

## REVIEW

# Ion channels, phosphorylation and mammalian sperm capacitation

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Sexually reproducing animals require an orchestrated communication between spermatozoa and the egg to generate a new individual. Capacitation, a maturational complex phenomenon that occurs in the female reproductive tract, renders spermatozoa capable of binding and fusing with the oocyte, and it is a requirement for mammalian fertilization. Capacitation encompasses plasma membrane reorganization, ion permeability regulation, cholesterol loss and changes in the phosphorylation state of many proteins. Novel tools to study sperm ion channels, image intracellular ionic changes and proteins with better spatial and temporal resolution, are unraveling how modifications in sperm ion transport and phosphorylation states lead to capacitation. Recent evidence indicates that two parallel pathways regulate phosphorylation events leading to capacitation, one of them requiring activation of protein kinase A and the second one involving inactivation of ser/thr phosphatases. This review examines the involvement of ion transporters and phosphorylation signaling processes needed for spermatozoa to achieve capacitation. Understanding the molecular mechanisms leading to fertilization is central for societies to deal with rising male infertility rates, to develop safe male gamete-based contraceptives and to preserve biodiversity through better assisted fertilization strategies.

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## INTRODUCTION

On September 2010, Dr Robert Edwards was awarded the Nobel Prize for the development of human *in vitro* fertilization (IVF) therapy. Since the first successful ‘Test-Tube’ baby was born in 25 July 1978,<sup>1</sup> over four million babies were conceived with this methodology. This outstanding landmark represented by Louise Joy Brown’s birth was achieved almost 100 years after the first IVF attempts in mammals. The difficulties to attain mammalian IVF were inherent to a lack of knowledge on how gametes behave in *in vitro* systems. It was not until the early 1950s that Austin<sup>2</sup> and Chang<sup>3</sup> independently demonstrated that sperm had to reside in the female reproductive tract for a period of time before becoming able to fertilize an egg. The physiological changes that occur to the sperm in their transit toward the egg were collectively defined as ‘sperm capacitation’. The discovery of capacitation was essential for IVF because this finding made researchers aware that to acquire fertilizing capacity, mammalian sperm need to be in contact with molecules present in the female. Less than 10 years later, this discovery was followed by the first successful mammalian IVF when Chang showed that eggs from a black rabbit fertilized *in vitro* by capacitated sperm from a black male, and transferred to a white rabbit female, resulted in black offspring.<sup>4</sup>

The relevance of sperm capacitation for IVF increased the need to further study this process at the molecular level. Inherent to the initial studies on capacitation was the understanding that this process

comprises multiple signaling events. For example, as soon as sperm are ejaculated, they become actively motile; however, they are still unable to penetrate the egg layers. In part, this motility activation is regulated by changes in the ionic environment surrounding the sperm. Later on, sperm undergo other modifications in their motility pattern known as hyperactivation. Although the exact role of hyperactivation is not clear yet, this process is believed to be related to the release of sperm from the oviduct reservoirs as well as to help sperm to penetrate the extracellular matrix of the egg.<sup>5</sup> Besides changes in the flagellum, capacitation is also related to the ability of sperm to undergo a physiologically induced acrosome reaction. Interestingly, these processes occurring respectively in the tail and the head are temporally coordinated and are activated by similar signaling pathways. Finally, although the acrosome reaction is needed for fertilization and initially this process was considered part of the capacitation process, current views considered that the acrosome reaction is a post-capacitation event.<sup>6</sup>

In the last years, state-of-the-art techniques are being used to elucidate the molecular basis of sperm capacitation. Among them it is possible to highlight: (i) genetically modified mice models; (ii) global approaches to identify proteins present in different sperm structures; and (iii) whole cell patch clamp techniques to directly measure ion transport in differentiated sperm. Although this review will mention some of the most critical discoveries in the field of capacitation, it will

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be mostly focused on the analysis of two aspects of capacitation: (i) the regulation of the capacitation-associated changes in ion transport including those that are involved in the modulation of the sperm plasma membrane potential ( $E_m$ ); and (ii) the regulation of the capacitation-associated changes in phosphorylation. For more information, some other excellent reviews on capacitation have been published in recent years.<sup>7–12</sup>

## REGULATION OF ION FLUXES DURING SPERM CAPACITATION

Cells expend a significant portion of their energetic resources building and maintaining ion concentration gradients across the membrane through the use of pumps and ion transporters. They use these stored resources to decode information from their surroundings and their interior. Ion channels and certain transporters capitalize these resources as their activation can change the electrical potential of the cell and the concentrations of second messengers within a wide time range, depending on the modes of channel regulation. In particular, ion channels can be opened to allow the flow of millions of ions per second across the membrane down their electrochemical gradient by small conformational changes induced by voltage, ligands, phosphorylation changes, membrane pressure and other signaling events.<sup>13</sup>

As part of their transit through the epididymis, sperm are exposed to conditions very different to the ones that they will encounter in the seminal fluid and in the female tract. In comparison with other body fluids, the epididymis lumen contains lower levels of  $\text{Na}^+$ , higher levels of  $\text{K}^+$  and lower levels of  $\text{Cl}^-$ . Osmolarity is compensated by organic anions such as carnitin, phosphocoline and glycerylphosphorylcholine.<sup>14</sup> Most interestingly, the epididymal fluid is maintained at a low pH by a finely regulated epithelial release of  $\text{H}^+$  and uptake of  $\text{HCO}_3^-$ . This reduced pH is essential to maintain functionally competent sperm.<sup>15</sup>

After ejaculation, when spermatozoa are transferred to and navigate through the female tract, extracellular ion concentrations and osmolarity change again. In this case, extracellular  $[\text{K}^+]_e$  ( $[\text{K}^+]_e$ ) is reduced and  $[\text{HCO}_3^-]_e$  and  $[\text{Na}^+]_e$  are increased in relation to seminal fluid ion concentrations.  $\text{HCO}_3^-$  increases from  $\sim 4 \text{ mmol l}^{-1}$  in the epididymis<sup>16,17</sup> to  $> 20 \text{ mmol l}^{-1}$  in the oviduct<sup>18</sup> immediately activating mouse sperm flagellar beating. In this new milieu, sperm undergo capacitation, another extra testicular maturational change. As mentioned in the introductory paragraphs, discovery of capacitation made possible mammalian IVF. Initial experiments on this front focused in development of incubation media that support capacitation *in vitro*. Although each species has specific requirements, there is a consensus that certain components of the capacitation media are universal and close to the composition of the oviductal fluid. Among them, all capacitation media contained close-to-serum levels of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ .<sup>19,20</sup> In addition, these media contain energy metabolites (such as glucose, pyruvate and lactate) and a protein source that usually is serum albumin. Although the precise role of these molecules in capacitation is still under study, work from several groups is starting to elucidate some of their actions. A schematic representation of the roles and interrelationships between the ion transporters suspected to participate in capacitation is illustrated in **Figure 1**.

### Intracellular $\text{Ca}^{2+}$ and pH changes

$\text{Ca}^{2+}$  and  $\text{H}^+$  play a preponderant role during capacitation,<sup>20</sup> and both  $[\text{Ca}^{2+}]_i$  and  $\text{pH}_i$  increase during this process.<sup>21–26</sup> Though usually capacitating media contain  $\sim 2 \text{ mmol l}^{-1} \text{Ca}^{2+}$ ,  $100\text{--}200 \text{ }\mu\text{mol l}^{-1}$  is enough to capacitate mouse sperm.<sup>27,28</sup> Recent work exploring the  $[\text{Ca}^{2+}]_e$  requirement for the  $\text{HCO}_3^-$  stimulation of sperm motility

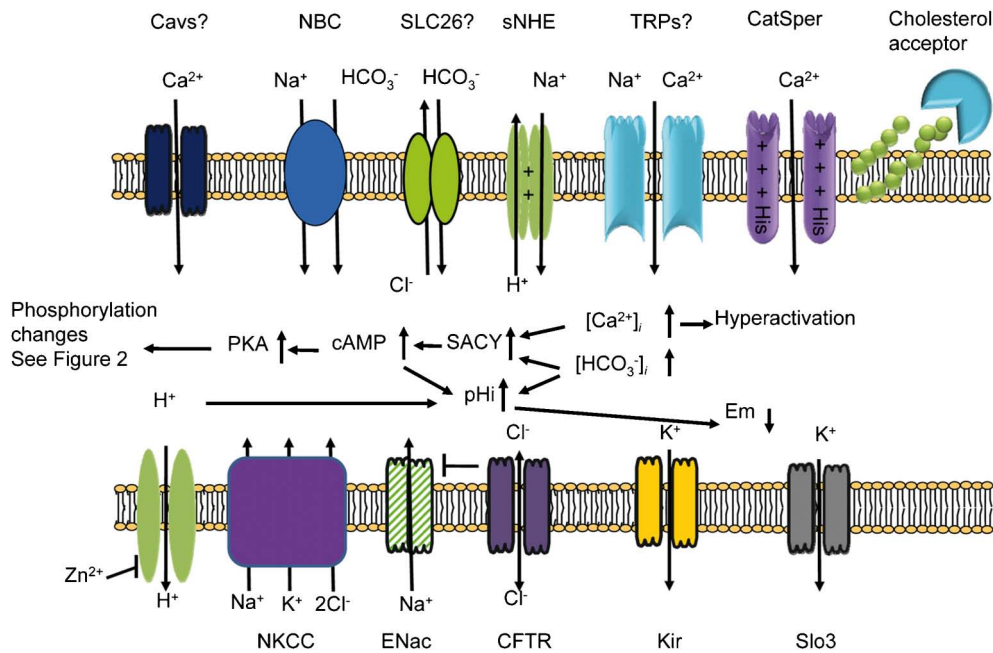
revealed that additionally to its critical role as second messenger,  $\text{Ca}^{2+}$  can act as a first messenger. A  $\text{Ca}^{2+}$  sensing receptor has been cloned<sup>29</sup> and proposed to participate in the  $\text{HCO}_3^-$ -induced acceleration of mouse sperm flagellar beat frequency.<sup>30</sup> However, how the  $[\text{Ca}^{2+}]_i$  increase required for capacitation occurs is not fully answered (**Figure 1**). Some transporters appear as main candidates responsible for  $\text{Ca}^{2+}$  influx:  $\text{Ca}_v$  and CatSper channels,  $\text{Na}^+/\text{Ca}^{2+}$  exchangers,  $\text{Ca}^{2+}$  ATPases and the novel STIM and ORAI. On the other hand,  $\text{Ca}^{2+}$  released from intracellular stores must also be considered. In this regard, the main candidates would be: IP3 receptors, ryanodine receptors, TPC proteins, SERCA and SPM ATPases. We will briefly describe the characteristics and possible relation of CatSper and  $\text{Ca}_v$ s to sperm capacitation.

### $\text{Ca}^{2+}$ channels

**CatSper.** Bioinformatics and gene-targeting strategies have revealed that mouse spermatozoa possess sperm-specific subunits of a new family of cation channels named CatSper channels (cation channel of spermatozoa). In addition to mammals, these channels are also present in reptiles, tunicates, echinoderms and cnidarians.<sup>31,32</sup> This novel class of  $\text{Ca}^{2+}$  channels has been shown to be essential for mammalian hyperactivated sperm motility, sperm detachment from the female reproductive tract<sup>33</sup> and egg coat penetration and fertility.<sup>32,34,35</sup> CatSper mutations in humans have been associated to infertility.<sup>36</sup> At present, the family has four members (CatSper1–4), all most likely found in the principal piece of the flagella, plus two additional auxiliary subunits, CatSperB and G. These later subunits are transmembrane proteins, have large extracellular domains and have unknown functions.<sup>34,37,38</sup> Interestingly, it has been noted that as in other voltage-dependent ion channels, extracellular ligand binding to auxiliary subunits could modulate CatSper's activity.<sup>39</sup>

All four CatSper subunits (1–4) are needed for functional expression in mouse sperm, as knockout of any one of them results in absence of the other subunits in mature sperm with the consequent observation of male infertility and the lack of the CatSper associated currents recorded by sealing on cytoplasmic droplet of epididymal sperm.<sup>34,35,40</sup> *CatSper1* null male mice also display defects in cAMP and depolarization-induced  $\text{Ca}^{2+}$  entry.<sup>34,41</sup> The pore-forming subunits of CatSper channels are like those of voltage-dependent  $\text{K}^+$  channels displaying six transmembrane spanning domains with a P-loop between transmembrane domains S5 and S6. It is thought that the channel would be formed by four of these subunits. However, their selectivity filter sequences are closer to those of  $\text{Ca}_v$  channels constituted by a single polypeptide with 24 transmembrane domains. CatSper channels are constitutively active, weakly voltage-dependent,  $\text{Ca}^{2+}$  selective and strongly potentiated by intracellular alkalization.<sup>34,42</sup> Unfortunately, it has been impossible to heterologously express CatSper channels; thus their characterization has been restricted to optical, electrophysiological and biochemical experimental strategies in mouse and human sperm.

The high  $\text{pH}_i$  sensitivity of CatSper is likely due to its CatSper1 subunit which contains an intracellular C-terminal domain rich in histidines. Although not yet demonstrated, capacitation-associated  $\text{pH}_i$  changes may activate this channel with the consequent increase in  $[\text{Ca}^{2+}]_i$ . Related to the  $\text{pH}_i$  regulation might be the well-described cyclic nucleotide-induced  $[\text{Ca}^{2+}]_i$  increase,<sup>43</sup> as neither cAMP nor cGMP elevate  $[\text{Ca}^{2+}]_i$  in sperm from CatSper null mice. It is worth noting that none of the CatSper subunits have a cyclic nucleotide-binding sequence.



**Figure 1** Ion permeability model. This figure illustrates a schematic representation of the roles and interrelationships between the ion transporters suspected to participate in capacitation. Capacitation and hyperactivation take place during sperm transit through the female tract and have parallel but also intercrossing pathways. Cholesterol removal from the sperm plasma membrane by albumin in the female tract modifies several membrane properties. Albumin can also directly stimulate CatSper channels, increasing  $[Ca^{2+}]_i$ . The  $HCO_3^-$  increase experienced by sperm upon entering the female reproductive tract elevates intracellular  $HCO_3^-$  possibly through a NBC which would cause a hyperpolarization and stimulation of SACY, producing cAMP. In addition to triggering the phosphorylation cascades (see **Figure 2**), cAMP may activate the sNHE which together with the proton channel ( $H_V$ ), possibly stimulated by  $Zn^{2+}$  removal in the female reproductive tract,<sup>35</sup> would raise  $pH_i$  and activate CatSper and Slo3 channels. cAMP would also activate CFTR and participate in closing ENaCs which together with  $K^+$  channels hyperpolarize sperm. The  $[Ca^{2+}]_i$  increase may influence glycolysis and the axoneme activity promoting hyperactivated motility. Several  $Ca^{2+}$  mobilizing pumps (PMCA4 and SPCA1) and channels (IP3R, RyR and TRPs) may also participate during  $Ca^{2+}$  signaling. Not all sperm species undergo an Em hyperpolarization associated to capacitation like mouse; the channels and transporters that may contribute to this hyperpolarization are indicated. CFTR, cystic fibrosis transmembrane regulator; Em, membrane potential; IP3R, IP3 receptor; NBC,  $Na^+/HCO_3^-$  cotransporter; NKCC,  $Na^+/K^+/Cl^-$  cotransporter; PKA, protein kinase A; RyR, ryanodine receptor; SACY, soluble adenyllyl cyclase; sNHE, sperm  $Na^+/H^+$  exchanger; TRP, transient receptor potential.

Alternatively, cyclic nucleotides may regulate CatSper increasing  $pH_i$  by activating a sperm specific  $Na^+/H^+$  exchanger (sNHE) (see below).<sup>32,34</sup> Finally, recent work from the Ren's laboratory suggests that CatSper may contribute to the  $[Ca^{2+}]_i$  increase that accompanies the acrosome reaction.<sup>44</sup> However, though sperm from CatSper null mice lack a zona pellucida (ZP)-induced  $Ca^{2+}$  uptake component, they undergo tyrosine phosphorylation,<sup>41</sup> a ZP-induced acrosome reaction and they can fertilize zona-free eggs.<sup>34,35</sup> Interestingly, within the time resolution of  $Ca^{2+}$  measurements in this work, the ZP-induced  $Ca^{2+}$  increase starts in the flagellum where CatSper are present. How ZP proteins regulate CatSper in the flagellum is not yet known.

*Ca<sub>v</sub>s*. These  $Ca^{2+}$  channels convert Em changes into  $Ca^{2+}$  signals. The subunit of these channels contains the ion permeation pathway which is encoded by a family of at least 10 genes. *Ca<sub>v</sub>* channels have been cataloged in two major functional classes: high voltage-activated and low voltage-activated. Though transcripts and proteins for both types of *Ca<sub>v</sub>* have been detected in mammalian sperm,<sup>45</sup> electrophysiological evidence has mainly revealed the functional presence of *Ca<sub>v</sub>3* channels in mouse and human spermatogenic cells<sup>46–48</sup> and in mouse testicular sperm.<sup>49</sup> Weak depolarizations can open *Ca<sub>v</sub>3* channels which are encoded by the *Ca<sub>v</sub>3* subfamily of genes (*Ca<sub>v</sub>3.1* to *Ca<sub>v</sub>3.3*).<sup>49,50</sup> However, *Ca<sub>v</sub>3* channels begin inactivating at relatively hyperpolarized potentials (about  $-60$  mV); at the Em of non-capacitated sperm (about  $-40$  mV) they are inactivated.<sup>47,51,52</sup> As the pharmacology of the mouse sperm acrosome reaction and at least part

of the  $Ca^{2+}$  influx associated to it, are consistent with that of *Ca<sub>v</sub>3* channels, it was proposed, though now debated,<sup>34</sup> that they participate in this important reaction.<sup>53</sup> It turns out that *Ca<sub>v</sub>3.1* and *Ca<sub>v</sub>3.2* null mice are fertile<sup>54,55</sup> and though *Ca<sub>v</sub>3* currents have been recorded in mouse testicular sperm,<sup>49</sup> no *Ca<sub>v</sub>* currents have been detected in epididymal sperm.<sup>34</sup> These findings certainly question the role of *Ca<sub>v</sub>3* and in general *Ca<sub>v</sub>* channels in sperm physiology. Resolving this matter requires further studies since  $[Ca^{2+}]_i$  measurements and their pharmacology are consistent with the presence and participation of certain *Ca<sub>v</sub>* channels in sperm physiology.<sup>56–58</sup>

If *Ca<sub>v</sub>* channels were involved in the acrosome reaction, sperm Em should hyperpolarize during capacitation to remove their inactivation before they could open and trigger this process.<sup>59</sup> Indeed, single-cell Em measurements indicated that the Em of  $\sim 50\%$  of mouse sperm remain close to that of uncapacitated sperm, while the rest hyperpolarize to  $-80$  mV, a potential that can remove inactivation from low voltage-activated  $Ca^{2+}$  channels.<sup>51</sup> These experiments suggest that only the hyperpolarized population can undergo the AR when exposed to solubilized ZP (see the section on 'Em changes').

#### Intracellular pH regulation

As discussed earlier, mammalian sperm capacitation requires  $HCO_3^-$ . Uptake of this anion contributes to the  $pH_i$  increase that accompanies this process.<sup>21,22,60</sup> In addition,  $HCO_3^-$  influx hyperpolarizes mouse sperm depending on the concentration of external  $Na^+$ , suggesting the

involvement of an electrogenic  $\text{Na}^+/\text{HCO}_3^-$  cotransporter during capacitation.<sup>60</sup>

Regulation of sperm  $\text{pH}_i$  is not only fundamental for capacitation, but also for motility and the acrosome reaction. As mentioned, sperm express a specific member of the mammalian NHE superfamily of  $\text{Na}^+/\text{H}^+$  exchangers, sNHE.<sup>61–63</sup> It is localized on the principal piece of the sperm flagellum and predicted to have 14 membrane-spanning helices. sNHE contains a nucleotide-binding domain close to its intracellular C-terminus and four putative transmembrane helices analogous to the voltage sensor of voltage-dependent channels.<sup>64</sup> These distinctive characteristics suggest that sNHE may be regulated by cyclic nucleotides and Em,<sup>65</sup> in addition to phosphorylation and protein interactions.<sup>61</sup> Sperm from sNHE null male mice are immotile and infertile, and can be rescued adding permeable analogs of cAMP or  $\text{NH}_4\text{Cl}$ . sNHE and the soluble adenylyl cyclase (SACY) may form a transduction complex to efficiently regulate  $\text{pH}_i$ ,  $\text{HCO}_3^-$ , cAMP and sperm motility, as the exchanger is required for the full length expression of SACY.<sup>63,65</sup>

Recent evidence has revealed that mammalian sperm, particularly those of human, express  $\text{H}_V$  channels in the flagella.<sup>35</sup> These channels may participate in the  $\text{pH}_i$  increase necessary for capacitation. Since protein kinase C (PKC) regulates this type of channel in other cell types, an interesting possibility would be that this regulation is conserved in sperm.<sup>66</sup> In mouse sperm, it has been shown that  $\text{H}_V$  channels do not play a preponderant role in  $\text{pH}_i$  regulation;<sup>35</sup> therefore, it is likely that  $\text{pH}_i$  relies on the regulation of sNHE as suggested by the sterile phenotype of sNHE null mice.<sup>63</sup> In this respect, analysis of its sequence predicts that the sNHE is voltage-dependent suggesting that the hyperpolarization that occurs during mouse sperm capacitation could stimulate this exchanger.

### Em changes

In sperm, as in most cells, the internal ion concentrations are markedly different from those in the extracellular medium. These differences result from the relative ion permeability of the plasma membrane to each of the ions found in the inner and outer media given by the specific ion channels and transporters present in the cell, to the gradients they establish and the metabolic state of the cell. At rest, the balance of these fluxes, gradients and permeabilities results in an electric potential, known as the resting Em. Before capacitation mouse sperm are relatively depolarized (Em: about from  $-35$  to  $-45$  mV) and become hyperpolarized (about  $-70$  mV) during capacitation.<sup>51,60,67,68</sup> This change results from a combination of electrogenic ion permeability alterations that shift the Em towards the  $\text{K}^+$  equilibrium potential (about  $-90$  mV).<sup>60,69</sup> Several types of  $\text{K}^+$  channels have been detected in testis, spermatogenic cells and sperm using molecular, electrophysiological and biochemical tools (see the section on ' $\text{K}^+$  channels').<sup>67,70–73</sup> It has been mentioned that capacitation-associated hyperpolarization may increase  $\text{pH}_i$ -stimulating sNH and consequently CatSper.<sup>32</sup> However, a  $\text{pH}_i$  increase shifts the CatSper current–voltage curve toward more negative membrane potential values.<sup>42</sup> Considering that CatSper is activated by membrane depolarization, hyperpolarization may have a dual effect on this channel regulated by a subtle balance between these variables, further influenced by cAMP levels. Because capacitation prepares the sperm to undergo the AR, it has been proposed that the capacitation-associated hyperpolarization could also regulate the ability of sperm to generate transient  $[\text{Ca}^{2+}]_i$  elevations during the AR induced by physiological agonists (e.g., ZP).<sup>46,47,51</sup>

In the absence of bovine serum albumin and  $\text{HCO}_3^-$ , mouse sperm at pH 7.2 do not hyperpolarize which suggests that cholesterol removal and  $\text{HCO}_3^-$  somehow regulate the sperm Em changes that occur during capacitation. As *in vitro* capacitation also depends on the presence of  $\text{Na}^+$  and  $\text{Cl}^-$  in the medium,<sup>74,75</sup> other ion channels and transporters are likely to regulate sperm Em and intervene in this maturational process. Furthermore,  $50 \text{ mmol l}^{-1}$  external KCl inhibits hyperpolarization and capacitation in mouse sperm.<sup>51</sup>

### $\text{Na}^+$ influences the sperm Em

*ENaCs*. Addition of  $\text{Na}^+$  to the external media depolarizes mouse sperm more before than after capacitated. This depolarization is very sensitive to amiloride. Indeed, ENaCs, amiloride-sensitive  $\text{Na}^+$  channels that are expressed in many invertebrate and vertebrate cell types, are present in mouse and human sperm.<sup>76</sup> These channels may be regulated by pH,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$  and phosphorylation,<sup>77</sup> parameters that change during capacitation. The ENaC  $\alpha$ - and  $\delta$ -subunits were detected in the sperm flagellar mid-piece and acrosome region, respectively.<sup>76</sup> These channels are  $\text{Na}^+$ -selective, amiloride-sensitive and contribute to the resting Em in cells by displacing it towards the  $\text{Na}^+$  equilibrium potential.<sup>78</sup> Both mouse spermatogenic cells<sup>76</sup> and testicular sperm display amiloride-sensitive inward  $\text{Na}^+$  currents whose characteristics match those of ENaCs. Notably, permeable cAMP analogs which induce *in vitro* capacitation decrease the sperm depolarization induced by addition of external  $\text{Na}^+$ . These experiments are consistent with the hypothesis that elevation of cAMP levels during sperm capacitation decreases ENaC activity and contribute to the hyperpolarization needed for this process to occur.

### $\text{K}^+$ channels

As mentioned earlier, mouse sperm hyperpolarize from about  $-40$  to about  $-70$  mV during capacitation.<sup>51,67</sup> The involvement of  $\text{K}^+$  channels in this change was proposed since it was affected by external  $\text{K}^+$  and  $\text{K}^+$ -channel blockers.<sup>34,51,67,70</sup> Spermatogenic cells and sperm have been reported to possess  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels,<sup>71</sup> voltage-gated  $\text{K}^+$  channels,<sup>72,73</sup> two-pore domain  $\text{K}^+$  channels,<sup>79</sup> inwardly rectifying  $\text{K}^+$  (Kir) channels<sup>34,67,70</sup> and Slo3  $\text{K}^+$  channels.<sup>49,80–84</sup> Below we will discuss the  $\text{K}^+$  channels that have been proposed to participate in capacitation.

*Kirs*. Kir channels move  $\text{K}^+$  easier into the cell than out. The Kir family is constituted by seven subfamilies (Kir1–7). These subfamilies can be further divided into four functional groups: constitutively active classical Kir channels (Kir2), G protein-gated Kir channels (Kir3), ATP-sensitive  $\text{K}^+$  channels (Kir6) linked to cellular metabolism and  $\text{K}^+$  transport channels (Kir1, 4, 5 and 7). Among many cell functions, Kir channels are important in maintaining Em and  $\text{K}^+$  homeostasis, membrane excitability, vascular tone, heart rate and insulin secretion. Intracellular substances such as  $\text{Mg}^{2+}$  and polyamines block the pore causing inward rectification. Ions, phospholipids and proteins modulate Kir channel activity. A Kir subunit is basically made up of two transmembrane helices with cytoplasmic  $\text{NH}_2$  and  $\text{COOH}$  termini and an extracellular loop which folds back to form the pore-lining ion selectivity filter. Four of these homo- or heteromeric subunits constitute a functional Kir channel.<sup>80</sup>

Patch-clamp recordings in mouse spermatogenic cells revealed Kir currents sensitive to  $\text{Ba}^{2+}$ , glucose, tolbutamide and glibenclamide.<sup>34,67,70</sup> More recently these compounds were shown to block  $\text{K}^+$  currents in mouse testicular sperm;<sup>81</sup> they also inhibited the hyperpolarization associated to mouse sperm capacitation and, at least partially the AR.<sup>34,67,70</sup>

Tolbutamide and glibenclamide are sulfonylureas known to block Kir channels regulated by ATP ( $K_{ATP}$  channels).  $K_{ATP}$  channels are formed from Kir6 subunits and ATP-binding cassette sulfonylurea receptor (SUR) proteins.<sup>85</sup> Transcripts for the  $K_{ATP}$  channel subunits SUR1, SUR2, Kir6.1 and Kir6.2 were detected in elongated spermatids and the expressed proteins immunolocalized in mature mouse sperm.<sup>70</sup>

Kir channels are in general inhibited by low  $pH_i$ .<sup>80</sup> As sperm  $pH_i$  is relatively acidic before capacitation, it could maintain Em depolarized keeping  $Ca_v$  channels inactivated and unable to cause unregulated  $Ca^{2+}$  entry and the acrosome reaction.<sup>22</sup> In spite of the structural similarities between different Kirs, cholesterol affects them differentially. Its depletion increases Kir2 current density, while it inhibits Kir4 channels.<sup>86</sup> Sperm capacitation is accompanied by activation of the cAMP/protein kinase A (PKA) signaling pathway, cholesterol depletion and a  $pH_i$  increase.<sup>87</sup> It is therefore feasible that Kir channels such as the  $K_{ATP}$  channels contribute to the capacitation-associated hyperpolarization in mouse sperm.

**Slo3.** The Slo gene family of  $K^+$  channels are formed by an  $\alpha$  subunit with a large cytosolic C-terminal containing potential ligand regulation sites and a  $\beta$ -auxiliary subunit. There are four  $\alpha$  subunits, where each type can apparently form a functional tetramer,<sup>88</sup> and four different  $\beta$ -subunits that regulate the function and expression of Slo channels.<sup>89</sup> Specific cytosolic ions regulate each of the subunits:  $Ca^{2+}$  for Slo1,  $Na^+$  for both Slo2.1 (Slick) and Slo2.2 (Slack), and  $H^+$  for Slo3.<sup>90</sup>

The Slo3 subunit, cloned many years ago and expressed only in spermatogenic cells and sperm,<sup>80,82</sup> has only recently been shown to be fundamental for sperm function;<sup>49,83</sup> Slo3 null male mice are infertile.<sup>84</sup> Slo3 currents in mouse testicular sperm are similarly activated by intracellular alkalinization, depolarization and cAMP, blocked by  $1 \text{ mmol l}^{-1} \text{ Ba}^{2+}$  and high [TEA] ( $60 \text{ mmol l}^{-1}$ ), and poorly  $K^+$  selective ( $PK^+/PNa^+ : \sim 8$ ), as they are when heterologously expressed in oocytes.<sup>49</sup> These currents are absent in testicular sperm from Slo3 null mice, confirming their identity.<sup>84</sup>

As discussed earlier, under capacitating conditions wild-type mouse sperm hyperactivate, their  $pH_i$  increase and their Em hyperpolarize. In contrast, sperm from Slo3 null mice are unable to swim progressively, to hyperpolarize and to undergo the acrosome reaction. Remarkably, these sperm cannot acrosome react even when exposed to  $Ca^{2+}$  ionophore. These results show that Slo3 channel activation is pivotal for the capacitation-associated processes necessary for fertilization. These considerations also point to this channel as an attractive target for male contraception.<sup>84</sup>

Recent findings revealed that Slo3 channels heterologously expressed are stimulated by PIP2. This also occurs to the corresponding currents in epididymal mouse sperm. These Slo3 currents are inhibited by EGF acting through the EGF receptor, in a PIP2-dependent manner.<sup>91</sup> Further studies are needed regarding the selectivity, voltage dependence and  $\beta$ -subunit regulation of Slo3 channels in mammalian sperm to fully understand their participation in capacitation and fertilization.

**$HCO_3^-$ ,  $Cl^-$  and cystic fibrosis transmembrane regulator (CFTR)**  $HCO_3^-$  influences mouse sperm Em in addition to its role regulating SACY. Upon addition of  $HCO_3^-$ , the electrogenic influx of this anion causes a temporal hyperpolarization of mouse sperm that is distinct from the slowly developing hyperpolarization associated to capacitation. In these experiments,  $Na^+$  replacement from the external media by the non-permeant cation choline<sup>+</sup> does not allow

the hyperpolarization induced by adding  $HCO_3^-$ , and inhibits the capacitation-associated increase in protein tyrosine phosphorylation and the preparation for the ZP-induced acrosome reaction. Addition of permeable analogs of cAMP to the choline incubation medium restores protein tyrosine phosphorylation. These findings suggest that a  $Na^+/HCO_3^-$  cotransporter is present in mouse sperm and is coupled to events regulating capacitation.<sup>60</sup>

ENaCs and the CFTR have been found to regulate each other and colocalize in several cell types.<sup>92</sup> Cystic fibrosis, the most widespread human genetic disease, is caused by mutations in CFTR, a cAMP-modulated, ATP-dependent  $Cl^-$  channel.<sup>93</sup> ENaCs and CFTRs have been detected in the mid-piece of mouse and human sperm flagella.<sup>69,94</sup> Em measurements have indicated that cAMP may close ENaCs by activating CFTR and thus contribute to the capacitation associated hyperpolarization.<sup>69</sup>

$Cl^-$  transport across the plasma membrane is important for the regulation of cell Em, volume,  $pH_i$  and for  $HCO_3^-$  fluxes. Different  $Cl^-$  channels, exchangers and cotransporters move this anion.<sup>13</sup> Sperm capacitation and fertilization require external  $Cl^-$ .<sup>95,96</sup> Evidence indicates that  $[Cl^-]_i$  increases during capacitation;<sup>69</sup> its influx may be necessary to allow a raise in intracellular  $HCO_3^-$  that would stimulate SACY through  $Cl^-/HCO_3^-$  antiporters.<sup>69,96</sup> As sperm Em is close to the  $Cl^-$  equilibrium potential, opening  $Cl^-$  channels would not allow accumulation of this anion. These considerations point out that it is likely that other  $Cl^-$  transport systems are present in sperm. From a battery of  $Cl^-$  transport antagonists tested in capacitation assays, only general  $Cl^-$  transport blockers that do not allow identification of specific transporters, such as stilbenes (i.e., DIDS), inhibited tyrosine phosphorylation, hyperactivation and fertilization. These findings are consistent with the presence and importance of  $Cl^-$  transporters in sperm physiology. Furthermore, as observed in external media without  $Cl^-$ , bumetanide and furosemide, two  $Na^+/K^+/Cl^-$  cotransporter (NKCC) inhibitors, reduced sperm tyrosine phosphorylation and hyperactivation. It is worth noting that higher concentrations were needed for this inhibition than those required to antagonize NKCCs.<sup>97</sup> In addition, external  $K^+$ , an ion needed for NKCC function, was not required for the increase in tyrosine phosphorylation associated to capacitation. This and the high bumetanide concentrations needed to inhibit may indicate that this antagonist could affect other targets in addition to NKCC.<sup>96</sup>

On the other hand, the ZP-induced acrosome reaction which depends on external  $Cl^-$ ,  $K^+$  and  $Na^+$ , is inhibited at a much lower concentration of bumetanide and furosemide, suggesting that NKCC might participate in the capacitation-dependent preparation of sperm for the acrosome reaction or directly in this reaction. The messenger for this cotransporter and its protein were detected in mouse spermatogenic cells and sperm, respectively.<sup>96</sup> Interestingly, other conditions that do not inhibit either hyperactivation or the increase in tyrosine phosphorylation, such as CFTR antagonists DPC and inh-172, can block the ZP-induced acrosome reaction. These results suggest that capacitation requires nonlinear signaling paths. In accordance with this hypothesis, although sperm from SACY null mice fail to hyperactivate and to increase their tyrosine phosphorylation, they are able to undergo the ZP-induced acrosome reaction.<sup>98</sup> Noteworthy, the general  $Cl^-$  channel inhibitor niflumic acid inhibited the mouse acrosome reaction initiated by different stimuli, including ZP and glycine.<sup>99</sup> In addition, the presence of glycine receptor/ $Cl^-$  channel has been shown to be involved in the human zona-induced acrosome reaction.<sup>100</sup> The

inhibitor strychnine was also able to inhibit the  $\text{Ca}^{2+}$  influx associated with addition of recombinant human ZP3,<sup>101</sup> further substantiating the key role of  $\text{Cl}^-$  influx in the acrosome reaction.

### Is sperm hyperpolarization needed for capacitation and if so why?

Originally it was argued that mouse sperm needed to hyperpolarize during capacitation to remove inactivation from  $\text{Ca}_V$  channels so they could open when stimulated by ZP to trigger the acrosome reaction.<sup>46,47,51</sup> However, the functional involvement of  $\text{Ca}_V$  channels in sperm is being questioned. It is worth considering that this hyperpolarization could be required to activate sNHE and increase  $\text{pH}_i$ , which would in turn stimulate Slo3 and CatSper channels,<sup>32</sup> and possibly SACY.<sup>65</sup> In addition, a hyperpolarization would increase the driving force for  $\text{Ca}^{2+}$  uptake through other  $\text{Ca}^{2+}$  permeable channels such as transient receptor potentials, some of which are activated by alkalinization.

## PHOSPHORYLATION EVENTS LINKED TO SPERM CAPACITATION

### cAMP, $\text{HCO}_3^-$ and $\text{Ca}^{2+}$ in SACY regulation

As mentioned, one critical change in the sperm surrounding milieu after ejaculation is the change in  $\text{HCO}_3^-$  concentration. In addition to the aforementioned relevance of this anion to regulate  $\text{pH}_i$  and Em, there is a connection between  $\text{HCO}_3^-$  and the cAMP pathway.<sup>102</sup> This landmark observation indicated that  $\text{Ca}^{2+}$  upregulated cAMP levels only when  $\text{HCO}_3^-$  was present in the incubation medium. This initial finding was followed by experiments in boar sperm showing that  $\text{HCO}_3^-$  in the seminal fluid was needed for motility initiation<sup>17</sup> and that this anion was able to increase cAMP levels through direct stimulation of a unique type of adenylyl cyclase present in sperm. More than a decade later, this unique cyclase was cloned from rat testis. Since it is found in a soluble fraction of the testis, it was called SACY.<sup>103</sup> Although it is not clear whether SACY has a role in germ cell differentiation, SACY knockout mice are sterile.<sup>104</sup> The sterility phenotype is mapped to lack of capacitation; in particular sperm from the SACY null mice are not able to move actively and cannot hyperactivate.<sup>105</sup> Unexpectedly, these sperm have no obvious problems with their ability to undergo a ZP-induced acrosome reaction, suggesting that this event can be dissociated from other capacitation-associated processes.<sup>104</sup>

### Role of PKA as a target of cAMP

Over the years the role of cAMP in the regulation of sperm has been supported by biochemical and pharmacological approaches. It is well established that inhibitors of PKA such as H89, rpScAMP and the peptide protein kinase inhibitor<sup>106,107</sup> block sperm motility and other downstream signaling pathways (see below). Although cAMP has multiple targets, these experiments suggested that PKA has a relevant role in capacitation. Consistent with this hypothesis, mammalian sperm contain a specific splicing variant of the PKA catalytic subunit alpha,  $\text{C}\alpha 2$ .<sup>108,109</sup> When the unique  $\text{C}\alpha 2$  exon is eliminated by homologous recombination, mice are sterile without presenting other problems. The sterility is due to defects in sperm capacitation that are phenotypically similar to the ones observed for the SACY knockout.<sup>109</sup> More recently, the same group has engineered another mouse model in which exon 5 of the PKA catalytic  $\alpha$ -subunit was replaced with a mutant form displaying a point mutation in the ATP-binding pocket.<sup>110</sup> Although this mutation is silent and PKA in this mice is able to perform its normal functions, this mutant, named  $\text{C}\alpha\text{M120A}$ , is

now sensitive to a bulky inhibitor such as 1NM-PP1, which is inactive against the wild-type PKA. Using sperm of these mice *in vitro*, the authors clearly showed PKA is required for the activation of flagellar beat and for the flagellar waveform asymmetry associated with hyperactivation.<sup>110</sup>

### Events downstream of PKA activation

Using the mouse as experimental model, it was shown that sperm capacitation is associated with an increase in protein tyrosine phosphorylation.<sup>111</sup> This work indicated that  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  and bovine serum albumin (or other cholesterol acceptors) were needed to support capacitation as well as the increase in tyrosine phosphorylation. Following studies demonstrated that glucose,<sup>112</sup>  $\text{Na}^+$  and  $\text{Cl}^-$  were also essential for capacitation and for the increase in phosphorylation<sup>76,96</sup>. Interestingly, the lack of protein tyrosine phosphorylation in the absence of each of these capacitation medium elements was reversed by activation of a cAMP pathway.<sup>96</sup> Moreover, in complete medium, PKA inhibitors such as H89 or Rp-cAMPS were able to block the increase in tyrosine phosphorylation.<sup>107</sup> Altogether, these data indicated that the increase in protein tyrosine phosphorylation was downstream of cAMP synthesis and activation of PKA. The role of cAMP in the increase in tyrosine phosphorylation has been further analyzed using genetically modified mice models.

SACY,  $\text{C}\alpha 2$  and AKAP4 are three genes essential for the function of the cAMP/PKA pathway in sperm. Elimination of each of these genes resulted in a male sterile phenotype.<sup>113</sup> All these null mice presented defects in the regulation of the protein tyrosine phosphorylation pathway as well as in several other capacitation-related events such as the onset of hyperactivated motility.<sup>104,114,115</sup> In addition, sperm lacking AKAP4 presented morphological flagellar defects likely due to the role of this protein in tail assembly.<sup>115</sup> Consistent with a role of PKA upstream the capacitation-associated increase in tyrosine phosphorylation, the aforementioned sperm from  $\text{C}\alpha\text{M120A}$ -expressing mouse lines did not undergo capacitation-associated changes in tyrosine phosphorylation when the mutated PKA was blocked by the bulky inhibitor 1NM-PP1.<sup>110</sup>

Despite the fact that many groups have shown similar regulatory pathways in sperm from other species, there is still a limited knowledge on the identity and the role of proteins phosphorylated in tyrosine residues. One of the first proteins to be identified as a substrate for tyrosine phosphorylation was human AKAP4.<sup>116</sup> Tyrosine phosphorylation of this protein was also demonstrated in hamster sperm<sup>117</sup> and the exact sites of tyrosine phosphorylation were mapped.<sup>118</sup> Other proteins known to be tyrosine phosphorylated in mammalian sperm are the valosin-containing protein, AKAP3 and CABYR,<sup>118,119</sup> a sperm-specific aldolase and pyruvate hydrogenase.<sup>74,120</sup> The role of these phosphorylations has not yet been elucidated; however, it is speculated that tyrosine phosphorylation may regulate either the activity or the location of these enzymes.

### Regulation of tyrosine phosphorylation: role of Src kinase family and ser/thr phosphatases

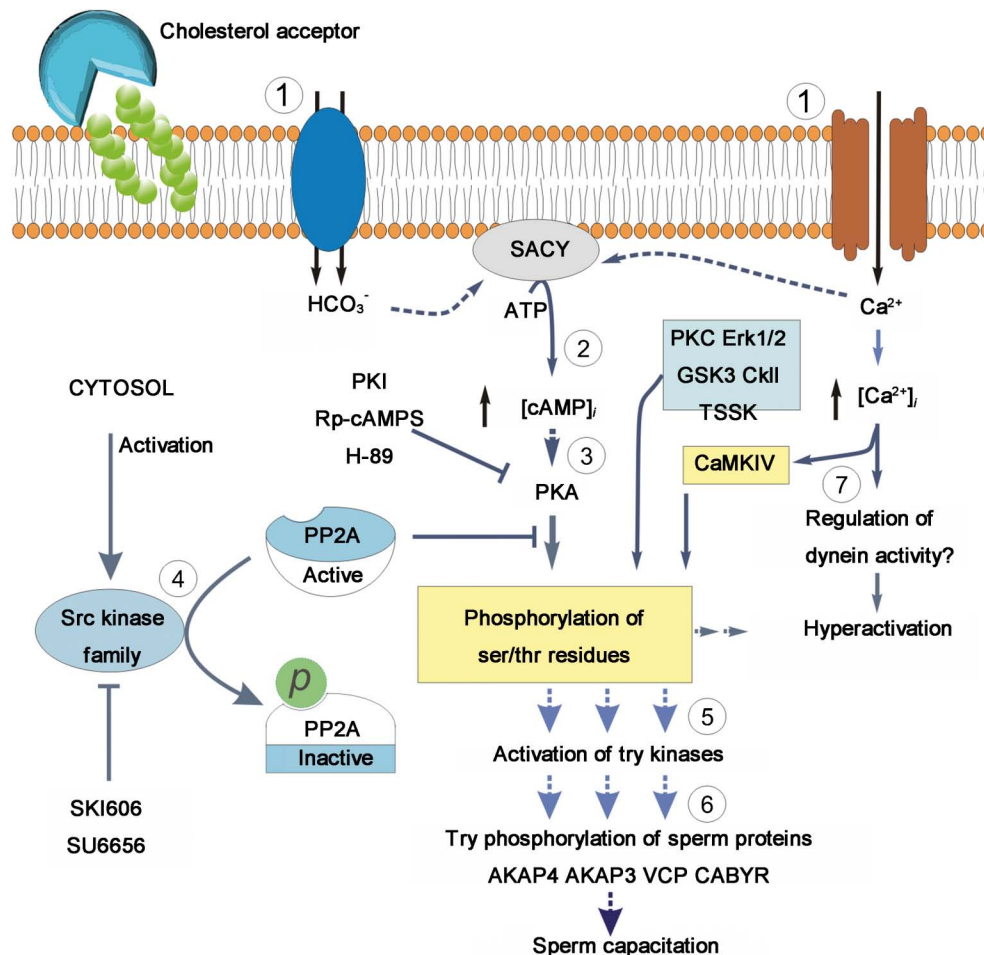
Another area of inquiry in the last years has been the identification of the tyrosine kinase(s) responsible for the observed changes in protein tyrosine phosphorylation accompanying the capacitation process. Recently, several groups have demonstrated the presence of cSrc in mouse and human sperm.<sup>121–124</sup> These publications also presented evidence that cSrc inhibitors blocked the capacitation-associated increase in tyrosine phosphorylation. These results were consistent with the hypothesis that cSrc plays a

role in the regulation of tyrosine phosphorylation in sperm. Challenging this conclusion, experiments conducted with sperm from cSrc null mice demonstrated that at least in this species, cSrc either is not essential for the increase in tyrosine phosphorylation or it can be replaced by other kinase in the cSrc null sperm.<sup>125</sup> Other results in this work indicated that cSrc family kinase inhibitors such as Bosutinib (SKI606) and SU6656 significantly reduced sperm motility parameters, and blocked IVF. These compounds blocked the increase in tyrosine phosphorylation; although this action was expected, these inhibitors also prevented direct phosphorylation by PKA, an event upstream of the increase in tyrosine phosphorylation. This observation was not explained by an unspecific effect of the cSrc inhibitors on PKA activity as shown using an *in vitro* kinase assay. Altogether, these results suggested the participation of a member of cSrc family kinase in the regulation of capacitation; however, they also indicated that a cSrc family member was upstream or parallel to the PKA activation and not an effector of this signaling pathway. It has been

established that cSrc kinases can regulate the activity of ser/thr phosphatases. In particular, the PP2A catalytic subunit exhibits a cSrc consensus phosphorylation site (TPDYFL) in its C-terminal domain that when phosphorylated downregulates its activity. If this pathway was active in sperm, it is predicted that inhibition of cSrc would result in upregulation of PP2A activity with a consequent dephosphorylation of ser/thr-phosphorylated substrates. Indeed, addition of low concentrations of okadaic acid or calyculin, two specific ser/thr phosphatase inhibitors, completely reversed the action of the cSrc inhibitors strongly suggesting that the role of a cSrc family kinase was mediated by a ser/thr phosphatase<sup>125</sup> (Figure 2).

### Other phosphorylation pathways observed during sperm capacitation

Although most is known about the regulation of the cAMP/PKA pathway and the downstream increase in tyrosine phosphorylation, several other protein kinases are present in sperm and are likely to play a role



**Figure 2** Model of the regulation of phosphorylation pathways during mammalian sperm capacitation. The overall pathway is modulated by cholesterol removal of sperm plasma membrane. ① Influx of  $\text{HCO}_3^-$  and  $\text{Ca}^{2+}$  stimulates an SACY. ② Increasing intracellular cAMP concentrations. ③ High cAMP levels activate PKA. This activation can be blocked *in vitro* by addition of the inhibitors H-89, Rp-cAMPS and the peptide PKI. ④ *In vivo*, phosphorylation of PKA substrates is subjected to regulation by a ser/thr phosphatase, likely PP2A. This phosphatase is in turn downregulated by a member of the Src kinase family by still unknown mechanisms. Activation of Src kinase family members can be blocked by *in vitro* addition of the inhibitors SU6656 or SKI-606 (Bosutinib). The onset of PKA substrates phosphorylation is followed by activation of unidentified tyrosine kinases ⑤ and the promotion of tyrosine phosphorylation of sperm proteins ⑥ (e.g., AKAP4, AKAP3, VCP and CABYR). ⑦ Intracellular  $\text{Ca}^{2+}$  increase might affect the axoneme directly, through selective regulation of dyneins. In addition,  $\text{Ca}^{2+}$  upregulates CaMKIV, leading to hyperactivation. PKA, protein kinase A; PKC, protein kinase C; PKI, protein kinase inhibitor; SACY, soluble adenylyl cyclase; VCP, valosin-containing protein.

in the regulation of sperm capacitation. Different PKC isoforms have been described in mammalian sperm.<sup>126,127</sup> For example, phorbol esters stimulate these kinases and change sperm motility,<sup>128</sup> the acrosome reaction<sup>129</sup> and cAMP levels.<sup>130</sup> Even though these phorbol ester effects are likely to be mediated by PKC, the possibility that other phorbol ester receptors such as chimaerins are present in sperm cannot be discarded.<sup>131</sup> Other ser/thr protein kinases reported in sperm are: Erk1/2,<sup>132,133</sup> GSK3,<sup>134</sup> CaMKIV,<sup>135</sup> and casein kinase II,<sup>136</sup> members of the testis-specific serine kinase family.<sup>137</sup> Interestingly, proline-directed phosphorylation appears to be regulated by cholesterol efflux.<sup>138</sup> Altogether, this evidence suggests that in addition to PKA, other kinases are involved in sperm capacitation; however, more research is needed to explain how they interact with other capacitation-associated pathways.

### Model of the regulation of phosphorylation during capacitation

During the last years, the textbook model of sperm capacitation has been linear. In this model,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  were involved in the activation of a cAMP/PKA pathway through the regulation of SACY. The finding that cSrc inhibitors obliterated phosphorylation of PKA substrates, without inhibiting PKA activation, has the implication that capacitation is also dependent on the activation of a second pathway. As part of this pathway, it is predicted that capacitation will also require the activation of a cSrc family kinase and that this activation will result in phosphorylation and downregulation of a ser/thr phosphatase. In parallel, activation of SACY with the consequent increase in cAMP synthesis will activate PKA. Both processes are needed to induce the increase in phosphorylation observed during sperm capacitation. How different components of the capacitation media interact to activate these pathways warrant future investigation (Figure 2).

## LIPIDS IN SPERM CAPACITATION

### Phospholipid transferases

It is now well established that phospholipids in the bilayer structure of cell membranes are distributed asymmetrically.<sup>139</sup> Under normal conditions, lipid distribution among the inner and the outer leaflet of the plasma membrane is maintained by the combined activity of several phospholipid transferases: an aminophospholipid transferase ('flipase') that moves phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet;<sup>140</sup> a nonspecific phospholipid transferase ('floppase') that transfers phospholipids from the inner to the outer leaflet;<sup>141</sup> and a bidirectional carrier ('scramblase') that moves all phospholipids in both directions across the membrane bilayer.<sup>142</sup> Under certain conditions, the normal phospholipid asymmetry maintained throughout cell life collapses, with the result that phosphatidylserine and phosphatidylethanolamine appear in the outer leaflet. Among other situations, such collapse (also known as 'scrambling') is seen during lymphocyte activation, red blood cell ageing and apoptosis.<sup>143-145</sup>

In sperm, scrambling is observed as an early capacitation event.<sup>146,147</sup> Analysis of phospholipid movements strongly suggests that the collapse of phospholipid asymmetry in these cells is due to an increase in the activity of a sperm scramblase.<sup>146</sup> Moreover, the change in phospholipid distribution appears to be stimulated by  $\text{HCO}_3^-$ , mediated by a cAMP/PKA pathway and enhanced by protein phosphatase inhibitors.<sup>146,147</sup> Importantly, while scrambling is a well-known characteristic of apoptosis (programmed cell death), there is evidence suggesting that the  $\text{HCO}_3^-$ -induced scrambling in sperm is not related to this process.<sup>148</sup> Although the role of phospholipid

scrambling in sperm capacitation is not well understood, it is likely to be connected with the need for serum albumin in this process. Phospholipid scrambling may facilitate albumin-mediated cholesterol extraction.<sup>146,149</sup> Though serum albumin may have other roles during capacitation such as promoting  $\text{Ca}^{2+}$  influx,<sup>150,151</sup> its ability to facilitate cholesterol efflux is required for capacitation. Capacitation is blocked by addition of cholesterol and/or cholesterol analogs to the capacitation medium.<sup>152</sup> Furthermore, serum albumin can be substituted by cholesterol-binding compounds such as high-density lipoproteins<sup>152,153</sup> and  $\beta$ -cyclodextrins<sup>154-157</sup> to induce capacitation *in vitro*.

### Cholesterol removal and lipid rafts

In spite of the fact that cholesterol efflux is involved in sperm capacitation, how this efflux modulates sperm physiology is not well understood. Possibly cholesterol concentrates in specialized sperm plasma membrane microdomains, also known as rafts.<sup>154-159</sup> Despite controversies regarding the methods used to obtain these rafts,<sup>160</sup> alternative approaches have emerged to identify proteins in these domains.<sup>161</sup> Several protein markers for these structures have been found in the sperm such as caveolin1 and cavelin2, as well as flotilin 1 and 2.<sup>161-164</sup> Most interestingly, sphingolipids such as GM1 and GM3 are also present in these cells. In the case of GM1, a capacitation-associated movement of this sphingolipid has been observed.<sup>165,166</sup> Using fluorescent cholera toxin-B subunit to track GM1 in mouse sperm may allow following capacitation in the female tract.<sup>166</sup> In addition, it has recently been proposed that GM1 binds decapacitation factors that are released as part of the capacitation process.<sup>167</sup>

In other cell types, lipid rafts bring protein assemblies together; it is predicted that changes in the cholesterol/phospholipid ratio within these structures can modulate their size and behavior.<sup>168,169</sup> In somatic cells, cholesterol-binding reagents such as beta-cyclodextrins, are capable of activating signaling pathways, for example, those involving tyrosine kinases, G proteins and/or other molecules.<sup>170-172</sup> As mentioned, the release of cholesterol accompanies sperm capacitation and correlates with an increase in protein phosphorylation. A working hypothesis consistent with this observation is that activation of raft complexes by cholesterol release and the regulation of phosphorylation pathways are coordinated and form part of the same signaling pathway. Alternatively or complementary to this hypothesis, changes in lateral mobility of membrane proteins as a consequence of cholesterol loss might be responsible for capacitation.<sup>173</sup>

## FINAL REMARKS

In view of the notion that the maturational process undergone by mammalian sperm in the female reproductive tract known as capacitation is essential for fertilization, there is a need to unravel the molecular processes involved in this complex and fundamental process. The discovery of sperm capacitation over 50 years ago opened new possibilities and a whole new era of reproductive medicine. However, while key aspects of capacitation including ionic transport, PKA stimulation and tyrosine phosphorylation of sperm proteins have significantly advanced, their functional interrelation is still not clearly understood. An initial fast bicarbonate-induced PKA activation appears to trigger capacitation both in the female tract and in *in vitro* systems. However, further work is needed to identify the ionic fluxes involved, the spatial PKA substrate arrangements and their signaling roles, as well as the sperm cAMP-dependent processes that are PKA-independent. Exciting new imaging, proteomic and electrophysiological tools are allowing researchers to tackle these and other open



questions in the field: What are the identities of the sperm proteins involved in capacitation that change their phosphorylation state? Which kinase(s) and phosphatases are responsible for modulating their function and interaction, and how are they regulated? Which are the identities of the transporters responsible for the key ion fluxes necessary for capacitation and how are they intertwined with the modulation of second messengers and phosphorylation changes? Are hyperactivation and capacitation parallel or independent and what is the role of the sperm-specific ionic channels CatSper and Slo3 in both processes? Is the concept of capacitation extensive to other species? Addressing such unsolved questions will bring fundamental progress into this fascinating field helping to set a better ground base for developing new infertility treatments, diagnostics and contraceptive techniques.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interest.

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