Partition of the organochlorine insecticide lindane into the human sperm surface induces membrane depolarization and Ca^{2+} influx

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The effects of the insecticide lindane (the γ-isomer of 1,2,3,4,5,6-hexachlorocyclohexane) on membrane potential, cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and surface biophysical properties were studied in human spermatozoa. The insecticide induces rapid, transient and reproducible membrane depolarization and opening of voltage-dependent Ca^{2+} channels leading to an increase in $[Ca^{2+}]_i$. In contrast with the effect in somatic cells, lindane did not affect γ-aminobutyric acid receptor-linked Cl^- currents. Ca^{2+} and K^+ currents were found to drive lindane-induced membrane depolarization and repolarization respectively, whereas Na^+ and Cl^- fluxes appear not to have a role in the phenomenon. The insecticide was still able to produce membrane depolarization both in the combined absence of extracellular Ca^{2+} and Na^+ and in high-K^+ buffer, suggesting that lindane alters the membrane dipole potential. In agreement with this, Laurodan and Prodan fluorescence spectroscopy revealed that lindane partition into the sperm plasma membrane lowers water molecular dynamics in the uppermost region of the membrane external leaflet, probably as the result of reordering of water dipoles. We propose that the first effect of lindane partitioning into the sperm plasma membrane is a change in the membrane dipole potential, which results in the activation of membrane-located Ca^{2+}-influx pathways.

INTRODUCTION

Environmental pollution is a well-established cause of reproductive damage [1] possibly responsible in part for the progressive lowering of the male fertility potential in occidental countries [2]. However, very little information is available on fertility impairment by environmental pollutants. In this paper we report that, and show how, human spermatozoa respond to the insecticide lindane (the γ-isomer of the polyhalogenated cyclic hydrocarbon hexachlorocyclohexane), a typical member of the vast family of organochlorine compounds broadly used as pesticides. Lindane is the active compound of various parasiticidal solutions for both human and animal medication [3]. Even though the use of lindane has been restricted for many years in developed countries, the global distillation effect and chemical persistence means that it is still ubiquitously present in the environment [4]. The substance has proved to be very harmful to many biological functions [5–11], including reproduction [12]. The molecular mechanisms through which lindane (and cognate toxicants as well) exerts its effects are poorly understood. In neuronal cells, lindane both inhibits γ-aminobutyrate (GABA)-gated Cl^- currents by interacting with GABA receptors [13] and depresses glucose uptake and phosphatidylinositol synthesis [14]. The structural similarity of lindane to Ins(1,4,5)P_3 [15] has been suggested to account for the insecticide-induced Ca^{2+} release from intracellular InsP_3-sensitive stores in macrophages [16] and in myometrial smooth-muscle cells [17]. It has also been hypothesized that Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VOCCs) may have a role in the mechanism(s) of lindane effects [18]. Owing to its high lipophilicity, the insecticide is incorporated into biomembranes [19]. When the effects of lindane on lipid fluidity were evaluated by means of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, a probe that senses the bilayer hydrophobic core, no apparent perturbation of either the lipid phase or bulk membrane fluidity was observed [20]. However, when lipid fluidity was investigated using the excimer to monomer fluorescence intensity ratio of 1,3-di-(1-pyrenyl)propane, a probe that senses the upper, less hydrophobic regions of the bilayer, lindane was found to exert an ordering effect [21].

In the present work the spectroscopic properties of the fluorescent probes Laurodan (2-dimethylamino-6-lauroylnaphthalene) and Prodan (2-dimethylamino-6-propionylnaphthalene) have been exploited to investigate the effects of lindane on the biophysical properties of the sperm membrane surface. These probes localize at the hydrophilic/hydrophobic interface of the bilayer, with the naphthalene fluorescent moiety placed at the level of the glycerol backbone and the hydrophobic tail situated in the phospholipid acyl chain region [22]. The different lengths of the hydrocarbon tails (12 and 3 carbon atoms for Laurodan and Prodan respectively) means that the Laurodan anchor is stronger and extends deeper into the bilayer than that of Prodan [23]. The spectroscopic properties of Laurodan and Prodan are extremely susceptible to both the number and kinetics of water molecules in the probe environment and are insensitive to the polar head charge and pH [24]. In comparison with other spectroscopic techniques used to probe membrane structure and dynamics, Laurodan and Prodan fluorescence spectra collected by steady-state spectroscopy allow the lipid-phase state as well as the polarity and molecular dynamics at the lipid/water interface to be detected and quantified in a sensitive and relatively simple way, avoiding the use of polarizers [25].

Very recently, by using Laurodan spectroscopy we demonstrated that, within the temperature range 10–42 °C, the human sperm surface is characterized by a single highly ordered liquid crystalline lipid phase [26]. In the present study, using the intracellular Ca^{2+} chelator fura 2 acetoxymethyl ester, the hydrophobic potential-sensitive probe bisoxonol, and steady-state fluorescence spectroscopy of Laurodan and Prodan, we found that the administration of lindane to human spermatozoa *in vitro*

Abbreviations used: VOCC, voltage-operated Ca^{2+} channel; TEA, tetraethylammonium; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; K_{Ca} channels, Ca^{2+}-activated K^+ channels; GPex, excitation generalized polarization; GPem, emission generalized polarization; GABA, γ-aminobutyric acid.

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induces both transient and sustained effects, the former consisting of re-inducible membrane depolarization and voltage-dependent Ca^{2+} influx, and the latter represented by an evident progressive decrease in molecular dynamics at the membrane surface. Within the lindane concentration range used, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane depolarization peaks increased with lindane concentration to saturation, and the membrane molecular dynamics decreased linearly with increasing lindane concentration. The hypothesis is put forward that lindane partitioning into the sperm surface alters the intrinsic membrane potential, thereby triggering all the subsequent ionic and electric events observed.

MATERIALS AND METHODS

Chemicals

The buffers used in this study were as follows: 30 mM Hepes, 1.19 mM $MgSO_4$,$7H_2O$, 5 mM KCl, 122 mM NaCl, 1.5 mM $CaCl_2$, 5.5 mM D-glucose, pH 7.4 (regular buffer); Na^+-free and Cl^--free buffers were prepared by equimolar replacement of NaCl with choline chloride (choline buffer) and chloride salts with gluconate salts (gluconate buffer) respectively; Ca^{2+}-free buffer was prepared by omitting $CaCl_2$ and adding 0.5 mM EGTA (EGTA buffer). The fluorescent probes fura 2 acetoxymethyl ester, bisoxonol, Laurodan and Prodan were obtained from Molecular Probes (Eugene, OR, U.S.A.). Lindane and all other chemicals were purchased from Sigma Chemical Co. The water used in this study was deionized (> 18 KΩ) with a Milli-Q water system (Millipore).

Spermatozoa

Seminal fluids obtained from healthy fertile donors with normal semen characteristics [27] were collected into plastic containers and allowed to liquefy at room temperature (20–22 °C) in the dark. Spermatozoa were isolated by a swim-up procedure [28] and washed twice (500 ***g***, 2 min each) with regular buffer. The final suspension (30×10^6–40×10^6 cells/ml) was maintained at room temperature in the dark up to the time of use. Sperm motility and viability were assessed by phase-contrast microscopy and propidium iodide exclusion [29] respectively.

Fluorescence assays

For $[Ca^{2+}]_i$ measurement, spermatozoa (20×10^6–25×10^6 cells/ml) were loaded at 37 °C in the dark for 45 min with the acetoxymethyl ester derivative of the Ca^{2+} chelator fura 2 at 2 μM final concentration (1 μl/ml of a 2 mM stock solution in DMSO). The cells were then washed (500 ***g***, 2 min), suspended in regular buffer, transferred (70 μl aliquots, corresponding to about 3.5×10^6 cells) into a series of 1.5 ml Eppendorf tubes each containing 1 ml of regular buffer and maintained at room temperature in the dark. Just before fluorescence readings, the spermatozoa were sedimented (500 ***g***, 2 min) and transferred to the spectrofluorimeter cuvette (acryl 67.755; Sarstedt) containing 1.5 ml of the selected buffer (final concentration 2.4×10^6 cells/ml). Fura 2 fluorescence intensity values (excitation/emission wavelength pair 340/505 nm) were converted into $[Ca^{2+}]_i$ as previously described [30].

In the present study we did not make use of electrophysiological techniques as they cannot be applied to cells as small as spermatozoa unless isolated membrane fragments are incorporated into an artificial lipid layer [31], or the entire cell surface area is extended by hypotonic stress [32], two methods that obviously produce dramatic alterations in native membrane configuration. Therefore the potential-sensitive dye, bisoxonol, a fluorescent probe suitable for investigating membrane potential variations in living human sperm [33], was used. In order to make the results from the various experiments comparable, membrane potential was evaluated in fura 2-loaded spermatozoa. Bisoxonol was added to the cell suspension at 200 nM final concentration (1.5 μl of a 200 μM stock solution in DMSO) and fluorescence readings were started after 1–2 min, the length of time necessary to allow transmembrane probe equilibration; bisoxonol was excited at 540 nm and fluorescence emission was read at 580 nm. Except for dose-dependence experiments, lindane was added to the sperm suspension at 40 μM final concentration (1.5 μl of a 40 mM stock solution in DMSO). In inhibition experiments, the spermatozoa were treated for 20 min at 37 °C in the presence of the organic VOCC blockers nicardipine, diltiazem and verapamil (5 μM each), and fluorescence assays were performed in their continuous presence. Fluorescence measurements were carried out with a computer-driven L-type spectrofluorimeter (Kontron SFM25), with continuous gentle stirring at 37 ± 0.1 °C. Cell autofluorescence was always less than 4 % and did not need to be subtracted.

In order to analyse membrane lipid dynamics, the spermatozoa were loaded with Laurodan or Prodan by adding 1 μl of probe stock solution (0.175 mM in DMSO, 0.117 μM final concn.) to 7.5×10^6 spermatozoa in 1.5 ml of regular buffer (final concentration 5×10^6 cells/ml). The incubation was allowed to proceed in the dark at 37 °C for 15 and 2 min for Laurodan and Prodan respectively. Fluorescence excitation (from 320 nm to 420 nm) and emission (from 420 nm to 520 nm) spectra were collected at an emission of 490 nm and an excitation of 370 nm respectively, and elaborated in terms of emission and excitation generalized polarization (GPem and GPex respectively) according to the equations:

$$\mathrm{GPem} = (I_{410} - I_{340})/(I_{410} + I_{340})$$

where I_{410} and I_{340} are the intensity at excitation wavelength of 410 nm and 340 nm respectively using an emission wavelength of 490 nm;

$$\mathrm{GPex} = (I_{435} - I_{490})/(I_{435} + I_{490})$$

where I_{435} and I_{490} are the intensity at emission wavelength of 435 nm and 490 nm respectively using an excitation wavelength of 370 nm [34].

Both GPem and GPex were calculated after subtraction of sperm and unpartitioned probe autofluorescence. The 490 nm emission wavelength for the excitation spectrum and the 370 nm excitation wavelength for the emission spectrum were chosen as being the wavelengths at which fluorescence intensity was maximal and least affected by autofluorescence subtraction. The fluorescence spectra were collected over a 40 min period at 2 min intervals. Lindane from a stock solution in DMSO was added to Laurodan- (or Prodan-) loaded spermatozoa at various concentrations (from 5 to 50 μM; final DMSO concentration never exceeded 0.1 %). DMSO (0.1 %) was added to control samples (no lindane added).

Best line fits to the experimental data were obtained by using the computer program TableCurve 2D® (Jandel Scientific Software), and the biologically relevant parameters were estimated visually from the graphs.

RESULTS

A sperm population characterized by more than 90 % regularly motile and viable cells was obtained by the isolation procedure. In the course of the experiments, cell viability did not vary to a

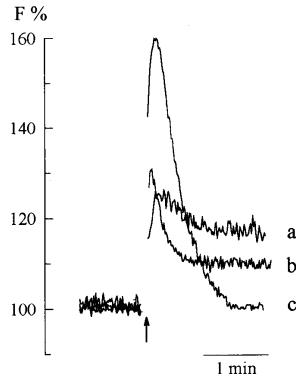

Figure 1 Effect of intracellular Ca^{2+} buffering on lindane-induced membrane depolarization in human spermatozoa

Human sperm were suspended in regular buffer and incubated with 200 nM bisoxonol before (a) and after cell loading with 2 μM fura 2 (b) and 20 μM quin 2 (c). The changes in fluorescence intensity were monitored using the excitation–emission pair 540–580 nm and are expressed as percentage variation in the basal signal (F, fluorescence arbitrary units). Arrow indicates lindane (40 μM) addition. The traces are representative of three consistent experiments.

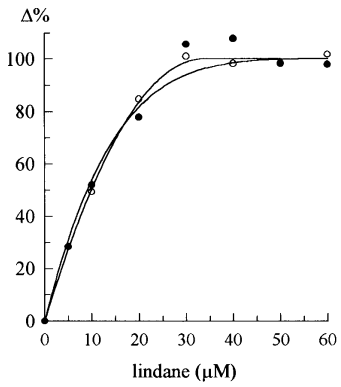

Figure 2 Effect of increasing concentrations of lindane on membrane potential and $[Ca^{2+}]_i$ in human sperm

The changes in fluorescence elicited by lindane were monitored in fura 2-loaded spermatozoa suspended in regular buffer in the presence or absence of 200 nM bisoxonol using the excitation–emission pairs 540–580 nm for bisoxonol (○) and 340–505 nm for fura 2 (●). The data points correspond to the peak values normalized assuming that the maximal response = 100. Each data point is the mean value of the data obtained from four preparations. The curves are the best line fits to the experimental data.

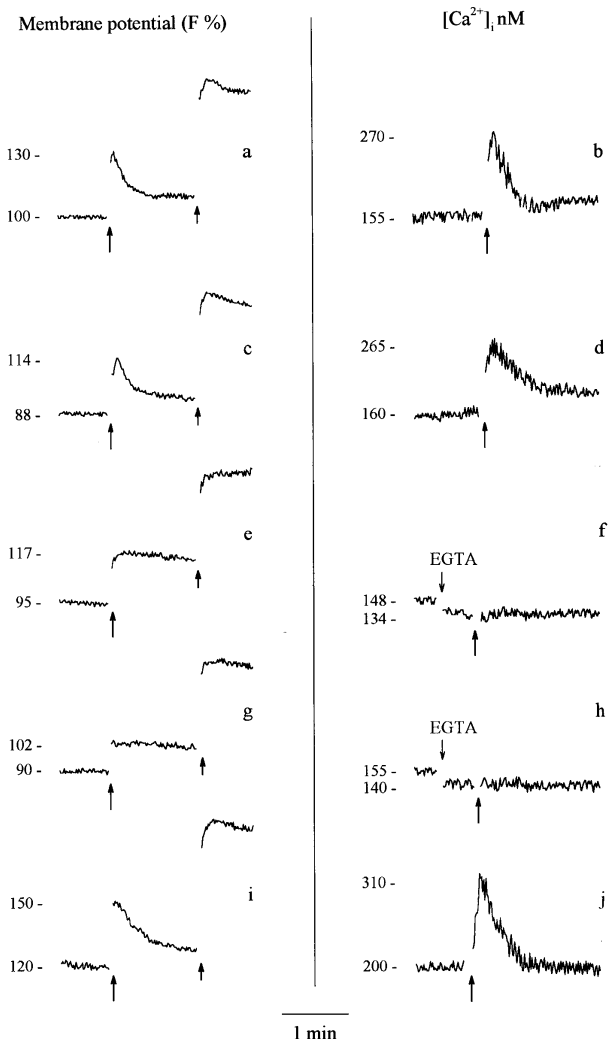

Figure 3 Membrane potential (left traces) and $[Ca^{2+}]_i$ (right traces) responses elicited by lindane in human spermatozoa suspended in buffers with different ion compositions

The changes in fluorescence were monitored in fura 2-loaded spermatozoa suspended in the different buffers in the presence or absence of 200 nM bisoxonol as described in Figure 2. (**a**) and (**b**) Regular buffer (122 mM NaCl, 1.5 mM $CaCl_2$); (**c**) and (**d**) Na^+-free buffer (equimolar replacement of NaCl with choline chloride); (**e**) and (**f**) Ca^{2+}-free buffer {no Ca^{2+} added, supplemented with 0.5 mM EGTA (extracellular $[Ca^{2+}] \approx 20$ nM)}; (**g**) and (**h**) Na^+-free/Ca^{2+}-free buffer {equimolar substitution of NaCl with choline chloride; no Ca^{2+} added, supplemented with 0.5 mM EGTA (extracellular $[Ca^{2+}] \approx 20$ nM)}; (**i**) and (**j**) gluconate buffer (equimolar substitution of NaCl, KCl and $CaCl_2$ with the corresponding gluconate salts). Long arrows indicate the addition of 40 μM lindane; short arrows indicate the addition of 60 mM KCl. F%, bisoxonol fluorescence intensity normalized assuming that the control basal signal (collected in regular buffer) = 100. The traces are representative of at least five experiments.

significant extent. In spermatozoa labelled with bisoxonol, lindane induced a steep membrane depolarization that reached its maximum at 20 s and was followed by a partial repolarization (Figure 1, trace a); however, when the bisoxonol signal was measured in fura 2-loaded sperm, the repolarization phase was more pronounced (Figure 1, trace b). These different responses of the bisoxonol signal may be ascribed to intracellular Ca^{2+} buffering by the fluorescent chelator, which delays the activation of repolarizing Ca^{2+}-activated channels thus allowing us to distinguish more efficiently between the two phases of membrane potential variation. In fact, when lindane was administered to spermatozoa loaded with quin 2, a Ca^{2+} probe with a greater buffering capacity than fura 2 and the property that it is easier to load into cells at high concentrations, the phenomenon was even more evident (Figure 1, trace c). Although the alteration of the kinetics and/or amplitude of $[Ca^{2+}]_i$ changes by quin 2 could be turned into a useful tool in properly controlled conditions [35], the potentially perturbing effects of quin 2 on cell functions [36] and the inhibition of various Ca^{2+}-dependent processes by this chelator prompted us to use fura 2-loaded spermatozoa throughout the study.

In regular buffer, lindane induced a dose-dependent increase in both the $[Ca^{2+}]_i$ and bisoxonol signals with maximal and half-maximal stimulating concentrations of 30 and 10 μM respectively for both phenomena (Figure 2). For the sake of clarity, the effects of lindane on $[Ca^{2+}]_i$ and membrane potential are reported

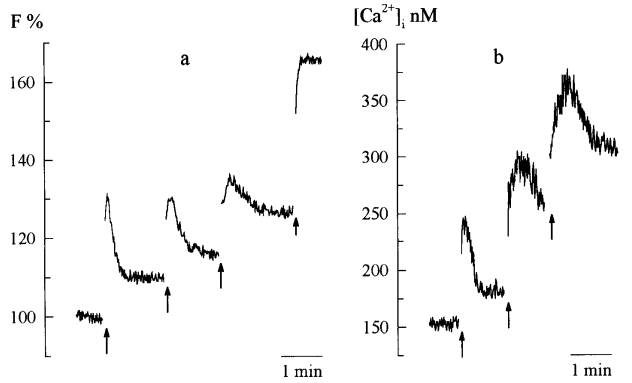

Figure 4 Effect of repeated additions of lindane on membrane potential (a) and $[Ca^{2+}]_i$ (b) responses in human spermatozoa

Fura 2-loaded sperm suspended in regular buffer in the presence or long absence of 200 nM bisoxonol were stimulated with subsequent readditions of 40 μM lindane (arrows). Short arrow indicates the addition of 60 mM KCl. Fluorescence changes were monitored as described in Figure 2. F%, normalized bisoxonol fluorescence intensity. The traces are representative of three experiments.

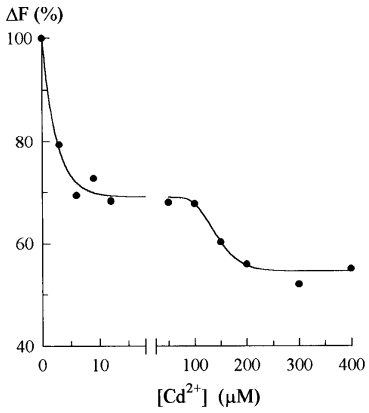

Figure 5 Effect of the VOCC inhibitor Cd^{2+} on the membrane potential response induced by lindane in human spermatozoa

Fura 2-loaded sperm suspended in regular buffer containing 200 nM bisoxonol were incubated in the presence of increasing concentrations of extracellular Cd^{2+}. The lindane-induced variations in the bisoxonol signal are expressed as percentage variation from the control (no Cd^{2+} added) of the peak values of fluorescence emission. The changes in fluorescence induced by 40 μM lindane were measured as described in Figure 2. Each data point is the mean value for data obtained from at least three preparations. The curves are the best line fits to the experimental data.

Table 1 Effect of VOCC inhibitors on the membrane potential and $[Ca^{2+}]_i$ responses induced by lindane in human spermatozoa

Fura 2-loaded sperm were suspended in regular buffer and incubated (20 min, 37 °C) in the presence of the organic Ca^{2+}-channel blockers nicardipine, diltiazem and verapamil (5 μM each). For membrane potential experiments, 200 nM bisoxonol was added to the sperm suspension. Lindane-induced membrane depolarization is expressed as percentage variation from the control (100%, no inhibitors added) of the bisoxonol fluorescence peak value; lindane-induced $[Ca^{2+}]_i$ increase is expressed as percentage variation from the control (100%, no inhibitors added) of the nanomolar peak value. Fluorescence changes induced by 40 μM lindane were monitored as described in Figure 2 in the continuous presence of the Ca^{2+} inhibitors. Each value is the mean value $\pm$ S.D. for data obtained from at least three preparations. $^*P < 0.01$.

	Nicardipine	Diltiazem	Verapamil
$[Ca^{2+}]_i$ increase (%)	$55 \pm 3.5^*$	$87 \pm 5.9^*$	96 ± 5.5
Depolarization (%)	$83 \pm 3.1^*$	98 ± 4.7	100 ± 6.5

grouped on the basis of the ion composition of the extracellular medium.

Ca^{2+}/Na^+ buffer (regular buffer)

On the addition of lindane, both sperm membrane potential and $[Ca^{2+}]_i$ rose from resting levels to reach a peak within approx. 15 s, and thereafter decreased to a plateau level in the subsequent 60 s (Figures 3a and 3b). Up to 6 mM extracellular tetraethylammonium chloride (TEA), an inhibitor of Ca^{2+}-activated K^+ channels (K_{Ca} channels), failed to alter membrane repolarization, suggesting that TEA-sensitive K_{Ca} channels are not involved (not shown). On subsequent re-addition of lindane, both of the sperm responses were reproduced, characterized by progressively decreasing peak intensities (Figures 4a and 4b). Lindane-induced $[Ca^{2+}]_i$ increase was 45 and 13% inhibited by treatment with nicardipine and diltiazem respectively whereas membrane depolarization was lowered by 17% only by nicardipine. The phenylalkylamine verapamil failed to affect either $[Ca^{2+}]_i$ or membrane potential responses to the insecticide (Table 1). These findings suggest that L-type and, because of the known inhibitory properties of nicardipine [37], also perhaps T-type VOCCs have a role in the sperm response to lindane, and confirm the absence of verapamil-sensitive Ca^{2+} channels in human sperm [38,39]. Further indication of VOCC opening by lindane was obtained by using Cd^{2+} [40]; this inorganic Ca^{2+} channel blocker, in fact, inhibited in a dose-dependent distinctly biphasic manner the lindane-induced membrane depolarization (with an IC_{50} of 2 and 150 μM for each phase respectively) (Figure 5). Unfortunately, Cd^{2+} binds to fura 2 with much higher affinity than do Ca^{2+} ions and causes fluorescence enhancing; therefore even a minute leakage of Cd^{2+} ions through Ca^{2+} channels produces a significant fluorescence increase making the fluorescence measurements unreliable [41]. As Cd^{2+} exerts a much stronger inhibition on L-type than T-type channels (K_d 7 μM and 160 μM respectively) [40], the above finding further suggests that human spermatozoa express T-type VOCCs. The inhibition of membrane depolarization by nicardipine and Cd^{2+} shows that VOCC-mediated inward Ca^{2+} current plays a role in the lindane-evoked electric phenomena.

Na^+-free buffer (choline buffer)

When extracellular Na^+ was iso-osmotically replaced by choline, the basal level of the bisoxonol signal was lower than that measured in regular buffer, reflecting, in accordance with the equation for the resting potential [42], membrane hyperpolarization. In this buffer, the timing and curve shape of membrane depolarization brought about by lindane were similar to those recorded in regular buffer (Figure 3c). Sperm $[Ca^{2+}]_i$ response to lindane was similar in shape and slightly reduced in magnitude ($< 10\%$) compared with that measured in regular buffer (Figure 3d). The lack of effect of Na^+ removal on the magnitude of membrane depolarization and the trace profile of the Ca^{2+} response would appear to rule out the involvement of either Na^+ influx or Ca^{2+}/Na^+ exchange.

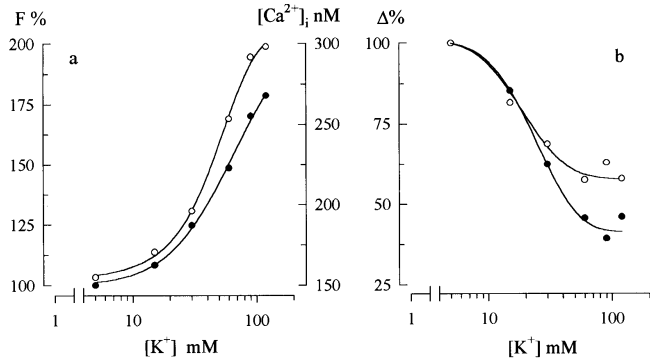

Figure 6 Effect of high extracellular $[K^+]$ on basal and lindane-affected sperm membrane potential and $[Ca^{2+}]_i$

Fura 2-loaded sperm were suspended in a regular buffer-based medium in which increasing concentrations of extracellular $[K^+]$ were compensated by proportionally lowering the NaCl concentration to ensure iso-osmolarity. For membrane potential experiments, 200 nM bisoxonol was added to the sperm suspension. The changes in fluorescence were monitored as described in Figure 2. The data are expressed as percentage variation from the control (regular buffer, $[K^+] = 5$ mM) of the basal (**a**) and 40 μM lindane-induced peak (**b**) values of the bisoxonol signal (●) and $[Ca^{2+}]_i$ (○). Each data point is the mean value for data obtained from at least four preparations. The curves are the best line fits to the experimental data.

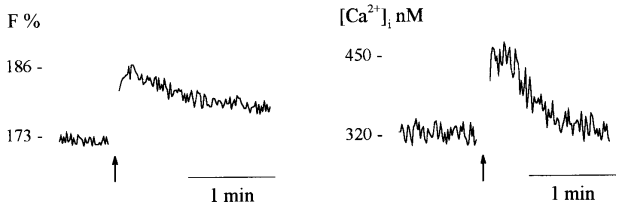

Figure 7 Membrane potential (left traces) and $[Ca^{2+}]_i$ (right traces) responses elicited by lindane in human spermatozoa suspended in high-K^+ buffer

Fura 2-loaded spermatozoa were suspended in iso-osmolar 120 mM K^+ buffer in the presence or absence of 200 nM bisoxonol. The changes in fluorescence were monitored as described in Figure 2. Arrows indicate the addition of 40 μM lindane. F%, bisoxonol fluorescence intensity normalized assuming that the control basal signal (collected in regular buffer) = 100. The traces are representative of at least four experiments.

Ca^{2+}-free buffer (EGTA buffer)

In this buffer, lindane-induced membrane depolarization was about 20 % lower than that elicited in regular buffer and showed no tendency to return to the basal level (Figure 3e). This finding confirms that the lindane-promoted inward Ca^{2+} current has an electrogenic role in the depolarization phenomenon, and indicates that the $[Ca^{2+}]_i$ increase is necessary for membrane repolarization. In the absence of extracellular Ca^{2+}, lindane did not alter $[Ca^{2+}]_i$ (Figure 3f), indicating that the increase in $[Ca^{2+}]_i$ depends totally on an influx from the extracellular medium.

Ca^{2+}-free/Na^+-free buffer (EGTA/choline buffer)

On the addition of lindane, reduced membrane depolarization was observed, corresponding to approx. 45 % of that obtained in regular buffer (Figure 3g). As expected, $[Ca^{2+}]_i$ did not change to any appreciable extent (Figure 3h). Comparable results were obtained when sucrose was iso-osmotically substituted for Na^+ (not shown), ruling out a role for choline in the membrane depolarization observed.

Cl^--free buffer (gluconate buffer)

When extracellular Cl^- was iso-osmotically replaced by gluconate, the basal levels of both the bisoxonol signal and $[Ca^{2+}]_i$ were higher than those observed in regular buffer. This finding indicates a more positive membrane potential [42], probably accounting in turn for the opening of some VOCCs. Membrane depolarization and $[Ca^{2+}]_i$ increase brought about by lindane were slightly smaller than those measured in regular buffer (Figures 3i and 3j), making the participation of Cl^- ions in the phenomenon unlikely.

High-K^+ buffer

On increasing extracellular $[K^+]$, concurrent increases in both the bisoxonol signal and $[Ca^{2+}]_i$ were measured, further supporting the above evidence that spermatozoa possess voltage-operated mechanisms of Ca^{2+} influx (Figure 6a). In the presence of increasing extracellular $[K^+]$, both membrane potential and $[Ca^{2+}]_i$ responses to lindane were progressively attenuated (Figure 6b) and were not further inhibitable at extracellular $[K^+]$ higher than 60 mM. These results confirm the voltage-dependence of the ionic events elicited by the insecticide, and show that a portion of the sperm responses to lindane (accounting for 57 and 43 % of $[Ca^{2+}]_i$ increase and membrane depolarization observed in regular buffer respectively) is independent of VOCC opening induced by collapsing the transmembrane potential. Rather unexpectedly, lindane was still able to produce further membrane depolarization and Ca^{2+} influx in the maximally depolarizing buffer ($[K^+] = 120$ mM) (Figure 7), and the electric response to lindane was still produced when Ca^{2+} was omitted from this buffer (not shown).

The addition of either 50 μM GABA or 50 μM picrotoxin (a rather specific inhibitor of GABA-receptor-linked Cl^- channels [43]) to spermatozoa suspended in regular buffer did not alter either the basal or lindane-regulated membrane potential and $[Ca^{2+}]_i$; therefore it may be ruled out that GABA receptors or GABA-receptor-linked Cl^- channels are functionally active or lindane-stimulatable in sperm (not shown). The tyrosine kinase inhibitor genistein, the Protein G inhibitor pertussin toxin and the protein kinase C inhibitor staurosporin failed to modify either membrane potential or $[Ca^{2+}]_i$ responses to lindane (not shown), making it unlikely that lindane activates molecular events belonging to the usual receptor-linked signal-transduction pathways in human spermatozoa.

Molecular dynamics of the membrane surface

Prodan and Laurodan fluorescence spectra collected in unstimulated cells are reported in Figure 8. Both Prodan and Laurodan showed excitation spectra with a single emission peak centred at 370 nm and emission spectra with two emission peaks centred at 435 nm and 490 nm. Laurodan showed a higher spectral fluorescence intensity, i.e. a higher quantum yield, and a higher shoulder of the emission profile in the blue region of the excitation spectrum. This spectroscopic behaviour suggests that Laurodan molecules were populating a less polar environment than Prodan [34]. In basal conditions, Prodan and Laurodan GPex values, which express the amount of dipolar relaxations, were comparable (0.390 ± 0.002 and 0.385 ± 0.004 respectively), whereas the Prodan GPem value was higher than that for Laurodan (-0.15 ± 0.012 and -0.21 ± 0.010 respectively; $P < 0.01$), confirming that Prodan was sensing a more polar environment. On the addition of lindane, a rapid increase in both

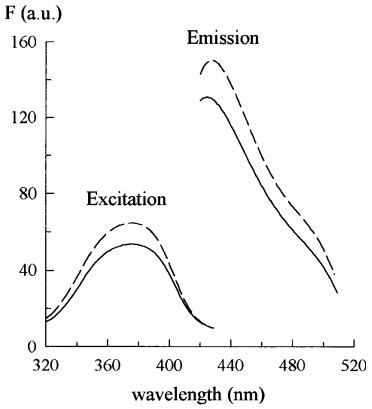

Figure 8 Excitation and emission spectra of Prodan- and Laurodan-loaded sperm

Human spermatozoa suspended in regular buffer were loaded with Prodan (——) or Laurodan (– – – –) as described in the Materials and methods section. The excitation spectra were collected at an emission wavelength of 490 nm, and the emission spectra were collected at an excitation wavelength of 370 nm. The traces are the best line fits to the experimental data and are representative of at least four experiments. Fluorescence is expressed in arbitrary units.

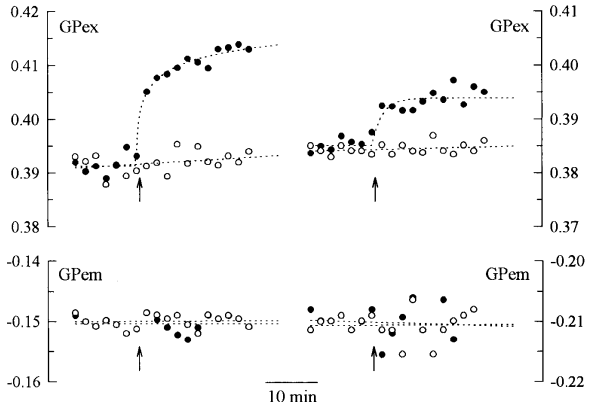

Figure 9 Effect of lindane on sperm Prodan and Laurodan GP values

GPex (upper traces) and GPem (lower traces) values obtained in control (DMSO addition at arrow; ○) and in lindane-supplemented (33 μM at arrow; ●) sperm suspended in regular buffer and loaded with Prodan (left traces) or Laurodan (right traces) as described in the Materials and methods section. The GP values were calculated from fluorescence spectra collected as described in Figure 8 and 2 min intervals after subtraction of sperm and unpartitioned probe autofluorescence. The traces are the best line fits to the experimental data and are representative of four consistent experiments.

Prodan and Laurodan GPex values occurred with a maximum after approx. 5 min, the GPem values remaining constant (Figure 9). The effect was dose-dependent and increased linearly with increasing lindane concentration (Figure 10). The greater variation of Prodan GPex than Laurodan GPex indicates that lindane brought about a larger decrease in probe dipolar relaxations in the most polar region of the bilayer. From these findings it may be inferred that lindane interacts with the sperm membrane by intercalating into the external leaflet of the bilayer at the lipid/water interface, thereby locally reducing water molecular dynamics.

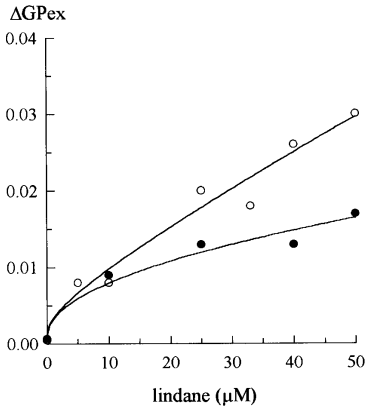

Figure 10 Effect of increasing concentrations of lindane on sperm Prodan and Laurodan GPex

Sperm suspended in regular buffer were loaded with Prodan (○) or Laurodan (●) as described in the Materials and methods section, and GPex values were calculated from fluorescence spectra as described in Figure 9. The data presented are the differences between GPex values obtained before and 5 min after the addition of lindane and are representative of at least four experiments. The traces are the best line fits to the experimental data.

DISCUSSION

The administration of lindane to human spermatozoa *in vitro* induced significant effects consisting of transient membrane depolarization, Ca^{2+} influx and reduction of the molecular dynamics at the lipid/water interface, as assessed by bisoxonol, fura 2 and Laurodan/Prodan fluorescence spectroscopy respectively.

The membrane depolarization induced by lindane in spermatozoa suspended in regular buffer was 20% reduced in the absence of extracellular Ca^{2+} and was not followed by repolarization, a finding that indicates that the Ca^{2+} influx participates in both membrane depolarization and repolarization. The evidence that membrane depolarization and repolarization were not affected by eliminating either Na^+ or Cl^- from the extracellular medium rules out an electrogenic role for these ions. However, the electric response to lindane was reduced by 55% in the combined absence of extracellular Na^+ and Ca^{2+}, indicating that a Na^+ influx was responsible in part for the depolarization observed in the absence of extracellular Ca^{2+}. The evidence that an influx of either Na^+ or Ca^{2+} *per se* produced membrane depolarization, along with the lack of additivity between the depolarizing potencies of these cations, might be interpreted as activation by lindane of membrane channels admitting both Ca^{2+} and Na^+ ions, i.e. non-specific cation channels. Indeed, the presence of such a channel in human spermatozoa has been reported [33]. However, neither an increase in Ca^{2+} influx nor increased membrane depolarization was elicited by lindane in the absence of extracellular Na^+ or Ca^{2+} respectively, apparently excluding the possibility that lindane activates non-specific cation channels. Instead, we feel that the electric events brought about by the insecticide are probably mainly driven by the Ca^{2+} influx. We found that increasing extracellular $[K^+]$ induced both membrane depolarization and $[Ca^{2+}]_i$ increase, meaning that voltage-operated Ca^{2+} influx pathways are expressed at the sperm surface. In high-K^+ buffer, both membrane potential and $[Ca^{2+}]_i$ responses to lindane were depressed, indicating that the ionic events elicited by the insecticide are mediated by voltage-dependent mechanisms. The partial inhibition of both $[Ca^{2+}]_i$ increase and

membrane depolarization by the Ca^{2+} antagonists nicardipine, diltiazem and Cd^{2+} demonstrates that lindane activates sperm VOCCs and that the membrane depolarization is, at least in part, produced by the Ca^{2+} flux through these channels. The rank of inhibition potency exerted by the organic antagonists on Ca^{2+} influx and the distinctly biphasic dose-dependence of the Cd^{2+} effect on membrane depolarization suggest that both L- and T-type VOCCs open on lindane interaction with human sperm. From all of the above, the Na^+ influx observed on lindane addition in the absence of extracellular Ca^{2+} could be explained by the known ability of VOCCs to pass univalent cations (Na^+ in this case) if extracellular Ca^{2+} is omitted or chelated [44]. The slight reduction in $[Ca^{2+}]_i$ increase induced by lindane in Na^+-free conditions was not unexpected, as the increased resting membrane potential caused by the absence of extracellular Na^+ [42] lowers the probability of VOCC opening.

We found that membrane repolarization was promoted by the increase in $[Ca^{2+}]_i$. Since the removal of Cl^- from the extracellular medium did not inhibit the phenomenon, it is likely that K_{Ca} channels are involved, and the insensitivity of membrane repolarization to extracellular TEA suggests that such channels probably belong to either the intermediate or small conductance subclasses of K_{Ca} channels [45].

In the presence of high extracellular K^+, lindane evoked additional membrane depolarization and Ca^{2+} influx. Since the electric response was still induced by the insecticide when extracellular Ca^{2+} was omitted, it could be ruled out that the above depolarization is driven by the inward Ca^{2+} current. Moreover, a membrane depolarization, accounting for approx. 45% of that obtained in regular buffer, was still induced by lindane in the complete absence of extracellular permeant cations. All these pieces of evidence, along with the finding that the insecticide did not modify the membrane permeability to Cl^-, strongly suggest that electric events independent of ion fluxes participate in lindane-evoked depolarization. In reference to this, let us recall that, apart from the transmembrane potential ($\Delta\Psi$) arising from the separation of net charge across the entire thickness of the bilayer, the electrostatic membrane potential is concurrently determined by (i) the surface charge density contributed by fixed ionized groups of membrane components exposed at the membrane/solution interface (surface potential, Ψ_S), and (ii) the intramembrane arrays of oriented dipoles localized just below the membrane/water interface (dipole potential, Ψ_D) [46]. Since the lindane molecule is electroneutral, it cannot alter Ψ_S; consequently, we hypothesize that lindane alters the membrane dipole potential. Indeed, as a result of the dipole moment given by the asymmetrical distribution of chlorine atoms in the molecule [47], lindane is probably able to modify Ψ_D directly and/or by reorienting membrane intrinsic dipoles. Since Ψ_D changes produce changes in the binding and translocation rates of hydrophobic ions [48], bisoxonol, a slightly lipophilic anion, might change its membrane/water distribution (mirrored by changes in emitted fluorescence intensity) as a result of a change in dipole potential. Consequently, the increase in bisoxonol fluorescence intensity measured on the addition of lindane to spermatozoa in which $\Delta\Psi$ had been collapsed by 120 mM K^+ could reasonably be ascribed to a change in Ψ_D. Earlier data showing that various membrane-active compounds alter the dipole potential in lipid bilayers lend further support to this hypothesis [49–51]. The evidence that the insecticide was still able to induce an increase in $[Ca^{2+}]_i$ in depolarizing conditions suggest that Ψ_D modifications may affect the conductance of membrane ion channels, as previously surmised on theoretical grounds [52,53].

Our hypothesis that lindane affects membrane Ψ_D is also in good agreement with the evidence from Laurodan and Prodan spectroscopy that the insecticide intercalates in the uppermost region of the bilayer external leaflet, locally reducing the molecular dynamics of water dipoles. At least two possibilities exist of how this effect is produced: (i) lindane partition induces an increase in membrane lipid acyl chain molecular order resulting in hindered mobility of water molecules and/or (ii) the water molecules reorient on the lindane dipole, thereby reducing their degree of mobility freedom. Since Prodan molecules populate a more exterior more polar membrane region than Laurodan [23], the finding that lindane produces a larger increase in Prodan GPex than the increase in Laurodan GPex favours the latter hypothesis.

Collectively, the data presented in this paper provide evidence that lindane partitions into the sperm membrane, alters the dipole potential and stimulates Ca^{2+} influx, at least partially, through VOCCs. This Ca^{2+} current causes the transmembrane potential to reach the gating threshold of additional VOCCs, thereby increasing $[Ca^{2+}]_i$ and activating repolarization of K_{Ca} channels.

Our data bring to light marked differences between the mechanism of action of lindane in somatic cells and in spermatozoa. In neuronal cells, lindane interacts with and inhibits GABA-receptor-linked Cl^- channels [13]; however, this seems not to be the case in human spermatozoa where, although the $GABA_A$ receptor α-subunit was identified [54], neither GABA nor picrotoxin, a putative inhibitor of GABA-receptor-linked Cl^- channel, was able to alter either the basal or lindane-regulated membrane depolarization and $[Ca^{2+}]_i$. The insecticide has been suggested to release Ca^{2+} from $InsP_3$-sensitive intracellular stores in macrophages and myometrial smooth-muscle cells [16,17]. Although $InsP_3$-gated Ca^{2+} channels selectively localized to the acrosome of mammalian spermatozoa have been reported [55], our data show that the lindane-induced increase in $[Ca^{2+}]_i$ in human sperm is totally dependent on an influx from the extracellular medium. Finally, the evidence that both membrane depolarization and intracellular Ca^{2+} transients are reproduced on subsequent readdition of lindane, along with the lack of effect of several inhibitors of receptor-coupled signal-transduction pathway components, suggest that lindane interacts with human spermatozoa through a non-regulatable non-receptor-mediated mechanism. Interestingly, the findings of this study could give a rationale to those published in a recent paper in which lindane was reported to exert its convulsant effect through the activation of L-type VOCCs by a no-better-defined GABA-receptor-independent mechanism [18].

From a general point of view, this work highlights that the alteration of the membrane dipole potential produced by the partition of a hydrophobic molecule bearing a dipole moment is able to affect the conductance and/or the threshold potential of ion channels. Studies into how this until now rather neglected but evidently huge component of membrane potential might influence cell behaviour will presumably be of help in understanding better some still obscure aspects of cell physiology.

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