. 9. Effect of bicarbonate, Ca^{2+} and albumin on merocyanine fluorescence intensity of live acrosome-intact boar spermatozoa. Spermatozoa were added to variants of HBT at 38°C; all media contained PNA-F and Yo-Pro 1 to discriminate between live acrosome-intact cells and deteriorated and/or acrosome-reacted cells. At intervals, samples from the suspensions were mixed briefly with merocyanine and subjected to flow cytometric analysis to estimate the proportion of high merocyanine-staining cells in the live acrosome-intact subpopulation (see Fig. 8 for definition). (A) Data from spermatozoa incubated in media without BSA; (B) data from sperm incubated in media containing 3 mg/ml BSA. Control, HBT alone; Ca, HBT supplemented with 2 mM $CaCl_2$; Bic, HBT containing 15 mM bicarbonate/5% CO_2; Bic/Ca, HBT containing 15 mM bicarbonate/5% CO_2 and supplemented with 2 mM $CaCl_2$. Values are means ± s.d. from 5 independent experiments.

although some additive effects of BSA and/or calcium could be detected. These results, closely comparable to the findings of Harrison et al. (1996), demonstrated that merocyanine-detectable changes took place in live acrosome-intact cells in the absence of BSA. (Most of the studies of Harrison and colleagues were performed in the presence of BSA, whereas BSA was necessarily omitted from the present experiments because of its ability to bind C6NBD-phospholipids and thus interfere with our investigations.)

The effects of modulators of the cAMP/PKA signaling system on the merocyanine fluorescence intensity of sperm cells were then compared with their effects on C6NBD-phospholipid behavior (Table 1). In essence, changes in transbilayer behavior of C6NBD-phospholipids were mirrored closely by increases in merocyanine fluorescence intensity. Activators of the adenylyl cyclase/cAMP/PKA pathway induced the generation of a subpopulation of highly stained cells, whereas treatments designed to block PKA activity also blocked induction of high merocyanine fluorescence intensity (Table 1). While confirming more extensive investigations of the involvement of PKA in the control of merocyanine fluorescence intensity (Harrison and Miller, 2000), these findings, crucially, linked the parameter closely with changes in phospholipid transbilayer behavior.

DISCUSSION

Bicarbonate plays a major role in the induction of sperm capacitation in vitro (Boatman and Robbins 1991; Suzuki et al., 1994; Shi and Roldan, 1995; Visconti et al., 1995a,b). In the pig, bicarbonate brings about modifications in sperm surface coating (Ashworth et al., 1995), an increase in plasma membrane lipid disorder (Harrison et al., 1996), and an increase in the ability of live acrosome-intact cells to bind zona pellucida components (Harkema et al., 1998). Of particular interest has been the bicarbonate-inducible increase in lipid disorder (detected as an increase in merocyanine staining). In other cell types, an increase in merocyanine stainability has been interpreted as indicating a change in the transbilayer distribution of the plasma membrane phospholipids (Verhoven et al., 1995; Williamson and Schlegel, 1994). Our present results show clearly that bicarbonate does indeed induce in boar spermatozoa important changes in membrane phospholipid distribution.

Although the use of C6NBD-labeled phospholipid probes in combination with flow cytometry is an excellent way to study transbilayer phospholipid behavior in living cells, it also presents a number of potential pitfalls that could invalidate conclusions. In an earlier publication, we presented protocols specifically designed to obviate these pitfalls (Gadella et al., 1999). In the present study, we have added further refinements to our procedure in order to take account of subsidiary capacitation-induced changes in sperm that would interfere with assessment of C6NBD-phospholipid distribution. It is well documented that in vitro capacitation of sperm cells not only leads to their activation but also causes increased occurrence of 'spontaneous' acrosome reactions, as well as increased cell death (see Harrison et al., 1993; Yanagimachi, 1994). Spontaneous exocytosis of the acrosome would expose to the external milieu relatively large areas of previously sequestered membrane and thereby allow increased labeling with C6NBD-labeled lipids; these lipids would also be accessible to dithionite NBD-reducing action. Such resultant increased labeling and relative dithionite sensitivity would lead to overestimation of 'outer leaflet' phospholipid distribution. Thus we added fluorochrome-conjugated PNA to the sperm suspensions prior to flow cytometry in order to detect sperm with discharged or disrupted acrosomes and eliminate them from the data set (see Figs 1-3). PNA is a very sensitive probe of acrosomal integrity. It has affinity only for glycosidic residues on the luminal face of the outer acrosomal membrane (Flesch et al., 1998) and in unfixed sperm suspensions only labels sperm sites if the luminal face of the acrosomal membrane has become exposed to the exterior (e.g. Ashworth et al., 1995). Such exposure will begin as soon as fusion pores form between the plasma membrane and the outer acrosomal membrane. Therefore, in fact this assay is detecting the earliest signs of the initiation of acrosome reaction (or deterioration). We noted during preliminary multiple labeling experiments that, if capacitating sperm suspensions were cooled down from 38°C even to about 30°C, they suffered a significant increase in cell deterioration and spontaneous acrosome reactions. All analyses were therefore carried out in a flow cytometer that had been thoroughly equilibrated to 38°C. As a result of these extra precautions, we believe our data to be truly representative of the behavior of C6NBD-phospholipids in living acrosome-intact spermatozoa.

Compared with a control (bicarbonate-free) environment, bicarbonate/CO_2 affected the four classes of phospholipids in differing ways. While not appearing to alter greatly the eventual steady-state distribution of the aminophospholipids C6NBD-PE and C6NBD-PS, incubation in the presence of bicarbonate markedly slowed down their inward movements (Fig. 4) and speeded up their outward movements (Fig. 5). Almost complete translocation of C6ND-PS to the inner leaflet of the sperm plasma membrane was achieved within 20 minutes in the absence of bicarbonate, whereas in its presence at least 60 minutes was required. The steady-state distribution of C6NBD-PE, largely located in the inner membrane leaflet, was achieved after some 60 minutes labeling in the absence of bicarbonate whereas in the presence of bicarbonate steady-state distribution was far from complete at this time (Fig. 4). By contrast, prolonged incubation of sperm cells in the presence of bicarbonate led to an increase of the proportions of C6NBD-PC and C6NBD-SM in the inner plasma membrane leaflet (30% versus 15% for C6NBD-PC and 20% versus 5% for C6NBD-SM: Fig. 4). At the same time, bicarbonate accelerated the outward translocation kinetics of all four phospholipid classes (Fig. 5). Thus slow-down of net inward movement of aminophospholipids but speed-up of their outward movement was associated with increased inward and outward translocation of the choline phospholipids. The simplest interpretation of our findings (but not the only one) is that bicarbonate in some way activates a non-specific bidirectional phospholipid translocase (i.e. the so-called 'scramblase'). Others have reported that scramblase activation is Ca^{2+}-dependent (see Bevers et al., 1998). In our experiments addition of extracellular Ca^{2+} did not affect the NBD-phospholipid transbilayer movements; however, media such as HBT contain 10-50 μM residual Ca^{2+} (Yanagimachi, 1982; Fraser, 1987) even without Ca^{2+} supplement, levels that are

more than 200-fold higher than reported resting intracellular levels (Florman et al., 1999).

Several studies have deduced that bicarbonate appears to act on sperm principally through its ability to stimulate directly a sperm-specific adenylyl cyclase (Okamura et al., 1985; Garty and Salomon, 1987), whence the resultant increased intracellular cAMP activates PKA to initiate protein phosphorylation events in one or more as yet undefined signaling cascades (Visconti and Kopf, 1998). Recent investigations in one of our laboratories have shown that bicarbonate induces a merocyanine-detectable change in boar sperm plasma membrane lipid architecture via a cAMP-dependent protein phosphorylation pathway (Harrison, 1997; Harrison and Miller, 2000). The experiments of the present paper (Table 1) indicated that a similar pathway is involved in bicarbonate's induction of changes in C6NBD-phospholipids transbilayer behavior. (i) Either cAMP analogues or PDE inhibitors could replace bicarbonate in causing the changes (PDE inhibitors block on-going catabolism of endogenous nucleotides whence endogenous cAMP levels rise); the cyclic nucleotide requirement appeared specific for cAMP because 8Br-cAMP was a competent inducer of the transbilayer re-organization whereas 8Br-cGMP had no effect. (ii) Bicarbonate induction was abolished by inclusion of the PKA inhibitors H89 (Chijiwa et al., 1990) or Rp-cAMPS (De Wit et al., 1984). (iii) Changes in transbilayer phospholipid behavior could be induced by the protein phosphatase inhibitors okadaic acid or calyculin, in accord with the hypothesis that decreasing on-going protein dephosphorylation processes would enhance net levels of a putative active phosphorylated protein form.

It is well established that increased merocyanine stainability is positively related to membrane lipid packing disorder (Williamson et al., 1983; Langner and Hui, 1993). However, although increases in merocyanine staining of living cells have been shown to accompany changes in the cells' plasma membrane phospholipid distribution (see Williamson and Schlegel, 1994), a causative link has not been satisfactorily explained. In the present experiments, we assessed in parallel C6NBD-phospholipid asymmetry and merocyanine stainability in boar sperm suspensions incubated with a variety of effectors (Table 1). We detected increases in merocyanine stainability in all conditions leading to alterations in C6NBD-phospholipid distribution, while staining changes were not observed in situations in which changes in phospholipid distribution were not significant. As reported by Harrison and Miller (2000), merocyanine stainability was apparently controled by a cAMP-dependent protein phosphorylation signaling system, which in the present experiments was essentially indistinguishable from that controling phospholipid asymmetry. However, the merocyanine stainability changes show two features not seen in the changes in phospholipid asymmetry. Firstly, merocyanine always detects two clearly different populations of live cells, those that are stained weakly and those that are stained strongly; bicarbonate induces a change from a weakly staining state to a strongly staining state, and the concentration-dependent response essentially involves a shift in population from one state to the other: few cells of intermediate classification are observed (Fig. 8; see also Harrison et al., 1996). Flow cytometric assessments of C6NBD-phospholipid behavior, on the contrary, never revealed similar separable subpopulations of live cells. Secondly, the speed with which bicarbonate induces the changes differs between the two assays. Changes in merocyanine-binding ability were essentially complete in less than 15 minutes (Fig. 9; see also Harrison et al., 1996), whereas changes in phospholipid distribution clearly took much longer (Figs 4, 5). The following offers a possible explanation of these differences and relating of the two parameters. The molecular fluorescence intensity shown by merocyanine is dependent on its environment; translocation from a hydrophilic to a hydrophobic environment results in dramatically increased intensity. If the fatty acyl chains of a lipid leaflet are closely packed, the dye is prevented from intercalating into the leaflet's hydrophobic core; a decrease in the packing order, however, will allow such intercalation. Thus merocyanine staining of a cell whose plasma membrane outer leaflet lipid is highly ordered will be low, whereas staining of a cell with less-ordered outer leaflet lipid will be high (Smith et al., 1984; Allan et al., 1989; Verkman, 1987; Lagerberg et al., 1995). Because of merocyanine's intrinsic properties, a small decrease in membrane lipid packing sufficient to allow entry of the merocyanine molecule into the hydrophobic leaflet core will result in a large change in fluorescence. Thus merocyanine staining of cell suspensions will tend to reveal two populations, cells whose membrane lipid packing is too high for significant intercalation, and cells whose lipid packing has fallen below a 'threshold' at which intercalation becomes energetically favorable. The C6NBD-phospholipid asymmetry assay, on the contrary, will indicate changes in phospholipid distribution within a 'linear' continuum. If bicarbonate activates the scramblase as our results appear to indicate, the physical increase in phospholipid interchange between the two leaflets may of itself cause packing disorder and thus allow merocyanine intercalation; the change in scramblase activity (and putative concomitant increase in merocyanine staining) would necessarily precede detectable net changes in phospholipid distribution.

In a previous paper, we demonstrated the distorting effects of catabolism of C6NBD-phospholipids on the asymmetry assay (Gadella et al., 1999). Briefly, endogenous sperm phospholipases break down C6NBD-phospholipids to products that are still fluorescent but do not share the membrane transbilayer behavior of their parent C6NBD-phospholipids. In our previous study, we showed that inclusion of PMSF in the incubation systems was sufficient to inhibit the catabolic phospholipases. In the present study, while we were able to show that PMSF remained an effective phospholipase inhibitor in the presence of bicarbonate, we observed that, in the absence of PMSF, bicarbonate induced increased phospholipase catabolism of the C6NBD-phospholipids (Fig. 7; Table 2). Activation of phospholipase A_2 (production of C6NBD-FA) was relatively mild, activation of phospholipase C (production of C6NBD-DAG) was greater (especially in C6NBD-PC labeled cells), while activation of sphingomyelinase (production of C6NBD-Cer) was considerable (some five-fold). Not only do these findings demonstrate the importance of preventing endogenous phospholipid catabolism in studies of phospholipid behavior in spermatozoa, they are also of considerable physiological significance. Roldan and colleagues (Roldan and Murase, 1994; Roldan and Vazquez, 1996) have already emphasized the importance of phospholipid metabolites in the induction of the acrosome reaction and have detected

bicarbonate activation of phospholipase A_2 in boar sperm; moreover, Ruiz-Arguello et al. (1998) have reported that sphingomyelinase and phospholipase C, when acting in concert, induce fusion in artificial vesicles of mixed SM, PC, PE and cholesterol composition. Our evidence for a broad enhancement of phospholipase activity by bicarbonate is therefore fully in line with current concepts of capacitative processes. It suggests that increased provision of fusogenic phospholipid catabolites in the sperm plasma membrane render the sperm more responsive to physiological inducers of acrosomal exocytosis. The reason for the increase in phospholipase activity is unclear. Recent studies in other cell types have shown that cellular phospholipases activities can be modulated by direct phosphorylation of the enzyme molecules (Palmier et al., 1996; Gijon et al., 1999; Houle and Bourgoin, 1999). However, all types of phospholipase activity responded to signaling activators and inhibitors essentially in parallel with each other as well as with the changes in phospholipid distribution (compare Tables 1 and 2). This close parallelism is not easy to reconcile with individual phosphorylation control of each of the phospholipases types. It seems more likely that the observed general increases in phospholipase activity are due to increased accessibility of the enzymes to their substrates brought about by scramblase activation: continual redistribution and presentation of the various phospholipid species (e.g. increased levels of SM and PC in inner leaflet), also perhaps by the increase in lipid packing disorder (to allow attack of bonds previously protected by close physical packing of the lipid molecules). Scrambling of phospholipids (Bevers et al., 1998) and ceramide formation (Tepper et al., 1999) are general characteristics of the apoptotic execution phase. Moreover, C6NBD-Cer formation in apoptotic Jurkat cells was shown to be the result of C6NBD-SM scrambling to the inner leaflet during CD95-induced apoptosis where it becomes accessible for a neutral sphingomyelinase (A. D. Tepper, personal communication). Therefore, the observed changes in sperm cells seem to parallel surface phenomena in apoptotic cells.

The bicarbonate-induced alterations in transbilayer behavior of C6NBD-phospholipids, increases in lipid packing disorder and increases in phospholipase catabolism appear to us highly relevant to sperm capacitation. (i) Partial loss of phospholipid asymmetry, already associated with exocytotic events in secretory cells (Müller et al., 1996), may of itself increase the fusibility of the sperm plasma membrane in readiness for the two major fusion events in which the spermatozoon participates at fertilization (i.e. exocytosis of the acrosome and fusion with the egg itself). (ii) A decline in phospholipid asymmetry is also associated with increased cell-cell adhesion (Schlegel et al., 1985), and may therefore assist sperm binding to epithelial cells of the oviduct or to the zona pellucida prior to the onset of the acrosome reaction (Harkema et al., 1998). (iii) Increased phospholipase activity may provide fusogenic lipids to enhance exocytotic response to physiological inducers of the acrosome reaction (see Roldan and Murase, 1994; Roldan and Vazquez, 1996). In addition to bicarbonate, BSA and Ca^{2+} are also implicated in in vitro capacitation (Yanagimachi, 1994). Although the bicarbonate-induced changes described in the present paper are not dependent on these latter components, the overall capacitation process is lengthy and undoubtedly comprises a sequence of events. The alterations in phospholipid behavior take place relatively rapidly after commencement of incubation under capacitating conditions. They can therefore be viewed as representing early steps in the capacitation process, whereas both BSA and Ca^{2+} appear to be involved largely in later events (Neill and Olds-Clarke, 1987; Andrews and Bavister, 1989; Fraser, 1993). We believe that the changes in phospholipid behavior and the increase in lipid packing disorder are permissive processes. For example, several studies have indicated that cholesterol removal from the plasma membrane is an important factor in capacitation: one role of BSA may thus be to act as an in vitro acceptor of cholesterol (Cross, 1998). From these observations, one may speculate that the bicarbonate-induced changes described in this paper provide a lipid environment in the plasma membrane bilayer that is permissive for albumin-mediated cholesterol efflux. In its turn, the cholesterol efflux may allow further modifications of the membrane surface such as lateral redistributions of glycolipids (Gadella et al., 1994, 1995) and integral membrane proteins (Aguas and Pinto da Silva, 1989, Suzuki and Yanagimachi, 1989), and the unmasking of surface lectin-binding epitopes (Ashworth et al., 1995).

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REFERENCES

Aguas, A. P. and Pinto da Silva, P. P. (1989) Bimodal redistribution of surface transmembrane glycoproteins during Ca^{2+}-dependent secretion (acrosome reaction) in boar spermatozoa. *J. Cell Sci.* **93,** 467-479.

Allan, D., Hagelberg, C., Kallen K. -J. and Haest, C. W. M. (1989) Echinocytosis and microvesiculation of human erythrocytes induced by insertion of merocyanine 540 into the outer membrane leaflet. *Biochim. Biophys. Acta* **986,** 115-122.

Andrews, J. C. and Bavister, B. D. (1989) Hamster zonae pellucidae cannot induce physiological acrosome reactions in chemically capacitated hamster spermatozoa in the absence of albumin. *Biol. Reprod.* **41,** 117-122.

Ashworth, P. J. C., Harrison, R. A. P., Miller, N. G. A., Plummer, J. M. and Watson, P. F. (1995) Flow cytometric detection of bicarbonate-induced changes in lectin binding in boar and ram sperm populations. *Mol. Reprod. Dev.* **40,** 164-176.

Aussel, C., Bernard, G., Breittmayer, J. P., Pelassy, C., Zoccola, D. and Bernard, A. (1993) Monoclonal antibodies directed against the E2 protein (MIC2 gene product) induce exposure of phosphatidylserine at the thymocyte cell surface. *Biochemistry* **32,** 10096-10101.

Bailey, A. L. and Cullis, P. R. (1994) Modulation of membrane fusion by asymmetric transbilayer distributions of amino lipids. *Biochemistry* **33,** 12573-12580.

Bevers, E. M., Comfurius, P., Dekkers D. W. C., Harmsma, M. and Zwaal R. F. A. (1998) Transmembrane phospholipid distribution in blood cells: control mechanisms and pathophysiological significance. *Biol. Chem.* **379,** 973-986.

Boatman, D. E. and Robbins, R. S. (1991) Bicarbonate:carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol. Reprod.* **44,** 806-813.

Bogdanov, A., Verhoven, B., Schlegel, R. A. and Williamson, P. (1993) Asymmetry in trans-bilayer lateral pressure may drive expansion of the secretion fusion pore. *Biochem. Soc. Trans.* **21,** 271-275.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), of PC12D pheochromocytoma cells. *J. Biol. Chem.* **265,** 5267-5272.

Cross, N. L. (1998) Role of cholesterol in sperm capacitation. *Biol. Reprod.* **59,** 7-11.

De Wit, R. J. W., Hekstra, D., Jastorff, B., Stec, W. J., Baraniak, J., van Driel, R. and van Haastert P. J. M. (1984) Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases. *Eur. J. Biochem.* **142,** 255-260.

Flesch, F. M., Voorhout, W. F., Colenbrander, B., van Golde, L. M. G. and Gadella, B. M. (1998) Use of lectins to characterize plasma membrane preprations from boar spermatozoa: a novel technique for monitoring membrane purity and quantity. *Biol. Reprod.* **59**, 1530-1539.

Florman, H. M., Arnoult, C., Kazam, I. G., Li, C. and O'Toole, C. M. B. (1999) Calcium channels of mammalian sperm: properties and role in fertilization. *In The Male Gamete. From Basic Science to Clinical Applications. C.* (ed. C. Gagnon) pp. 187-193. Vienna, IL: Cache River Press.

Fraser, L. R. (1987) Minimum and maximum extracellular Ca^{2+} requirements during mouse sperm capacitation and fertilization *in vitro*. *J. Reprod. Fertil.* **81,** 77-89.

Fraser, L. R. (1993) Calcium channels play a pivotal role in the sequence of ionic changes involved in initiation of mouse sperm acrosomal exocytosis. *Mol. Reprod. Dev.* **36,** 368-376.

Gadella, B. M., Gadella T. W. J. Jr., Colenbrander, B., van Golde, L. M. G. and Lopes-Cardozo, M. (1994) Visualization and quantification of glycolipid polarity dynamics in the plasma membrane of the mammalian spermatozoon. *J. Cell Sci.* **107,** 2151-2163.

Gadella, B. M., Lopes-Cardozo, M., van Golde, L. M. G., Colenbrander, B. and Gadella T. W. J. Jr. (1995) Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction: evidence for a primary capacitation event. *J. Cell Sci.* **108,** 935-946.

Gadella, B. M., Miller, N. G. A., Colenbrander, B., van Golde, L. M. G. and Harrison, R. A. P. (1999) Flow cytometric detection of transbilayer movement of fluorescent phospholipid analogues across the boar sperm plasma membrane: elimination of labeling artifacts. *Mol. Reprod. Dev.* **53,** 108-125.

Garty, N. B. and Salomon, Y (1987) Stimulation of partially purified adenylate cyclase from bull sperm by bicarbonate. *FEBS Lett.* **218,** 148-152.

Gijon, M. A., Spencer, D. M., Kaiser, A. L. and Leslie, C. C. (1999) Role of phosphorylation sites and the C2 domain in regulation of cytosolic phospholipase A_2. *J. Cell Biol.* **145,** 1219-1232.

Harkema, W., Harrison, R. A. P., Miller, N. G. A., Topper, E. K. and Woelders, H. (1998) Enhanced binding of zona pellucida proteins to the acrosomal region of the intact boar spermatozoa in response to fertilizing conditions: a flow cytometric study. *Biol. Reprod.* **58,** 421-430.

Harrison, R. A. P. (1996) Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. *Reprod. Fertil. Dev.* **8,** 581-594.

Harrison, R. A. P. (1997) Sperm plasma membrane characteristics and boar semen fertility. *J. Reprod. Fertil.* (Suppl.) **52,** 195-211.

Harrison, R. A. P., Ashworth, P. J. C. and Miller, N. G. A. (1996) Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.* **45,** 378-391

Harrison, R. A. P., Mairet, B. and Miller, N. G. A. (1993) Flow cytometric studies of bicarbonate mediated Ca^{2+} influx in boar sperm populations. *Mol. Reprod. Dev.* **35,** 197-208

Harrison, R. A. P. and Miller, N. G. A. (2000) cAMP-dependent protein kinase control of plasma membrane lipid architecture in boar sperm. *Mol. Reprod. Dev.* (in press).

Houle, M. G. and Bourgoin, S. (1999) Regulation of phospholipase D by phosphorylation-dependent mechanisms. *Biochim. Biophys. Acta* **1439,** 135-150.

Lagerberg, J. W. M., Kallen, K. J., Haest, C. W. M., Vansteveninck, J. and Dubbelman, T. M. A. R. (1995) Factors affecting the amount and the mode of merocyanine 540 binding to the membrane of human erythrocytes. A comparison with the binding to leukemia cells. *Biochim. Biophys. Acta* **1235,** 428-436.

Langner, M., and Hui, S. W. (1993) Merocyanine interaction with phosphatidylcholine bilayers. *Biochim. Biophys. Acta* **1149,** 175-179.

Lucy, J. A. (1993) Loss of phospholipid asymmetry in cell fusion. *Biochem. Soc. Trans.* **21,** 280-285.

McEvoy, L., Schlegel, R. A., Williamson, P. and Del Buono, B. J. (1988) Merocyanine 540 as a flow cytometric probe of membrane lipid organization in leukocytes. *J. Leukocyte Biol.* **44,** 337-344.

Mower, D. A., Peckham, D. W., Illera, V. A., Fishbaugh, J. K., Stunz, L. L. and Ashman R. F. (1994) Decreased membrane phospholipid packing and decreased cell size precede DNA cleavage in mature mouse B cell apoptosis. *J. Immunol.* **152,** 4832-4842.

Müller, K., Pomorski, T., Müller, P., Zachowski, A. and Hermann, A. (1994) Protein-dependent translocation of aminophospholipids and asymmetric transbilayer distribution of phospholipids in the plasma membrane of ram sperm cells. *Biochemistry* **33,** 9968-9974.

Müller P, Pomorski, T., Porwoli, S., Tauber, R. and Herrmann, A. (1996) Transverse movement of spin-labeled phospholipids in the plasma membrane of a hepatocytic cell line (HepG2): implications for biliary secretion. *Hepatology* **24,** 1497-1503.

Neill, J. M. and Olds-Clarke, P. (1987) A computer-assisted assay for mouse sperm hyperactivation demonstrates that bicarbonate but not bovine serum albumin is required. *Gamete Res.* **18,** 121-140.

Nolan, J. P., Magargee, S. F., Posner, R. G. and Hammerstedt, R. (1995) Flow cytometric analysis of transmembrane phospholipid movement in bull sperm. *Biochemistry* **33,** 9968-9974.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H. and Sugita, Y. (1985) Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J. Biol. Chem.* **260,** 9699-9705.

Palmier, B., Leiber, D. and Harbon, S. (1996) Pervanadate mediated an increased generation of inositiol phosphates and tension in the rat myometrium. Activation and phosphorylation of phospholipase Cγ1. *Biol. Reprod.* **54,** 383-1389.

Roldan, E. R. S. and Murase, T. M. (1994) Polyphosphoinositide-derived diacylglycerol stimulates the hydrolysis of phosphatidylcholine by phospholipase C during exocytosis of the ram sperm acrosome. Effect is not mediated by protein kinase C. *J. Biol. Chem.* **269,** 23583-23589.

Roldan, E. R. S. and Vazquez, J. M. (1996) Bicarbonate/CO_2 induces rapid activation of phospholipase A_2 and renders boar spermatozoa capable of undergoing acrosomal exocytosis in response to progesterone. *FEBS Lett.* **396,** 227-232.

Ruiz-Arguello, M. B., Goni, F. M. and Alonso, A. (1998) Vesicle membrane fusion induced by the concerted activities of sphingomyelinase and phospholipase C. *J. Biol. Chem.* **273,** 22977-22982.

Sandberg, M., Butt, E., Nolte, C., Fischer, L., Halbrügge, M., Beltman, J., Jahnsen, T., Genieser, H. G., Jastorff, B. and Walter, U. (1991) Characterization of Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3',5'-monophosphorothionate (Sp-5,6-DCl-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. *Biochem. J.* **279,** 521-527.

Schlegel, R. A., Prendergast, P. W. and Williamson, P. (1985) Membrane phospholipid asymmetry as a factor in erythrocyte-endothelial cell interaction. *J. Cell. Physiol.* **123,** 215-218.

Seamon, K. B., Padgett, W. and Daly, J. W. (1981) Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. USA* **78,** 3363-3367.

Shi, Q. X. and Roldan, E. R. S. (1995) Bicarbonate/CO_2 is not required for zona pellucida- or progesterone-induced acrosomal exocytosis of mouse spermatozoa but is essential for capacitation. *Biol. Reprod.* **52,** 540-546.

Smith, J. C., Graves, J. M. and Williamson, P. (1984) The interaction of the potential-sensitive molecular probe merocyanine 540 with phosphorylating beef heart submitochondrial particles under equilibrium and time-resolved conditions. *Arch. Biochem. Biophys.* **231,** 430-453.

Suzuki, F. and Yanagimachi, R. (1989) Changes in the distribution of intramembranous particles and filipin-reactive membrane sterol during in vitro capacitation of golden hamster spermatozoa. *Gamete Res.* **23,** 335-347.

Suzuki, K., Ebihara, M., Nagai, T., Clarke, N. G. E. and Harrison, R. A. P. (1994) Importance of bicarbonate/CO2 for fertilization of pig oocytes in vitro, and synergism with caffeine. *Reprod. Fertil. Dev.* **6,** 221-227.

Tajima, Y., N. Okamura, and Y. Sugita. (1987). The activating effects of bicarbonate on sperm motility and respiration at ejaculation. *Biochim. Biophys. Acta* **924,** 519-529.

Tepper A. D., de Vries E., van Blitterswijk W. J., Borst J. (1999) Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. *J. Clin. Invest.* **103,** 971-978.

Verhoven, B., Schlegel, R. A. and Williamson, P. (1992) Rapid loss and restoration of lipid asymmetry by different pathways in resealed erythrocyte ghosts. *Biochim. Biophys. Acta* **1104,** 15-23.

Verhoven, B., Schlegel, R. A. and Williamson, P. (1995) Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J. Exp. Med.* **182**, 1597-1601.

Verkman, A. S. (1987) Mechanism and kinetics of merocyanine 540 binding to phospholipid membranes. *Biochemistry* **26,** 4050-4056.

Visconti, P. E., Bailey, J. L., Moore, G. D, Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995a) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121,** 1129-1137.

Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P. and Kopf, G. S. (1995b) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP dependent pathway. *Development* **121,** 1139-1150.

Visconti, P. E. and Kopf, G. S. (1998) Regulation of protein phosphorylation during sperm capacitation. *Biol. Reprod.* **59,** 1-6.

Williamson, P., Mattocks, K. and Schlegel, R. A. (1983) Merocyanine 540, a fluorescent probe sensitive to lipid packing. *Biochim. Biophys. Acta* **732,** 387-393.

Williamson, P. and Schlegel, R. A. (1994) Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. *Mol. Membrane Biol.* **11,** 199-216.

Yanagimachi, R. (1982) Requirement of extracellular calcium ions for various stages of fertilization and fertilization-related phenomena in the hamster. *Gamete Res.* **5,** 323-344.

Yanagimachi, R. (1994) Mammalian fertilization. In *The Physiology of Reproduction* (ed. E. Knobil and J. D. Neill), pp. 189-317. New York: Raven Press