Journal of Andrology, Vol. 25, No. 1, January/February 2004

Lack of Voltage-Dependent Calcium Channel Opening During the Calcium Influx Induced by Progesterone in Human Sperm. Effect of Calcium Channel Deactivation and Inactivation

ALBERTO MARTÍN GUZMÁN-GRENFELL AND MARCO T. GONZÁLEZ-MARTÍNEZ

From the Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, México, D.F., México.

ABSTRACT: Progesterone induces calcium influx and acrosomal exocytosis in human sperm. Pharmacologic evidence suggests that voltage-dependent calcium channels (VDCCs) are involved. In this study, membrane potential (Vm) and intracellular calcium concentration ($[Ca^{2+}]_i$) were monitored simultaneously to assess the effect of VDCC gating on the calcium influx triggered by progesterone. Holding the Vm to values that maintained VDCCs in a deactivated (−71 mV) closed state inhibited the calcium influx induced by progesterone by approximately 40%. At this Vm, the acrosomal reaction induced by progesterone, but not by A23187, was inhibited. However, when the Vm was held at −15 mV (which maintains VDCCs in an inactivated closed state), the progesterone-induced calcium influx was stimulated. Furthermore, the progesterone and voltage-dependent calcium influxes were additive. These findings indicate that progesterone does not produce VDCC gating in human sperm.

Key words: Intracellular calcium, mammalian sperm, membrane potential, membrane potential–sensitive dyes.

J Androl 2004;25:117–122

In capacitated human sperm, prior the morphologic changes that define the acrosomal reaction (which occurs in minutes), progesterone induces a calcium influx, which increases the intracellular calcium concentration ($[Ca^{2+}]_i$), through nongenomic receptors (Thomas and Meizel, 1989; Blackmore et al, 1990, 1991; Yanagimachi, 1994). The increase in $[Ca^{2+}]_i$ is transient; it reaches a peak in seconds and is followed by a sustained influx that maintains the $[Ca^{2+}]_i$ at a level that is slightly higher than the resting state.

The molecular identity of the calcium transport systems that are activated by progesterone remains unknown. The possible role of voltage-dependent calcium channels (VDCCs) has been extensively debated. Inhibitory effects of some calcium channel blockers on the acrosomal reaction and/or the $[Ca^{2+}]_i$ increase induced by progesterone in human sperm (O'Toole et al, 1996; Shiomi et al, 1996; Blackmore, 1998; García and Meizel, 1999; Morales et al, 2000; Senuma et al, 2000) and other mammalian sperm (Kobori et al, 2000; Cordoba and Beconi, 2001; Bai et al, 2003) have been observed. A recent study shows that nifedipine inhibits the late, sustained $[Ca^{2+}]_i$ increase induced by progesterone on single cell recordings, which suggests that VDCCs participate in the calcium influx induced by the hormone (Kirkman-Brown et al, 2003). On the other hand, it has been shown that the progesterone-induced calcium influx is not affected at inhibitor concentrations that block the T-type VDCC when analyzed by a patch clamp technique in mouse spermatogenic cells (Bonaccorsi et al, 2001). Accordingly, we have observed that the calcium influx induced by progesterone is not related to membrane potential (Vm) changes that are capable of inducing the gating of VDCCs (González-Martínez et al, 2002). Rather, these observations suggest that VDCCs are not involved in the calcium influx induced by progesterone.

Indirect evidence indicates that human sperm are endowed with functional VDCCs. Indeed, the induction of depolarization with potassium (in the presence of the potassium-ionophore valinomycin) induces a $[Ca^{2+}]_i$ increase (detected with fura-2) (Linares-Hernández et al, 1998). These putative VDCCs open slowly (in seconds), inactivate in about 1.5 minutes in medium without calcium, and are insensitive to nifedipine and verapamil but are blocked by nickel (Linares-Hernández et al, 1998). Since these putative channels are insensitive to organic blockers, they could be classified as R-type (resistant)

Supported by grant IN202702 from the Programa de Apoyo a Proyectos de Investigación e Innovación tecnológica (PAPIIT) of DGAPA, UNAM (Mexico).

Correspondence to: Dr Marco T. González-Martínez, Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, CP 04510, Apartado Postal 70-297, México, D.F., México (e-mail: tuliog@servidor.unam.mx).

Received for publication July 7, 2003; accepted for publication September 10, 2003.

VDCCs (Hille, 2001). The role of these channels in human sperm physiology is not known.

On the basis of the functional properties of VDCCs, we have collected additional evidence regarding the role that VDCCs play in calcium influxes induced by progesterone in human sperm. We assessed the progesterone-induced calcium influx under conditions in which VDCCs are held in a closed state. To do this, Vm and $[Ca^{2+}]_i$ were simultaneously measured in capacitated human sperm populations, and the Vm was chemically held with the potassium-ionophore valinomycin at Vm values that maintain VDCCs either in a deactivated or an inactivated state.

Methods

Fura-2 AM (acetoxymethyl ester) and diisopropylthiodicarbocyanine iodide ($diSC_3$[5]) were obtained from Molecular Probes (Eugene, Ore); ionomycin and valinomycin were obtained from Sigma Chemical Company (St Louis, Mo). The other reagents were obtained from Sigma, Merck (Darmstadt, Germany), or Baker (Phillipsburg, NJ). Capacitating human sperm medium (HSM) had the following composition (Suarez et al, 1986), in mM: NaCl 117.5, KCl 8.6, $CaCl_2$ 2.5, NaH_2PO_4 0.3, $MgCl_2$ 0.49, Na-pyruvate 0.3, Na-lactate 25, glucose 2, $NaHCO_3$ 25, and bovine serum albumin (BSA) 3 mg/mL (pH 7.6–7.7). Fluorescence recordings were performed in the above medium except that $NaHCO_3$ was replaced by 25 mM HEPES (HSM-H), and the pH was adjusted to 7.5–7.6. BSA was removed from this medium since 3 mg/mL interferes with $diSC_3(5)$ fluorescence.

Sperm Purification, Fura-2 Loading, and Capacitation

Human semen was obtained from a panel of 10 healthy 18- to 22-year-old donors. Normal samples were selected according to World Health Organization protocol as detailed previously (Linares-Hernández et al, 1998). Sperm purification was performed using Percoll gradients according to Suarez et al (1986) with minor modifications (Linares-Hernández et al, 1998). Simultaneous recordings of Vm and $[Ca^{2+}]_i$ were performed with samples of sperm incubated between 4.0 and 6.0 hours in HSM medium. At this incubation time, the physiologic processes that are identified with sperm capacitation occurred: 1) an induction of the acrosomal reaction by progesterone (Osman et al, 1989; Blackmore et al, 1990), 2) an increase in the intracellular pH from 6.75 plus or minus 0.02 to 6.84 plus or minus 0.02 (n = 7, SE) (detected with BCECF [2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein] fluorescence) (Cross and Razy-Faulkner, 1997), 3) an increase in the resting $[Ca^{2+}]_i$ of about 100 nM (Baldi et al, 1991; Blackmore, 1993; Mendoza and Tesarik, 1993), and 4) a marked enhancement of the $[Ca^{2+}]_i$ influx (~2.5-fold) induced by progesterone compared to noncapacitated sperm (Baldi et al, 1991; Blackmore, 1993; Mendoza and Tesarik, 1993; Tesarik et al, 1996; García and Meizel, 1999) (data not shown). In some experiments, noncapacitated sperm were used (incubated up to 1 hour in HSM).

Measurement of $[Ca^{2+}]_i$ and Vm

Vm and $[Ca^{2+}]_i$ were simultaneously detected and calibrated with the fluorescent probes $diSC_3(5)$ and fura-2, respectively, as described in Linares-Hernández et al (1998) with some modifications. Briefly, 2–3 mL of fura-2–loaded sperm ($1–2 \times 10^7$ cells) in capacitating medium was centrifuged at $300 \times g$ for 5 minutes. The pellet was resuspended in approximately 100 μL and immediately added to the fluorescence cuvette containing 2.5 mL HSM-H plus 500 nM $diSC_3(5)$ for simultaneous recordings. Mitochondrial potential does not contribute to the $diSC_3(5)$ signal in human sperm (Linares-Hernández et al, 1998; Guzmán-Grenfell et al, 2000), which is consistent with anaerobiosis (Makler et al, 1992) and mitochondrial inhibitors (Hong et al, 1983) having no effect on sperm motility. To calibrate the fluorescence signal, valinomycin was added to bring the Vm to the Nernst potential for potassium distribution ($E_K = -61.54$ mV log $[K_i]/[K_{ext}]$, where $[K_i] = 120$ mM). The addition of appropriate amounts of KCl depolarized the Vm to known values, and this induced concomitant increases in $diSC_3(5)$ fluorescence (Linares-Hernández et al, 1998). There is a linear relationship between the fractional change of fluorescence, f/f_0 (where f is the actual value of fluorescence and f_0 is the fluorescence value obtained with valinomycin in HSM-H) and the Vm measured as the E_K. The Vm fluorescence signal was converted to Vm values according to the following equation (González-Martínez, in press):

$$Vm = 1/m(f/f_0 - 1 - b)$$

where m and b are the slope and the intersection of the y-axis at 0 mV, respectively, of the calibration curve mentioned above.

The $[Ca^{2+}]_i$ was calibrated in the presence of 500 nM $diSC_3(5)$ as reported (Linares-Hernández et al, 1998).

Determination of the Acrosomal Reaction

Samples of capacitated sperm were incubated in 2 mL HSM with different additives for 15 minutes, and the percentage of sperm that showed the acrosomal reaction was determined. *Pisum sativum* lectin conjugated with fluorescein isothiocyanate was used to quantify acrosome-reacted and acrosome-intact sperm according to Tesarik et al (1993). Complete and partial acrosomal reactions (DasGupta et al, 1993) were both counted as a positive index of the acrosomal reaction. Sperm with a band of fluorescence present exclusively at the equatorial acrosomal segment (''pattern B'') were counted as having shown complete acrosomal reactions. The sperm that showed a partial acrosomal reaction had the equatorial fluorescence as well as a diffuse fluorescence in the acrosomal region (DasGupta et al, 1993) that were clearly less intense than the pattern observed in acrosomally intact sperm.

Statistical Analysis

Numeric results are expressed as mean plus or minus standard error; n denotes the number of individuals tested. A 1-way analysis of variance and the Dunnett test for multiple comparisons were used. Two-tailed P-values $<.05$ were considered statistically significant.

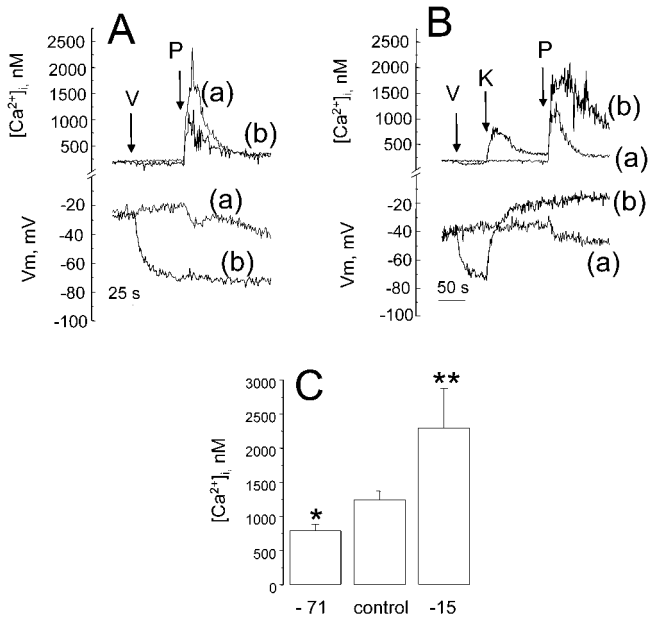

Figure 1. **Panel A**: Effect of progesterone on intracellular calcium concentration ($[Ca^{2+}]_i$) and plasma membrane potential (Vm) detected simultaneously in capacitated sperm maintained at −71 mV. A hyperpolarization to −71 mV was induced with valinomycin (V, 0.4 μM) 1 minute before the addition of 4 μM progesterone (P) (traces labeled [b]). **Panel B**: Sperm were first hyperpolarized to −71 mV with V (0.4 μM), and 1 minute later, 60 mM KCl was added to depolarize the sperm at −15 mV. Two minutes later, 4 μM P was added (traces labeled [b]). Both **(A)** and **(B)** show control experiments performed in the absence of V and KCl (traces labeled [a]). In **(C)**, the average calcium increment induced by P at unmodified Vm (control, no V or potassium added), at −71 mV (n = 8, $*P < .05$ compared to control), and at −15 mV ($**P < .01$ compared to control).

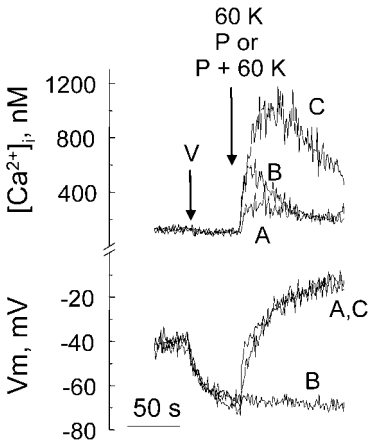

Figure 2. Effect of the simultaneous induction of calcium influx by progesterone and voltage-dependent calcium channel (VDCC) opening in noncapacitated sperm. In trace C, 4 μM progesterone plus 60 mM KCl (depolarization to −15 mV) were simultaneously added to a sperm sample previously hyperpolarized to −71 mV with 0.4 μM valinomycin. Control experiments show the effect of progesterone (trace B) and 60 mM KCl (trace A) added separately to valinomycin-treated cells. Traces are representative of 5 experiments.

Results

The effect of progesterone on $[Ca^{2+}]_i$ was assessed maintaining the Vm either at −71 or −15 mV in capacitated sperm. At these Vm values, VDCCs are closed, in a deactivated and inactivated state, respectively (Linares-Hernández et al, 1998). To change and maintain the Vm, we added valinomycin, a K-ionophore that brings the Vm to the E_K (ie, approximately −71 mV) (Linares-Hernández et al, 1998). Holding the Vm at −71 mV with valinomycin significantly inhibited (~40%) the $[Ca^{2+}]_i$ increase induced by progesterone (Figure 1A and C) compared to the control trace.

When capacitated sperm were depolarized by bringing the Vm down from −71 mV (valinomycin potential) to −15 mV (potential with 60 mM KCl), a $[Ca^{2+}]_i$ increase of 527 plus or minus 67 nM (n = 5) was observed (Figure 1B and C). This depolarization-induced increase in $[Ca^{2+}]_i$ was always smaller than the one obtained by saturating the sperm with 4 μM progesterone (1231 ± 184, n = 5). Once depolarized, after the peak, the sperm $[Ca^{2+}]_i$ returned to a value close to a resting state in about 1.5 minutes (Figure 1B). This is an indication of the inactivation of VDCCs, which occurs in around 1.5 minutes (Linares-Hernández et al, 1998), and the beginning of processes that remove calcium from the cell. Thus, after depolarization to −15 mV, a considerable fraction of VDCCs should be inactivated and not available for being opened by a presumptive progesterone-induced depolarization. When progesterone was added 2 minutes after depolarization, no changes in Vm were observed (because of the ''clamping'' effect of valinomycin), but a calcium influx was triggered that was significantly higher than the one obtained at the unmodified resting Vm (Figure 1B and C).

Similar results were obtained in noncapacitated cells, although, expectedly, the progesterone-induced $[Ca^{2+}]_i$ increase was much lower. In control experiments (without valinomycin), the calcium influx induced by 4 μM progesterone in noncapacitated sperm was 476 plus or minus 70 nM, whereas that at −71 and −15 mV was 360 plus or minus 48 and 956 plus or minus 236 (n = 5, SE), respectively (representative traces not shown).

To further explore the role of VDCCs in the progesterone-induced $[Ca^{2+}]_i$ increase, we compared the calcium influx induced by depolarization from −71 to −15 mV and by progesterone, when both inducers were applied concurrently, with the influx induced by each inductor when they were applied separately. The simultaneous addition of progesterone and 60 mM KCl, 1 minute after valinomycin, caused a $[Ca^{2+}]_i$ increase that was, at the peak, nearly the sum of the calcium increase induced by each inductor added separately (Figure 2), which suggests that the permeability pathways were independent of each other. Moreover, simultaneous depolarization and proges-

terone addition caused a remarkably sustained $[Ca^{2+}]_i$ increase that was higher than the sum of each added separately. Note that these experiments were performed on noncapacitated cells, since the progesterone response of these cells is less than that seen for capacitated cells. For capacitated cells, the additive effects frequently resulted in a $[Ca^{2+}]_i$ increase that saturated fura-2 (data not shown).

The acrosomal reaction was investigated under the experimental conditions in our study (Figure 3). In noncapacitated sperm, depolarization to −15 mV induced an acrosomal reaction, the extent of which was approximately 82% of that obtained with the calcium ionophore A23187. Valinomycin at normal potassium concentrations (which holds the Vm at −71 mV) blocked the acrosomal reaction induced by progesterone in capacitated sperm. As expected, valinomycin did not affect the acrosomal reaction induced by the calcium ionophore A23187 in normal medium. This suggested that the inhibitory mechanism of valinomycin on the acrosomal reaction induced by progesterone was related to its effect on Vm.

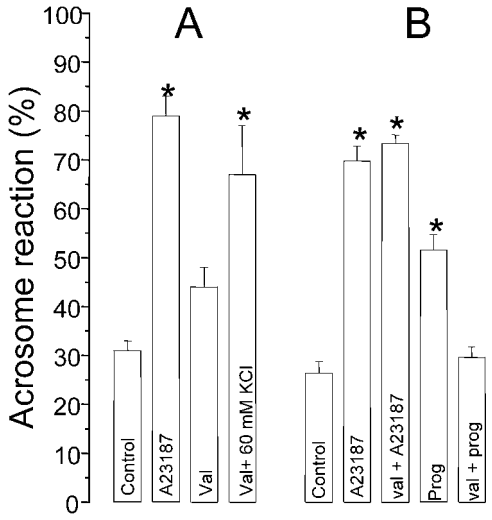

Figure 3. Determination of the acrosomal reaction of human sperm under different experimental conditions. **(A)** Effect of membrane potential (Vm) depolarization from −71 to −15 mV on the percentage of sperm undergoing an acrosomal reaction in noncapacitated human sperm. Valinomycin (1 μM) was added 1 minute before 60 mM KCl, and the acrosomal reaction was determined as described in ''Methods.'' The effects of 1 μM valinomycin and 10 μM A23187 are included. In control experiments, 5 μL dimethylsulfoxide (DMSO) was added. **(B)** Effect of valinomycin on the percentage of sperm undergoing an acrosomal reaction induced by progesterone in capacitated sperm. Valinomycin (1 μM) was added 1 minute before either 10 μM A23187 or 4 μM progesterone. The effects of progesterone and A231787 and 5 μL DMSO (control), without valinomycin, are included. Experimental procedures were performed as described in ''Methods.'' The values are means plus or minus standard errors, n = 5. *$P < .05$ compared to the control group.

Discussion

The possible participation of the VDCC in the increase of the $[Ca^{2+}]_i$ and the acrosomal reaction induced by progesterone in human sperm has been debated (O'Toole et al, 1996; Shiomi et al, 1996; Benoff, 1998; Blackmore, 1998; García and Meizel, 1999; Kobori et al, 2000; Morales et al, 2000; Senuma et al, 2000; Bonaccorsi et al, 2001; Cordoba and Beconi, 2001; González-Martínez et al, 2002; Bai et al, 2003; Kirkman-Brown et al, 2003). The use of inhibitory drugs that block the VDCC in other cell types has provided evidence suggesting that the calcium influx induced by progesterone involves VDCC gating. In this work, we observed that a depolarization from −71 to −15 mV induces acrosomal reactions in noncapacitated sperm. Thus, in principle, the calcium influx triggered through VDCC gating induces a $[Ca^{2+}]_i$ increase sufficient to trigger acrosomal exocytosis. In this study, we explored the contribution of VDCCs to the progesterone-induced calcium influx by examining only the VDCC properties of human sperm.

The opening of the VDCC requires appropriate Vm changes. If VDCC gating is involved in the calcium influx induced by progesterone, the hormone should induce a depolarizing pulse from a sufficiently negative resting value. Alternatively, if the Vm is depolarized, a voltage prepulse to negative values should be required to remove VDCC inactivation. A negative value, beyond −70 mV, allows the VDCC to be in a deactivated closed state (inactivation removed) and susceptible to being opened by a depolarizing pulse. Accordingly, in human sperm, valinomycin stimulates the opening of the VDCC, possibly because this ionophore induces a potassium permeability that hyperpolarizes the membrane from a resting state to −71 mV (the E_K). This hyperpolarization would remove the VDCC from the partially inactivated state that is present at a resting Vm (approximately −40 mV) (Linares-Hernández et al, 1998) to a deactivated closed state. In this regard, it has been observed that mouse sperm hyperpolarize during capacitation at Vm values that would remove the inactivation from a T-type VDCC (Arnoult et al, 1999). As for human sperm, the resting Vm remains relatively depolarized (Brewis et al, 2002; González-Martínez et al, 2002; Patrat et al, 2002) at Vm values that are not negative enough to fully remove the VDCC inactivation and therefore limit the ability of the VDCC to be gated by depolarization.

The physiologic depolarizing pulse that progesterone should induce to gate the VDCC could involve the opening of sodium channels, the opening of chloride channels (since the E_{Cl} is close to −20 mV) (Bray et al, 2002), or even the closing of potassium channels. The presence of sodium and chloride channels (γ-aminobutyric acid or glycine receptors) in sperm plasma membranes has been documented (Shi and Ma, 1998; Bray et al, 2002). Regardless of the mechanism, some reports show that pro-

gesterone is able to trigger a fast cation-dependent depolarization (Foresta et al, 1993; Brewis et al, 2000). However, other reports indicate that progesterone induces no changes (Blackmore, 1998), no immediate depolarization (Brewis et al, 2000), or a small depolarization (15 mV) that is followed by hyperpolarization (Patrat et al, 2002). We have observed that the calcium influx induced by progesterone may occur even in the absence of Vm changes (González-Martínez et al, 2002; this study), indicating a lack of contribution by the VDCC. However, since these measurements were performed in sperm populations, it could be argued that a small fraction of the population triggers Vm changes able to gate the VDCC but that these are hidden within the entire population's fluorescence signal.

In the present work, the use of valinomycin ensured that the entire population sample had nearly the same Vm, since this ionophore causes a chemical clamp that holds the Vm to the E_K. Using this approach, we found that VDCCs are not opened during the calcium influx triggered by progesterone in human sperm according to the evidence that follows. 1) The voltage step from −71 to −15 mV caused the opening and inactivation of a considerable fraction of the VDCC (Linares-Hernández et al, 1998), and when this occurred, the $[Ca^{2+}]_i$ increase (at −15 mV) that was induced by progesterone was significantly enhanced; moreover, the calcium influx through the VDCC resulted in a $[Ca^{2+}]_i$ increase that was about 50% lower than the one induced by progesterone saturation. Thus, even a large opening of the VDCC (induced by a depolarization of 56 mV, from −71 to −15 mV) would result in a partial contribution to the $[Ca^{2+}]_i$ increase that is triggered by progesterone. 2) The simultaneous depolarization (from −71 to −15 mV) and progesterone addition caused a $[Ca^{2+}]_i$ increase that, at the peak, was nearly the sum of each inductor added separately, indicating that they were distinct calcium permeability pathways. Similar results were observed by Kumar et al (2000); however, in that study, valinomycin was not used to modify the Vm with KCl, nor were Vm measurements performed.

Holding the Vm at −71 mV with valinomycin reduced by approximately 30% the $[Ca^{2+}]_i$ increment induced by progesterone. This latter effect may be related to the observed inhibitory effect of valinomycin on the acrosomal reaction induced by progesterone. Even though these findings may suggest that the VDCC participates in the progesterone-induced $[Ca^{2+}]_i$ increase and induction of the acrosomal reaction, the points discussed above indicate that the gating of the VDCC is not involved. That progesterone induced an enhanced $[Ca^{2+}]_i$ increase at −15 mV raises the possibility that the still unidentified calcium transport mechanism triggered by progesterone has some voltage dependence. Nonvoltage-dependent calcium channels may exhibit some voltage dependence, as occurs with the nicotinic acetylcholine receptor (Chabala, 1992) and the calcium release–activated calcium channels (CRACs) (Bakowski and Parekh, 2002). Functional store-operated calcium channels have been found in human sperm (Blackmore, 1993), and the presence of transient receptor potential proteins that have been proposed as candidates for CRAC channels have also been detected in human sperm (Castellano et al, 2003). In this regard, the inhibitory effect of nickel may suggest a role for these channels in progesterone responses, since this divalent cation inhibits the calcium influx induced by the hormone in human sperm (Blackmore and Eisoldt, 1999), although at lower concentrations than those that inhibit the store-operated calcium channels stimulated by zona pellucida glycoprotein 3 in mouse sperm (O'Toole et al, 2000).

As for the effect observed at −15 mV, another hypothesis, based on the $[Ca^{2+}]_i$–dependent responsiveness to progesterone (Baldi et al, 1991; Mendoza and Tesarik, 1993), may explain the stimulatory effect on the calcium influx. The $[Ca^{2+}]_i$ increase induced by VDCC gating at −15 mV could enhance the progesterone-induced $[Ca^{2+}]_i$ increase by switching on progesterone receptors (Mendoza and Tesarik, 1993). Additionally, progesterone induces an increase in tyrosine phosphorylation activity that in turn enhances the calcium influx only in human capacitated sperm (Tesarik et al, 1996). Whether or not the effects of Vm on the calcium influx induced by progesterone are related to tyrosine phosphorylation remains to be established.

Acknowledgments

We thank Consuelo Carreño and Juan José Fraire-Zamora for the excellent technical assistance.

References

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, Florman HM. Control of the low voltage–activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proc Natl Acad Sci U S A.* 1999;96:6757–6762.

Bai JP, Shi YL, Fang X, Shi QX. Effects of demethylzeylasteral and celastrol on spermatogenic cell Ca^{2+} channels and progesterone-induced sperm acrosome reaction. *Eur J Pharmacol.* 2003;464:9–15.

Bakowski D, Parekh AB. Monovalent cation permeability and Ca^{2+} block of the store-operated Ca^{2+} current I(CRAC)in rat basophilic leukemia cells. *Pflugers Arch.* 2002;443:892–902.

Baldi E, Casano R, Falsetti C, Krausz C, Maggi M, Forti G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J Androl.* 1991;12:323–330.

Benoff S. Voltage dependent calcium channels in mammalian spermatozoa. *Front Biosci.* 1998;3:D1220–1240.

Blackmore PF. Thapsigargin elevates and potentiates the ability of progesterone to increase intracellular free calcium in human sperm: possible role of perinuclear calcium. *Cell Calcium.* 1993;14:53–60.

Blackmore PF. News and views for non-genomic progesterone receptors on spermatozoa. *Andrologia.* 1998;30:255–261.

Blackmore PF, Beebe SJ, Danforth DR, Alexander N. Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem.* 1990;265:1376–1380.

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.

Blackmore PF, Neulen J, Lattanzio F, Beebe SJ. Cell surface-binding sites for progesterone mediate calcium uptake in human sperm. *J Biol Chem.* 1991;266:18655–18659.

Bonaccorsi L, Forti G, Baldi E. Low-voltage–activated calcium channels are not involved in capacitation and biological response to progesterone in human sperm. *Int J Androl.* 2001;24:341–351.

Bray C, Son JH, Kumar P, Harris JD, Meizel S. A role for the human sperm glycine receptor/Cl(−) channel in the acrosome reaction initiated by recombinant ZP3. *Biol Reprod.* 2002;66:91–97.

Brewis IA, Morton IE, Mohamad SN, Browes CE, Moore HD. Measurement of intracellular calcium concentration and plasma membrane potential in human spermatozoa using flow cytometry. *J Androl.* 2000; 21:238–249.

Castellano LE, Trevino CL, Rodriguez 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.

Chabala LD. Voltage dependence of acetylcholine receptor channel gating in rat myoballs. *J Gen Physiol.* 1992;100:729–748.

Cordoba M, Beconi MT. Progesterone effect mediated by the voltage-dependent calcium channel and protein kinase C on noncapacitated cryopreserved bovine spermatozoa. *Andrologia.* 2001;33:105–112.

Cross NL, Razy-Faulkner P. Control of human sperm intracellular pH by cholesterol and its relationship to the response of the acrosome to progesterone. *Biol Reprod.* 1997;56:1169–1174.

DasGupta S, Mills CL, Fraser LR. Ca(2+)-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. *J Reprod Fertil.* 1993;99:135–143.

Foresta C, Rossato M, Di Virgilio F. Ion fluxes though the progesterone-activated channel of the sperm plasma membrane. *Biochem J.* 1993; 294:279–283.

García MA, Meizel S. Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. *Biol Reprod.* 1999;60:102–109.

González-Martínez MT. Induction of a sodium dependent depolarization by external calcium removal in human sperm. *J Biol Chem.* 2003; 278:36304–36310.

González-Martínez MT, Bonilla-Hernández MA, Guzmán-Grenfell AM. Stimulation of voltage dependent calcium channels during capacitation and by progesterone in human sperm. *Arch Biochem Biophys.* 2002;408:205–210.

Guzmán-Grenfell AM, Bonilla-Hernández MA, González-Martínez MT. Glucose induces a Na^+,k^+-atpase-dependent transient hyperpolarization in human sperm. I. Induction of changes in plasma membrane potential by the proton ionophore CCCP. *Biochim Biophys Acta.* 2000; 1164:188–198.

Hille B. *Ion Channels of Excitable Membranes.* 3rd ed. Sunderland, Mass: Sinauer; 2001.

Hong CY, Chiang BN, Wei YH. Mitochondrial respiration inhibitors and human sperm motility: implications in the development of spermaticydes. *Br J Clin Pharmacol.* 1983;16:487–490.

Kirkman-Brown JC, Barratt CL, Publicover SJ. Nifedipine reveals the existence of two discrete components of the progesterone-induced $[Ca^{2+}]_i$ transient in human spermatozoa. *Dev Biol.* 2003;259:71–82.

Kobori H, Miyazaki S, Kuwabara Y. Characterization of intracellular Ca(2+) increase in response to progesterone and cyclic nucleotides in mouse spermatozoa. *Biol Reprod.* 2000;63:113–120.

Kumar S, Ying YK, Maddaiah VT. Potassium increases intracellular calcium simulating progesterone action in human sperm. *Arch Androl.* 2000;44:93–101.

Linares-Hernández L, Guzmán-Grenfell AM, Hicks-Gomez JJ, González-Martínez MT. Voltage dependent calcium influx in human sperm assessed by simultaneous detection of intracellular calcium and membrane potential. *Biochim Biophys Acta.* 1998;1372:1–12.

Makler A, Makler-Shiran E, Stoller J, Lissak A, Abramovici H, Blumenfeld Z. Use of a sealed mini-chamber to investigate human sperm motility in real time under aerobic and anaerobic conditions. *Arch Androl.* 1992;29:255–261.

Mendoza C, Tesarik J. A plasma-membrane progesterone receptor in human sperm is switched on by increasing intracellular free calcium. *FEBS Lett.* 1993;330:57–60.

Morales P, Pizarro E, Kong M, Kerr B, Ceric F, Vigil P. Gonadotropin-releasing hormone-stimulated sperm binding to the human zona is mediated by a calcium influx. *Biol Reprod.* 2000;63:635–642.

Osman RA, Andria ML, Jones AD, Meizel S. Steroid induced exocytosis: the human sperm acrosome reaction. *Biochem Biophys Res Comm.* 1989;160:828–833.

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: 171–184.

O'Toole CM, Roldan ER, Fraser LR. Role of Ca^{2+} channels in the signal transduction pathway leading to acrosomal exocytosis in human spermatozoa. *Mol Reprod Dev.* 1996;45:204–211.

Patrat C, Serres C, Jouannet P. Progesterone induces hyperpolarization after a transient depolarization phase in human spermatozoa. *Biol Reprod.* 2002;66:1775–1780.

Senuma M, Yamano S, Nakagawa K, Irahara M, Kamada M, Aono T. Progesterone accelerates the onset of capacitation in mouse sperm via T-type calcium channels. *Arch Androl.* 2000;47:127–134.

Shi YL, Ma XH. Ion-channels reconstituted into lipid bilayer from human sperm plasma membrane. *Mol Reprod Dev.* 1998;50:354–560.

Shiomi H, Yamano S, Shono M, Aono T. Characteristics of calcium ion influx induced by human follicular fluid in individual human sperm. *Arch Androl.* 1996;37:79–86.

Suarez SS, Wolf DP, Meizel S. Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. *Gamete Res.* 1986;14:107–121.

Tesarik J, Carreras A, Mendoza C. Single cell analysis of tyrosine kinase dependent and independent Ca^{2+} fluxes in progesterone induced acrosome reaction. *Mol Hum Reprod.* 1996;2:225–232.

Tesarik J, Mendoza C, Carreras A. Fast acrosome reaction measure: a highly sensitive method for evaluating stimulus-induced acrosome reaction. *Fertil Steril.* 1993;59:424–430.

Thomas P, Meizel S. Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca2+ influx. *Biochem J.* 1989;264:539–546.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neils D, eds. *Physiology of Reproduction.* New York, NY: Raven Press; 1994:189–317.