Action of Ionophore A23187 at the Cellular Level

SEPARATION OF EFFECTS AT THE PLASMA AND MITOCHONDRIAL MEMBRANES*

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Bovine epididymal spermatozoa incubated aerobically in vitro in the presence of 0.1 to 0.2 mM CaCl₂ accumulate 25 to 50 nmol of calcium/10⁸ cells. The addition of low concentrations of the ionophore A23187 (0.01 to 0.5 nmol/mg of sperm protein) induces efflux of this accumulated calcium. At high ionophore concentrations (0.5 to 5.0 nmol/mg of sperm protein), calcium release is followed by an influx of up to 25 nmol of calcium/10⁸ cells that is not dependent on mitochondrial energization. A selective increase in the permeability of the sperm plasma membrane produced by treatment with the polyene antibiotic, filipin, results in the release of that calcium which is accumulated in the presence of high concentrations of A23187. Sperm first treated with filipin possess the ability to accumulate and retain calcium (in the presence of an oxidizable substrate) but release Ca²⁺ without subsequent reaccumulation after the addition of 3 nmol of A23187/mg of protein. These observations are explained by the existence of competing calcium pumps operating within the mitochondrial and plasma membranes of the spermatozoan. Treatment with high concentrations of A23187 allows calcium influx into a non-mitochondrial compartment of the sperm cell as a consequence of the equilibration of this cation across both mitochondrial and plasma membranes. The amount of calcium uptake and its sensitivity to filipin indicate that calcium binding to soluble, intracellular components is also involved.

The ability of low concentrations of A23187 to induce calcium efflux is explained as a result of the continued operation of the plasma membrane pump coincident with ionophore-induced decay of the concentration gradient across the mitochondrial membrane. This hypothetical action of low levels of the ionophore on the mitochondria is supported by the observation of net movements of calcium with filipin-treated cells and the respiratory responses and movements of phosphate and membrane-associated calcium with intact sperm. It is suggested that the basis of this apparent selectivity of ionophore action lies in the relative activities and kinetic properties of the competing calcium pumps in the plasma and mitochondrial membranes of these cells.

Ionophore-induced influx of calcium into the extramitochondrial space results in a stimulation of respiration and kinetic activity of the sperm. This activation of motility is observed also with cells made entirely dependent upon glycolysis (by treatment with respiratory inhibitors) and suggests a direct involvement of calcium in the regulation of flagellar function.

The carboxylic acid ionophores X537A and A23187 have attracted considerable attention since the demonstration of their ability to transport divalent cations across biological membranes (1–4). Information originally obtained from bulk phase extraction experiments was consistent with the formation of a neutral, lipophilic, 2:1 complex of ionophore A23187 and divalent metal ions (3–5). Detailed examination of the ¹³C-NMR spectra of A23187 and its metal complexes has since confirmed this stoichiometry and indicated that A23187 possesses sufficient conformational rigidity to explain its observed cation-size selectivity (6). Other aspects of the chemistry of A23187 and its metal complexes are under study in this laboratory.

The available data indicate that A23187 promotes an electroneutral exchange of protons and divalent metal ions across the membranes of cells and organelles. For instance, the uncoupling of respiration and oxidative phosphorylation in isolated mitochondria treated with A23187 is explained in terms of a cyclic, energy-dissipating calcium flux produced by opposing actions of the ionophore and the mitochondrial calcium pump (1–3). A similar explanation obtains for the stimulation of sarcoplasmic reticulum ATPase activity which follows ionophore treatment (7). In the erythrocyte, which normally maintains a low intracellular calcium concentration,
A23187 induces calcium influx from the medium and a rapid release of protons. The present study consists of observations made with spermatozoa, in which action of the ionophore at the plasma and mitochondrial membranes can be separately or simultaneously examined.

A large number of reports have described the activation by ionophores X537A and A23187 of those cellular processes relating to motility (1, 6-10), secretion (11-14), and division (15-18) which are thought to be regulated by intracellular Ca$^{2+}$ concentrations. However, in general, the functional alterations produced by treatment of intact cells and tissues with these ionophores have been explained only in vague terms as the result of redistribution of calcium across cellular membranes, often with no experimental justification. The experiments presented here indicate some of the complexities that may in fact underly ionophore-induced activation of cellular processes and provide a partial explanation of A23187 action on cellular calcium fluxes.

Earlier reports from this laboratory indicated that treatment with either phosphodiesterase inhibitors (19-21) or ionophore A23187 (1) affects the kinetic activity of bovine spermatozoa. Also offered herein are objective documentation of these effects and a description of the requirements for ionophore-mediated activation of sperm motility.

### EXPERIMENTAL PROCEDURE

Chemicals were obtained from the following sources: morpholino-propanesulfonic acid, triethanolamine, and sodium D-β-hydroxybutyrate from Sigma Chemical Co.; filipin from the Upjohn Co.; polyvinylpyrrolidone from Nutritional Biochemicals; rotenone from S. P. Penick Co.; chlorotetracycline and carbonylcyanide-m-chlorophenylhydrazone from Calbiochem; A23187 from Eli Lilly Co.; murexide from Eastman Organic; $^{45}$CaCl$_2$ and $^{32}$P from Amersham/Searle. Procedures for sperm preparation and incubation in vitro were as previously described (22) except that magnesium salts were deleted and all media and solutions of mannitol/sucrose were deionized, adjusted to pH 7.4 with triethanolamine, and stored at 4°. A23187 was stored as a 10 mM solution in dimethylformamide/ethanol (3/1) at -20°. The determination of $^{45}$P, uptake was also performed as before and $^{45}$Ca uptake studies were identical in procedure except that $^{45}$CaCl$_2$ (700 cpm/nmol) replaced $^{32}$P, as the radioisotope and the washing media contained 1 mM CaCl$_2$ rather than 10 mM sodium phosphate. An Aminco DW-2 spectrophotometer was employed for monitoring murexide (23) absorbance changes using the wavelength pair A$_{385}$/A$_{525}$ and an Amino-Bowman spectrotitruometer was used to follow chlorotetracycline fluorescence (24, 25), with excitation and emission wavelengths of 385 and 525 nm, respectively. Sperm motility was assessed by a time-lapse (2-s exposure) photomicroscopic procedure (26) in Petroff-Hauzer counting chambers (E. A. Hauser, Blue Bell, Pa.) at 30°. Motility was determined from the percentage of total cells which showed flagellar motion in each of six successive fields (each containing approximately 75 cells). The average swimming speed was calculated from the measurement of tracks left by those cells which showed forward progression. The total protein content of bovine sperm was taken as 2.2 mg/10$^8$ cells. Mitochondrial protein was estimated as 0.11 mg/10$^8$ cells, 60% of the reported dry mass of the bovine sperm midpiece (27).

### RESULTS

**Calcium Accumulation by Sperm**—Bovine epididymal spermatozoa have an endogenous content of 5 to 10 nmol of calcium/10$^8$ cells (28). Fig. 1 shows that during incubation in vitro, epididymal sperm accumulated 3 to 6 times this amount of calcium (under appropriate conditions. calcium uptake approached 150 nmol/10$^8$ cells). In these experiments, addition of 0.17 mM CaCl$_2$ resulted in an initial decrease of optical difference absorbance due to the formation of the calcium-murexide complex (23). As the sperm accumulated calcium, the absorbance returned as a result of the reappearance of free murexide. This influx of calcium is accompanied by phosphate entry and the uptake of both ions is sensitive to inhibition by —SH reagents, ruthenium red and respiratory inhibitors (22). It is most likely that calcium and phosphate accumulation by sperm is a result of mitochondrial sequestration.

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**Fig. 1 (left).** Dose-dependent effects of A23187 on sperm calcium fluxes. Washed bovine epididymal spermatozoa (1.42 x 10$^8$ cells) were suspended in 3.0 ml of medium containing 200 mM mannitol/70 mM sucrose/10 mM sodium morpholinopropanesulfonate, pH 7.4/0.1 mM murexide/3 mM sodium D-β-hydroxybutyrate/0.3 mM sodium phosphate. CaCl$_2$ was added to a final concentration of 0.17 mM. A23187 was added in 10 μl dimethylformamide/ethanol (3/1) to the final amounts indicated at 7.5 min after CaCl$_2$ addition.

**Fig. 2 (center).** The effect of A23187 on calcium influx and efflux rates and oxygen consumption by sperm. Bovine sperm (1.50 x 10$^8$/ml) were incubated as in Fig. 1 for measurement of initial rates of calcium efflux (O). For calcium influx (●), 0.5 nmol of A23187/mg of protein was added and 1 min later, A23187, to give the final concentrations indicated. Rates of influx were determined immediately after the second addition of ionophore. The respiratory rate after the final addition of ionophore (A) was followed with a vibrating platinum electrode.

**Fig. 3 (right).** Separation of calcium uptake and release induced by A23187. Bovine sperm (1.17 x 10$^8$ cells) were incubated as in Fig. 1. Additions were made at the indicated times to give final concentrations of: CaCl$_2$, 0.13 mM; A23187, 3 and 30 μM; C1CCP, 1 μM.
**A23187 and Mg**\(^{2+}\) Effects on Calcium Flux and Respiration—**Fig. 1** also shows the effect of increasing concentrations of A23187 on net calcium flux for intact bovine spermatozoa. Even very low concentrations of A23187 caused a gradual but complete release of accumulated calcium. Larger quantities of ionophore increased the rate of release. However, further increases in A23187 concentration resulted in a diminution of net Ca\(^{2+}\) efflux followed by a progressively more rapid reaccumulation of this cation.

**Fig. 2** shows the initial rates of calcium release and uptake in response to various dosages of A23187, along with the associated respiratory rates observed after ionophore addition. Since the lipophilic ionophore preferentially partitions into cellular membranes, its effects on calcium fluxes were a function of the ratio of ionophore to cell concentrations and comparable rates of calcium flux were obtained by varying either of these parameters.

Cellular influx and efflux were in equilibrium in the presence of 5 pmol of A23187/mg of protein. The rate of ionophore-induced calcium efflux increased until apparent saturation was reached at approximately 0.5 nmol of A23187/mg of protein. In these experiments, the maximum calcium efflux rate was approximately 10 times that of the influx observed in the absence of ionophore. However, in other experiments it was found that the rate of calcium efflux induced by the ionophore depended very little on the initial external calcium concentration, whereas the rate of influx observed in untreated cells was strongly dependent on calcium concentration.

The rate of calcium influx that occurred in the presence of high levels of the ionophore likewise exceeded the influx rate observed in untreated cells and was proportional to A23187 concentration in the range 0.5 to 3 nmol of A23187/mg of protein (Fig. 2). In other experiments, it was found that both the rate and amount of calcium uptake that were induced by addition of A23187 depended on external calcium concentration. Maximum uptake approached 25 nmol of calcium/10\(^6\) cells as the external calcium concentration was increased to 0.35 mM.

Oxygen consumption increased in the presence of ionophore concentrations that caused calcium influx. The maximum respiratory stimulation of approximately 3-fold was achieved at A23187 concentrations in excess of 1 nmol/mg of protein. Oxygen consumption did not increase after A23187 addition if EGTA was present to chelate external calcium. Storey (29) similarly observed that respiratory activation of rabbit epididymal spermatozoa by A23187 required the simultaneous presence of ionophore and external calcium.

The described effects of A23187 on sperm calcium fluxes were obtained in both mannitol/sucrose and modified Ringer buffers. Magnesium had no effect on calcium uptake by untreated sperm. In the presence of A23187, the rate of calcium efflux was reduced to 40% by the inclusion of 0.8 mM MgCl\(_2\), but the rate of calcium influx was only 6% of the influx rate in magnesium-free medium. Presumably these observations reflect the accessibility of A23187 to extracellular Mg\(^{2+}\) which competes with Ca\(^{2+}\) for complex formation with the ionophore (5).

**Separation of A23187 Induced Efflux and Influx**—The calcium influx and efflux induced by A23187 can be temporally separated. Efflux produced by low concentrations of ionophore (Fig. 3A) was followed by influx when higher concentrations of ionophore were later added (Fig. 3B). No calcium accumulation occurred in cells that were treated with respiratory uncouplers such as CCCP prior to calcium addition (Fig. 3D), although calcium influx was observed when CCCP and high concentrations of A23187 were both added to calcium-depleted cells (Fig. 3C). A23187 also induced calcium influx into cells which had not been subjected to a prior cycle of accumulation and depletion (Fig. 3D). It should be noted that addition of low concentrations of ionophore, which produced only calcium efflux in control preparations, resulted in calcium influx for cells treated with uncoupler (cf. Fig. 3, B and C; Fig. 3, A and D). The ionophore-induced influx in uncoupler-treated sperm was confirmed with \(^{45}\)Ca radioisotope measurements (Table I). Together, these experiments indicated that calcium uptake in the presence of ionophore does not require mitochondrial participation and is in fact potentiated by agents which interfere with mitochondrial functions.

Experiments employing the polyene antibiotic filipin support the lack of mitochondrial involvement in the calcium influx induced by the ionophore. Filipin forms a complex with cholesterol in free solution and in biological membranes (30, 31). This antibiotic selectively increases the permeability of the bovine sperm plasma membrane while leaving the mitochondria of these cells functionally intact (22, 32). This is presumably reflecting a differential cholesterol content of the respective membranes. When sperm were treated with filipin, sperm mitochondria accumulated calcium if an oxidizable substrate was also supplied, and while low ionophore concentrations still produced calcium efflux, high concentrations of A23187 failed to induce calcium influx (Fig. 4A). Moreover, if ionophore-induced influx was allowed to occur in intact sperm, subsequent disruption of the integrity of the plasma membrane by filipin resulted in calcium release (Fig. 4B).

Previous experiments utilizing sulfhydryl-directed reagents indicated that the plasma membrane of sperm limits calcium and phosphate entry into sperm mitochondria (29). This contention is born out in the present experiments (Fig. 4) showing an increased rate of calcium uptake after filipin treatment due in part to the higher concentration of Ca\(^{2+}\) accessible to the mitochondria of treated cells. Oxygen consumption almost doubled after addition of 0.17 mM CaCl\(_2\) to filipin-treated cells and the further addition of 0.3 nmol of A23187/mg of protein resulted in nearly maximal respiratory stimulation. The respiratory responses of filipin-treated sperm to CaCl\(_2\) and A23187 were similar to the effect of these agents upon mitochondria isolated from other sources (3). In contrast, additions of CaCl\(_2\) and low levels of ionophore to intact cells produced only slight increases in respiratory activity. Significant stimulation of oxygen consumption occurred only when calcium influx was induced by high concentrations of ionophore (cf. Fig. 2).

Results from experiments utilizing the fluorescent probe, chlorotetraacycline (24, 25, 33) also suggest a bimodal action of A23187 on cellular calcium distribution. Addition of chlorotetraacyline to intact sperm produced a time-dependent increase in the fluorescence signal, presumably as a consequence of accumulation of the antibiotic with resulting fluorescence enhancement in the apolar environment of the cellular membranes (Fig. 5). A further fluorescence increase paralleled the rapid, early calcium influx observed with the murexide technique. Similar observations with isolated mitochondria are interpreted as reflections of the interaction of chlorotetraacyline and membrane-associated calcium (33, 34). No further investigations were carried out.

1 The abbreviation used is: CCCP, carbonylcyanide-m-chloro phenylhydrazone.
TABLE I

<table>
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Bovine sperm (0.92 x 10^6 cells/ml) were incubated as in Fig. 1 with or without 3 μM CICCP and 0, 10, or 100 μM A23187 for the indicated times prior to addition of 0.15 mM CaCl_2 containing 700 cpm of ^44Ca/nmol. After 12 min incubation, 0.1-ml samples were removed, collected on glass fiber filters, and washed four times with 1 ml of medium containing 1 mM CaCl_2.

Concentration occurred during the later, linear phase of calcium accumulation which apparently corresponds to deposition of calcium in the mitochondrial matrix (24, 33). The complete efflux of calcium induced by addition of 0.3 nmol of A23187/mg of protein is accompanied by a corresponding loss of the fluorescence signal. Note however that there is no return of fluorescence during influx of calcium induced by high concentrations of ionophore. Apparently, interaction of chlorotetracycline with Ca^2+ occurs only at mitochondrial membranes of the sperm. Visual observation of tetracycline fluorescence in treated sperm indicates that this may be a result of preferential distribution of the antibiotic in the mitochondria of these cells (35). In this regard it has been reported that bacteria actively accumulate chlorotetracycline (36).

We have previously established that bovine sperm accumulate phosphate during incubation in the presence of calcium salts (22). Fig. 6 shows that treatment of sperm with A23187 at concentrations that caused calcium efflux also released accumulated phosphate. Note however that no further phosphate movement followed subsequent addition of a quantity of ionophore sufficient to induce calcium influx. This latter observation was interpreted as a probable reflection of an electroneutral exchange of protons and calcium across the plasma membrane promoted by high concentrations of the ionophore. The parallel release of calcium and phosphate however suggests that other mechanisms intervene in the calcium efflux induced by treatment with low levels of the ionophore. The possible movement of other ions cannot be excluded.

**A23187 Effect on Motility**—Bovine caudal epididymal spermatzoa were almost completely immotile when suspended in...
from other sources (approximately 1500 nmol of calcium/mg of mitochondrial protein) (37), we tentatively accept the hypothesis that nearly all sperm mitochondria accumulate calcium under our experimental conditions. Granting this qualification, the action of ionophore A23187 on sperm calcium fluxes is most simply explained in terms of the operation of opposing calcium pumps residing in the mitochondrial and plasma membranes of these cells, responsible for maintenance of low cytosolic calcium concentrations. In fact, an uncoupler-sensitive, ouabain-resistant ATPase stimulated by Mg$^{2+}$ and Ca$^{2+}$ is present in human sperm (38). In membranous fragments possibly derived from bull sperm, a similar activity exceeds 1 μmol of P/min/mg of protein (39).

The net cellular efflux of calcium which follows exposure to small quantities of A23187 apparently results from ionophore-induced release of this cation into the extramitochondrial space in response to the concentration gradient existing across the mitochondrial membrane. No respiratory stimulation is associated with the ionophore-induced calcium efflux from intact sperm. In contrast, with filipin-treated sperm and with isolated rat liver mitochondria (3), respiratory acceleration is observed at ionophore concentrations even lower than those required for net calcium release. The presence of chelating agents enhances the ability of low levels of A23187 to induce calcium efflux from liver mitochondria but prevents cyclical reuptake of this ion and the associated increases in oxygen consumption (3). We conclude that low levels of ionophore are ineffective in activating the respiration of intact spermatozoa because the plasma membrane calcium pump plays a comparable role in preventing energy-dissipative, cyclic fluxes of calcium and is finally responsible for expulsion of this ion to the medium. It is unclear if the release of phosphate which accompanies calcium efflux is a reflection of the reversal of the uptake process occurring at the plasma membrane in which movement of these 2 ions is closely linked (22) or whether phosphate movement is necessary to balance the movement of other cations possibly transported by A23187 (40, 41).

We have shown that following treatment of sperm with high concentrations of ionophore A23187, the resulting influx and retention of calcium requires an intact plasma membrane but that mitochondrial participation is not necessary. Apparently ionophore-mediated calcium influx is also a consequence of ionophore-induced decay of a concentration gradient, in this case the one which still exists across the plasma membrane. Calcium binding to normally inaccessible intracellular components may be involved since calculations based on the water space of 16 μm$^3$ for the bovine spermatozoan (42) and the observed uptake of approximately 200 amol/cell indicate that calcium is present in the sperm at greater than 50 times its concentration in the medium. Since filipin releases calcium from sperm which have accumulated this cation in response to A23187 treatment, these binding components must also be released (and diluted), i.e. they are soluble and of relatively low molecular weight.

The respiratory increases associated with the ionophore-induced calcium uptake result, at least in part, from the calcium which is then available for cyclical flux across the mitochondrial membrane. It is thus concluded that high concentrations of A23187 operate at both mitochondrial and plasma membranes of the sperm. The ability of respiratory uncouplers to potentiate ionophore-mediated calcium influx is most easily explained as a consequence of depletion of the

<table>
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<th>Rotenone</th>
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**Discussion**

Implicit in the interpretation which follows is the assumption that the sperm populations studied are homogeneous in their calcium uptake properties. Direct measurement of calcium distributions among individual spermatozoa are in progress. In the meantime, since the maximum amount of calcium uptake observed with either intact or filipin-treated sperm equals or exceeds the total capacity of mitochondria isolated a mannitol/sucrose medium and observed in the motility chamber employed in the studies shown in Table II. Only an occasional cell showed any flagellar motion whatsoever. The addition of caffeine resulted in the activation of the flagellar contractile apparatus of the vast majority of cells (19). Since the heads of more than 90% of the sperm were apparently attached to the glass surface of the chamber or to its cover, only the small remaining portion of cells were capable of progressive swimming behavior. From the movements of these an average swimming speed of 20 μm/s was calculated.

Motility was affected very little by the addition of either low levels of A23187 or high levels of the ionophore in the absence of external calcium or in the presence of calcium and an excess of EGTA. Motility was stimulated only under conditions where ionophore-mediated calcium influx from the medium could occur. These conditions paralleled those required for respiratory stimulation (Fig. 2). It should be noted however that calcium influx and motility activation were also observed after A23187 addition to cells that were made dependent upon glycolysis by treatment with the respiratory inhibitor, rotenone.

A much higher basal motility rate was observed when subjective motility evaluations were made on concentrated cell suspensions in either a modified Ringer buffer or the sucrose/mannitol medium. Such observations confirmed the correlation between motility activation and ionophore-induced calcium influx under conditions where these parameters were simultaneously examined. Studies of this type also indicated that motility remained activated for periods greater than 2 hours following ionophore treatment, that subsequent addition of excess EGTA suppressed motility to the basal rate, and that EDTA addition resulted in a slow but complete loss of motility. At optimal conditions (corresponding to those found in Fig. 2) at 2 nmol of A23187/mg of protein) motility activation approached that observed after caffeine treatment. Small changes in the ionophore or calcium concentration resulted in activation of a lesser degree or more transient nature.
energy supply required by the competing plasma membrane pump.

To account for the observed selective action of low concentrations of ionophore upon mitochondrial calcium flux one could envision differences in chemical composition of cellular membranes resulting in preferential distribution of the lipid-soluble ionophore into mitochondrial membranes. However, attempts to quantitate partitioning of A23187 into various sperm membrane fractions have met with only limited success. Alternative explanations may be found in the relative activities and kinetic properties of the competing mitochondrial and plasma membrane calcium pumps. Interpretation of the generality of a model of A23187 action at the cellular level is hampered by the paucity of data concerning effects of the ionophore on calcium fluxes in other cellular systems. It has been noted that 5 μM A23187 produces calcium influx from hemolysates (49) and influx into uropyles (17). We would predict that, in fact, such influx is preceded by partial or complete depletion of endogenous mitochondrial calcium stores. A biphasic action of A23187 on the contraction of isolated smooth muscle cells has been reported (44) and the sensitivities of the described responses to external chelators are consistent with the interpretation that low levels of A23187 produce mitochondrial calcium efflux followed by influx from the medium. Careful titration with A23187 in a cell type where free cytoplasmic Ca\(^{2+}\) can be directly measured (45) would be particularly informative and would allow a more detailed analysis of ionophore action.

The role of calcium in the regulation of muscle contraction is relatively well understood (46) and elegant studies with cilia and flagella possess highly similar ultrastructure, calcium addition to permeabilized, reactivated sperm produces only deleterious effects on motility (49). Since the data presented here show clearly that the calcium influx mediated by A23187 has a dramatic stimulatory effect on flagellar activity, the possibility must be considered that detergent treatment results in the loss of a calcium-sensitive component involved in the regulation of flagellar function. A calcium-binding protein which is the major, soluble, acidic component involved in the regulation of flagellar function. A calcium-binding protein which is the major, soluble, acidic component involved in the regulation of flagellar function.

Recent results indicated that A23187 enhances Ca\(^{2+}\) influx and cyclic AMP concentrations in fetal rat bone cells (51). However, in blow fly salivary glands, treatment with A23187 stimulates fluid secretion without concomitant changes in intracellular cAMP (52). Future investigations will include studies of the relationship between Ca\(^{2+}\) and cyclic nucleotides in the regulation of the flagellar activity of spermatozoa.

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