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Ecto-Cyclic AMP Independent Protein Kinase-A Potent Regulator of L-type Calcium Channel and Forward Motility of Goat (*Capra indicus*) Epididymal Spermatozoa

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Abstract

Objective: Caprine (Capra indicus) spermatozoa possess a novel ecto-cAMP independent protein kinase (ecto-CIK) on their membrane surface. This enzyme showed remarkable alteration through the maturation process of spermatozoa in epididymis. We investigated the role of CIK in the regulation of forward motility of epididymal spermatozoa by controlling the intracellular level of $[Ca^{2+}]$.

Method: Cauda epididymal mature sperm cells were treated with CIK antibody and maximum inhibition of enzyme activity (85%) was observed at 120 minutes of exposure. To analyze the calcium uptake mechanisms cells were exposed to $^{45}Ca^{2+}$ after treatment with CIK antibody and pretreated with different calcium channel regulators. The intracellular $[Ca^{2+}]_i$ signal was determined fluorometrically by using fura 2-AM. The computerized spectrophotometric assay method was used to measure the percentage of forward motility.

Result: It was shown that the uptake of calcium through the L-type voltage-dependent Ca^{2+} channels of the plasma membrane is regulated by CIK. Pretreatment of sperm cells with varapamil (20 μM), nifedipine (20 μM) significantly inhibited the increased intracellular $[Ca^{2+}]_i$ induced by the CIK antibody. Whereas calmodulin antagonists trifluoperazine and w_{13} (*N*-(4-Aminobutyl)-5-chloro-2 naphthalenesulfonamide hydrochloride and sodium azide, a potent mitochondrial inhibitor, showed no effect on this calcium entry. The treatment with ca ionophore A123187 of the CIK antibody treated cells showed channel inhibitor insensitive increase of calcium uptake. It was observed that verapamil (20 μM) has significant role in decreasing (~50%) forward motility of CIK-antibody treated spermatozoa.

Conclusion: We concluded that CIK is an important regulator of forward motility in epididymal spermatozoa and the regulation may be activated partly by the intracellular $[Ca^{2+}]_i$ level the extent of which is functionally imparted by the voltage gated L type calcium channel.

Keywords: cAMP independent protein kinase (CIK); Forward motility; Epididymis; Spermatozoa; Calcium channel; L type; Voltage gated

Introduction

Testicular spermatozoa are immotile and infertile. During transit through different parts of the epididymis they acquire the potential for forward motility and fertility. In spermatozoa, intracellular calcium has a regulatory role in the control of motility [l-6]. A natural consequence of the selection of Ca^{2+} as an intracellular second messenger is the necessity for precise regulation of their fertility potential. In mammalian spermatozoa, the systems that regulate intracellular Ca^{2+} concentration involve the ATP-dependent Ca^{2+} pump [7-9] Naf/Ca^{2+} exchanger [l0-12] and the voltage dependent Ca^{2+}channels [13] of the plasma membrane and the mitochondria [14-20]. Depending on tissue type, this transport mechanism has been implicated in either the entry or removal of cell Ca^{2+} and is therefore thought to function in Ca^{2+} homeostasis [21,22]. It was demonstrated that the signaling pathway leading to tyrosine phosphorylation in mouse spermatozoa is negatively regulated by $[Ca^{2+}]_i$, and that maturation mechanisms that control $[Ca^{2+}]_i$ within the spermatozoon are critically important during epididymal transit [23]. The calcium antagonists include at least three distinct classes of drugs like nifedipin, verapamil and benzothiazepine [24] having high affinity to distinct but allosterically interacting receptor sites on the core subunit of L-subtype voltage dependent calcium channel which is present in a wide range of cells [25-27]. Mammalian spermatozoa possess a mechanism for calcium entry that has the same pharmacological sensitivity as the L-type voltage dependent calcium channel of mammalian cardiac and skeletal muscle [13,28,29]. Studies from our laboratory have provided several lines of evidences for the occurrence of a cyclic AMP-independent protein kinase (ecto-CIK) on the external surface of caprine (*Capra indicus*) cauda epididymal mature spermatozoa that causes phosphorylation of the membrane-bound phosphoproteins [30-32]. Our recent studies using caprine sperm as the model have described for the first time the purification to apparent homogeneity of an ectoprotein kinase [33] as well as its major phosphoprotein substrate: MPS [34] located on the sperm external surface. Ecto-CIK is a 115 kDa protein made up of two subunits: 63 kDa and 55 kDa. It is a strongly basic protein with strong immunogenic property. The ectokinase has nearly 30 times greater affinity for MPS as compared to casein-the

most potent exogenous protein substrate. Thereby, it demonstrates that CIK is a unique membrane protein-specific kinase, which specifically phosphorylates serine and threonine residues of the sperm outer cell-surface phosphoproteins [35]. It was further demonstrated that ecto-CIK plays a vital role in the regulation of sperm forward progression and velocity through its substrate protein (MPS) [33-36]. The aim of this present study is to investigate the role of the phosphorylation event mediated by the CIK on the activation of forward motility in the epididymis through plasma membrane bound voltage dependent L type calcium channel.

Materials and Methods

The following reagents were obtained from Sigma Chemical Company (St. Louis, MO): Polyethylene glycol (average molecular weight 20 kDa), 32P-labeled ATP, ethylenediaminetetra-acetic acid (EDTA), phenyl methyl sulphonyl fluoride (PMSF), casein-sepharose-4B, PBE-118, triethyl amine-HCl, β-mercapto ethanol, glycerol, Triton X-100, DEAE cellulose, HRP-conjugated anti-rabbit IgG, sodium chloride, potassium chloride, potassium phosphate, penicillin, complete Freund's adjuvant, incomplete Freund's adjuvant, and ammonium sulphate. Phosphate buffer saline (PBS), orthophenylenediamine (OPD), citrate phosphate buffer, H_2O_2, H_2SO_4, BSA, fetuin, casein, ovalbumin, calcium chloride, magnesium sulphate, glucose, sodium bicarbonate, sucrose.

Isolation of caprine epididymal spermatozoa

Goat epididymal spermatozoa were isolated within 2 hours of slaughter [37]. To obtain the mature sperm, cauda epididymis was cut into several pieces and suspended in a modified Ringer's solution (RPS medium: 119 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 10 mM glucose, 16.3 mM potassium phosphate, and 50 U penicillin mL–1, pH 6.9) with gentle stirring. Maturing spermatozoa were derived from caput and corpus epididymis. The spermatozoa were then filtered through 4 to 5 layers of cheese cloth and sedimented by centrifugation at 500 g for 5 minutes and then washed in RPS medium.

Isolation of sperm plasma membrane

Plasma membrane was isolated and purified from the mature cauda and maturing corpus and caput spermatozoa by an aqueous two-phase-polymer method [37-39]. Membrane purity was examined by estimating marker enzymes: alkaline phosphatase, 5-nucleotidase, acrosin, cytochrome C-oxidase, and glucose-6-phosphatase and by electron microscopic study. The specific activities of the plasma membrane bound 5-nucleotidase and alkaline phosphatase were 10-14-fold higher in the isolated PM than in the cell debris, indicating marked membrane enrichment. There was no detectable amount of acrosin and glucose-6-phosphatase in the isolated PM and specific activity of cytochrome oxidase was very low (approx 6.58 times) in membrane than in the cell debris. The data show that there is little contamination of PM with acrosome, mitochondria and endoplasmic reticulum. Electron microscopic studies also showed high degree of purity of the isolated sperm PM. The membrane preparation was finally dispersed in 25 mM potassium phosphate buffer, pH 7.0, containing 1 mM PMSF, 2 mM β-mercaptoethanol, 1 mM EDTA 30% (v/v) glycerol and were stored at -20°C. The protein content of the plasma membrane was estimated using BSA standard [40]

Purification of membrane-bound Ecto-CIK

The ecto-CIK was purified to apparent homogeneity from the plasma membrane of mature cauda epididymal spermatozoa [41]. Isolated plasma membrane was treated with 1% Triton X-100 at 4°C for 1 hour for the solubilization of proteins. Treated plasma membrane was centrifuged at 27000 g for 90 minutes at 4°C. The resulting supernatant was loaded on DEAE-Cellulose column. The activity peak was eluted in unretained fraction and the proteins of unretained fraction were chromatographed on casein- sepharose 4B affinity column. The activity peak was eluted with 0.2 M NaCl and loaded on PBE-118 column equilibrated with 0.025 M triethyl amine-HCl, pH 11.0. The activity peak was eluted depending on its PI. The active fractions were concentrated and passed through casein Sephsrose-4B column again to remove the eluent buffer. The active fraction was eluted as mentioned before, dialysed and concentrated. The concentrated protein was preserved in buffer A with 50% glycerol at -20°C until used.

Production of antibody

Antiserum against the purified CIK was raised in rabbit by 4 successive injections at the 1st, 7th, 15th, and 21st days as described earlier [41]. First injection was given subcutaneously using 500 µg of CIK in complete Freund's adjuvant. Second and third injections comprised 200 µg of CIK in incomplete Freund's adjuvant. Fourth injection contained 400 µg of CIK in incomplete Freund's adjuvant. Blood was collected from the ear vein on the 27th day of inoculation, and serum was prepared and stored at −70°C. Preimmune blood serum was collected from the same animal before starting the inoculation programme [42]. The immunoglobulin of the immune serum was precipitated twice with 50% ammonium sulfate. The final precipitate was dissolved in 0.25 M PBS (pH 8.0) and dialyzed overnight against the same buffer. The immunoglobulin fraction obtained after the ammonium sulfate fractionation was subjected to DEAE-cellulose chromatography. Unbound protein peak containing IgG was collected with 0.01 M phosphate buffer, pH 7.0, and stored at -20°C.

Assay of CIK

The standard assay system contained 200 nmol of ATP containing 20×10^4 cpm to 50×10^4 cpm, 2 µmol of magnesium chloride, 1 mg of casein, and 200 ng of isolated enzyme in a total volume of 0.2 ml of 50 mM tris-HCl buffer pH 9.0. The incubation was carried out at 37°C for 5 min. When casein was used as substrate, the reaction was stopped by adding 0.1 ml 0.5% casein as carrier protein containing 250 mM K-phosphate-10 mM ATP and 2 ml 10% TCA [41]. The radiolabelled protein was recovered by filtration through whatman no 1 filter paper washed with 5% TCA dissolved in scintillation fluid and counted for radioactivity. Standard assay system for intact cell ecto-CIK activity contained 250 µg of casein, 20 nmol of ATP containing 50×10^4 cpm to 80×10^4 cpm, 1 mol of magnesium chloride and intact spermatozoa (7×10^6 cells to 8×10^6 cells) in a total volume of 0.2 ml RPS and incubation were carried out at 37°C for 3 min. The reaction was stopped with addition of 0.1 ml of 1.5% casein containing 250 mM potassium phosphate and 10 mM ATP and 2 ml of 10% TCA. The resulting protein suspensions were then processed and enzyme activity was measured as mentioned above. Preimmuned rabbit serum incubated intact cells were used as control preparation. One unit of CIK activity has been defined as the amount of the enzyme that catalyzes transfer of 10 pmole f^{32} P from [γ^{32} P] ATP to casein.

Spectrophotometric assay of sperm flagellar motility

Forward motility of spermatozoa was estimated using spectrophotometric method [41,43,44]. The microscopic method of motility assay described above takes into consideration the number of cells with forward progression but not their velocity, whereas the spectrophotometric method reflects the percentage of forward motile cell as well as their velocity. The weakly motile cells are not detected in this method. In case of antibody treatment, cells were extracted in RPS medium and incubated with different concentration of antibody at 37°C for 2 hrs and whereas in control system cells were treated with control sera. The method includes the layering 50 µl of freshly extracted cauda epididymal spermatozoa (200×10^6/ml), mixed with 10% Ficoll in a total volume of 0.5 ml RPS medium (with and without calcium), with a Hamilton syringe at the bottom of a standard cuvette containing 1.3 ml of RPS medium (was sufficient to cover the entire width of the light beam). Vigorously motile spermatozoa that moved upward and crossed the light beam at any particular time, were registered continuously as an increase of absorbance at 545 nm with a Gilford spectrophotometer equipped with the recorder. At the level of maximum absorbance (A_{eq}), the content of the cuvette was mixed and the absorbance for the mixture was noted (A_t). The percentage of the cells that showed vigorous forward motility was calculated as $A_{eq}/A_{tx}100$. The change of velocity after treatment with CIK antibody was measured according to change of forward motility activity. One unit of forward motility activity of the most vigorous group of spermatozoa (responsible for the first slope) was defined as an initial linear increase of absorbance of 0.01/min under standard assay condition. Specific activity of sperm forward motility was expressed as units of forward progression per 10^7spermatozoa.

$^{45}Ca^{2+}$ uptake measurements

The sperm cells were incubated in presence of $^{45}Ca^{2+}$ (specific activity 50000 cpm/nmole) in 0.2 ml RPS with or without added calcium. Each assay medium contains highly motile 5×10^6 cauda epididymal spermatozoa. In case of antibody treatment cell were extracted in RPS medium and incubated with different concentration of antibody at 37°C for 2 hrs and in case of control system cell were treated with control sera. After incubation cells were washed thoroughly with RPS-Ca^{2+}. After incubation, each assay medium was centrifuged at 500 g for 10 minutes. The cell pellet was washed thoroughly with RPS containing 10 mM EGTA for 5 times to 6 times and finally suspended in 0.1 ml of 1% Triton X-100 and left overnight at 37°C [45]. Sample transferred to scintillation vial and added with 10 ml cocktail T (scintillation fluid) and counted for radioactivity. The result was correlated for the background obtained when cell free medium was subjected to the above procedure.

Fluorimetric measurement of free intracellular $[Ca2+]_i$

Cauda epididdymal spermatozoa were washed and suspended in RPS medium. Loaded with the intracellular Ca^{2+} indicator Fure 2 AM (1 µm) and incubated with gentle stirring at 37°C for 40 minutes (modified from Thomas and Meizel 1989) [46]. In case of antibody treatment cell were extracted in RPS medium and incubated with different concentration of antibody at 37°C for 2 hrs and in case of control system cell were treated with control sera. The cell was centrifuged at 2000 rpm and washed thoroughly with RPS and resuspended. Aliquots containing approximately 5×10^6 cells were taken in cuvette and their fluorescence was monitored in a F4010 Hitachi spectrofluorometer. The fluorescence signal is displayed as the ratio of fluorescence for the 340 nm and 380 nm excitation wavelength after background elimination. Emitted fluorescence was recorded at each excitation wavelength. The intracellular Ca^{2+} concentration was estimated by the equation $[Ca^{2+}]_I=Kd(F-F_{min})/F_{max}-F$. The F_{max} was obtained by lysing spermatozoa with 0.1% Triton X-100 in Tris-Cl buffer pH 7.5 subsequently 20 mM EGTA was added to obtain F_{min} value. A dissociation constant of kd of 224 nm was assumed for the Ca^{2+}/FURA 2 system as suggested by Grynkiewiez et al. in 1985 [47].

Results

Effects of antibody on the CIK activity on the external surface of cauda epididymal spermatozoa

CIK antibody inhibited the enzyme activity drastically in the intact cell when casein was used as the exogenous substrate for phosphorylation. The loss of activity was about 85% in antibody treated cells within two hours of incubation (Figure 1).

Figure 1: Western blotting of CIK showing strong innumogenicity. Lane 1: purified CIK after silver staining on gel. Lane 2: 10 µg of CIK-antibody reaction on PVDF membrane Lane 3: 25 µg of CIK-antibody reaction on PVDF membrane.

As the inhibition pattern of CIK activity was same at 1: 100 and 1:500 dilutions, the higher dilution level (i.e., 1: 500 dilution) was taken as the experimental antibody dilution in all the inhibition reaction as mentioned earlier [41]. The control rabbit serum from non-immunized rabbit did not have any significant effect on the kinase reaction. So this polyclonal antibody can be used to modulate the membrane CIK activity to monitor different physiological functions.

Measurement of intracellular calcium level

When CIK antibody treated cells were exposed to RPS medium (119 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 10 mM glucose, 16.3 mM potassium phosphate, and 50 U penicillin mL–1, pH 6.9) containing extracellular Ca^{2+}, a steady increase in intracellular calcium level was observed. The entry of calcium was completely dependent on availability of extracellular calcium. A linear increase in calcium entry

was also observed when extracellular Ca^{2+} concentration was increased from 10 nm to 200 nm (Figure 2).

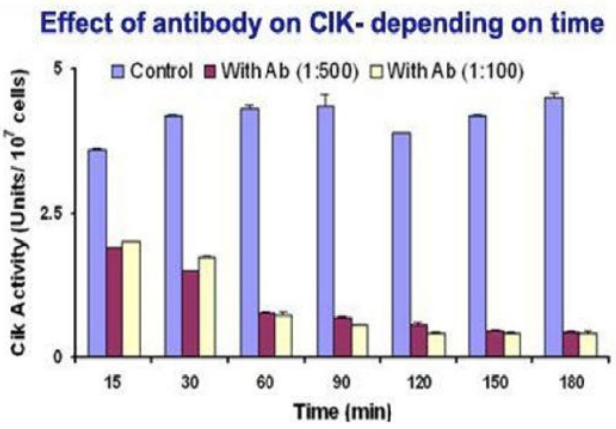

Figure 2: Effect of CIK-antibody on kinase activity of intact cauda epididymal sperm cells. Standard assay system for ecto-CIK activity in intact cells was used. Preimmune rabbit serum incubated intact cells were used as control preparation Bar represent mean ± SD (n=6) $p<0.05$).

The uptake of calcium was time dependent event. Increase in uptake was observed until 60 minutes of incubation and maximum uptake was found at 150 minutes in presence of extracellular calcium (at concentration of 200 nM) (Figure 3).

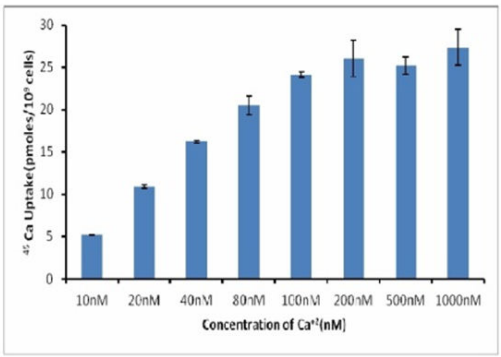

Figure 3: Effect of extracellular calcium on the $^{45}Ca^{2+}$ uptake of cauda epididymal intact spermatozoa. Standard assay method was used as mentioned in the method section. Bar represent mean ± SD (n=5).

We examined the change of intracellular $[Ca2+]_i$ level when the cells were treated with different dilution of CIK antibody. It was also observed that calcium uptake by antibody treated cells was increased about 6 times to 7 times compared with the control serum treated cells. The most significant increase was observed at 1:500 dilution of antibody (Figure 4).

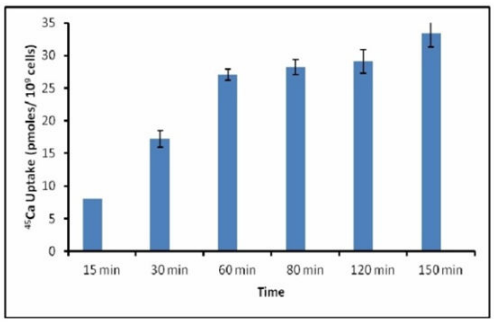

Figure 4: Time dependent uptake of $^{45}Ca^{2+}$ by cauda spermatozoa. Standard assay method was used bar represent mean ± SD (n=5).

Effects of calcium channel antagonists

To dissect out the mechanism of calcium uptake in CIK antibody treated cauda epididymal spermatozoa, cells were treated with antibody and exposed to verapamil, a voltage sensitive calcium channel blocker (Table 1) (Figure 5). The uptake of calcium was decreased about 85% when treated with 20 μM of verapamil.

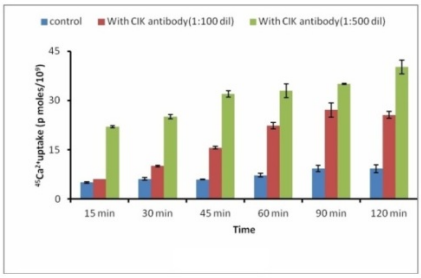

Figure 5: Representative tracings depicting changes in Ca^{2+}/fura 2-AM fluorescence induced by CIK antibody in the presence of extracellular Ca^{2+} in cauda epididymal mature spermatozoa. The standard assay method as mentioned in the method section was used. Changes in $[Ca^{2+}]i$ recorded as the ratio (F_{340}/F_{380}) of fura 2 fluorescence at excitation wavelengths of 340 and 380. The result is a representative of at least 3 such results.

There is dose dependent effect of verapamil on the entry of calcium through plasma membrane.

As the cells were incubated in RPS with high concentration of K^+ (5 mM), the membrane depolarization cannot be ignored.

So, in presence of K^+ increased uptake of calcium was observed which is concurrent with the increased basal intracellular calcium or $[Ca^{2+}]i$ (Figure 6).

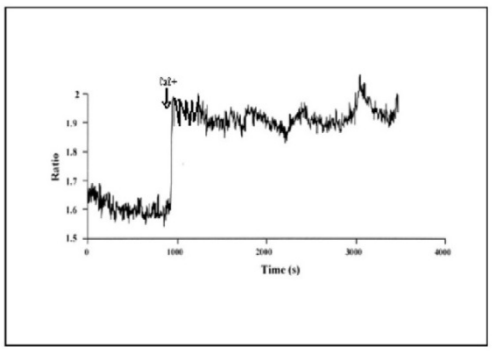

Figure 6: Effect of CIK antibody on the $^{45}Ca^{2+}$ uptake by caprine cauda epididymal spermatozoa. Preimmune sera treated sperm were used as control system. Bar represent mean ± SD (n=6) $p<0.05$.

Changes in intracellular level of calcium were also monitored by measuring the variation in fluorescence of intracellular calcium indicator Fura2AM (1 μM) loaded cells. Figure 5 shows that $[Ca^{2+}]_i$ changes occurred when Fura 2 loaded cells were treated with antibody and simultaneously exposed to extracellular calcium (at the concentration of 200 nM). The results suggest that $[Ca^{2+}]_i$ signals induced by CIK antibody in epididymal spermatozoa involve voltage-operated calcium channel as the increase of intracellular calcium can be prevented when the cells were added with verapamil (20 μM) in presence of extracellular calcium (Figure 6).

We investigated the effects of the L-type voltage-operated Ca^{2+} channels blockers pharmacologically with the help of nifedipin on the calcium uptake of CIK-antibody treated cells. The cauda epididymal mature cells were pretreated with specific L-type voltage-operated Ca^{2+} channel blocker nifedipine (20 μM), and exposed to extracellular calcium, a decrease in calcium uptake (70%) was observed in comparison to the control serum treatment (Table 1). While calmodulin antagonists trifluoperazine and w_{13} (*N*-(4-Aminobutyl)-5-chloro-2-naphthalenesulfonamide hydrochloride) did not show any effect on the entry of Ca2+ into antibody treated cells. Moreover, sodium azide, a potent inhibitor of mitochondrial function also showed no effect on the entry at about 2 mM concentration. Another set of experiments showed that exposure of antibody treated cells with calcium ionophore A123187 at conc of 10 μM resulted in the strong induction of calcium uptake and cause a massive accumulation of Ca^{2+} into the extramitochondrial compartment of sperm (about 100 times in comparison with CIK antibody treated cells (Table 1).

Different effectors	Concentration (μM)	Ca^{2+} uptake in pmole/10^9 Cell
Verapamil	5	7.5 ± 0.02
	10	1.42 ± 0.1
	20	1.1 ± 0.09
Nifedipine	5	9.81 ± 0.03
	10	5.41 ± 0.2
	20	3.1 ± 0.01
Na-azide (NaN3)	1	14.8 ± 1.92
	2	14 ± 2.91
Trifluoperizine	10	14.22 ± 0.91
	20	15 ± 0.2
	50	15.3 ± 0.9
	20	15 ± 0.2
	50	15.3 ± 0.9
W13 (N-[4 amino butyl]- 5 chloro-2 napthaline sulfonamide-Hydrochloride)	10	15 ± 0.9
	20	17 ± 2.1
	50	13 ± 1.0
A 123187 (calcium ionophore)	5	1400 ± 24.5
	10	16 ± 2.3
I Control (Cells treated with CIK antibody 1:500)	-	16 ± 2.3

II Control (Cells without any treatment)	-	4.5 ± 1.10

Table 1: Effect of different calcium channel regulator calcium uptake by epididymal mature spermatozoa.

It was also observed that when Fura 2 loaded cells ware treated with nifedipin (20 μM) intracellular level of increased calcium was depleted in antibody treated cells and if the cells were added with calcium ionophore A123187, the effect of nifedipin was ignored and intracellular calcium level was increased at least 20 times in comparison to the basal value (Figure 7).

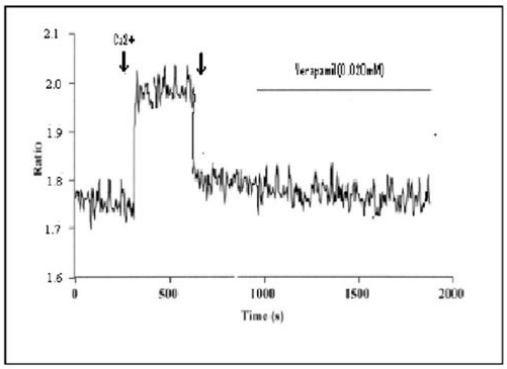

Figure 7: Effects of verapamil (20 μM), a voltage dependent L type calcium channel blocker on the elevations of $[Ca^{2+}]i$ induced by CIK antibody in the presence of extracellular calcium. Changes in $[Ca^{2+}]i$ were recorded as the ratio (F_{340}/F_{380}) of fura 2 fluorescence at excitation wavelengths of 340 and 380. The tracings are representative of 4 such results.

Assessment of forward thrust of cauda epididymal spermatozoa

The movement of the cells in the vertical plane was measured using the automated computerized spectrophotometric system [43] as all the motility analyzers till date, including CASA, never considered the importance of motility in vertical direction which is otherwise crucial for fertilization in female system.

The computerized spectrophotometric assay method (Figure 8A) showed a drastic inhibition in the percentage of forward motility with time when treated with CIK antibody as compared to control serum treated sperm At 1:500 dilution of antibody forward motility percentage decreased about 30%, 50%, 80% after 30 min, 1 hour, 2 hours of incubation respectively in the presence of extracellular calcium (200 nM) (Figure 8B).

The effect of preabsorbed antibody (with antigen CIK) on percentage of motility also indicated the specificity of antigen-antibody binding and presence of the antibody as the specific motility inhibitor in this system (data not shown).

It was the point to note that when the pretreated cells (with CIK antibody) were incubated with verapamil (20 μM) before exposure to extracellular calcium, decrease in forward motility was partially prevented (~50%) (Figure 8B).

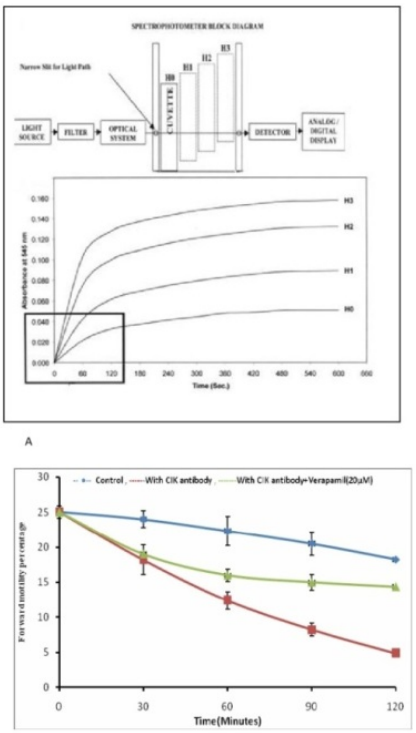

Figure 8: A: Schematic diagram showing the principle of spectrophotometric assay of velocity of forward motile spermatozoa. B: Effect of CIK-antibody on the velocity of forward motile spermatozoa in presence of extracellular calcium. The effect of verapamil (20 μM) was also shown in CIK antibody treated spermatozoa where cell was exposed to varapamil before added with calcium (200 nM) in the extracellular medium. Preimmune sera treated sperm were taken as the control system. Standard assay system was followed as mentioned in the Method section. The data are shown as mean ± SD (n=4).

Discussion

Controlling the permeability of the plasma membrane is one of the main mechanisms by which cells can regulate their cytoplasmic $[Ca^{2+}]_i$. In many instances, the plasma membrane contains voltage sensitive calcium channels which can regulate a no of cellular functions.

The concentration and distribution of intracellular Ca^{2+} play an important role in the regulation of contractile and secretory functions in many different types of cells as reviewed by Berridge in 1975; Rasmussen and Goodman in 1977 [48,49]. Intracellular Ca^{2+} has been implicated as a regulatory mediator in spermatozoa as well.

Increase in the permeability of sea urchin sperm membrane to Ca2+ are associated with alteration of sperm motility that are required for fertilization [50-52].

Recently it has been found that activation of motility in mammalian sperm follow treatments that induce influx of extracellular Ca2+ into these cells. A molecular basis for the action of increased intracellular $[Ca^{2+}]_i$ in the regulation of cellular functions has not been established for either invertebrate or mammalian sperm.

It is also unknown till date how the lateral movements of proteins/enzymes on the surface of spermatozoa can be linked with intracellular calcium level and forward motility of spermatozoa. The cyclic AMP/PKA signaling pathway is generally recognized as the major pathway in the regulation of mammalian sperm motility [53-56].

However, studies in our laboratory focused on the activity of an ecto-cAMP independent protein kinase (CIK) on the outer surface of plasma membrane which acts in concert with a PPases as a coupled enzyme system to modulate the phosphorylated states of the ecto phosphoprotein substrates [57-59].

The specific activity of these ecto-CIK and PPases have been found to increase markedly during forward progression of spermatozoa, suggesting thereby that these ecto-enzymes and their substrates have a role in modulating sperm motility [57,60]. It was also found that highly purified plasma membrane isolated from goat cauda epididymal spermatozoa possesses CIK that phosphorylates several membranes bound phosphoproteins.

This event is not dependent on the endogenous cAMP-dependent proten kinase [61]. Rapid turnover of these cell surface phosphoproteins exhibits the characteristics of regulatory proteins [61,62].

The observation that the epididymal sperm maturation event is associated with a remarkable lateral movement of CIK leading to the realignment on the cell surface, strengthened this view.

Ecto-CIK has been purified from cauda sperm plasma membrane. Inhibition of cauda sperm flageller motility by ecto-CIK antibody can be the direct evidence of the involvement of this kinase as motility regulators [41].

The result of spectrophotometric motility assessment shows that treatment of the motile spermatozoa with ecto-CIK antibody causes marked fall in velocity of the cells because the number of spermatozoa that swim upward direction against the gravity to enter the light path decreases as indicated by time dependent absorbance lowering of forward motility index or velocity of most vigorously motile population of spermatozoa (Figure 9). One of the important findings of the present study is that the regulation of L-type voltage-gated Ca^{2+} channel associated with the inactivation of plasma membrane surface cAMP independent protein kinase (CIK) is independent of calcium-calmodulin regulated channels and mediation of mitochondria (Table 1). The presence of voltage-gated Ca^{2+} channel in mature sperm cell is very important for many reasons and has wide implications. The induction of forward motility in the mature epididymal spermatozoa is an important event for the cell to reach the ova in the female tract and these functions have been shown to be triggered via ion channels which either provide Ca^{2+} entry pathways or provide the driving force for Ca^{2+} influx through these pathways.

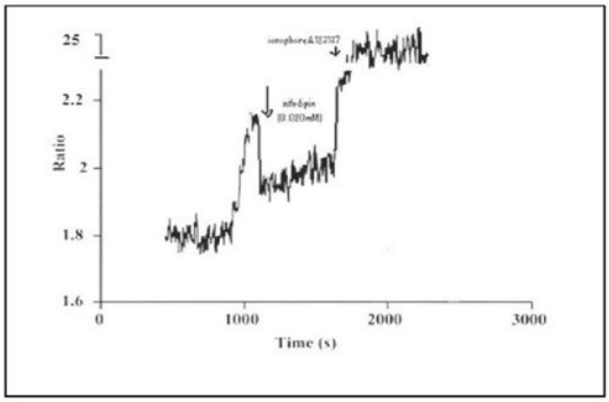

Figure 9: Effects of nifedipine (10 μM), a voltage dependent L type calcium channel blocker on the elevations of $[Ca^{2+}]_i$ induced by CIK antibody in the presence of extracellular calcium. A change of intracellular calcium was also shown in the presence of calcium ionophore A 123187(5 μM) on the same assay system. Changes in $[Ca^{2+}]_i$ were recorded as the ratio (F_{340}/F_{380}) of fura 2 fluorescence at excitation wavelengths of 340 and 380. The tracings are representative of 4 such results.

Caput sperm had higher $[Ca^{2+}]_i$ than caudal cells and decreasing the concentration from caput to cauda cells through maturation may be critically important [23]. The decreasing expression of ecto-CIK from caput to cauda cells may be related to this controlling mechanism in natural way. If the ecto CIK in the forward motile mature cauda cells is blocked with antibody the phosphorylation of its endogenous surface phosphoproteins (i.e., MPS) is also decreased as shown in previous publications [35]. As a result, the entry of the calcium through the voltage gated calcium channel is opened up (Figures 4 and 7). But the presence of voltage-gated ion channels has been difficult to reconcile with the slow and often small changes in membrane potential. However, this incidence of low-current voltage-gated ion channels which is not detectable in relation to common calcium channels as opened by ionophore A123187 (Figure 7), may be related to the CIK-phophorylated-MPS regulatory mechanism of forward mobility (Figure 9) of mature spermatozoa. It was pointed to note that blocking of L type calcium channel by verapamil (20 μM) solely cannot reframe the membrane structures which can maintain normal percentage of velocity of cauda cells after treatment with CIK antibody. So, it can be concluded although ecto cAMP independent protein kinase/phosphatase/phosphoprotein substrates are the part of the membrane bound L type calcium channel but there are also other factors in additions to this system that are critically important for the regulation of forward progression of epididymal spermatozoa.

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