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# Human Sperm Thermotaxis Is Mediated by Phospholipase C and Inositol Trisphosphate Receptor $Ca^{2+}$ Channel[1]

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## ABSTRACT

**Capacitated human and rabbit spermatozoa can sense temperature differences as small as those within the oviduct of rabbits and pigs at ovulation, and they respond to them by thermotaxis (i.e., by swimming from the cooler to the warmer temperature). The molecular mechanism of sperm thermotaxis is obscure. To reveal molecular events involved in sperm thermotaxis, we took a pharmacological approach in which we examined the effect of different inhibitors and blockers on the thermotactic response of human spermatozoa. We found that reducing the intracellular, but not extracellular, $Ca^{2+}$ concentration caused remarkable inhibition of the thermotactic response. The thermotactic response was also inhibited by each of the following: $La^{3+}$, a general blocker of $Ca^{2+}$ channels; U73122, an inhibitor of phospholipase C (PLC); and 2-aminoethoxy diphenyl borate, an inhibitor of inositol 1,4,5-trisphosphate receptors ($IP_3R$) and store-operated channels. Inhibitors and blockers of other channels had no effect. Likewise, saturating concentrations of the chemoattractants for the known chemotaxis receptors had no effect on the thermotactic response. The results suggest that the $IP_3R$ $Ca^{2+}$ channel, located on internal $Ca^{2+}$ stores, operates in sperm thermotaxis, and that the response is mediated by PLC and requires intracellular $Ca^{2+}$. They also suggest that the thermosensors for thermotaxis are not the currently known chemotaxis receptors.**

*sperm chemotaxis motility and transport, sperm thermotaxis*

## INTRODUCTION

During the last decade, it became clear that mammalian spermatozoa possess two guidance mechanisms: thermotaxis and chemotaxis (for a review, see Eisenbach and Giojalas [1]). Although the characteristics of sperm chemotaxis are gradually being revealed, little information is available about the more recently discovered process of sperm thermotaxis. Furthermore, unlike sperm chemotaxis, which is known to occur in a large number of nonmammalian species [2–4], sperm thermotaxis has been hitherto demonstrated in mammals only [5].

Earlier studies in rabbits [6] and pigs [7] found that at ovulation, a temperature difference of 1°C–2°C exists between the sperm storage site and the fertilization site, the latter being warmer than the former. Later, Bahat et al. [8] demonstrated that the temperature difference in the rabbit's oviduct is created by a temperature drop at the storage site. They further showed that this difference is time dependent and is built up at ovulation (10.5–11.0 h after mating), rising from 0.8°C ± 0.2°C before ovulation to 1.6°C ± 0.1°C after ovulation. These findings, taken together with the observations that mammalian spermatozoa are capable of sensing small temperature differences and swim toward the warmer temperature [5], led to the suggestion that this ovulation-dependent temperature gradient guides spermatozoa by thermotaxis toward the warmer fertilization site [9]. This means that spermatozoa should possess a very sensitive thermosensing capability (0.16°C/cm in the case of rabbits [8, 9]). As in sperm chemotaxis [10–12], only capacitated spermatozoa are thermotactically responsive [5], restricting sperm thermotaxis to ready-to-fertilize cells only. Accordingly, it was suggested that capacitated spermatozoa are first guided from the cooler storage site toward the warmer fertilization site by thermotaxis [5, 9], and then, when in close proximity to the egg, by chemotaxis toward the steroid progesterone secreted from the cumulus cells surrounding the oocyte [13–15], followed by chemotaxis within the cumulus mass toward a yet unknown chemoattractant(s) secreted from the oocyte [16].

Nothing is known about the molecular mechanism underlying sperm thermotaxis. From two of the systems in which thermotaxis was most investigated, *Escherichia coli* bacteria and the nematode *Caenorhabditis elegans*, it appears that thermotaxis shares a high degree of commonality with chemotaxis, both at the behavioral and molecular levels. In bacteria, the chemotaxis receptors are also the thermosensors for thermotaxis [17, 18], and the rest of the signal transduction pathway is common. Furthermore, a temperature stimulus and a chemotactic stimulus trigger similar changes in swimming behavior [19]. In *C. elegans*, the thermosensory neuron is distinct from the chemosensory neurons [20, 21], but there are several molecular components that are used both in thermosensation and chemosensation [22, 23].

The mechanisms of thermosensing in eukaryotic cells appear to involve temperature-sensitive ion channels, mostly from the transient receptor potential (TRP) family. This large family includes, among others, six known temperature-sensitive channels: four heat-gated ion channels (TRPV1–TRPV4; for reviews, see Montell [24] and Patapoutian et al. [25]) and two cold thermosensors (TRPM8 and TRPA1) [26, 27]. Of these channels, TRPV1 was detected by immunocytochemical analysis on the head of boar spermatozoa [28], TRPV4 was shown to be expressed in the rat testis [29], and TRPM8 was identified on the head and flagellum of human spermatozoa and was shown to cause elevation of intracellular $Ca^{2+}$ ($Ca^{2+}_{in}$) in response to temperature decrease or ligand (menthol) binding [30]. It is not known whether the other TRPV channels exist in spermatozoa. Several members of the TRPC subfamily have been identified on the head and the flagellum of mouse and human spermatozoa [31–33]. Howev-

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er, there is no indication that these TRPC $Ca^{2+}$ channels can function as thermosensors. Other channels reported as thermosensors (but not belonging to the TRP family) are heat-activated and cold-activated $Ca^{2+}$ channels in the ciliate *Paramecium*, each with a different ion selectivity [34], a heat-activated $K^+$ channel in sponges [35], and a heat-activated background channel (KCNK2 or TREK-1) in the peripheral sensory neurons and central hypothalamic neurons [36].

It is well known that $Ca^{2+}$ signaling in spermatozoa is critical for fertilization, because it is a major player in motility and hyperactivation [37–39], capacitation [40], and the acrosome reaction [41]. It also plays an important role in mammalian [39, 42, 43] and nonmammalian [44–47] sperm chemotaxis. In addition, $Ca^{2+}$ ions were found to be involved in the signal-transduction pathways of chemotaxis and thermotaxis in several unicellular species [48–50], and $Ca^{2+}$ channels were found to play a major role in thermotaxis of several eukaryotic cells [25]. Human spermatozoa are known to possess two types of $Ca^{2+}$ stores as well as a range of channels, pumps, and transporters, allowing the spermatozoa to generate $Ca^{2+}$ signals [51].

In the present work, we looked for molecular events that mediate human sperm thermotaxis. Our approach was restricted to pharmacologically testing candidates known to act as thermosensors or signaling molecules in other systems for their possible involvement in human sperm thermotaxis. This restriction evolved from our finding that probes used to monitor intracellular ions, at least $Ca^{2+}{}_{in}$, are themselves sensitive to temperature changes in the temperature range of thermotaxis, and therefore cannot be used for studying $Ca^{2+}{}_{in}$ changes induced by a temperature jump (Bahat and Eisenbach, unpublished observations). In the absence of molecular markers for thermotaxis, our pharmacological study, consisting of studying the effects of different inhibitors and blockers on the thermotactic response, was further restricted to drug concentrations that did not inhibit motility, and thereby allowed the determination of thermotaxis by swimming spermatozoa. In spite of these restrictions, we identified the involvement of $Ca^{2+}{}_{in}$, phospholipase C (PLC), and inositol 1,4,5-trisphosphate receptors ($IP_3R$) in sperm thermotaxis.

## MATERIALS AND METHODS

### Chemicals

The $Ca^{2+}$ ionophore A23187, choline chloride, nickel chloride, progesterone, propidium iodide, dimethyl sulfoxide, 2-aminoethoxydiphenyl borate, ethylene glycol-bis (β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and all of the compounds for enriched Biggers, Whitten, and Whittingham (BWW) medium (each of the highest purity available) were obtained from Sigma (St. Louis, MO); BAPTA-AM from AnaSpec (San Jose, CA); mouse anti-human CD46-fluorescein isothiocyanate (CD46-FITC) from Serotec (Oxford, UK); human serum albumin (HSA) solution from Irvine Scientific (Santa Ana, CA); *N*-(4-tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide (BCTC) and bourgeonal from BIOMOL (Plymouth Meeting, PA); and U73122 from Calbiochem (Gibbstown, NJ). Lanthanum chloride (Merck) was a gift from M. Segal, tetraethylammonium and ruthenium red (both from Sigma) were a gift from E. Reuveny, and nifedipine (Sigma) was a gift from M. Walker, all of whom are at the Weizmann Institute of Science, Rehovot, Israel.

### Media

For handling and capacitating human spermatozoa, we used either the commercially available Flushing Medium (MediCult, Jyllinge, Denmark) supplemented with 0.2% HSA (bringing the total HSA concentration to 0.3%), or enriched BWW (BWW medium [95 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 20 mM sodium lactate, 5 mM glucose, 0.25 mM sodium pyruvate, and 25 mM $NaHCO_3$, pH 7.4] [52] enriched with 40 mM Hepes [pH 7.4] and 0.3% HSA). Oocyte-cumulus-conditioned medium was obtained from the in vitro fertilization unit in Barzilai Medical Center (Ashkelon, Israel) and was prepared by incubating oocyte-cumulus complexes in standard P-1 medium (Irvine Scientific), as described in Sun et al. [16].

### Spermatozoa

Human semen samples were obtained from healthy donors after 3 days of sexual abstinence. Informed consent was obtained from each donor, and the study was approved by the Weizmann Institute of Science. Semen samples with normal sperm density, motility, and morphology (according to WHO guidelines [53]) were allowed to liquefy for 30–60 min at room temperature. Human spermatozoa were separated from the seminal plasma by centrifugation ($120 \times g$, 10 min, twice) with capacitating medium (either enriched BWW or Flushing Medium supplemented with additional 0.2% HSA). After this procedure, the sperm concentration was adjusted to $5 \times 10^6$ to $40 \times 10^6$ cells/ml. Each sperm sample was analyzed for motility parameters using a Makler counting chamber (Sefi Medical Instruments Ltd.) and a computerized sperm analysis software program (Hobson Tracking System Ltd.) as described below. For capacitation, the sperm suspensions were incubated under an atmosphere of 5% $CO_2$ at 37°C for 2 h [12].

### Analysis of Sperm Kinetic Parameters

The analysis of sperm kinetic parameters was carried out by a computerized motion analysis system (Hobson Sperm Tracking System; 250 cells each analysis). The measured kinetic parameters were: curvilinear velocity (VCL; the time-averaged velocity of the sperm head along its actual trajectory); straight-line velocity (VSL; the time-averaged velocity of the sperm head along a straight line from its first position to its last position; also called *progressive velocity*); percent linearity (LIN; the ratio VSL:VCL, multiplied by 100); percent straightness (STR; the ratio between the straight line from the first point on the smoothed path to the last point on this path and the total distance along the smoothed path, multiplied by 100); percent motile cells (MOT); percent hyperactivated cells (HYP; cells that have a motility pattern characterized by increased velocity, decreased linearity, increased amplitude of lateral head displacement, and flagellar whiplash movement) [54, 55].

### Thermotaxis Assay

For detecting the thermotactic response, we used a thermoseparation device (developed and designed by ReproMed) consisting of two basic units: an electrical unit to maintain the temperature gradient (Fig. 1a) and a Lucite tube for the separation process (Fig. 1b). The tube was composed of two compartments separated by a thin disc (made of medical grade 316 stainless steel) having pores 40 μm in diameter. One compartment was filled with human spermatozoa ($40 \times 10^6$ cells/ml) that had been allowed to capacitate for 2 h, and the other compartment was filled with the same medium but with no spermatozoa. The tube was inserted to the thermoseparation device, forming a linear temperature gradient along the tube (verified experimentally; from 33°C at the edge of the sperm compartment to 37°C at the edge of the medium compartment). The tube was incubated in this device for 15 min, allowing the migration of spermatozoa from one compartment to the other. Finally, the spermatozoa were collected from the warmer compartment and counted by a hemacytometer. As a negative control for coincidental migration to the other compartment, the same procedure was repeated, but this time with a constant temperature (no gradient) along the tube, with both compartments being at the same temperature, 37°C. (In the first experiments, we also examined 33°C as the constant temperature. Because there was no significant difference between the two controls, we continued with the more stringent control, 37°C, at which the swimming speed of the spermatozoa is somewhat faster than at 33°C.) As a negative control for passive migration, the same procedure (with a temperature gradient) was repeated, but this time with dead spermatozoa substituting for the live cells.

### Examination of the Effect of Different Drugs on Thermotaxis

After the incubation for capacitation, different drugs (inhibitors, blockers, and chelators) were added to the sperm suspension and incubated at 37°C as indicated in the text. This drug-containing sperm suspension was then inserted to the thermoseparation tube. The same drug concentration was added also to the medium compartment so as to avoid a concentration gradient of the drug in the tube. As a negative control for the effect of each drug, we added to the cells the drug solvent and tested its effect on the thermotactic response (defined as 100% response). The drugs used in the experiments were verified to be active in other systems.

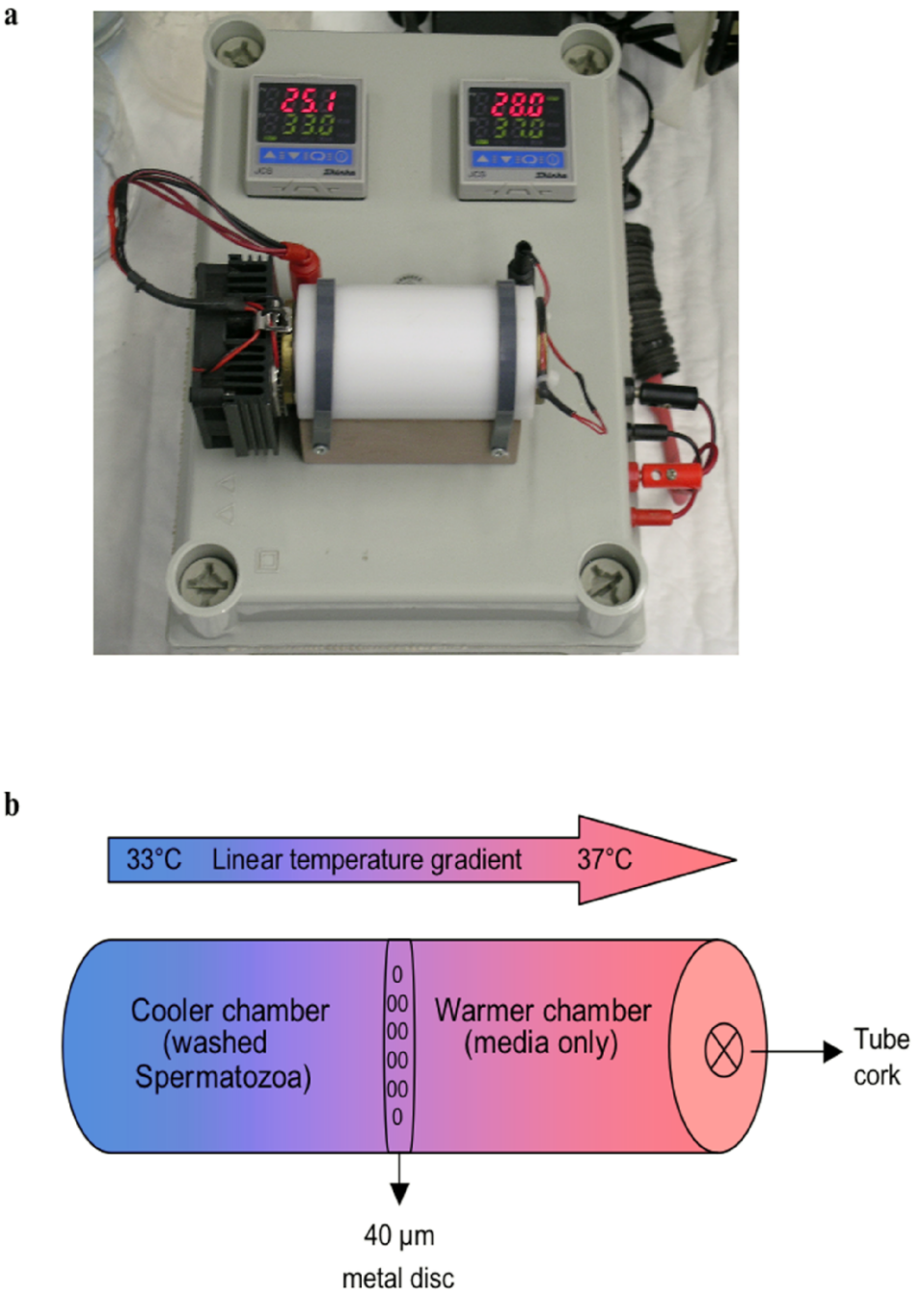


FIG. 1. A device for sperm thermoseparation. **a**) An electrical unit to maintain a temperature gradient. The numbers in green are the designate temperature, and the numbers in red indicate the current temperature during warming. **b**) A scheme of the thermotaxis separation tube.

### *Acrosome-Reaction Induction and Determination of the Fraction of Acrosome-Reacted Cells*

The fraction of capacitated spermatozoa was determined from the difference between the levels of acrosome-reacted spermatozoa before and after an acrosome-reaction induction [40, 56]. The acrosomal marker CD46-FITC was used to visualize the state of the acrosome. For stimulating the acrosome reaction, human spermatozoa ($5 \times 10^6$ to $20 \times 10^6$ cells/ml) were incubated for 30 min at 37°C with A23187 (10 µM from a stock solution in dimethyl sulfoxide [DMSO]; the final DMSO concentration was 0.2%) or, as a negative control, with DMSO. Subsequently, A23187 or DMSO remnants were washed off the cells by centrifugation ($300 \times g$, 10 min) with PBS, and the spermatozoa were incubated for 30 min at room temperature with 1% BSA to block nonspecific sites. After washing the blocker off with PBS by centrifugation ($300 \times g$, 10 min), anti-human CD46-FITC was added to the pellet (1:50) for 30 min at room temperature in the dark. The spermatozoa were again washed with PBS ($300 \times g$, 10 min), and the pellet was transferred to a 4-ml fluorescence-activated cell sorting (FACS) tube. Immediately prior to FACS analysis, the supravital probe propidium iodide (2.5 µg/ml final concentration) was added. Samples of 10 000 live cells were analyzed by a Becton Dickinson FACSort.

### *Statistical Analysis*

InStat 3 software package (Graph Pad Software, San Diego, CA) was used for statistical calculations. The significance of the difference between the treatments was calculated by Student *t*-test or ANOVA one-way test, as indicated.

## RESULTS

### *Setting a New Thermotaxis Assay*

To carry out the pharmacological study, we had to have a high-fidelity, relatively rapid thermotaxis assay. To this end, we used a thermoseparation device developed by ReproMed and described in *Materials and Methods*. Each treatment

consisted of a pair of thermoseparation tubes, one subjected to a temperature gradient and one to a constant temperature (no gradient). For each treatment, we calculated the gradient-dependent sperm accumulation (Fig. 2, net thermotaxis bar) by subtracting the number of cells that apparently arrived coincidently at the other compartment without temperature guidance (i.e., in the absence of a temperature gradient; Fig. 2, control bar) from the number of cells that arrived there in the presence of a temperature gradient (Fig. 2, middle bar). The net thermotactic response of nontreated (when no drug was added) capacitated human spermatozoa was usually in the range of 5%–9%, meaning that a little more than 5%–9% of the sperm population was temperature guided to the warmer compartment. (This ''little more,'' 0.2%–0.4% of responsive cells, resulted from the fact that the no-gradient control also contained potentially responsive cells that arrived coincidentally.)

### *The Involvement of $Ca^{2+}$ in the Mechanism of Sperm Thermotaxis*

To determine whether or not $Ca^{2+}$ is involved in the thermotactic response of spermatozoa, we reduced the extracellular and intracellular $Ca^{2+}$ concentrations (one at a time, termed $Ca^{2+}_{ex}$ and $Ca^{2+}_{in}$, respectively) of capacitated human spermatozoa to predetermined values by treating them with calculated concentrations of ethylene glycol tetraacetic acid (EGTA) and BAPTA-AM, respectively. We then examined their thermotactic response.

Because some $Ca^{2+}$ channels, particularly from the TRP family, are known to be somewhat permeable to $Na^+$ [57–59] and $Mg^{2+}$ [60] (although there is no evidence that both ions can functionally replace $Ca^{2+}$ within the cell), for the EGTA experiments we incubated the cells for capacitation in enriched BWW medium free of both $Na^+$ and $Mg^{2+}$ (with choline substituting for $Na^+$). After the 2-h incubation, we added EGTA to a final concentration of 2.5 mM (yielding calculated free $Ca^{2+}_{ex}$ of 51 nM when taking into consideration the original $Ca^{2+}$ concentration in the suspension)—the highest tested EGTA concentration that did not abolish sperm motility (the reduction in the speed of swimming and the percentage of hyperactivated cells were statistically insignificant; Table 1). Under these conditions, the thermotactic response after the chelator addition was not different from that in the absence of EGTA (Fig. 3 and Supplemental Fig. S1; all Supplemental Data are available online at www.biolreprod.org), suggesting that $Ca^{2+}_{ex}$ is not obligatory for sperm thermotaxis, at least not at concentrations higher than those required for motility.

We examined the effect of BAPTA-AM in enriched BWW medium that contained no added $Ca^{2+}$ and $Mg^{2+}$. This is because $Ca^{2+}_{ex}$ may enter the cells and somewhat compensate for the $Ca^{2+}$ ions chelated intracellularly by BAPTA [61], and $Mg^{2+}$ may potentially substitute for $Ca^{2+}$ [60]. BAPTA-AM at the indicated concentration strongly inhibited the thermotactic response (Fig. 3 and Supplemental Fig. S2) without affecting the capacitation level of the cells (Fig. 4) or their motility (Table 1). These results, taken together with the results obtained in the presence of EGTA (Fig. 3), suggest that $Ca^{2+}_{in}$ is required for thermotaxis.

In view of the fact that most thermosensors of eukaryotic cells are temperature-gated $Ca^{2+}$ channels [34, 62, 63], we examined whether $Ca^{2+}$ channels are involved in sperm thermotaxis. To that purpose, we studied the effect of $La^{3+}$—a potent $Ca^{2+}$ competitor [64] and, consequently, a general blocker of $Ca^{2+}$ channels [65]—on the thermotactic response. ($La^{3+}$ readily inhibits $Ca^{2+}$ channels on the cell membrane;

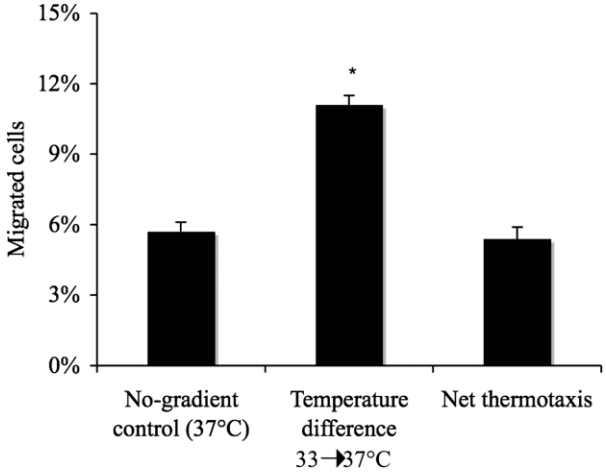


FIG. 2. Migration of human spermatozoa from 33°C to 37°C. The thermotaxis assay was carried out as described in *Materials and Methods*. Net thermotaxis was calculated by subtracting the no-gradient control from the sperm accumulation in a temperature gradient. The results are the mean ± SEM of eight determinations. The asterisk indicates a significant difference ($P < 0.0001$) relative to the no-gradient control, as determined by the unpaired Student *t*-test.

however, long enough incubation with relatively high $La^{3+}$ concentrations, as in this study, also inhibits $Ca^{2+}$ channels on intracellular membranes [66–70].) $La^{3+}$ significantly inhibited the thermotactic response (Fig. 3 and Supplemental Fig. S3) without affecting the motility parameters of the spermatozoa (Table 1) or the fraction of capacitated cells (Fig. 4). These results suggest that $Ca^{2+}$-associated proteins are involved in sperm thermotaxis. On the basis of analogy with other thermosensing systems, we assumed that $Ca^{2+}$ channels, rather than other $Ca^{2+}$ carriers and transporters, are involved in sperm thermotaxis. Therefore, we next tried determining what type of $Ca^{2+}$ channels might be involved. Although the involvement of $Ca^{2+}_{in}$ in thermotaxis (Fig. 3) suggested that the $Ca^{2+}$ channel, if it is indeed involved, would be an internal channel, the results could not exclude the possibility of involvement of an external channel that carries $Ca^{2+}$ out (e.g., TRPC3, TRPC6, TRPC7, TRPV1, TRPV2, TRPV4, and TRPM7—see Clapham et al. [71] for a review). Therefore, we did not limit the search to internal channels only.

### *Proteins Tested to Be Involved in Thermosensing*

*TRP $Ca^{2+}$ channels.* As mentioned above, most of the heat-gated $Ca^{2+}$ channels in eukaryotic cells belong to the TRPV subfamily. To examine whether TRPV channels are involved in sperm thermotaxis, we studied the effect of their general inhibitor, ruthenium red [25], on the thermotactic response. Ruthenium red, tested in a range of concentrations, did not affect the thermotactic response (Fig. 5a), suggesting lack of involvement of TRPV channels in sperm thermotaxis. The activity of this drug was verified by E. Garty and E. Reuveny (personal communication), demonstrating that the same batch of ruthenium red successfully blocked the electrical current produced by a TRPV1-expressing *Xenopus* oocyte.

Next, we examined whether TRPC channels, identified on mouse and human spermatozoa and known to be essential for nerve growth-cone guidance [72] and for detection of pheromonal signals by the vomeronasal (olfactory sense) organ [73], participate in sperm thermotaxis. Because these channels are activated through PLC [24, 74], we studied the effect of a PLC inhibitor on the thermotactic response. U73122, known to

TABLE 1. Kinetic parameters of human sperm motility in different media and in the presence of different chelators and inhibitors.[a]

| Tested medium and chelator | VCL (μm/sec) | VSL (μm/sec) | STR (%) | LIN (%) | MOT (%) | HYP (%) |
|---|---|---|---|---|---|---|
| Control (enriched BWW) | 91 ± 4 | 59 ± 4 | 83 ± 4 | 66 ± 7 | 60 ± 4 | 10 ± 3 |
| $Na^+$- and $Mg^{2+}$-free enriched BWW | 94 ± 14 | 50 ± 6 | 76 ± 11 | 58 ± 13 | 55 ± 14 | 25 ± 15 |
| EGTA (2.5 mM) in $Na^+$- and $Mg^{2+}$-free enriched BWW | 75 ± 13 | 40 ± 5 | 79 ± 9 | 58 ± 10 | 48 ± 12 | 17 ± 12 |
| Enriched BWW with 0.05% DMSO | 90 ± 9 | 63 ± 5 | 86 ± 6 | 72 ± 10 | 47 ± 11 | 11 ± 7 |
| $Ca^{2+}$ and $Mg^{2+}$-free enriched BWW with 0.05% DMSO | 82 ± 7 | 64 ± 3 | 91 ± 3 | 78 ± 6 | 59 ± 3 | 5 ± 2 |
| BAPTA-AM (50 μM) in $Ca^{2+}$- and $Mg^{2+}$-free enriched BWW | 76 ± 7 | 49 ± 6 | 85 ± 5 | 66 ± 7 | 37 ± 8 | 12 ± 7 |
| Control (Flushing medium) | 104 ± 6 | 61 ± 2 | 82 ± 2 | 59 ± 3 | 71 ± 4 | 7 ± 1 |
| $La^{3+}$ (0.4 mM) in Flushing medium | 103 ± 10 | 66 ± 3 | 89 ± 4 | 66 ± 7 | 68 ± 13 | 4 ± 4 |
| Control (Flushing medium) | 97 ± 1 | 61 ± 9 | 81 ± 6 | 63 ± 9 | 37 ± 3 | 19 ± 7 |
| U73122 (5 μM) in Flushing medium | 104 ± 5 | 50 ± 5 | 72 ± 6 | 50 ± 7 | 38 ± 6 | 27 ± 4 |
| Control (Flushing medium) | 84 ± 8 | 52 ± 5 | 76 ± 4 | 57 ± 4 | 69 ± 10 | 7 ± 3 |
| 2-APB (0.3 mM) in Flushing medium | 91 ± 12 | 25 ± 4[b] | 59 ± 5 | 28 ± 1[b] | 56 ± 16 | 29 ± 11 |

[a] The results shown are the mean ± SEM of three determinations (250 cells in each).
[b] Values are significantly different ($P \leq 0.014$) relative to the respective control, as determined by the unpaired Student *t*-test.

directly block PLC in a specific manner, inhibited the thermotactic response by 50% (Fig. 5b and Supplemental Fig. S4) without affecting significantly the fraction of capacitated cells (Fig. 4) or the kinematic parameters of the spermatozoa (Table 1). It is therefore possible that PLC and, perhaps, TRPC channels are involved in sperm thermotaxis.

Recently, De Blas et al. [30] demonstrated the presence of TRPM8 in human spermatozoa and suggested that it might have a function in sperm thermotaxis or chemotaxis. We therefore examined its possible involvement in human sperm thermotaxis, even though the likelihood of being involved was low because of being a cold-activated channel below the range of our experiments (≤25°C) [25]. We looked for an effect of its antagonist, BCTC, on sperm thermotaxis. BCTC had no effect (Fig. 5b and Supplemental Fig. S5), suggesting that TRPM8 is not involved in human sperm thermotaxis.

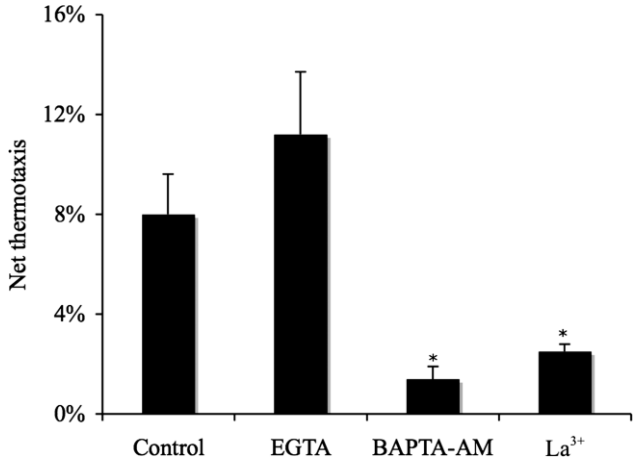


FIG. 3. The effects of $Ca^{2+}$ chelators and blocker on the thermotactic response. The results with each chelator or blocker as well as the SEM values were normalized according to the BAPTA-AM control, the normalizing factor being the ratio between the average accumulation in the control under a certain set of conditions and the average accumulation in the control in the presence of BAPTA-AM. The nonnormalized results with the respective individual controls for each set of conditions can be seen in Supplemental Figures S1–S3. The chelators and blocker were at equal concentration throughout the measuring thermotaxis tube. The results are the mean ± SEM of three to six determinations. The effect of 2.5 mM EGTA (free $Ca^{2+}_{ex}$ was ~51 nM, calculated by MaxChelator software (Stanford University, Stanford, CA)) was tested in $Mg^{2+}$-free and $Na^+$-free enriched BWW immediately after the chelator addition. The effect of 50 μM BAPTA-AM (the calculated $Ca^{2+}_{in}$ was ~0.4 nM, calculated for 37°C by MaxChelator software, assuming 50 μM intracellular BAPTA concentration, 120 nM $Ca^{2+}_{in}$, 0.5 mM $Mg^{2+}_{in}$, and $pH_{in}$ 6.6) was tested in $Ca^{2+}$-free and $Mg^{2+}$-free enriched BWW after preincubation of 30 min with the spermatozoa. The effect of 0.4 mM $La^{3+}$ was tested after 20 min of preincubation with the spermatozoa. Asterisks indicate a significantly different thermotactic response ($P < 0.01$) relative to the respective control ($Ca^{2+}$-free and $Mg^{2+}$-free enriched BWW supplemented with 0.05% DMSO in the case of BAPTA-AM, and Flushing Medium alone in the case of $La^{3+}$), as determined by the unpaired Student *t*-test.

*Voltage-gated $Ca^{2+}$ channels.* We also tested the possibility of voltage-gated $Ca^{2+}$ channel involvement in sperm thermotaxis by examining the effect of two selective blockers of such channels: $Ni^{2+}$, known to block T-type voltage-dependent $Ca^{2+}$ channels (activated by weak depolarization), and nifedipine, which blocks L-type voltage-dependent $Ca^{2+}$ channels (activated by strong depolarization) [75]. The blockers were at concentrations known to effectively block these voltage-dependent $Ca^{2+}$ channels in human spermatozoa [75, 76]. Nifedipine, freshly dissolved in DMSO in the dark before each experiment, was verified to be active by its inhibitory, concentration-dependent effect on sperm motility, the inhibition being complete at concentrations >0.2 mM. Because in certain cases the inhibitory effect of $Ni^{2+}$ is recognized only in the absence of $Ca^{2+}$ and $Mg^{2+}$ (because of their competition with $Ni^{2+}$ [34]), we tested the effect of $Ni^{2+}$ also in the absence of both of these ions. Within the experimental error, each of these blockers had no effect on the thermotactic response, independently of whether $Ni^{2+}$ was added to a medium that contained (Fig. 5b and Supplemental Fig. S6) or lacked (data not shown) $Ca^{2+}$ and $Mg^{2+}$. This suggests that the main voltage-gated $Ca^{2+}$ channels are uninvolved in thermotaxis.

*$K^+$ channels.* Because two similar $K^+$ channels are responsible for peripheral thermosensing by mammals and sponges [35, 36], we examined the involvement of $K^+$ channels in sperm thermotaxis. We employed two approaches. In one approach, we treated the cells with tetraethylammonium (TEA; a specific blocker of the six-transmembrane family of $K^+$ channels [77]) within the concentration range known to block $K^+$ channels in human spermatozoa [78]. TEA did not affect the thermotactic response (Fig. 5b and Supplemental Fig. S7). Because TEA does not inhibit other types of $K^+$ channels, we used an additional approach in which we tested the effect of $K^+$-free medium and a high external $K^+$ concentration (15 mM) on the thermotactic response, thus interfering with inward and outward $K^+$ fluxes, respectively. Again, no effect on the thermotactic response was observed (Fig. 5b and Supplemental Fig. S7), suggesting that the main $K^+$ channels are not involved in sperm thermotaxis.

*$IP_3R$ and store-operated $Ca^{2+}$ channels.* The experiments described until now suggest the possible involvement of PLC, known to hydrolyze phosphatidylinositol 4,5-bisphosphate

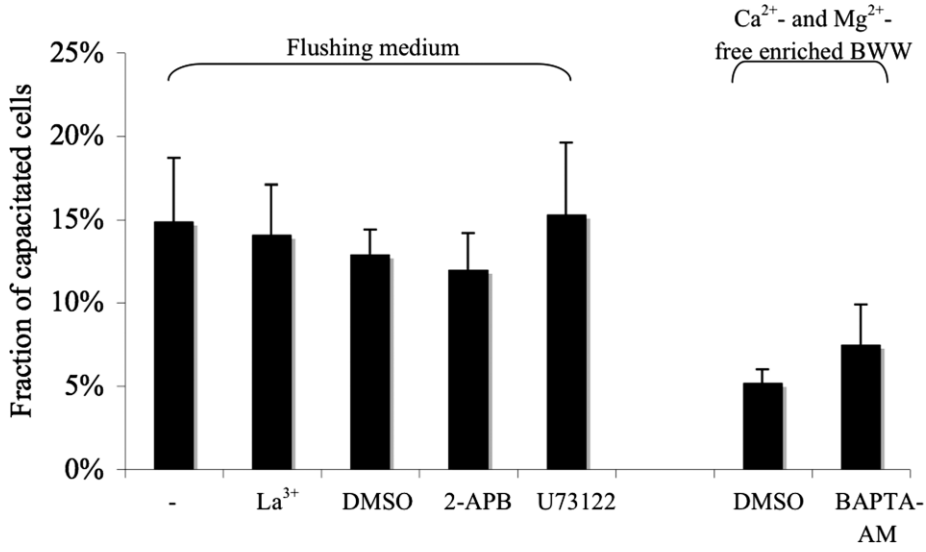


FIG. 4. The effect of inhibitors and blocker used in this study on the fraction of capacitated cells. The fraction of capacitated spermatozoa was determined from the difference between the levels of acrosome-reacted spermatozoa before and after the acrosome-reaction induction with the $Ca^{2+}$ ionophore A23187. Acrosome-reacted spermatozoa were identified by the acrosomal marker CD46-FITC. The different inhibitors and their solvents were incubated with capacitated spermatozoa (spermatozoa that had been incubated for 2 h under capacitating conditions) at 37°C as follows: $La^{3+}$ (0.4 mM), 0.5% DMSO (in Flushing Medium), 2-APB (0.3 mM), and U73122 (5 μM) for 20 min; and 0.05% DMSO (in $Ca^{2+}$-free and $Mg^{2+}$-free enriched BWW) and BAPTA-AM (50 μM) for 30 min. In the case of BAPTA-AM, 1.3 mM $Ca^{2+}$ was added together with the ionophore. The results are the mean ± SEM of three to five determinations. -, control.

($PIP_2$) to inositol $IP_3$ and diacylglycerol (DAG) [79]. $IP_3$ is known to bind to its receptor ($IP_3R$) on an internal $Ca^{2+}$ store to trigger $Ca^{2+}$ release and, consequently, $Ca^{2+}_{in}$ increase [80]. The depletion of $Ca^{2+}$ store may activate store-operated $Ca^{2+}$ channels (SOCs), with a resultant entrance of $Ca^{2+}_{ex}$ into the cell to refill the store (a mechanism known as the store-operated, or capacitative, $Ca^{2+}$ entry [81]). Therefore, we tested the possibility that $IP_3R$ and/or SOCs are involved in sperm thermotaxis. For this purpose, we used 2-aminoethoxydiphenyl borate (2-APB), a compound known to block both SOCs and $IP_3R$. Application of 2-APB (at a concentration within the effective range in other cell types [80]) to human spermatozoa strongly inhibited the thermotactic response (Fig. 6 and Supplemental Fig. S8) without affecting the fraction of capacitated spermatozoa (Fig. 4). The inhibitor decreased the linearity and velocity of swimming and increased the level of hyperactivated cells (Table 1). However, because such changes in these kinetic parameters do not affect the thermotactic response (Fig. 7), it is unlikely that they are the causes of the observed inhibition of sperm thermotaxis. This suggests that SOCs and/or $IP_3R$ may be involved in the thermotactic response. To examine whether SOCs alone are involved in thermotaxis, we treated the cells with $Gd^{3+}$, a blocker of SOCs when employed at low concentrations [82]. $Gd^{3+}$ had no effect on the thermotactic response (Fig. 6 and Supplemental Fig. S9). Together, these results suggest that $IP_3R$ $Ca^{2+}$ channels are probably involved in the thermotactic response of human spermatozoa.

### *Chemoreceptors*

To determine whether chemotaxis and thermotaxis of human spermatozoa share the same receptors, we saturated the sperm chemoreceptors with different known chemoattractants [1, 16, 43] and examined the thermotactic response of the spermatozoa, as described in *Materials and Methods*. Our notion was that if the same receptors were involved in these two processes, saturating a receptor with its ligand chemoattractant should render it insensitive to further stimulation, independently of whether the stimulation is chemical or temperature. We found that saturating concentrations of an oocyte-cumulus-conditioned medium (diluted 1:100; i.e., 100-fold more concentrated than the dilution that yields maximal chemotactic activity [16]), bourgeonal (10 μM), and progesterone (1 μM), all pretested and verified to be chemotactically active in our chemotaxis assays, had no effect on the thermotactic response (Fig. 8 and Supplemental Fig. S10). This suggests that the thermosensors are not chemotaxis receptors for the currently known chemoattractants.

## DISCUSSION

The aim of this study was to identify molecular events and players involved in the mechanism of human sperm thermotaxis. As discussed below, the results suggest the involvement of $Ca^{2+}_{in}$, PLC and $IP_3R$ in sperm thermotaxis (Fig. 9).

### *The Experimental System*

The thermoseparation device used in this study yielded highly stable and reliable results. Yet, the device measures sperm accumulation rather than changes in the direction of swimming. Although sperm accumulation can also be caused by trapping of any kind, not only by thermotaxis [42, 83], in this particular case we have already demonstrated that sperm accumulation faithfully reflects thermotaxis, where spermatozoa change their swimming direction to move up the temperature gradient [5]. Furthermore, all of the experiments of this study were carried out in a temperature range that did not affect the speed and swimming pattern of the cells, thus eliminating these factors as potential causes of trapping.

### *$Ca^{2+}_{in}$, PLC, and $IP_3R$ $Ca^{2+}$ Channels Are Apparently Involved in Sperm Thermotaxis*

$Ca^{2+}$ ions are major players in many sperm functions, including chemotaxis [42–46], and they are involved in thermotaxis of several different systems [25, 48–50]. The strong inhibition of the thermotactic response by $La^{3+}$ (a common $Ca^{2+}$ competitor [64]; Fig. 3 and Supplemental Fig. S3) is consistent with an involvement of a $Ca^{2+}$ channel(s) in sperm thermotaxis. Although $La^{3+}$ may inhibit almost any $Ca^{2+}$-associated response, its most pronounced effect is inhibition of $Ca^{2+}$ channels [64], independently of whether they reside on the cell surface [84], allowing $Ca^{2+}$ fluxes inwardly, or in internal membranes [66–70], releasing $Ca^{2+}$ within the cell from internal stores.

We distinguished between these possibilities by the $Ca^{2+}$ chelators, BAPTA-AM (a $Ca^{2+}_{in}$ chelator [85]; Fig. 3 and Supplemental Fig. S2) and EGTA (a $Ca^{2+}_{ex}$ chelator [86, 87]; Fig. 3 and Supplemental Fig. S1) that restricted the

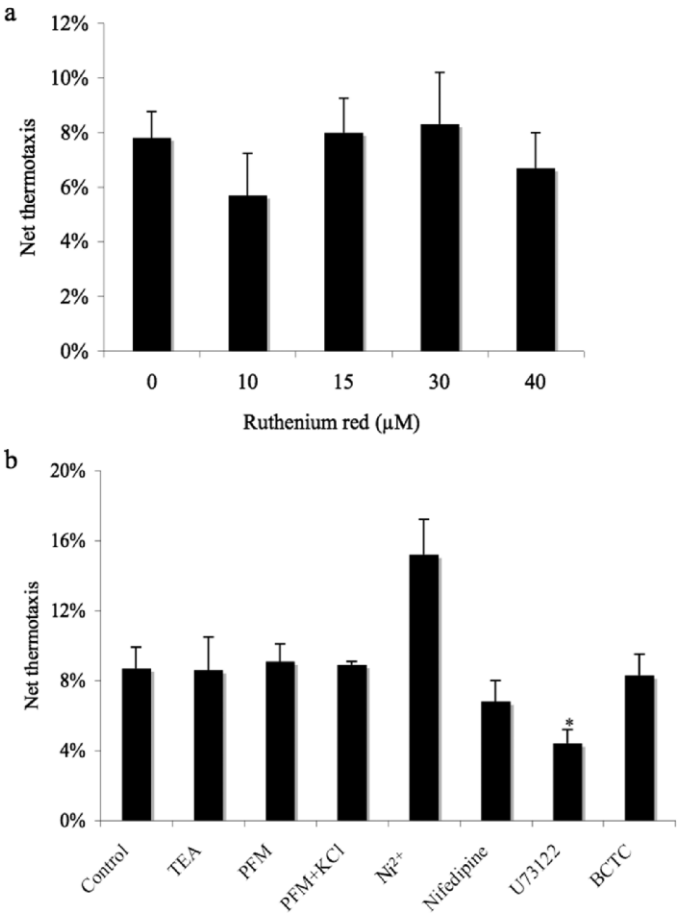


FIG. 5. Thermotactic response in the presence of different channel inhibitors and blockers. The results and SEM values were normalized according to the TEA control as described in the legend to Figure 3. The nonnormalized results with the respective individual controls for each set of conditions can be seen in Supplemental Figures S4–S7. Each inhibitor and blocker was at equal concentration throughout the measuring thermotaxis tube. The results are the mean ± SEM of 3–17 determinations. **a**) The effect of different concentrations of ruthenium red on thermotaxis. The blocker was preincubated for 40 min with the spermatozoa. The differences between the net thermotaxis values with or without ruthenium red (defined as zero) were not significant (determined by ANOVA one-way test). **b**) The effect of 3 mM TEA (preincubated for 40 min with the spermatozoa), $K^+$-free medium (PFM), PFM supplemented with 15 mM KCl (PFM + KCl), 1 mM $Ni^{2+}$, 0.2 mM nifedipine (preincubated for 30 min with the spermatozoa), 3.2 μM BCTC, and 5 μM U73122 (preincubated for 20 min with the spermatozoa) on thermotaxis. The asterisk indicates a significant difference ($P = 0.004$) relative to the control (0.4% DMSO in Flushing Medium) as determined by the unpaired Student *t*-test. Nifedipine at 0.2 mM slightly reduced sperm motility without affecting the thermotactic response.

involvement to $Ca^{2+}_{in}$ only, and therefore suggested the involvement of $Ca^{2+}$ channels on internal membranes. This observation is similar to the occurrence in human sperm chemotaxis, where progesterone—a sperm chemoattractant secreted from the cumulus cells [13–15]—was shown to initiate a store-mediated $Ca^{2+}_{in}$ signaling in human spermatozoa [88]. We cannot exclude, however, the possibility that a $Ca^{2+}_{ex}$ concentration similar to, or lower than, the $Ca^{2+}$ concentration required for motility is needed and sufficient for the thermotactic response. Such a possibility can only be tested when a motility-independent molecular marker for the thermotactic response becomes available.

The inhibition of the thermotactic response by 2-APB (an inhibitor of store-operated $Ca^{2+}$ channels and $IP_3R$ $Ca^{2+}$

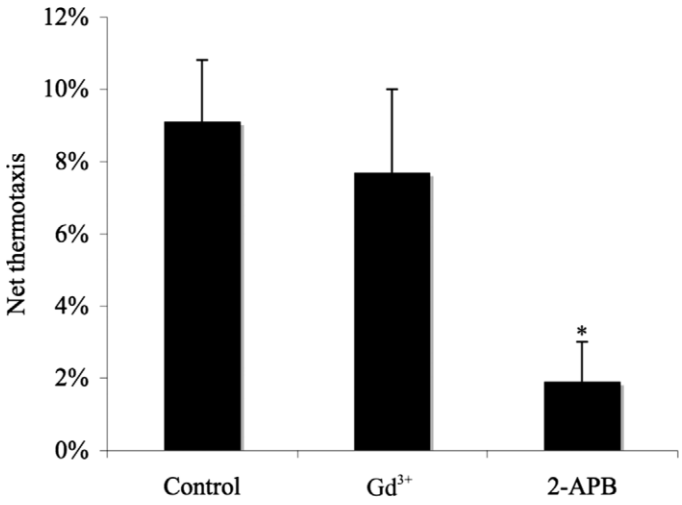


FIG. 6. The effect of 2-APB and $Gd^{3+}$ on the thermotactic response. The results and SEM values were normalized according to the $Gd^{3+}$ control as described in the legend to Figure 3. The nonnormalized results with the respective individual controls for each set of conditions can be seen in Supplemental Figures S8 and S9. The inhibitor and blocker were at equal concentrations throughout the measuring thermotaxis tube. The results are the mean ± SEM of five to eight determinations. 2-APB (0.3 mM) and $Gd^{3+}$ (40 μM) were preincubated for 20 min with the spermatozoa. The asterisk indicates a significant difference ($P = 0.0002$) relative to the control (0.3% DMSO in Flushing Medium), as determined by the unpaired Student *t*-test.

channels [80]; Fig. 6 and Supplemental Fig. S8) suggested that SOCs and $IP_3R$ $Ca^{2+}$ channels may be involved in thermotaxis. These channels are also known to participate in the chemotactic response of *Dictyostelium dictyostelium* [89], human neutrophils [90], and ascidian spermatozoa [91]. Store-operated $Ca^{2+}$ channels are $Ca^{2+}$ channels that reside on the plasma membrane and allow the entry of $Ca^{2+}_{ex}$ to the cell [82, 92]. Because our results suggest the involvement of $Ca^{2+}_{in}$, but not $Ca^{2+}_{ex}$, in the thermotactic response, it is likely that the inhibition by 2-APB is due to inhibition of $IP_3R$ $Ca^{2+}$ channels rather than SOCs. This conclusion and the possibility that

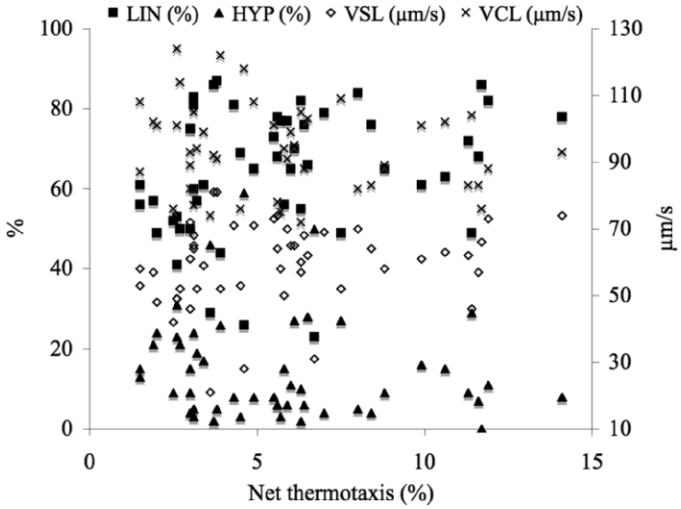


FIG. 7. Thermotactic response as a function of the percentage of HYP, VCL, VSL, and LIN of the cells. The net thermotactic response was calculated by subtracting the no-gradient control from the sperm accumulation in a temperature gradient (33°C–37°C). There is no relationship between the kinematics parameters of the cells and the level of their thermotactic response ($R^2 \leq 0.07$).

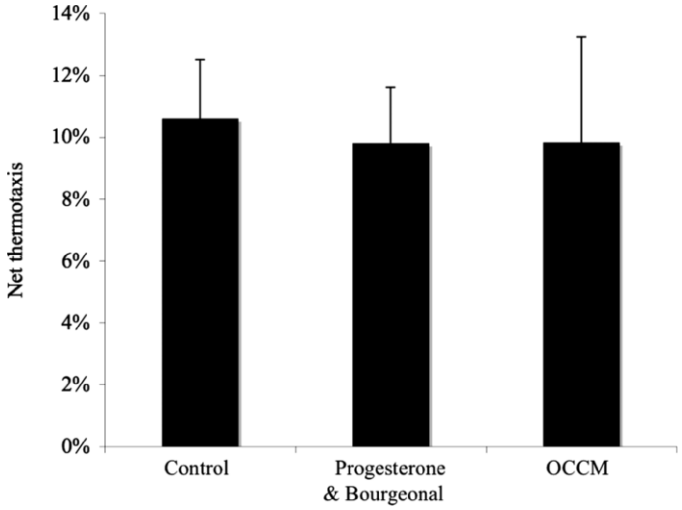


FIG. 8. Thermotactic response in the presence of different chemoattractants. The results and SEM values were normalized according to the oocyte-cumulus conditioned medium (OCCM) control as described in the legend to Figure 3. The nonnormalized results with the respective individual controls for each set of conditions can be seen in Supplemental Figure S10. The chemoattractants tested were: progesterone (1 μM), bourgeonal (10 μM), and OCCM (diluted 1:100). In each experiment, the chemoattractants were at an equal concentration throughout the measuring thermotaxis tube. The results are the mean ± SEM of four to six determinations.

internal $Ca^{2+}$ stores gated by $IP_3R$ channels are involved in the thermotactic response are supported by the observations that high $Ni^{2+}$ (Fig. 5b and Supplemental Fig. S6) and low $Gd^{3+}$ concentrations (Fig. 6 and Supplemental Fig. S9), both known to block SOCs in the plasma membrane [82, 92], did not inhibit the thermotactic response. The inhibitory effect of U73122 (an inhibitor of $IP_3$ production by PLC [93]) on sperm thermotaxis (Fig. 5b and Supplemental Fig. S4) substantiates our assumption that $IP_3R$ channels participate in sperm thermotaxis and further suggests the involvement of PLC in thermotaxis.

$Ca^{2+}$ can be released from internal stores not only via the $IP_3R$ $Ca^{2+}$ channels but also through the ryanodine receptor channels, believed to be localized to the internal store located at the junction between the caudal head and the midpiece [88]. However, they do not seem to be involved in thermotaxis, at least not exclusively, because ruthenium red, known to block these channels [25], did not inhibit the thermotactic response (Fig. 5a).

### *The Thermosensors Seem to Be Different from the Chemosensors*

The identity of the thermosensors in spermatozoa is an intriguing question. On face value it seems that, unlike in bacteria where the thermosensors for thermotaxis are the chemotaxis receptors [17, 18], in sperm the thermotaxis thermosensors and the chemotaxis chemoreceptors are distinct. This is because saturating the chemotaxis receptors with their ligands did not affect the thermotactic response (Fig. 8 and Supplemental Fig. S10). However, this conclusion should be taken with caution because it is possible that there are additional, yet unidentified chemotaxis receptors, which were still functional in our experiments with saturating concentrations of chemoattractants. Furthermore, it was argued that thermotaxis in mammalian spermatozoa should be extremely sensitive (0.16°C/cm threshold gradient detection in the case of rabbits [8, 9]). It is reasonable that such high sensitivity can only be achieved by a large repertoire of thermosensors, each being sensitive at another temperature range, the ranges being superimposed to some extent. Examples of superimposing sensing are well known in biology, with the olfactory [94], hearing [95], and immune systems [96, 97] being among the best known. The possible existence of multiple thermosensors makes their identification by pharmacological means very difficult. This is because pharmacological inhibition of a single thermosensor may be compensated for by other thermosensors, and therefore may not have any detectable effect on the thermotactic response.

With this in mind, we also tried examining the effects of combinations of inhibitors of different potential thermosensors. However, such combinations resulted in a considerable reduction of both sperm motility and the fraction of capacitated spermatozoa (only capacitated cells are thermotactically responsive [5]), making this combined pharmacological approach ineffective (Bahat and Eisenbach, unpublished observations). For all of the above reasons, we cannot rule out the possibility that the tested chemoreceptors are, after all, involved in thermosensing in concert with additional thermosensors.

### *Lack of Involvement of TRPV Channels, TRPM8 Channel, Voltage-Dependent $Ca^{2+}$ Channels, and $K^+$ Channels*

$Ca^{2+}$ channels from the TRPV family are recognized to be the main warm thermosensors in mammals [25, 26]. However, our studies suggest that these channels are not involved in

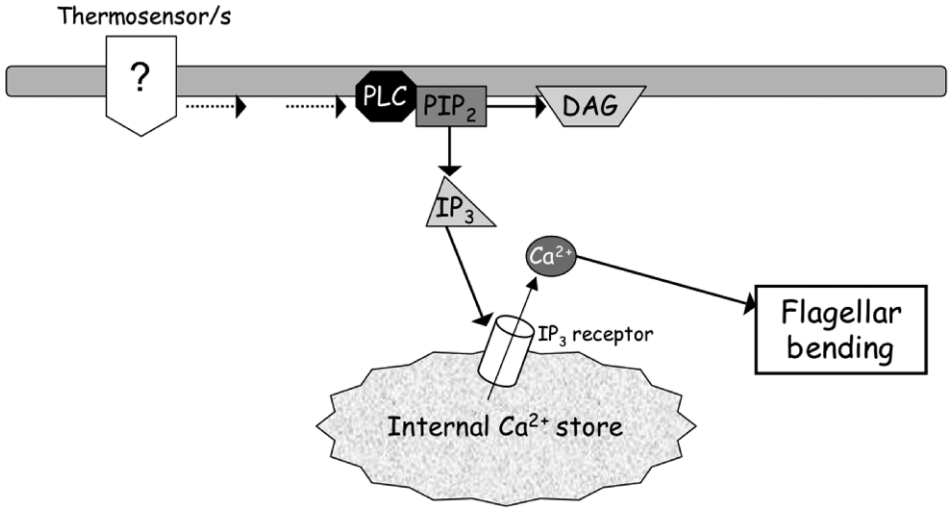


FIG. 9. Schematic model of thermotactic signaling events in human spermatozoa. Temperature stimulation activates PLC via one or more unidentified steps, causing hydrolysis of $PIP_2$ to $IP_3$ and DAG. $IP_3$ binds to its receptor on an internal $Ca^{2+}$ store and triggers $Ca^{2+}$ release that affects flagellar bending [99] and, consequently, the swimming direction.

thermotaxis (Fig. 5a), at least not exclusively. Similar results against exclusive involvement in thermotaxis were obtained for the cold-activated channel, TRPM8 (Fig. 5b and Supplemental Fig. S5), for the T- and L-type voltage-dependent $Ca^{2+}$ channels (Fig. 5b and Supplemental Fig. S6), and for heat-sensitive $K^+$ channels (Fig. 5b and Supplemental Fig. S7), known to be involved in thermosensing of mammalian neurons [36] and sponges [35]. We cannot exclude the possibility that multiple channels are involved in thermotaxis, and therefore inhibition of one of them does not abolish the thermotactic response.

Our findings that $Ca^{2+}{}_{in}$, PLC and $IP_3R$ are involved in sperm thermotaxis provide the first building stones toward revealing the molecular mechanism of this process, which appears to be essential for fertilization [1, 9]. It is likely that sperm thermotaxis acts through the PLC signaling pathway (Fig. 9), in which $IP_3$ production [79] results in the opening of $IP_3R$ channels and, consequently, in $Ca^{2+}$ release from internal stores [80]. Elevated $Ca^{2+}{}_{in}$ modifies flagellar bending and, consequently, the swimming pattern [98, 99].

## ACKNOWLEDGMENTS

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## REFERENCES

1. Eisenbach M, Giojalas LC. Sperm guidance in mammals—an unpaved road to the egg. Nat Rev Mol Cell Biol 2006; 7:276–285.
2. Miller RL. Sperm chemo-orientation in the metazoa. In: Metz CB, Monroy A (eds.), Biology of Fertilization, vol. 2. New York: Academic Press; 1985:275–337.
3. Cosson MP. Sperm chemotaxis. In: Gagnon C (ed.), Controls of Sperm Motility: Biological and Clinical Aspects. Boca Raton: CRC Press; 1990: 103–135.
4. Hildebrand E, Kaupp UB. Sperm chemotaxis: a primer. Ann N Y Acad Sci 2005; 1061:221–225.
5. Bahat A, Tur-Kaspa I, Gakamsky A, Giojalas LC, Breitbart H, Eisenbach M. Thermotaxis of mammalian sperm cells: A potential navigation mechanism in the female genital tract. Nat Med 2003; 9:149–150.
6. David A, Vilensky A, Nathan H. Temperature changes in the different parts of the rabbit's oviduct. Int J Gynaecol Obstet 1972; 10:52–56.
7. Hunter RHF, Nichol R. A preovulatory temperature gradient between the isthmus and the ampulla of pig oviducts during the phase of sperm storage. J Reprod Fertil 1986; 77:599–606.
8. Bahat A, Eisenbach M, Tur-Kaspa I. Periovulatory increase in temperature difference within the rabbit oviduct. Hum. Reprod 2005; 20:2118–2121.
9. Bahat A, Eisenbach M. Sperm thermotaxis. Mol Cell Endocrinol 2006; 252:115–119.
10. Fabro G, Rovasio RA, Civalero S, Frenkel A, Caplan SR, Eisenbach M, Giojalas LC. Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. Biol Reprod 2002; 67: 1565–1571.
11. Cohen-Dayag A, Ralt D, Tur-Kaspa I, Manor M, Makler A, Dor J, Mashiach S, Eisenbach M. Sequential acquisition of chemotactic responsiveness by human spermatozoa. Biol Reprod 1994; 50:786–790.
12. Cohen-Dayag A, Tur-Kaspa I, Dor J, Mashiach S, Eisenbach M. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. Proc Natl Acad Sci U S A 1995; 92: 11039–11043.
13. Teves ME, Barbano F, Guidobaldi HA, Sanchez R, Miska W, Giojalas LC. Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. Fertil Steril 2006; 86:745–749.
14. Oren-Benaroya R, Orvieto R, Gakamsky A, Pinchasov M, Eisenbach M. The sperm chemoattractant secreted from human cumulus cells is progesterone. Hum Reprod 2008; 23:2339–2345.
15. Guidobaldi HA, Teves ME, Unates DR, Anastasia A, Giojalas LC. Progesterone from the cumulus cells is the sperm chemoattractant secreted by the rabbit oocyte cumulus complex. PLoS ONE 2008; 3:e3040.
16. Sun F, Bahat A, Gakamsky A, Girsh E, Katz N, Giojalas LC, Tur-Kaspa I, Eisenbach M. Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants. Hum Reprod 2005; 20:761–767.
17. Imae Y. Molecular mechanism of thermosensing in bacteria. In: Eisenbach M, Balaban M (eds.), Sensing and Response in Microorganisms. Amsterdam: Elsevier; 1985:73–81.
18. Nara T, Kawagishi I, Nishiyama S, Homma M, Imae Y. Modulation of the thermosensing profile of the *Escherichia coli* aspartate receptor tar by covalent modification of its methyl-accepting sites. J Biol Chem 1996; 271:17932–17936.
19. Nara T, Lee L, Imae Y. Thermosensing ability of Trg and Tap chemoreceptors in *Escherichia coli*. J Bacteriol 1991; 173:1120–1124.
20. Mori I, Ohshima Y. Neural regulation of thermotaxis in *Caenorhabditis elegans*. Nature 1995; 376:344–348.
21. Samuel ADT, Silva RA, Murthy VN. Synaptic activity of the AFD neuron in *Caenorhabditis elegans* correlates with thermotactic memory. J Neurosci 2003; 23:373–376.
22. Hedgecock EM, Russell RL. Normal and mutant thermotaxis in the nematode *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 1975; 72: 4061–4065.
23. Mori I. Genetics of chemotaxis and thermotaxis in the nematode *Caenorhabditis elegans*. Annu Rev Genet 1999; 33:399–422.
24. Montell C. The TRP superfamily of cation channels. Sci STKE 2005; 2005:1–24.
25. Patapoutian A, Peier AM, Story GM, Viswanath V. ThermoTRP channels and beyond: mechanisms of temperature sensation. Nat Rev Neurosci 2003; 4:529–539.
26. Tominaga M, Caterina MJ. Thermosensation and pain. J Neurobiol 2004; 61:3–12.
27. Mizushima T, Obata K, Katsura H, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Mashimo T, Noguchi K. Noxious cold stimulation induces mitogen-activated protein kinase activation in transient receptor potential (TRP) channels TRPA1- and TRPM8-containing small sensory neurons. Neuroscience 2006; 140:1337–1348.
28. Maccarrone M, Barboni B, Paradisi A, Bernabo N, Gasperi V, Pistilli MG, Fezza F, Lucidi P, Mattioli M. Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. J Cell Sci 2005; 118:4393–4404.
29. Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell 2000; 103:525–535.
30. De Blas GA, Darszon A, Ocampo AY, Serrano CJ, Castellano LE, Hernandez-Gonzalez EO, Chirinos M, Larrea F, Beltran C, Trevino CL. TRPM8, a versatile channel in human sperm. PLoS One 2009; 4:e6095.
31. Jungnickel MK, Marrero H, Birnbaumer L, Lemos JR, Florman HM. Trp2 regulates entry of $Ca^{2+}$ into mouse sperm triggered by egg ZP3. Nat Cell Biol 2001; 3:499–502.
32. Trevino CL, Serrano CJ, Beltran C, Felix R, Darszon A. Identification of mouse trp homologs and lipid rafts from spermatogenic cells and sperm. FEBS Lett 2001; 509:119–125.
33. Castellano LE, Treviño CL, Rodríguez D, Serrano CJ, Pacheco J, Tsutsumi V, Felix R, Darszon A. Transient receptor potential (TRPC) channels in human sperm: expression, cellular localization and involvement in the regulation of flagellar motility. FEBS Lett 2003; 541:69–74.
34. Imada C, Oosawa Y. Thermoreception of *Paramecium*: different $Ca^{2+}$ channels were activated by heating and cooling. J Membr Biol 1999; 168: 283–287.
35. Zocchi E, Carpaneto A, Cerrano C, Bavestrello G, Giovine M, Bruzzone S, Guida L, Franco L, Usai C. The temperature-signaling cascade in sponges involves a heat-gated cation channel, abscisic acid, and cyclic ADP-ribose. Proc Natl Acad Sci U S A 2001; 98:14859–14864.
36. Maingret F, Lauritzen I, Patel AJ, Heurteaux C, Reyes R, Lesage F, Lazdunski M, Honoré E. TREK-1 is a heat-activated background $K^+$ channel. EMBO J 2000; 19:2483–2491.
37. Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock DF. CatSper1 required for evoked $Ca^{2+}$ entry and control of flagellar function in sperm. Proc Natl Acad Sci U S A 2003; 100:14864–14868.
38. Suarez S, Ho HC. Hyperactivated motility in sperm. Reprod Domest Anim 2003; 38:119–124.
39. Publicover SJ, Giojalas LC, Teves ME, de Oliveira GS, Garcia AA, Barratt CL, Harper CV. $Ca^{2+}$ signalling in the control of motility and guidance in mammalian sperm. Front Biosci 2008; 13:5623–5637.

40. Jaiswal BS, Eisenbach M. Capacitation. In: Hardy DM (ed.), Fertilization. San Diego: Academic Press; 2002:57–117.
41. Kirkman-Brown JC, Punt EL, Barratt CLR, Publicover SJ. Zona pellucida and progesterone-induced $Ca^{2+}$ signaling and acrosome reaction in human spermatozoa. J Androl 2002; 23:306–315.
42. Eisenbach M. Sperm chemotaxis. Rev Reprod 1999; 4:56–66.
43. Spehr M, Gisselmann G, Poplawski A, Riffell JA, Wetzel CH, Zimmer RK, Hatt H. Identification of a testicular odorant receptor mediating human sperm chemotaxis. Science 2003; 299:2054–2058.
44. Kaupp UB, Solzin J, Hildebrand E, Brown JE, Helbig A, Hagen V, Beyermann M, Pampaloni F, Weyand I. The signal flow and motor response controlling chemotaxis of sea urchin sperm. Nat Cell Biol 2003; 5:109–117.
45. Böhmer M, Van Q, Weyand I, Hagen V, Beyermann M, Matsumoto M, Hoshi M, Hildebrand E, Kaupp UB. $Ca^{2+}$ spikes in the flagellum control chemotactic behavior of sperm. EMBO J 2005; 24:2741–2752.
46. Cook SP, Brokaw CJ, Muller CH, Babcock DF. Sperm chemotaxis: egg peptides control cytosolic calcium to regulate flagellar responses. Dev Biol 1994; 165:10–19.
47. Kaupp UB, Hildebrand E, Weyand I. Sperm chemotaxis in marine invertebrates–molecules and mechanism. J Cell Physiol 2006; 208:487–494.
48. Dohrmann U, Fisher PR, Bruderlein M, Williams KL. Transitions in *Dictyostelium discoideum* behaviour: influence of calcium and fluoride on slug phototaxis and thermotaxis. J Cell Sci 1984; 65:111–121.
49. Norris V, Grant S, Freestone P, Canvin J, Sheikh FN, Toth I, Trinei M, Modha K, Norman RI. Calcium signalling in bacteria. J Bacteriol 1996; 178:3677–3682.
50. Zhang Y, Chou JH, Bradley J, Bargmann CI, Zinn K. The *Caenorhabditis elegans* seven-transmembrane protein ODR-10 functions as an odorant receptor in mammalian cells. Proc Natl Acad Sci U S A 1997; 94:12162–12167.
51. Jimenez-Gonzalez C, Michelangeli F, Harper CV, Barratt CL, Publicover SJ. Calcium signalling in human spermatozoa: a specialized 'toolkit' of channels, transporters and stores. Hum Reprod Update 2006; 12:253–267.
52. Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JD (ed.), Methods in Mammalian Embryology. San Francisco: Freeman; 1971:86–116.
53. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. New York: Cambridge University Press; 1993.
54. Davis RO, Siemers RJ. Derivation and reliability of kinematic measures of sperm motion. Reprod Fertil Dev 1995; 7:857–869.
55. Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. Hum Reprod Update 1997; 3: 403–439.
56. Mortimer D, Fraser L. Consensus workshop on advanced diagnostic andrology techniques. Hum Reprod 1996; 11:1463–1479.
57. Carbone E, Lux HD, Carabelli V, Aicardi G, Zucker H. $Ca^{2+}$ and $Na^{+}$ permeability of high-threshold $Ca^{2+}$ channels and their voltage-dependent block by $Mg^{2+}$ ions in chick sensory neurones. J Physiol 1997; 504(pt 1): 1–15.
58. Gonzalez-Martinez MT. Induction of a sodium-dependent depolarization by external calcium removal in human sperm. J Biol Chem 2003; 278: 36304–36310.
59. Minke B, Cook B. TRP channel proteins and signal transduction. Physiol Rev 2002; 82:429–472.
60. Huang CL. The transient receptor potential superfamily of ion channels. J Am Soc Nephrol 2004; 15:1690–1699.
61. De Blas G, Michaut M, Trevino CL, Tomes CN, Yunes R, Darszon A, Mayorga LS. The intraacrosomal calcium pool plays a direct role in acrosomal exocytosis. J Biol Chem 2002; 277:49326–49331.
62. Guatteo E, Chung KK, Bowala TK, Bernardi G, Mercuri NB, Lipski J. Temperature sensitivity of dopaminergic neurons of the substantia nigra pars compacta: involvement of transient receptor potential channels. J Neurophysiol 2005; 94:3069–3080.
63. Rosenzweig M, Brennan KM, Tayler TD, Phelps PO, Patapoutian A, Garrity PA. The *Drosophila* ortholog of vertebrate TRPA1 regulates thermotaxis. Genes Dev 2005; 19:419–424.
64. Van Breemen C. Blockade of membrane calcium fluxes by lanthanum in relation to vascular smooth muscle contractility. Arch Int Physiol Biochim 1969; 77:710–716.
65. Chinopoulos C, Gerencser AA, Doczi J, Fiskum G, Adam-Vizi V. Inhibition of glutamate-induced delayed calcium deregulation by 2-APB and $La^{3+}$ in cultured cortical neurones. J Neurochem 2004; 91:471–483.
66. Schultz JE, Schonefeld U, Klumpp S. Calcium/calmodulin-regulated guanylate cyclase and calcium-permeability in the ciliary membrane from *Tetrahymena*. Eur J Biochem 1983; 137:89–94.
67. Mills JS, Johnson JD. Metal ions as allosteric regulators of calmodulin. J Biol Chem 1985; 260:15100–15105.
68. Cervetto L, McNaughton PA. The effects of phosphodiesterase inhibitors and lanthanum ions on the light-sensitive current of toad retinal rods. J Physiol 1986; 370:91–109.
69. Reichling DB, MacDermott AB. Lanthanum actions on excitatory amino acid-gated currents and voltage-gated calcium currents in rat dorsal horn neurons. J Physiol 1991; 441:199–218.
70. Powis DA, Clark CL, O'Brien KJ. Lanthanum can be transported by the sodium-calcium exchange pathway and directly triggers catecholamine release from bovine chromaffin cells. Cell Calcium 1994; 16:377–390.
71. Clapham DE, Runnels LW, Strubing C. The TRP ion channel family. Nat Rev Neurosci 2001; 2:387–396.
72. Li Y, Jia YC, Cui K, Li N, Zheng ZY, Wang YZ, Yuan XB. Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 2005; 434:894–898.
73. Zufall F. The TRPC2 ion channel and pheromone sensing in the accessory olfactory system. Naunyn Schmiedebergs Arch Pharmacol 2005; 371: 245–250.
74. Kiselyov K, Kim JY, Zeng W, Muallem S. Protein-protein interaction and functionTRPC channels. Pflugers Arch 2005; 451:116–124.
75. Blackmore PF, Eisoldt S. The neoglycoprotein mannose-bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa. Mol Hum Reprod 1999; 5:498–506.
76. Kirkman-Brown JC, Barratt CL, Publicover SJ. Nifedipine reveals the existence of two discrete components of the progesterone-induced [Ca2+]i transient in human spermatozoa. Dev Biol 2003; 259:71–82.
77. Mathie A, Wooltorton JR, Watkins CS. Voltage-activated potassium channels in mammalian neurons and their block by novel pharmacological agents. Gen Pharmacol 1998; 30:13–24.
78. Yeung CH, Cooper TG. Effects of the ion-channel blocker quinine on human sperm volume, kinematics and mucus penetration, and the involvement of potassium channels. Mol Hum Reprod 2001; 7:819–828.
79. Hofmann SL, Majerus PW. Modulation of phosphatidylinositol-specific phospholipase C activity by phospholipid interactions, diglycerides, and calcium ions. J Biol Chem 1982; 257:14359–14364.
80. Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM. 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release. FASEB J 2002; 16:1145–1150.
81. Dutta D. Mechanism of store-operated calcium entry. J Biosci 2000; 25: 397–404.
82. Broad LM, Cannon TR, Taylor CW. A non-capacitative pathway activated by arachidonic acid is the major Ca2+ entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentrations of vasopressin. J Physiol 1999; 517(pt 1):121–134.
83. Ralt D, Manor M, Cohen-Dayag A, Tur-Kaspa I, Makler A, Yuli I, Dor J, Blumberg S, Mashiach S, Eisenbach M. Chemotaxis and chemokinesis of human spermatozoa to follicular factors. Biol Reprod 1994; 50:774–785.
84. Perez JF, Ruiz MC, Chemello ME, Michelangeli F. Characterization of a membrane calcium pathway induced by rotavirus infection in cultured cells. J Virol 1999; 73:2481–2490.
85. Tymianski M, Spigelman I, Zhang L, Carlen PL, Tator CH, Charlton MP, Wallace MC. Mechanism of action and persistence of neuroprotection by cell-permeant $Ca^{2+}$ chelators. J Cereb Blood Flow Metab 1994; 14:911–923.
86. Blackmore PF. Rapid non-genomic actions of progesterone stimulate $ca^{2+}$ influx and the acrosome reaction in human sperm. Cell Signal 1993; 5: 531–538.
87. Dragileva E, Rubinstein S, Breitbart H. Intracellular Ca(2+)-Mg(2+)-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. Biol Reprod 1999; 61:1226–1234.
88. Harper CV, Barratt CL, Publicover SJ. Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of [Ca(2+)](i) oscillations and cyclical transitions in flagellar beating. J Biol Chem 2004; 279:46315–46325.
89. Malchow D, Mutzel R, Schlatterer C. On the role of calcium during chemotactic signalling and differentiation of the cellular slime mould *Dictyostelium discoideum*. Int J Dev Biol 1996; 40:135–139.
90. Anderson R, Steel HC, Tintinger GR. Inositol 1,4,5-triphosphate-mediated shuttling between intracellular stores and the cytosol contributes to the sustained elevation in cytosolic calcium in FMLP-activated human neutrophils. Biochem Pharmacol 2005; 69:1567–1575.
91. Yoshida M, Ishikawa M, Izumi H, De Santis R, Morisawa M. Store-

operated calcium channel regulates the chemotactic behavior of ascidian sperm. Proc Natl Acad Sci U S A 2003; 100:149–154.
92. O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. $Ca^{2+}$ entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Mol Biol Cell 2000; 11:1571–1584.
93. Howell KP, Skipwith A, Galione A, Eckberg WR. Phospholipase C-dependent Ca2+ release by worm and mammal sperm factors. Biochem Biophys Res Commun 2003; 307:47–51.
94. Spehr M, Leinders-Zufall T. One neuron–multiple receptors: increased complexity in olfactory coding? Sci STKE 2005; 2005:pe25.
95. Kernan MJ. Mechanotransduction and auditory transduction in *Drosophila*. Pflugers Arch 2007: 454:703–720.
96. Nikolich-Zugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nat Rev Immunol 2004; 4:123–132.
97. Varla-Leftherioti M. The significance of the women's repertoire of natural killer cell receptors in the maintenance of pregnancy. Chem Immunol Allergy 2005; 89:84–95.
98. Brokaw CJ, Josslin R, Bobrow L. Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa. Biochem Biophys Res Commun 1974; 58:795–800.
99. Lindemann CB, Goltz JS. Calcium regulation of flagellar curvature and swimming pattern in Triton X-100–extracted rat sperm. Cell Motil Cytoskeleton 1988; 10:420–431.