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Original article

Permeability changes induced by polylysines in rat spermatids

Ramon A. Jorquera [a], Julio Berrios [a], Jorge Sans [b], Cecilia Vergara [c], Dale J. Benos [d], Juan G. Reyes [a,d,*]

[a]*Instituto de Quimica, Universidad Catolica de Valparaiso, Casilla 4059, Valparaiso, Chile*
[b]*Instituto de Ciencias Biomedicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[c]*Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile*
[d]*Department of Physiology and Biophysics, The University of Alabama at Birmingham, Birmingham, AL 35294, USA*

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Abstract

High molecular weight (HMW, >15 kDa) but not low molecular weight (LMW, <15 kDa) polylysines (PLs) bound and induced permeability changes in rat spermatid plasma membranes, estimated by Mn^{2+} quenching of intracellular indo-1 fluorescence ($K_{1/2} = 3.3 \pm 0.5$ μg/ml) and Co^{2+} quenching of intracellular calcein. The pharmacology of the Mn^{2+} entry pathway activated by HMW PL does not suggest that Ca^{2+} channels are involved in this phenomenon. Concentrations of HMW PL that induced divalent ion entry did not induce the entry of ethidium bromide, suggesting that HMW PL first bound and perturbed the plasma membrane structure inducing a non-specific increase in membrane permeability. High concentrations of HMW PL induced cell lysis ($K_{1/2} = 23$ μg/ml). The binding of HMW PL, initially homogenous on the cell surface, subsequently progressed to a segregated pattern resembling a clustering phenomenon.
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Keywords: Cationic peptides; Spermatogenic cells

1. Introduction

Polylysine (PL) has been used extensively as a cell adhesion molecule for cells growing on a solid support. However, this cationic homopeptide does not seem to behave as a passive cell adhesion molecule. Thus, it has been shown to induce Ca^{2+} entry in leukemia T cells (Galvanovskis et al., 1996), tracheal cells (Uchida et al., 1996) and spermatozoa (Wennemuth et al., 1998). PL has also been shown to mimic the action of heparin-binding growth factors (Aoyama et al., 1997; Baron et al., 1996; Fenstermaker et al., 1993). A marked cytotoxic action upon exposure of cells in culture to high concentrations of PL has been also demonstrated (e.g., Elferink, 1985). In our studies of Ca^{2+} homeostasis in spermatogenic cells, we observed that round spermatids progressively gained intracellular Ca^{2+} ([Ca^{2+}]i) without losing plasma membrane integrity when adhered to a PL-covered support. Given the importance that modulation of Ca^{2+} entry could have in cell differentiation (e.g., Whitaker and Patel, 1990), and the fact that spermatogenic cells possess T type Ca^{2+} channels (Arnoult et al, 1996; Lievano et al., 1996) as well as intracellular Ca^{2+} stores and intracellular Ca^{2+} channels (Berrios et al., 1998; Treviño et al., 1998), we explored the mechanisms involved in the changes in [Ca^{2+}]i induced by HMW PL in rat round spermatids. Our results indicate that HMW PL (>15 000 Da) but not low molecular weight (LMW) PL (4000 Da) can induce a non-specific cation permeability increase in round spermatids. In a concentration-dependent manner, this permeability increase can progress to cell lysis. HMW FITC-labeled PL bound to the cells homogeneously on the cell surface. The homogenous FITC-PL labeling progressed to a spotted pattern suggesting a clustering of membrane components on the cell surface.

* Corresponding author. Fax: +56-32-273-422.
E-mail address: jreyes@ucv.cl (J.G. Reyes).

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2. Materials and methods

2.1. Rat spermatogenic cell preparation

The Ethics Committee, Universidad Catolica de Valparaiso, approved the studies described in this paper. The animals used (Wistar rats) were obtained from the Universidad de Valparaiso Animal Facility and were maintained with free access to food and water on a 12 h light/dark cycle. Rat spermatids were prepared from the testicles of adult (60 d old) rats as described by Romrell et al. (1976). In brief, the rats were killed by cervical dislocation and the testicles were excised, decapsulated and digested at 33 °C for 20 min with 0.5 mg/ml collagenase and 2 μg/ml DNAse in a Krebs-Henseleit (KH) medium supplemented with 10 mM Hepes and 10 mM DL-lactate, pH 7.4 (KH-lactate). The resulting seminiferous tubules were washed three times in the same medium, digested with 0.2 mg/ml trypsin for 5 min, and then mechanically disrupted by repeated pipetting with a plastic pipette. The resulting cell suspension was filtered through a layer of cotton and 250 and 70 μm nylon meshes, and subsequently washed three times in KH-lactate medium. The round spermatid fraction was purified using sedimentation velocity in a 250 ml 2–4% BSA gradient in KH-lactate medium at unit gravity and at 16 ± 2 °C (Romrell et al., 1976). The cells were allowed to settle for 3 h, and the BSA gradient was collected in 3 ml fractions at approximately one fraction per minute. The round spermatid fraction (steps 1–7 spermatids) had a purity of 92 ± 3%.

2.2. Cellular identification

For single cell $[Ca^{2+}]i$ measurements, the non-intercalating bisbenzimide DNA probe, Hoechst 33342 (H_{33342}) (excitation wavelengths 330–380 nm, emission wavelengths >420 nm) was used to identify microscopically the rat round spermatids according to the morphology of their nucleus (Reyes et al., 1997). The cells were incubated for 10 min in KH-lactate in the presence of 4 μM H_{33342} and subsequently observed by fluorescence microscopy in a perifusing microscope chamber. Only round spermatids (steps 1–7) were used in the single cell $[Ca^{2+}]i$ measurements. Our method of vital cell identification does not allow making a subclassification of rat spermatids at these stages of development.

2.3. Measurements of $[Ca^{2+}]i$ in cells in suspension

For quantitative estimations of $[Ca^{2+}]i$ content in rat spermatids, we used ratiometric spectrofluorimetric determinations with the Ca^{2+} probe fura-2. Acetoxymethyl fura-2 (5 μM) was incubated with the purified spermatids in KH-lactate for 45 min at room temperature (18 ± 2 °C), the cells were subsequently washed thrice in KH-lactate and the fluorescence of the suspended cells (1 million/ml) was estimated at alternative excitation wavelengths of 340 and 380 nm, with emission wavelength at 510 nm in a PTI Deltascan spectrofluorimeter (Photon Technology International Inc., NJ). To calibrate the fura-2 signal, at the end of the experiments, the cells were lyzed with digitonin (20 μg/ml) in the absence of external Ca^{2+} (0.5 mM EGTA) and then 1 mM $CaCl_2$ was added. $[Ca^{2+}]i$ was calculated as described by Grynkiewicz et al. (1985).

2.4. Measurements of $[Ca^{2+}]i$ in single cells

The variations of $[Ca^{2+}]i$ in single cells were estimated using the fluorescent probe fluo-3 (excitation wavelengths 450–490 nm, emission wavelengths >510 nm). Acetoxymethyl fluo-3 (fluo-3 AM) (5 μM) was incubated with the cells in KH-lactate for 30 min at room temperature (18 ± 2 °C). The cell fluorescence was quantified using videomicroscopy in an inverted Nikon Diaphot microscope with epifluorescence (excitation wavelengths 450–490 nm, emission wavelength >510 nm) and a 40× fluo objective (NA 0.85). A cooled CCD, 12-bit videocamera (Spectrasource, Los Angeles, CA) was attached to the videoport of the microscope. The image analysis was performed with an appropriate software using a personal computer. The experiments performed in the nominal absence of external Ca^{2+} were made with KH medium without Ca^{2+}, and with 0.5 mM EGTA added. This condition gives an estimated free $[Ca^{2+}]$ of approximately 3 nM.

2.5. Measurements of Mn^{2+} quenching of intracellular indo-1

In order to determine the pharmacological properties of the permeability changes observed as $[Ca^{2+}]i$ increases induced by HMW PL in rat spermatids, we utilized the properties of indo-1 to be quenched by Mn^{2+} ions and the properties of this ion to substitute for Ca^{2+} in Ca^{2+} channel conduction. Thus, Mn^{2+} entry, evidenced as quenching of indo-1 fluorescence in the presence of Ca^{2+}, can be measured at the isosbestic point for the Ca^{2+}–indo-1 complex (excitation wavelength 345 nm) (Montero et al., 1990). Round spermatids were loaded with indo-1 by exposing them to 4 μM indo-1 AM for 40 min at room temperature (18 ± 2 °C). The isosbestic point for the intracellular Ca^{2+}–indo-1 complex was determined by taking the emission spectra of indo-1 loaded cells and modifying the $[Ca^{2+}]i$ by the addition of 10 μM ionomycin with 0.5 mM external Ca^{2+}. At the isosbestic point (447 nm, slit width: 3 nm), the decrease of the indo-1 fluorescence was dependent on the presence of extracellular Mn^{2+}, and independent of $[Ca^{2+}]i$ (not shown).

2.6. Measurements of Co^{2+} quenching of calcein

In order to determine the selectivity of the permeability changes observed as $[Ca^{2+}]i$ increases induced by HMW PL

in rat spermatids, we utilized the properties of some fluorescent probes to be quenched by divalent ions. Thus, calcein is a plasma membrane impermeant fluorescent probe (excitation wavelength 494 nm, emission wavelength 517 nm) relatively insensitive to changes in [Ca^{2+}] in the range of intracellular [Ca^{2+}] changes observed in this work (Haughland, 1996). However, Co^{2+}, a plasma membrane impermeant cation, can quench calcein, allowing estimation of non-specific plasma membrane permeability changes. The cells were loaded with calcein by exposing them to 0.2 μM calcein-AM for 30 min at room temperature. The cell fluorescence was estimated using videomicroscopy (excitation wavelengths 450–490 nm, emission wavelength >510 nm) by taking serial images of the cells subjected to different treatments.

2.7. Polylysine binding to rat spermatids

In order to visualize the binding of PL to rat spermatids adhered to a microscope chamber coverslip in KH-lactate media, we exposed these cells to 2.5 μg/ml poly-L-lysine-FITC (MW 30 000–70 000) for 5 min. The cells were then perifused with 10 ml of KH-lactate solution, 5 μM ethidium bromide were added to monitor cell lysis, and the cells were observed under light transmission and fluorescence microscopy (excitation wavelengths 450–490 nm, emission wavelength >510 nm). The pattern of cell fluorescence was followed for 45 min and timed photographs of the cells were recorded. Confocal microscopy of FITC-PL labeled rat spermatids was made in a Zeiss LSM 410 confocal microscope with a 100× oil immersion objective (NA 1.4) using a 488 nm laser source and a pinhole size of 56 μm.

2.8. Estimation of cell lysis

Rat spermatid lysis was estimated as the number of cell nuclei labeled with ethidium bromide (5 μM) or the loss of fluorescence from calcein-loaded cells when the spermatids were subjected to different concentrations of PL at room temperature (excitation wavelengths 450–490 nm, emission wavelength >510 nm).

2.9. Statistics

The data were analyzed using non-parametric statistics. Non-linear regression was performed with the ORIGIN™ software package.

2.10. Reagents

Fura-2 AM, Fluo-3 AM, indo-1 AM, calcein AM and H_{33342} were obtained from Molecular Probes (Eugene, OR). Poly-L-lysine, poly-D-lysine, poly-L-arginine, chondroitinase ABC, heparitinase and all the other reagents, enzymes and salts were obtained from Sigma Chem. Co. (St. Louis, MO).

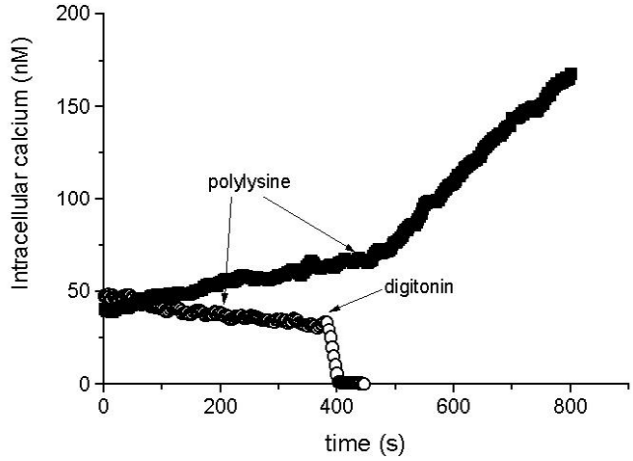


Fig. 1. [Ca^{2+}]i in rat spermatids in suspension. [Ca^{2+}]i was measured at 33 °C using fura-2, in the presence (0.5 mM) (•) and absence (5 mM EGTA) (○) of external Ca^{2+}. At the arrows, 10 μg/ml PL (70–150 kDa) or 20 μg/ml digitonin were added. These results are representative of three measurements performed in two different cell preparations.

3. Results

3.1. Polylysine triggers a [Ca^{2+}]i increase in rat spermatids

Addition of 5 μg/ml poly-L-lysine (HMW, 70,000–150,000) to a suspension of rat round spermatids loaded with fura-2 in the presence of external Ca^{2+} (0.5 mM) induced a sustained rise in [Ca^{2+}]i (Fig. 1). Similar experiments performed in the absence of external Ca^{2+} induced neither a rise in [Ca^{2+}]i nor a decrease in fura-2 fluorescence suggesting that the rise in [Ca^{2+}]i induced by PL was not due either to release of Ca^{2+} from intracellular stores or leakage of fura-2 from the cells (Fig. 1).

3.2. HMW polylysine effects on intracellular Ca^{2+} in single rat round spermatids

When HMW poly-L-lysine (5 μg/ml) was added to the microscope chamber that contained single cells loaded with fluo-3 in KH-lactate, it triggered an increase in [Ca^{2+}]i (Fig. 2A). A fraction of the cell population (20–30%) responded with a [Ca^{2+}]i increase at low concentrations of HMW PL (5 μg/ml). This increase in [Ca^{2+}]i was heterogeneous in magnitude and duration in different spermatids, and could be distinguished from a loss of membrane integrity because when that occurred, the cells suddenly lost >90% of the fluorescence (release of fluo-3). Similarly, rat spermatids adhered to coverslips treated with a 0.1% PL solution (MW 70 000–150 000 kDa) showed a steady increase in [Ca^{2+}]i (approximately 10–20 nM/min, Fig. 2B). These PL adhered cells excluded ethidium bromide indicating that their membranes were not disrupted. These data demonstrate that the

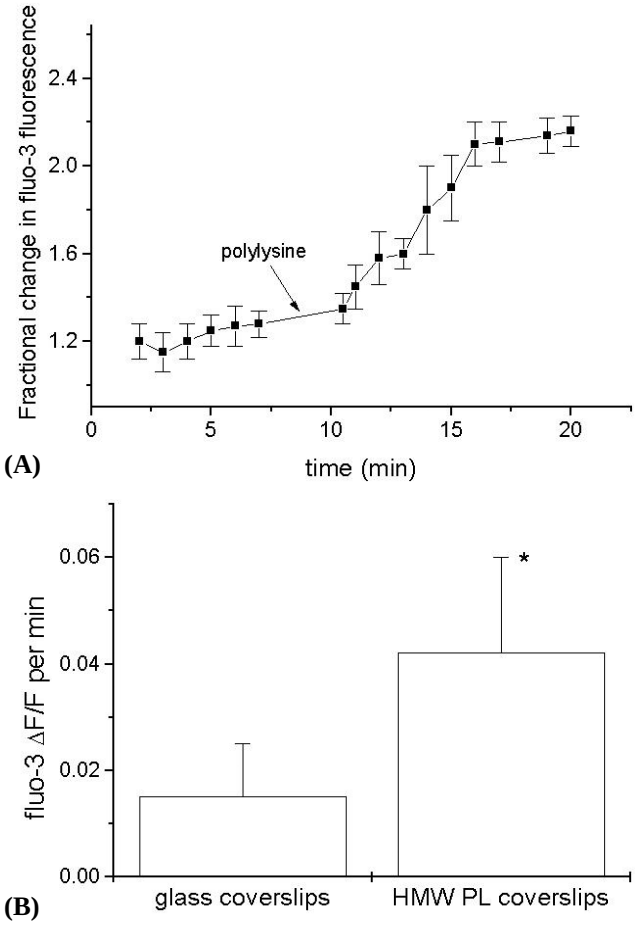


Fig. 2. (A) Fractional increase in fluorescence of single fluo-3 loaded rat spermatids. At the time indicated, 5 μg/ml PL were added to the microscope chamber. Images were taken every 2 min as described in the text. The results shown are expressed as the mean ± S.D. of 40 cell measurements performed in three different cell preparations. (B) Rate of fractional increase in the fluorescence signal of fluo-3 loaded rat spermatids adhered to glass or PL-covered glass coverslips. The data are shown as the mean ± S.D. ($N = 71$ and 37 for glass coverslips and glass coverslips treated with HMW PL, respectively). The rates of signal increase were significantly different at $P < 0.05$.

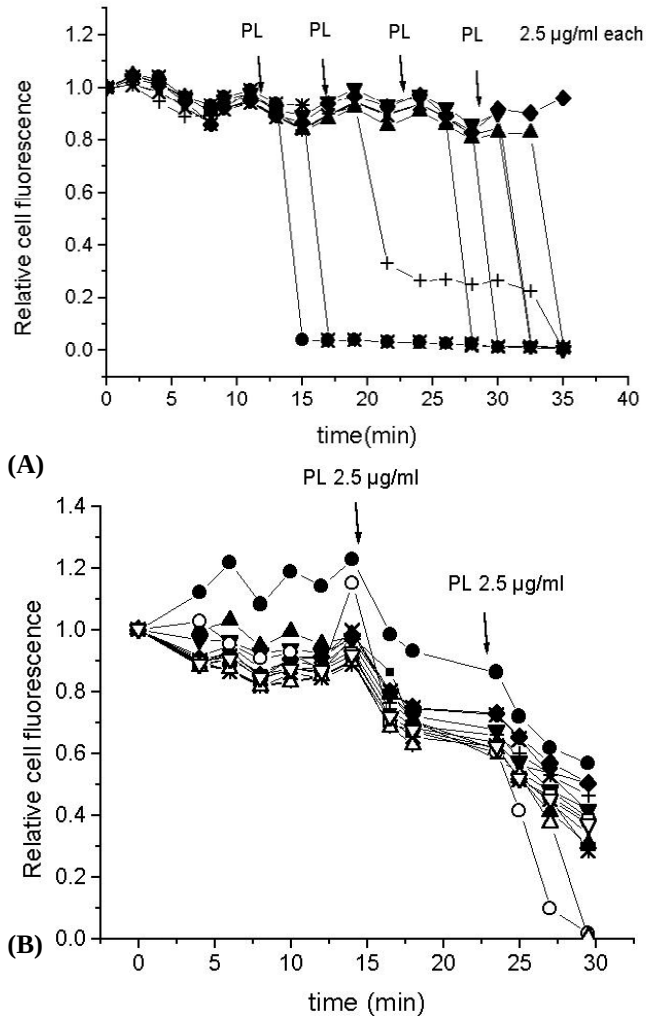


Fig. 3. (A) Fractional changes in fluorescence of single calcein-loaded rat spermatids in KH-lactate. At the times indicated, 2.5 μg/ml HMW PL were added to the microscope chamber. Images were taken every 2 min as described in the text. (B) Fractional changes in fluorescence of single calcein-loaded rat spermatids in KH-lactate supplemented with 100 μM $CoCl_2$. At the times indicated, 2.5 μg/ml HMW PL were added to the microscope chamber. Images were taken every 2 min as described in the text.

PL-induced Ca^{2+} entry in rat spermatids preceded the lysis of the cells and also that was not related only to cell–cell interactions triggered by the multidomain characteristic of PLs.

3.3. Calcein quenching by Co^{2+}

The data presented above demonstrated that interaction of HMW PLs with spermatids induced Ca^{2+} entry into the cells. In order to test whether divalent ion entry induced by PL was specific for Ca^{2+}, we utilized the ability of Co^{2+}, an impermeant ion, to quench calcein (Kendall and MacDonald, 1982). Thus, in cells loaded with calcein, it is expected that when the cell membrane integrity is lost (cell lysis), calcein and Co^{2+} would mix, quenching the fluorescence of the probe. The results shown in Fig. 3A indicate that when the cells loaded with calcein in the absence of external Co^{2+} were exposed to HMW PL, they maintained intracellular fluorescence until a sudden drop in fluorescence occurred indicating cell lysis. Instead, when Co^{2+} was externally present, HMW PL induced a steady decrease in intracellular fluorescence with a slower kinetic in agreement with activation of Co^{2+} entry by HMW PL in these cells (Fig. 3B). Cell lysis was occasionally observed at lower than 5 μg/ml HMW PL.

3.4. Activation of Mn^{2+} entry by polylysine

In order to characterize the properties of divalent ion entry into rat spermatids and determine initial velocities of divalent ion entry into the cells, a parameter that is expected

to be less affected by cell lysis, we monitored the entry of Mn^{2+} in spermatids loaded with indo-1. Addition of HMW poly-L-lysine to a suspension of rat spermatids loaded with indo-1 and with 100 μM $MnCl_2$ in the external media (KH-lactate), within 1 min induced entry of Mn^{2+} into the cells and the subsequent quenching of the indo-1 fluorescence measured at the isosbestic point for Ca^{2+} (Fig. 4A). Ultrafiltration (cut off > 10 000 kDa) of the HMW PL solution and testing of the ultrafiltrate and the supernatant showed that the activation of Mn^{2+} entry was not induced by low molecular weight compounds present in the HMW PL stock (tested in two different batches of PL) (Fig. 4A). Addition of PL in the absence of Mn^{2+} did not induce changes in indo-1 fluorescence (not shown), discarding the fact that the indo-1 fluorescence quenching could be due to an optical phenomenon of cell aggregation after PL addition to the cell suspension. The dose–response curve for the Mn^{2+} entry phenomenon triggered by PL showed a $K_{1/2}$ of 3.3 ± 0.5 μg/ml (hyperbolic non-linear fit) (Fig. 4B). Low molecular weight (LMW) poly-L-lysine (4000 or 7000 kDa) did not induce entry of Mn^{2+} into rat spermatids up to 40 μg/ml. The effect of an increase in Mn^{2+} entry was observed with poly-L-lysines of 25000 kDa or larger, and also with HMW poly-D-lysine and HMW poly-L-arginine (75–150 kDa, not shown). Lysine (10 μg/ml) did not induce or block the poly-L-lysine-induced Mn^{2+} entry in rat spermatids.

Spermatogenic cells have been shown to possess T type Ca^{2+} channels (Arnoult et al., 1996; Lievano et al., 1996). Although the entry of Co^{2+} induced by HMW PL strongly suggested that a non-specific increase in plasma membrane permeability was induced by the homopeptide, we wanted to further test the possible involvement of calcium channels in the PL-induced Mn^{2+} or Ca^{2+} entry. In order to do that, we examined the effects of several inhibitors of T type voltage-dependent calcium channels on this phenomenon. In Table 1, it is shown that the effects of nifedipine and Ni^{2+}, although showing a tendency to inhibit the effect of HMW PL on Mn^{2+} entry, did not reach statistical significance. Octanol significantly blocked the PL-induced Mn^{2+} entry into rat spermatids. An excess of heparin (20 μg/ml) was able to block completely the effect of PL on Mn^{2+} entry into the cells. Amiloride, an inhibitor of T-type Ca^{2+} channels, could not be used in this work, because at the concentrations required, it presented a strong light absorption at the indo-1 emission wavelength, as well as of the fluo-3 excitation wavelength.

The Mn^{2+} entry activating ability of PL was not shared by polycations such as spermine and spermidine even at concentrations of 1 mM (not shown). Spermine and spermidine also did not block the effects of PL on Mn^{2+} entry in rat spermatids.

Some of the actions of polycation peptides on cells have been shown to occur by the interactions with membrane polysaccharides (Donato et al., 1996). However, in rat

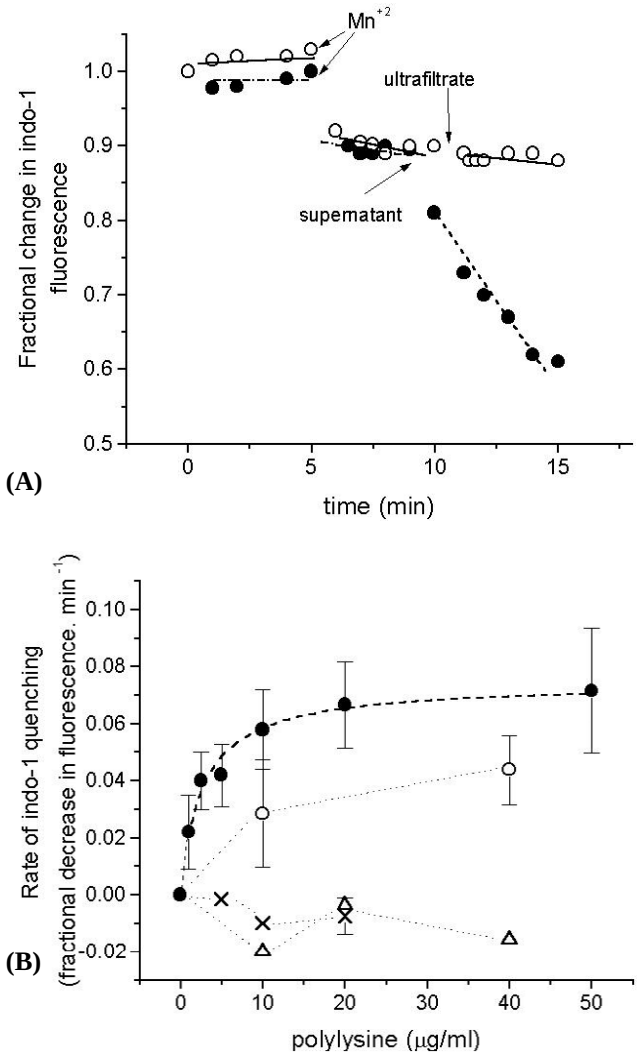


Fig. 4. (A) Plot of fractional fluorescence decrease of indo-1 loaded cells against time. The cells were suspended in 1 ml of KH-lactate buffer without phosphate. At the times indicated, Mn^{2+} (100 μM), 10 μl of the ultrafiltrate (○) or the supernatant (●) of a 1 mg/ml PL solution and 100 μM La^{3+}, were added to the 2 ml cell suspension at 33 °C. These results are representative of three different measurements performed in similar conditions with two different cell preparations and using two different batches of PL. The lines were drawn by eye and show the tendency of the indo-1 fluorescence as a function of time. (B) Rate of fractional Mn^{2+} quenching of indo-1 loaded spermatids at different concentrations of PLs of different molecular weights (●, 75–150 kDa; ○, 25 kDa; ×, 15 kDa; Δ, 4 kDa). The $K_{1/2}$ for PL action (75–150 kDa) was estimated to be 3.3 ± 0.5 μg/ml in a non-linear hyperbolic fit of the data (dashed line). The error bars represent the S.D. of four different measurements performed in three different cell preparations.

spermatids, heparitinase and chondroitinase treatment of the cells (2.4 and 1 units/ml, respectively, 60 min at 33 °C) did not significantly modify the ability of HMW PL to induce Mn^{2+} entry in round spermatids. Thus, the fractional quenching of indo-1 fluorescence in round spermatids by Mn^{2+} in control, heparitinase and heparitinase + choindroitinase treatment were 0.063 ± 0.022, 0.049 ± 0.017 and 0.056 ± 0.018, respectively. These results strongly suggest

Table 1
Inhibition of PL-activated Mn^{2+} entry in rat spermatids (%)

Addition [a]	Percentage inhibition	*N*
Control	3 ± 10	3
Nifedipine (30 μM)	77 ± 32	3
Ni^{2+} (100 μM)	57 ± 25	3
Octanol (0.5 mM)	98 ± 1 [b]	3
Heparin (20 μg/ml)	110 ± 9 [b]	**3**

[a] Nifedipine, Ni^{2+}, octanol and heparin were added 5–6 min before the addition of 10 μg/ml PL. The percentage inhibition was estimated as the paired fractional change in the rate of Mn^{2+} quenching of indo-1 loaded round spermatids. The data are expressed as the average ± SEM.
[b] Significantly different from control (Wilcoxon test, $P < 0.01$).

that negatively charged chondroitin sulfate and heparansulfate surface molecules were not involved in the mechanisms of activation of Mn^{2+} entry in rat spermatids.

3.5. *Effects of HMW poly-L-lysine on rat spermatids cell membrane integrity*

In order to characterize the lytic effects of PLs on rat spermatids, we tested for the entry of ethidium bromide as an indication of the loss of plasma membrane selective permeability. HMW poly-L-lysine, at concentrations higher than 10 μg/ml and after 10 min of exposure, induced a significant lysis of rat spermatids (Fig. 5). LMW PL (4000 MW) did not induce cell lysis even at concentrations of 40 μg/ml. The effect of PL on rat spermatid membrane integrity was blocked by heparin and was also observed in the absence of extracellular calcium.

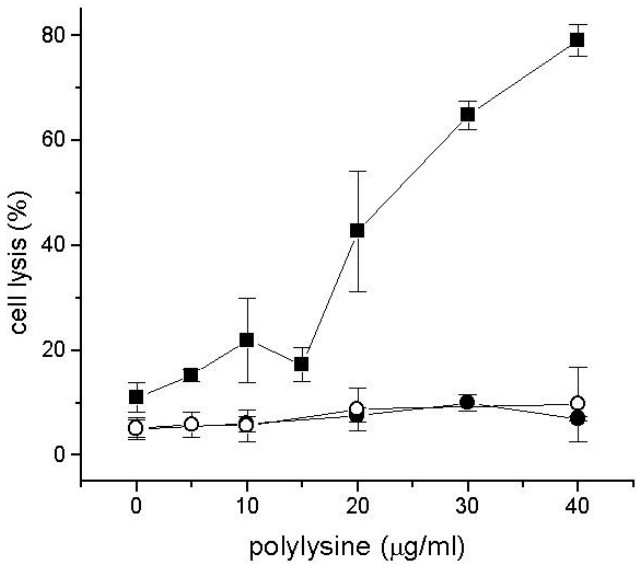


Fig. 5. Dose–response curve of PL-induced lysis in rat spermatids in the absence (○) and presence (●) of 20 μg/ml heparin. The $K_{1/2}$ for the PL effect in the absence of heparin was 23 μg/ml. The results shown are expressed as the mean ± S.D. of at least three different measurements for each PL concentration performed in three different cell preparations.

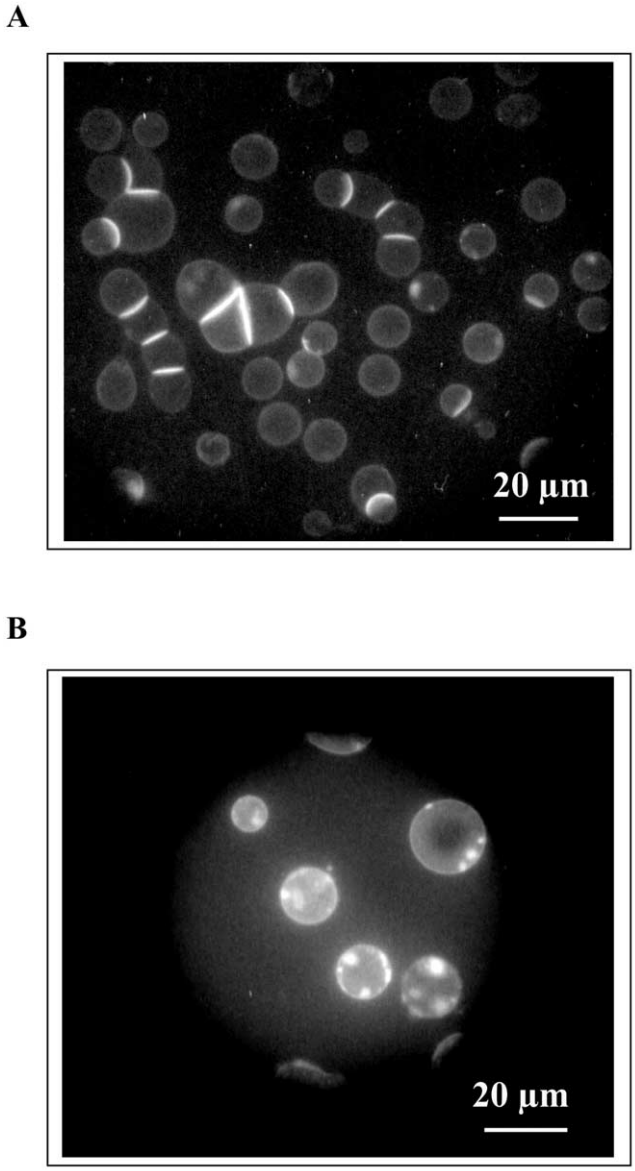
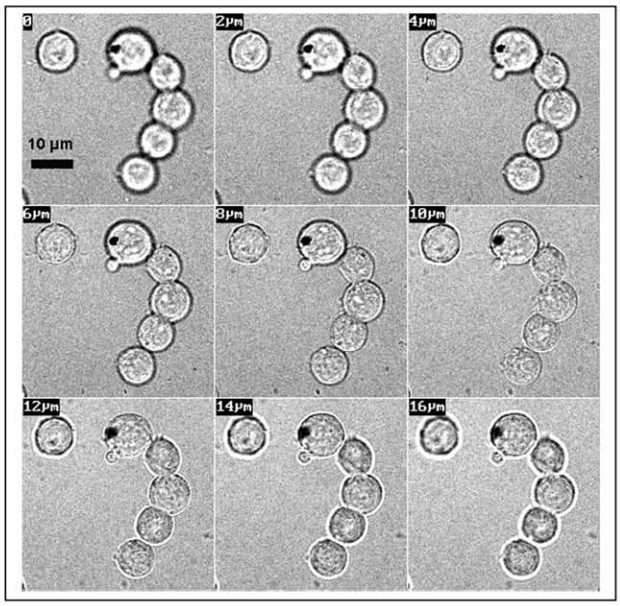


Fig. 6. (A) Fluorescence photomicrograph of FITC-PL labeled rat spermatogenic cells 10 min after FITC-PL addition at 18 ± 2 °C. The cells were previously allowed to settle at the bottom of the microscope chamber and washed with 10 ml of KH-lactate buffer. The bar represents 20 μm. (B) Fluorescence photomicrograph of FITC-PL labeled rat spermatogenic cells 30 min after FITC-PL addition. Other conditions were similar to (A). The cells show clearly visible clusters of fluorescence. The magnification was similar to (A).

3.6. *HMW poly-L-lysine binding to rat spermatids*

Direct visualization of PL-FITC labeling of rat spermatids showed that PL bound to the membrane of these cells (Fig. 6A,B). The zones of contact between cells present a progressive increase with the time of the FITC-labeled PL zone. However, these contact zones do not present intercellular GAP junction-like communication as evidenced by the absence of intracellular content mixture when two populations of cells labeled with H_{33342} (nuclei) or calcein (cyto-

A



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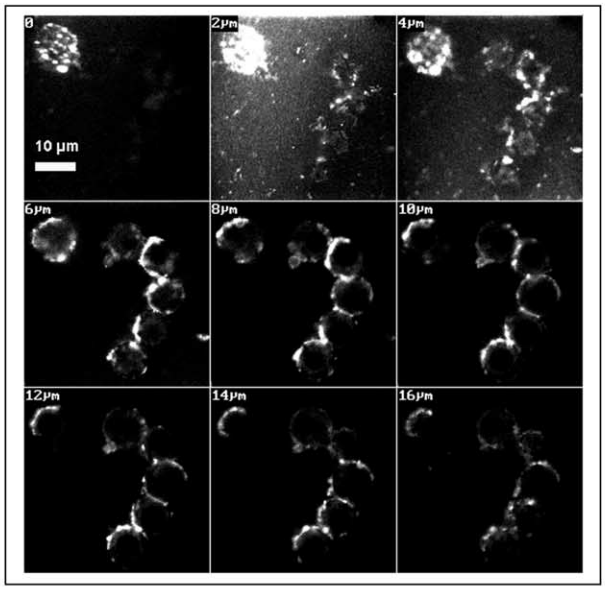

Fig. 7. (A) Transmission confocal sections of a group of rat spermatids. The bar represents 10 μm. (B) Fluorescence confocal sections of a group of rat spermatids labeled with FITC-PL after 40 min exposure to PL. The magnification was similar to (A).

sol) were put in contact in the presence of HMW PL (not shown). This FITC-PL binding was prevented by previous incubation with heparin (20 μg/ml). The bound PL was only slowly removed by the removal of FITC-PL from the external solution, suggesting a relatively strong binding of this molecule to rat spermatids. At short times after FITC-PL addition (5–10 min), 20 μg/ml heparin partially removed the bound PL from the surface of the cells (31 ± 15%, $N = 4$). Furthermore, the pattern of PL distribution on the cell surface progressively changed from a homogenous distribution at 10 min after FITC-PL addition (Fig. 6A) to a spotted pattern after 30–40 min of PL addition (Fig. 6B). Confocal microscopy of FITC-PL labeled cells showed that the spotted pattern was present on the surface of the cells (Fig. 7A,B), and hence it did not seem to originate from an endocytic activity of rat spermatids. As shown in the optical section at 4 μm (Fig. 7B), the site of cell adhesion to the coverslip also showed a spotted pattern, complementing the notion that HMW PL induced changes in plasma membrane structure in spermatids bound to a PL-covered support.

4. Discussion

In this work, we have shown that HMW PL bound to rat round spermatids, and as an early event, it induced divalent cation entry into these cells. The increase in membrane permeability to divalent cations was non-specific. Ca^{2+}, Mn^{2+} and Co^{2+} entry into rat spermatids was induced by HMW PL. Subsequently, HMW PL induced a redistribution of membrane molecules on the cell surface. At concentrations higher than 10 μg/ml, this cationic homopeptide induced cell lysis. The lytic action induced by HMW PL in rat spermatids does not seem to be mediated by the entry of Ca^{2+} into the cells because it was also observed in the absence of external Ca^{2+}. Heparin, a polyanion that binds and induces an alpha-helix conformation of the homopeptide in solution (Mulloy et al., 1996), inhibited the binding of PL to rat spermatids, the activation of Ca^{2+} entry and the lytic actions of HMW PL.

The action of PL on cation entry did not seem to be mediated only by PL-induced cell–cell interactions, since the entry of Ca^{2+} was also induced by HMW PL in single cells that do not have cell–cell contact. The effect of HMW PL on cation entry in rat spermatids did not seem to be mediated by the interactions with plasma membrane chondroitin or heparan sulfate groups.

In agreement with a non-specific increase in ion permeability induced by HMW-PL in spermatids, the pharmacology of the HMW PL-activated Mn^{2+} entry was not typical of T-type Ca^{2+} channels (e.g., Arnoult et al, 1996; Lievano et al., 1996). This type of Ca^{2+} channels has been described and characterized in rodent spermatids and spermatocytes (e.g., Arnoult et al., 1996; Lievano et al., 1996). Since the interaction of PL with membranes induced a destabilizing action (see below), the effects of octanol and the near significant inhibition by nifedipine can, in principle, be interpreted as a classical membrane stabilization phenomenon described for many amphiphylic molecules (Seeman, 1972).

A theoretical model developed to account for the surface potential effects of polycation peptides on lipid membranes predicted the formation of microdomains by the interaction of these polypeptides and the negatively charged lipids (Denisov et al., 1998). However, our data indicate that macrodomains can be also obtained in cell membranes by the effect of HMW PL. Thus, HMW PL bound to cell membranes, followed by permeability changes and then, this membrane effect propagated to a macroredistribution of plasma membrane components.

The differential effects of HMW vs. LMW PLs on spermatid cation permeability and cell lysis correlate with the ability of HMW but not LMW PLs to interact with model lipid membranes both electrostatically and by hydrophobic forces. Alpha-helices formation by the HMW but not LMW cationic homopeptides has been described (Hammes and Schullery, 1970; Lasch et al., 1998). These interactions of HMW-PL with lipid membranes induced changes in phase separation and modification of the polar head group in phospholipids that could lead to changes in dipole potential of the membranes (Carrier and Pezolet, 1986; Kleinschmidt and Marsh, 1997). These effects could produce changes in surface pressure, peptide conformation and membrane destabilization by HMW PL at concentrations where alpha-helix formation occurs, and would be more pronounced if negatively charged lipid microdomains exist in the membranes.

Our results demonstrate that HMW PL (or polyarginine) but not LMW PL can induce cation permeability changes that are non-selective and do not imply cell lysis. Our data add to the ample evidence showing that HMW PL can activate cell surface receptors or induce changes in plasma membrane structure and permeability. Hence, this molecule cannot be considered an inert cell adhesion molecule, and care must be used when interpreting physiological data of the cells adhered to substrates using this or other cationic homopeptides.

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References

Aoyama, H., Naka, D., Yoshiyama, Y., Ishii, T., Kondo, J., Mitsuka, M., Hayase, T., 1997. Isolation and conformational analysis of fragment peptide corresponding to the heparin-binding site of hepatocyte growth factor. Biochemistry 36, 10286–10291.

Arnoult, C., Cardullo, R.A., Lemos, J.R., Florman, H.M., 1996. Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. Proc. Natl. Acad. Sci. USA 93, 13004–13009.

Baron, V., Gaul, P., Alengrin, F., Van Obberghen, E., 1996. Role of the insulin receptor C-terminal acidic domain in the modulation of the receptor kinase by polybasic effectors. Eur. J. Biochem. 241, 186–192.

Berrios, J., Osses, N., Opazo, C., Arenas, G., Mercado, L., Benos, D.J., Reyes, J.G., 1998. Intracellular Ca^{2+} homeostasis in rat round spermatids. Biol. Cell 90, 391–398.

Carrier, D., Pezolet, M., 1986. Investigation of polylysine-dipalmitoylphosphatidylglycerol interactions in model membranes. Biochemistry 25, 4167–4174.

Denisov, G., Wanaski, S., Luan, P., Glaser, M., McLaughlin, S., 1998. Binding of basic peptides to membranes produces lateral domains enriched in the acidic lipids phosphatidylserine and phosphatidylinositol 4,5-bisphosphate: an electrostatic model and experimental results. Biophys. J. 74, 731–744.

Donato, J.L., Marcondes, S., Antunes, E., Nogueira, M.D., Nader, H.B., Dietrich, C.P., Render, F., de Nucci, G., 1996. Role of chondroitin-4-sulfate as a receptor for polycation-induced human platelet aggregation. Br. J. Pharmacol. 119, 1447–1453.

Elferink, J.G., 1985. Cytolytic effect of polylysine on rabbit polymorphonuclear leukocytes. Inflammation 9, 321–331.

Fenstermaker, R.A., Poptic, E., Bonfield, T.L., Knauss, T.C., Corsillo, L., Piskurich, J.F., Kaetzel, Ch.S., Jentoft, J.E., Gelfand, C., Dicorleto, P.E., Kaetzel, D.M., 1993. A cationic region of the platelet-derived growth factor (PDGF) A-chain (Arg^{159}–Lys^{160}–Lys^{161}) is required for receptor binding and mitogenic activity of the PDGF-AA homodimer. J. Biol. Chem 268, 10482–10489.

Galvanovskis, J., Sandblom, J., Bergquist, B., Golt, S., Hamneriers, Y., 1996. The influence of 50 Hz magnetic field on cytoplasmic Ca^{2+} oscillations in human leukemia-T cells. Sci. Total Environ. 180, 19–33.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Hammes, G.G., Schullery, S.E., 1970. Structure of macromolecular aggregates. II. Construction of model membranes from phospholipids and polypeptides. Biochemistry 9, 555–2563.

Haughland, P., 1996. Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes, sixth. Eugene, OR, USA, pp. 377–397.

Kendall, D.A., MacDonald, R.C., 1982. A fluorescence assay to monitor vesicle fusion and lysis. J. Biol. Chem. 257, 13892–13895.

Kleinschmidt, J.H., Marsh, D., 1997. Spin-label electron spin resonance studies on the interactions of lysine peptides with phospholipid membranes. Biophys. J. 73, 2546–2555.

Lasch, P., Schultz, C.P., Naumann, D., 1998. The influence of poly-(L-lysine) and porin on the domain structure of mixed vesicles composed of lipopolysaccharide and phospholipid: an infrared spectroscopy study. Biophys. J. 75, 840–852.

Lievano, A., Santi, C.M., Serrano, C.J., Treviño, C.L., Bellvé, A.R., Hernandez-Cruz, A., Darszon, A., 1996. T-type Ca^{2+} channels and α_{1E} expression in spermatogenic cells and their possible relevance to sperm acrosome reaction. FEBS Lett. 388, 150–154.

Montero, M., Alvarez, J., Garcia-Sancho, J., 1990. Uptake of Ca^{2+} and refilling of intracellular Ca^{2+} stores in Ehrlich-ascites-tumour cells and in rat thymocytes. Biochem J. 271, 535–540.

Mulloy, B., Crane, D.T., Drake, A.F., Davies, D.B., 1996. The interaction between heparin and polylysine: a circular dichroism and molecular modeling study. Brazilian J. Med. Biol. Res. 29, 721–729.

Reyes, J.G., Diaz, A., Osses, N., Opazo, C., Benos, D.J., 1997. On stage single cell identification of rat spermatogenic cells. Biol. Cell. 89, 1–10.

Romrell, L.J., Bellvé, A.R., Fawcett, D.W., 1976. Separation of mouse spermatogenic cells by sedimentation velocity. Dev. Biol. 19, 119–131.

Seeman, P., 1972. The membrane action of anesthetics and tranquilizers. Pharmacol. Rev. 24, 583–655.

Treviño, C.L., Santi, C.M., Beltran, C., Hernandez-Cruz, A., Darszon, A., Lomeli, H., 1998. Localization of IP_3 and ryanodine receptors during mouse spermatogenesis: possible functional implications. Zygote 6, 159–172.

Uchida, D.A., Irvin, C.G., Ballowe, C., Larsen, G., Gott, G.R., 1996. Cationic proteins increase the permeability of cultured rabbit tracheal cells: modification by heparin and extracellular Ca^{2+}. Exp. Lung Res. 22, 85–99.

Wennemuth, G., Eisoldt, S., Bode, H.P., Renneberg, H., Schiemann, P.J., Aumuller, G., 1998. Measurement of calcium influx in surface-fixed single sperm cells: efficiency of different immobilization methods. Andrologia 30, 141–146.

Whitaker, M., Patel, R., 1990. Calcium and cell cycle control. Development 108, 525–542.