Changes in lysosomal enzymes and M6P receptors related to sexual maturation in bull epididymis

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1 **Running head: lysosomal enzymes in bull epididymis**

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3 Changes in lysosomal enzymes and M6P receptors 4 related to sexual maturation in bull epididymis

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29 **Key Words:** reproductive tract, epididymis, lysosomal enzymes, mannose-6-
30 phosphate receptors, protein secretion.

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31
32 **Abstract**
33
34 One of the most striking features of mammalian epididymis is the secretion
35 of lysosomal enzymes (LE). These LEs might play a role in sperm maturation.
36 Here, we have studied the activity and distribution of four LE in bull epididymis at
37 two different ages, six months and four years, in order to know whether these
38 enzymes vary with sexual maturity. In young bulls (SI) we found high activity of LE
39 in the epididymal tissue that accounts for a developed and active lysosomal
40 apparatus. In contrast, low activity of LE was measured in sexually mature animals
41 (SM), and some of them were mostly secreted into the lumen. We also attempted
42 to correlate the LE distribution with the expression and functionality of mannose-6-
43 phosphate receptors (MPRs) which are thought to be involved in proper delivery to
44 lysosomes. The cation-dependent MPR (CD-MPR) was highly expressed in SI,
45 decreasing during adulthood, whereas the expression of the cation-independent
46 MPR (CI-MPR) was higher in the SM bulls. In addition, some LE from the
47 epididymal lumen were recognized differently by both MPRs at each age. We
48 conclude that the activity and distribution of LE in bull epididymis vary with sexual
49 maturity and that the distribution would be regulated differently by the two MPRs.
50 These findings could provide some molecular bases for male infertility.
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62 **Introduction**

63 The mammalian epididymis is a convoluted duct of the male reproductive
64 tract where spermatozoa acquire their progressive motility and fertilizing capacity
65 (Hermo and Robaire, 2002, Cornwall, 2009). During this process, gametes
66 undergo numerous molecular changes along the epididymis, including modification
67 of protein and carbohydrate composition of the plasmalemma (Dachaux et al.,
68 2005, Tulsiani and Abou-Haila, 2011). The active participation of the epididymis in
69 these events is mostly due to the endocytic and secretory activity of the epithelium
70 lining the duct. Although epididymal sperm maturation is a conserved process
71 among mammalians the most abundant proteins found in the lumen of the
72 epididymis are known to be species-specific (Dacheux et al., 2009).

73 In different mammalian species, among the proteins secreted into the
74 lumen, acid hydrolases can be found at a high concentration (Mayorga and Bertini,
75 1985, Gupta and Setty, 1995, Tulsiani et al., 1998, Belmonte et al., 2002, Hermo
76 and Robaire, 2002, Dachaux et al., 2005, Tulsiani and Abou-Haila, 2011), although
77 the function of these enzymes in the epididymis is still controversial. Moreover, it
78 has been demonstrated that the secretion of some hydrolytic enzymes in the
79 epididymis is influenced by changes in the levels of androgenic hormones (Gupta
80 and Setty, 1995, Robaire and Viger, 1995, Abou-Haila et al., 1996, Belmonte et al.,
81 2002, Ezer and Robaire, 2002, Robaire and Hamzeh, 2011). The acid hydrolases
82 β-galactosidase (β-Gal) and N-acetyl-β-D-glucosaminidase (β-NAG), have been
83 found in high concentrations in rat epididymal fluid, and at a lesser extent, α-
84 mannosidase (α-Man) and β-glucuronidase (β-Glu) (Mayorga an Bertini, 1985,
85 Tulsiani et al., 1998, Belmonte et al., 1998, 2002). In addition, these enzymes are
86 distributed differently in epididymis of this specie and they are strongly influenced
87 by hormones. However, the mechanism for enzyme secretion in the epididymis is
88 still unknown. In order to understand the functionality of these enzymes, it is
89 necessary to elucidate the mechanisms of epididymal secretion and its possible
90 involvement in sexual maturity and fertility.

91 Typically, acid hydrolases exert their activity in the acidic environment of
92 lysosomes. Many lysosomal enzymes acquire a mannose-6-phosphate residue

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during their synthesis and maturation to be later recognized by specific receptors (mannose-6-phosphate receptors, MPRs), and transported selectively to lysosomes (Hille-Rehfeld, 1995, Dahms et al., 2008). To date, two types of MPRs have been described, the cation-dependent (CD-MPR) and the cation-independent MPR (CI-MPR), which co-exist in most of mammalian cell types (Dahms et al., 2002, Nadimpalli and Amancha, 2010), though the relevance of that co-existence has not been conclusively explained.

Proteomic analyses have demonstrated that an active secretion occurs in bull epididymis and this could be related to the reproductive capacity. Thus, cathepsin D and α-L-fucosidase were more predominant in high-fertility bulls (Moura et al., 2006). Moreover, hydrolytic enzymes such as α- and β-mannosidase, β-hexosaminidase, and cathepsins A and D have been found in the epididymal lumen of high-fertility bulls. The presence of the latter enzymes is known to be the result of epididymal secretion (Belleannée et al., 2011).

The aims of this study were to determine whether the activity and distribution of enzymes in bull epididymis are related with sexual maturation and if MPRs might be responsible for enzyme distribution in the different regions of the organ. To this end, we have studied the activity and distribution of four LE~~lysosomal enzymes~~: α-mannosidase (α-MAN), N-acetyl-β-D-glucosaminidase (β-NAG), β-galactosidase (β-Gal) and β-glucuronidase (β-Glu) in bull epididymis at two ages (six months and four years), and attempted to correlate such activity and distribution with the expression of CD-MPR and CI-MPR.

Materials & Methods

Reagents

The rabbit polyclonal anti-CI-MPR antiserum was kindly provided by Dr. Nancy Dahms (Wisconsin University, USA), and the rabbit polyclonal anti-CD-MPR serum was kindly provided by Dr. A. Hille-Rehfeld (Stuttgart, Germany). The rabbit anti-actin antiserum (A2668) was purchased from Sigma Chemical Co. (St. Louis,

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124 MO). The biotin-conjugated anti-rabbit IgG (whole molecule, Cat. B-7389) and
125 horseradish peroxidase-conjugated avidin were also purchased from Sigma. The
126 4-methylumbelliferyl derivatives employed as substrates for α-mannosidase (α-
127 MAN), N-acetyl-β-D-glucosaminidase (β-NAG), β-galactosidase (β-Gal) β-
128 glucuronidase (β-Glu), and the mannose-6-phosphate (M6P, disodium salt,
129 cat.M6876) were all purchased from Sigma. The chemiluminescent reagent was
130 prepared with 1.25 mM luminol, and 198 μM *p*-coumaric acid in 100 mM Tris-HCl
131 (pH 8.5) according to Mruk and Yan Cheng (2011).
132

133 ***Biological samples***

134 Epididymis from reproductively mature bulls (SM; ~4 years old) (Abeerdeen
135 Angus) were obtained from a local slaughterhouse (María del Carmen, Corralitos,
136 Mendoza) and processed up to 2 h after slaughtering. Under these conditions the
137 integrity of the epithelium was preserved. Epididymis from reproductively immature
138 bulls (SI; 6 months old) (Abeerdeen Angus) were obtained by castration. Animals
139 (four at each age) were fed with diets primarily composed of pasture provided from
140 the same farm located in Córdoba (Argentina). The epididymis (one from each
141 animal) were carefully dissected and the three main epididymal segments (caput,
142 corpus and cauda) were processed separately. Tissues (from caput to cauda) were
143 cut into small pieces with a stainless steel blade. Samples were then suspended
144 (1:3 w/v) in Hank´s solution at 37 ºC for 30 min with gentle manual agitation. The
145 minced tissue was left for 10 min at 4 ºC for sedimentation. The supernatant was
146 then centrifuged a 400 *xg* for 5 min and spermatozoa were separated from the
147 tissue supernatant (assumed as epididymal fluid). The remaining epididymal tissue
148 was weighed and suspended (1:5 w/v) in buffer H (10mM Tris–acetate buffer, pH
149 7.2, containing 0.25M sucrose, 1 % EDTA, 1 mM PMSF, 0.02 % sodium azide, and
150 5 mM glycerophosphate) and homogenized with a Teflon/glass homogenizer.
151 Homogenates were centrifuged at 800 *xg* for 20 min at 4 °C. The resulting post-
152 nuclear supernatant was centrifuged at 100,000 *xg* for 45 min, at 4 °C to pellet a
153 membrane-enriched fraction. Pellets (source of MPRs) were then resuspended in

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154 0.05 M Tris-HCl buffer, pH 7.2, (containing 0,5 % saponin, 50 mM EDTA, and 1mM
155 PMSF) and stored, as well as the epididymal fluids, at -20°C until use.
156
157 ***Immunoblotting***
158 All the procedures were carried out according to Romano et al. (2002).
159 Briefly, 30-45 µg of proteins from the epididymal membranes (SI and SM bulls)
160 were run on SDS–PAGE gels (7.5% or 10% acrylamide/bisacrilamide for CI–MPR
161 or CD-MPR, respectively) and electrotransferred to nitrocellulose (NC) membranes
162 (pore size 0.45 µm, GE Healthcare) at 250 V for 4 h (CI-MPR) or 50 min (CD-
163 MPR). CD-MPR and CI-MPR were detected using the corresponding specific
164 antibodies. After transferring, the non-specific binding sites were blocked for 1 h at
165 37 °C with 6 % skimmed milk in phosphate-buffered saline containing 0.05%
166 Tween 20 (PBS-T). Membranes were then washed three times with PBS-T and
167 incubated for 2 h with either the anti-CI-MPR (1:1000) or anti-CD-MPR (1:250)
168 diluted in PBS-T. Membranes were washed as previously described (Romano et
169 al., 2002) and incubated with the biotinylated anti-rabbit IgG antiserum (diluted
170 1:5000 in PBS-T) for 2 h. After washing as above, NC membranes were incubated
171 with peroxidase-conjugated streptavidin (1:10000 in PBS-T) for 1 h and washed
172 again. Specific bands were detected by the enhanced chemiluminescence method,
173 and quantified by densitometric scanning on the membranes, using an Image
174 Quant LAS 4000. Detection of actin was used as loading control. Detection of
175 either CD-MPR or CI-MPR was normalized to the data corresponding to actin.
176
177 ***Immunohistochemical techniques***
178 Immunohistochemical staining was performed using a streptavidin-biotin-
179 peroxidase complex method. Pieces of epididymal tissue (from corpus) were fixed
180 by immersion in Bouin's fluid for 24 h, then dehydrated, and embedded in paraffin.
181 Sagittal sections were cut at 5 µm and serial tissue sections were mounted on
182 gelatin-coated microscope slides (two or three sections per slide). Sections were
183 xylol-deparaffinized, hydrated in a decreasing alcohol series, washed in
184 phosphate-buffered saline (PBS; 0.01 M, pH 7.6), and treated with 3 % H_2O_2 to

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185 extinguish endogenous peroxidase activity. Sections were then washed with PBS
186 and incubated overnight in a moist chamber at 4 °C with either anti-CD-MPR
187 (1:300) or anti-CI-MPR (1:300). The visualization was carried out using the
188 BioGenex Super Sensitive Ready-to-Use Immunostaining Kit (BioGenex, San
189 Ramon, CA) at 20 °C. Slides were incubated with the diluted biotinylated anti-rabbit
190 IgG antiserum for 30 min, washed in PBS, and incubated for 30 min with
191 horseradish peroxidase-conjugated streptavidin. The reaction was revealed with
192 100 µl 3,3-diaminobenzidine tetrahydrochloride (DAB; Bio-Genex) in 2.5 ml PBS
193 and 50 µl H_2O_2. Slides were counterstained with hematoxylin for morphological
194 analysis, dehydrated, and mounted with coverslips. In order to confirm the
195 specificity of the immunoreactive procedures (negative control), adjacent sections
196 were processed according to the protocol described above, but omitting the
197 incubation with the primary antibodies.
198
199 ***Binding assays***
200 Membrane-enriched fractions, obtained as described above, were treated
201 with 0.6 M KCl (with sonication) for 10 min on ice in order to extract the
202 endogenous enzymes. After centrifugation at 80,000 *x g* for 30 min at 4 °C and two
203 washes with 10 mM Tris–acetate containing 0.5 mM EGTA (pH 7.2, buffer B),
204 pellets were resuspended in buffer B.
205 The binding assay was performed with 25 µg membrane proteins from either SI or
206 SM (from caput, corpus and cauda) and 250-1100 units (U) of β-glucuronidase (p-
207 GLU) purified from rat preputial gland (Tulsiani et al., 1975), either in the presence
208 or absence of 0.5 mM $CaCl_2$ and 0.5 mM $MnCl_2$, and in the presence or absence of
209 10 mM mannose-6-phosphate (M6P), as described by Romano et al. (2002).
210 Values of K_D and the number of binding sites (B_{max}) were estimated in each case
211 from the binding curves according to Romano et al. (2002).
212
213 ***Binding assay for endogenous enzymes***
214 Membrane proteins (25 µg) from the corpus of either SI or SM were
215 incubated with the corresponding cauda fluids (~~from cauda~~~ 2 mg protein/ ml)

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216 adjusted to 500 U of each of the following enzymes: α-Man, β-NAG, β-Gal or β-
217 Glu, either in the presence or in the absence of 0.5 mM $CaCl_2$ and 0.5 mM $MnCl_2$,
218 and in the presence or absence of 10 mM mannose-6-phosphate, as described by
219 Romano et al.(2002). The activity of bound enzymes was measured in the pellets
220 after centrifuging at 13,000 *xg* for 30 min, at 4 ºC. The total binding to MPRs was
221 determined in the presence of bivalent cations and M6P. The binding to CD-MPR
222 was estimated from the difference between the total binding and in the absence of
223 bivalent cations.
224

225 ***Measurements***

226 The activity of α-Man, β-NAG, β-Gal and β-Glu was measured
227 fluorometrically, using the corresponding 4-methyl-umbellyferyl substrate, as
228 described by Barrett and Heath (1977). One unit of enzymatic activity catalyzes the
229 release of 1 nmol of 4-methyl-umbellyferone/h. Protein concentration was
230 estimated according to Lowry et al. (1951).
231

232 ***Statistical analysis***

233 Data were analysed by one-way ANOVA followed by Tukey-Kramer's
234 multiple comparisons test, and the level of significance was set at $P < 0.05$ and~~.~~ P
235 < 0.01
236
237

238 **Results**

239

240 **Comparative activity and distribution of lysosomal enzymes in SI and SM**
241 **bulls**

242 We first studied the activity and distribution of some lysosomal enzymes
243 (LE) in bull epididymis and compared the results obtained between SI and SM
244 animals. It was observed that activities of β-Gal, β-NAG, β-Glu and α-Man were
245 significantly higher in the epididymes of SI animals with respect to SM (Fig. 1)~~Table~~
246 ~~1~~). In addition, the activity of some of these enzymes (β-Gal and β-Glu) increased

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247 progressively from the caput to the cauda in SI, whereas β-NAG and α-Man
248 displayed higher activities in the corpus (Fig. 1). These regional differences were
249 not observed in the SM bulls.
250 We also determined the distribution of the LE between the tissue and the lumen
251 (that includes vesicles and the fluid) of epididymis at both ages. The activity
252 associated to sperm in the SM bulls was not taken into account since it represents
253 less than 5 % of total activity. With the exception of α-Man, the enzymes were
254 mostly retained in the tissue of SI epididymis, whereas in SM, the enzymes tended
255 to be progressively secreted into the lumen (Fig. 2).
256
257 **Expression of MPRs in epididymis of SI and SM bulls**
258 We measured the expression of MPRs along the epididymal duct and
259 attempted to correlate it with the distribution of enzymes between the lumen and
260 the tissue in SI and SM animals. As shown in the Fig. 3 (A and B) the CD-MPR is
261 highly expressed in the tissue along the epididymis in SI and that expression
262 decreases drastically in SM animals. In addition, a similar expression of CD-MPR
263 was found in the three regions of the epididymis of SI animals (Fig. 3 B), whereas
264 in SM, the expression of this receptor was significantly higher in corpus as
265 compared to other regions (Fig. 3C). Surprisingly, the CD-MPRs obtained from the
266 corpus of both SI and SM, exhibited a slight change in its electrophoretic mobility
267 (Fig. 3 A and C). Unlike the CD-MPR, the expression of CI-MPR was higher in the
268 SM than in the SI (Fig. 4 A and B). In addition, the expression of CI-MPR did not
269 vary significantly between the different regions of the epididymis in SI, although a
270 progressive decrease from caput to cauda was observed in SM animals (Fig. 4).
271
272 **Distribution of MPRs in corpus of SI and SM bulls**
273 By immunohistochemical techniques, we observed that the CD-MPR is
274 mostly concentrated at the apical and supranuclear region in the SM bulls (Fig. 5
275 B), whereas a more scattered distribution in the cytoplasm and with some signal at
276 the apical area was observed in SI bulls (Fig. 5 A). In turn, the CI-MPR was mostly
277 located at the apical zone of principal cells at both ages and some staining in basal

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278 cells of SM bulls (Fig. 5 C and D). We have also observed abundant vesicles in
279 the lumen of the SI bulls, with some weak staining for both MPRs.
280

281 **Differential binding activity of MPRs in SI and SM bulls**

282 In order to determine if there was a correlation between the expression of
283 both receptors and the ability to bind phosphomannosyl ligands, we performed
284 binding assays using an exogenous enzyme, such as β-glucuronidase purified
285 from rat preputial gland (p-GLU, which is a polyvalent phosphomannosyl ligand),
286 either in the presence or in the absence of bivalent ions. In these assays, the
287 binding activity of CD-MPR was higher in SI than in SM (Fig. 6 and table 1~~2~~),
288 consistent with the high expression of this receptor in SI bulls, although this
289 difference was not as significant as in the case of its expression, indicating that an
290 important number of CD-MPR sites could be inactive in SI. In turn, the number of
291 active CI-MPR (in the absence of bivalent cations) did not vary between SI and SM
292 (except in caput) in discordance with the expression, indicating that a number of
293 CI-MPR could be inactive in SM bulls.
294

295 **Interaction of luminal enzymes with MPRs in bull epididymis**

296 We also attempted to determine if the enzymes that are found in the
297 epididymal fluid of SI or SM are ligands for MPRs and if these enzymes were able
298 to interact either with both MPRs or whether they interact differently with each one.
299 To this end, a binding assay was performed with membranes from corpus (SI or
300 SM), which contain similar active CD-MPR and CI-MPR binding sites (Table 1~~2~~),
301 and the corresponding crude fluids, either in the presence or in the absence of
302 bivalent ions and in the presence or absence of mannose-6-phosphate, assuming
303 that no changes in the behaviour of the enzymes occur between the regions and
304 taking into account possible competitive effects between them.

305 In the SI bulls we observed that β-Glu and β-NAG, were mostly recognized by the
306 CI-MPR. In the SM bulls, β-Glu interacts mostly with the CI-MPR, whereas β-Gal
307 and α-Man showed more affinity for CD-MPR, despite its low expression (Fig. 7).
308 This phenomenon would indicate that CD-MPR is the main recognition molecule in

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309 the SM bulls. It is noteworthy that only a minor fraction of α-Man interacts with
310 MPRs in both SI and SM (Fig. 7), suggesting the existence of an alternative
311 recognition system for this enzyme.
312

313 **Discussion**

314

315 The epididymis plays a major role in sperm maturation and acquisition of
316 gamete fertility. These processes involve the interaction of gametes with proteins
317 that are synthesized and secreted by the epididymal epithelium in a region-
318 dependent manner. The remodelling of the sperm is believed to be carried out by
319 lysosomal enzymes that modify glycoconjugates on the surface of the gametes
320 (Tulsiani, 2006). In addition, during the epididymal transit, some proteins become
321 associated with the membrane of bull spermatozoa, either from the fluid or
322 mediated by luminal vesicles called epididymosomes (Sullivan and Saez, 2013).
323 Thus, the acquisition of reproductive maturity could be related to changes in the
324 secretion of lysosomal enzymes in the mammalian epididymis. In bulls, an increase
325 of cathepsin D and α-L-fucosidase activity has been associated with the acquisition
326 of reproductive maturity (Moura et al., 2006).
327 In this study, we report a first comparative description on the activity and
328 distribution of four hydrolytic enzymes (β-Gal, β-Glu, β-NAG and α-Man) in bull
329 epididymis, at two different ages; six months (SI) and four years (SM), in order to
330 determine whether these enzymes are related to sexual maturity. We observed
331 that these enzymes were mostly retained in the epididymal tissue in SI, whereas
332 they tended to be secreted into the lumen in SM bulls.
333 In most cell types and tissues, lysosomal enzymes are typically delivered to
334 lysosomes via cation-dependent (CD-MPR) and cation-independent (CI-MPR)
335 mannose-6-phosphate receptor (Chao et al., 1990, Hille-Rehfeld, 1995). These
336 receptors have also been described in the epididymis of certain mammalian
337 species (Carvelli et al., 2010, 2013), although the incidence of MPRs in distribution
338 and secretion of acid hydrolases in this organ is still unknown.

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339 We also attempted to correlate the distribution of these enzymes with the
340 expression and distribution of MPRs along the epididymal duct.
341 The high activity and tissue retention of lysosomal enzymes in the six
342 months old animals (SI) could be related to the presence of a highly developed
343 lysosomal apparatus and to a high expression of CD-MPR at this age. In turn, a
344 low expression of the CD-MPR could explain the lower levels of retention of
345 enzymes found in the epididymis of SM bulls. However, it seems that the high
346 expression of CD-MPR in young animals correlates neither with the number of
347 active receptor sites (B_{max}) nor with the K_D (Table 12), suggesting that an important
348 population of this receptor may be inactive at this age. Although previous
349 evidences indicate that CD-MPR is involved in exporting enzymes to the
350 extracellular compartment (Chao et al., 1990) our results do not allow infering that
351 a similar phenomenon occurs in the epididymis of SM animals, even though an
352 increased apical distribution of this receptor was observed in the epithelium (Fig.
353 5).
354 Unlike the CD-MPR, the expression of CI-MPR was higher in SM than in SI
355 bulls, although an important number of binding sites for this receptor may be
356 inactive (Table 1). These data, together with a different distribution of both MPRs in
357 epithelial cells (Fig. 5), suggest that CD-MPR and CI-MPR could play different role
358 in the epididymis, according to the age and/or sexual maturity status of the
359 animals. Since the CI-MPR can recognize other ligands apart from lysosomal
360 enzymes (Kang et al., 1997, Braulke, 1999, Villevalois-Cam et al., 2003, Hawkes
361 and Kar, 2004), it could be suggested that this receptor might have additional
362 functions during sexual maturation. We also observed that β-Gal, β-Glu and β-NAG
363 from the epididymal fluid are ligands for MPRs at both ages and that they can be
364 recognized differently by both MPRs (Fig. 6).
365 Our data may also indicate the existence of subpopulations of enzymes in
366 bull epididymis; the predominance of either phosphomonoesters or
367 phosphodiesters on the enzyme molecule could be related to the differential
368 affinitiy for one or another MPR (Distler et al., 1991). In addition, a differential

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369 sorting of lysosomal enzymes has also been described in other biological models
370 (Ludwig et al.,1994).
371 While Belleannee (2011) indicates that the distal caput seems to be the
372 most active region in bulls, our results also suggest that corpus could be more
373 important than expected. Thus, we have observed a significant increase in the
374 expression of CD MPR in corpus of SM bulls as compared to the caput and cauda
375 regions. This increase is accompanied by an apparent change in the M*r* of the
376 receptor, which could be due to a post-translational modification that justifies a
377 difference in the binding activity. It is known that phosphorylation of the cytoplasmic
378 domain of CD-MPR induces redistribution of this receptor toward the plasmalemma
379 in MDCK cells (Breuer et al., 1996). However, it is unlikely that a simple
380 phosphorylation be responsible for such a change in M*r*.
381 In conclusion, the lysosomal apparatus would be more developed and active
382 in the epididymal epithelium of young bulls, while in mature animals this tissue
383 tends to secrete enzymes. Furthermore, the MPRs could be responsible for the
384 distribution of several enzymes in the epididymis of bulls, as it happens in other
385 tissues and in other species. Our findings would indicate that both receptors play
386 different roles during bull sexual maturity. The role of both receptors in the
387 distribution of lysosomal enzymes in the epididymis might provide the molecular
388 bases for some reproductive dysfunctions.
389
390
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556 **Legends to Figures**
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558 **Fig. 1.** Acid hydrolase activity of caput, corpus and cauda obtained from either SI
559 (black bars) or SM (grey bars) bull epididymis (n = 4 at each age). Bars represent
560 the means ± SE of specific activity (units/mg protein) of β-Gal, β-Glu, β-NAG, and
561 α-MAN as indicated. (*) (**) Significantly different ($p < 0.05$ and $p < 0.02$
562 respectively).
563
564

565 **Fig. 2.** Distribution of acid hydrolases in fluid and tissue of epididymis obtained
566 from either SI or SM bulls (as indicated on the top). Bars represent the means ± SE
567 of percentages of total activity measured in each region: caput (white bars), corpus
568 (grey bars) and cauda (black bars) from SI (n = 4) or SM (n=5) bulls. β-Gal, β-
569 Glu,β-NAG, and α-MAN activities were measured as indicated in Material and
570 Methods. (*) Significantly different from fluids obtained from the other regions ($p <$
571 0.05).
572

573 **Fig. 3.** (A) Immunoblotting of CD-MPR in epididymal tissue obtained from either
574 mature (SM) or immature (SI) bulls. (B) Quantification of the bands normalized to
575 expression of actin. Bars represent the means of relative optical density (R.O.D., =
576 $OD_{specific\ band}$ / $OD_{actin\ band}$) ± SE (from four animals at~~n = 4, for~~ each age). [(a,b,c)]
577 significantly different from [(a',b',c')] respectively ($p < 0.01$). (C) Immunoblotting of CD-
578 MPR in the epididymal tissue of SM (at longer a exposure time) and quantification
579 of the bands. Bars represent the means of R.O.D. ± SE from six measurements. (*)

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Significantly different from the other regions ($p < 0.05$). Cap: caput; Cor: corpus, Cau: cauda.

Fig. 4. (A) Immunoblotting of CI-MPR in epididymal tissue obtained from either SI or SM bulls. (B) Quantification of the bands from A, normalized to expression of actin. Bars represent the means of relative optical density (R.O.D.) ± SE (from four animals at~~n = 4, for~~ each age). [(a,b)] significantly different from [(a',b')], respectively (p < 0.01).(**) a´and b´ were significantly different from c´ ($p < 0.05$). Cap: caput; Cor: corpus, Cau: cauda.

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Fig. 5. Immunostaining of MPRs in corpus of either SI or SM bulls. Corpus~~i~~ were processed for immunohistochemistry as detailed in Materials and methods (A-B) CD-MPR and (C-D) CI-MPR.. Arrows indicate intraluminal vesicles. Ep: epithelium; Lu: lumen; Sp: spermatozoa. Inset: epithelium after incubation with the secondary antibody alone (as negative control).

Fig. 6. Binding of p-GLU to epididymal membranes from either SI or SM bulls. Membrane proteins (from each region) were incubated with 250-1100 U of enzyme, either in the absence (lower curve) or in the presence (upper curve) of bivalent cations. The non-specific binding in the presence of mannose-6-phosphate was subtracted from the curve. Each point in the curves represents the mean ± SD from three independent binding experiments (n =3 at each age).

Fig. 7. Binding of epididymal fluid acid hydrolases from cauda of SI or SM bulls to the CD-MPR or CI-MPR obtained from the respective epididymal corpus tissues. Values represent the percentages (means ± SD) of each enzyme bound to membrane (as indicated), either in the presence or in the absence of bivalent ions. The non-specific binding (in the presence of mannose-6-phosphate) was subtracted. EE: endogenous enzyme. (*), (**), (***) significantly different between both MPRs ($p < 0.05$)

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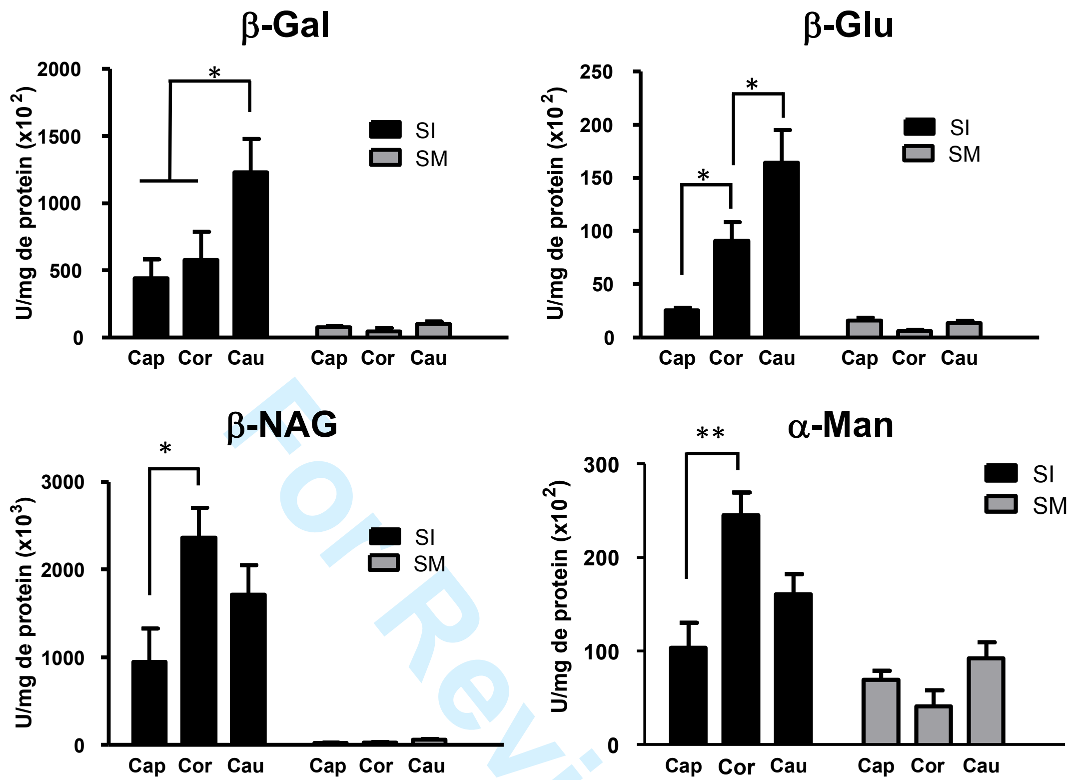

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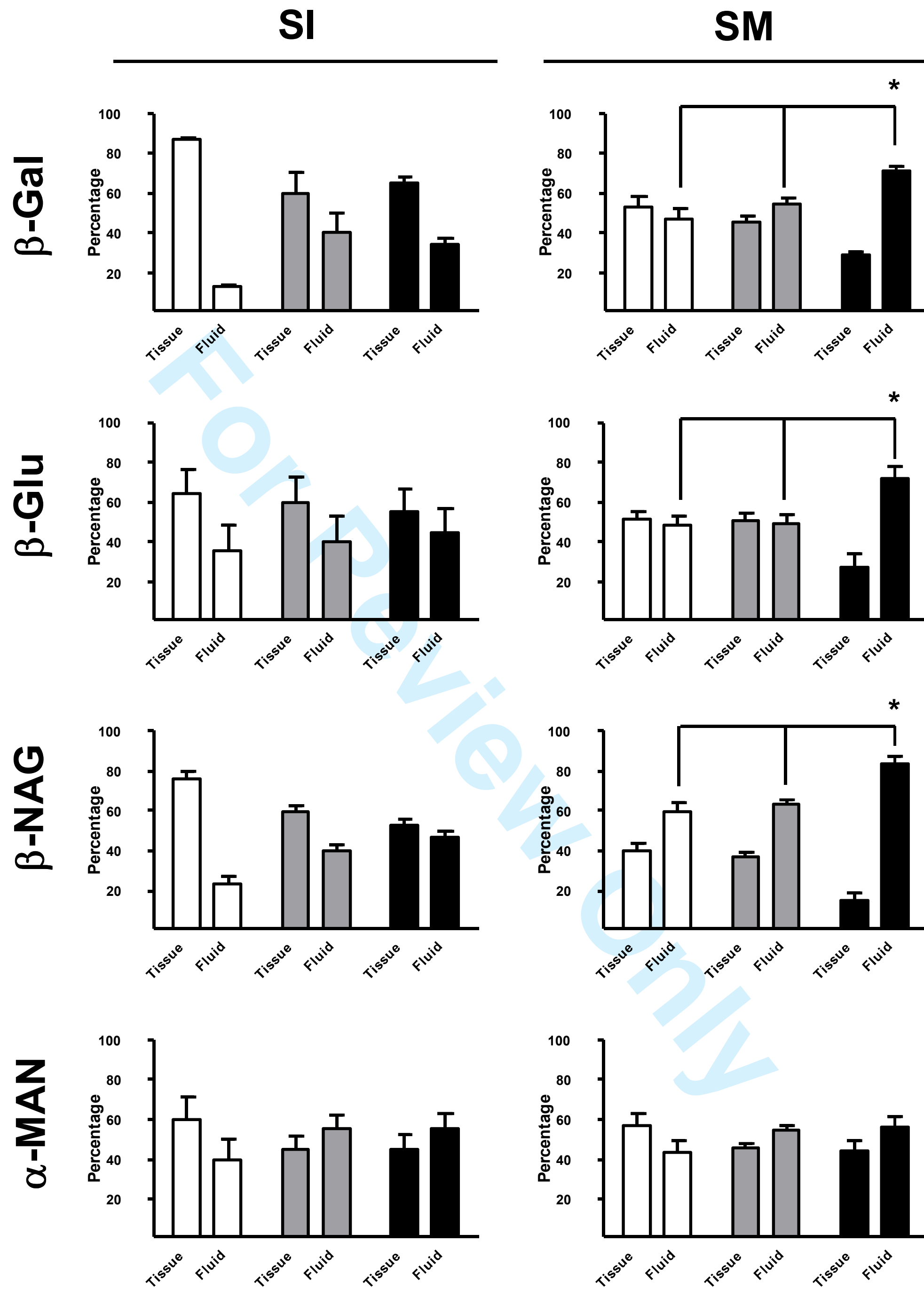

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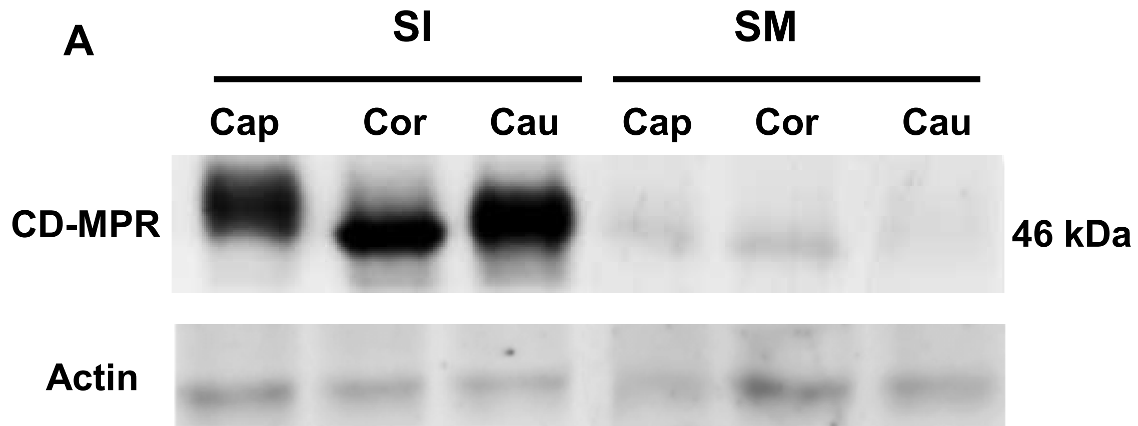

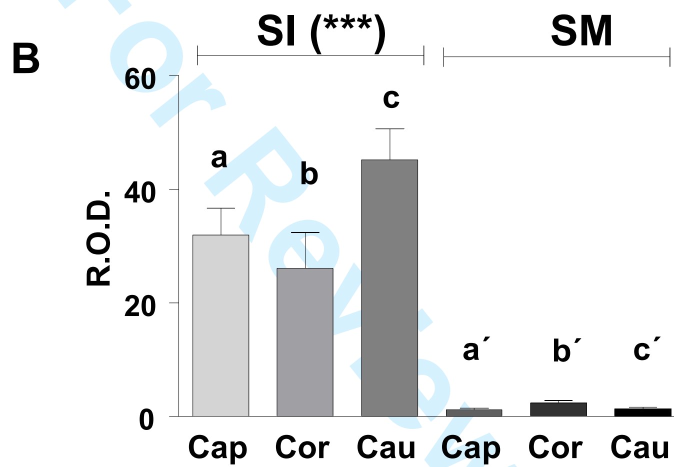

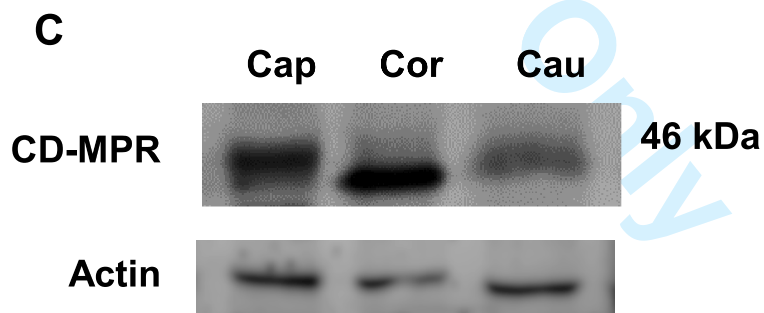

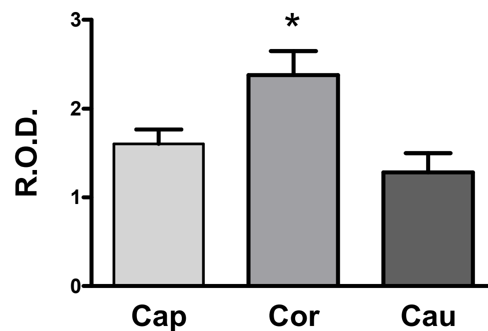

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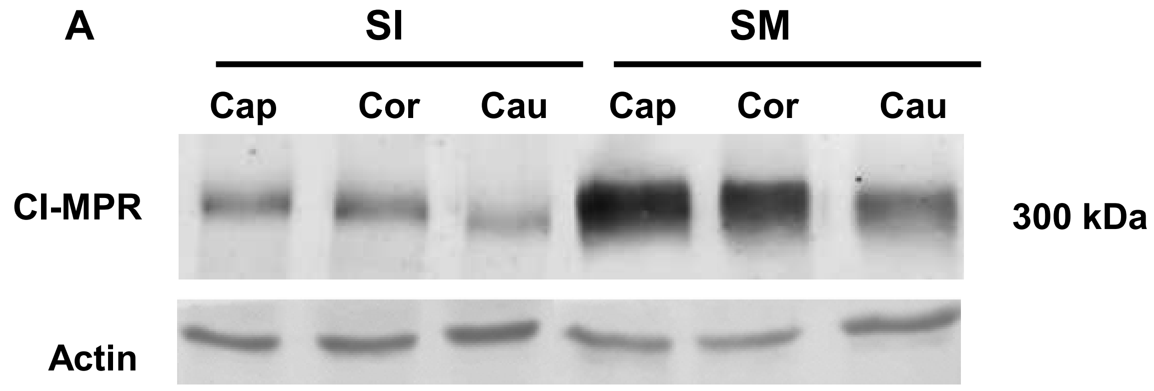

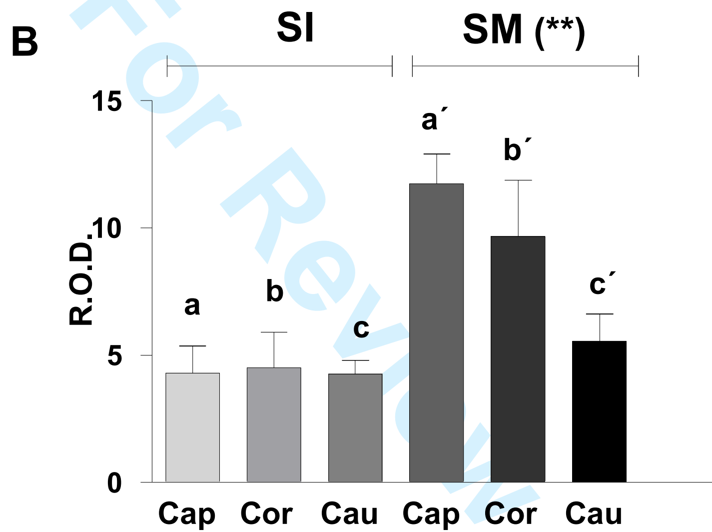

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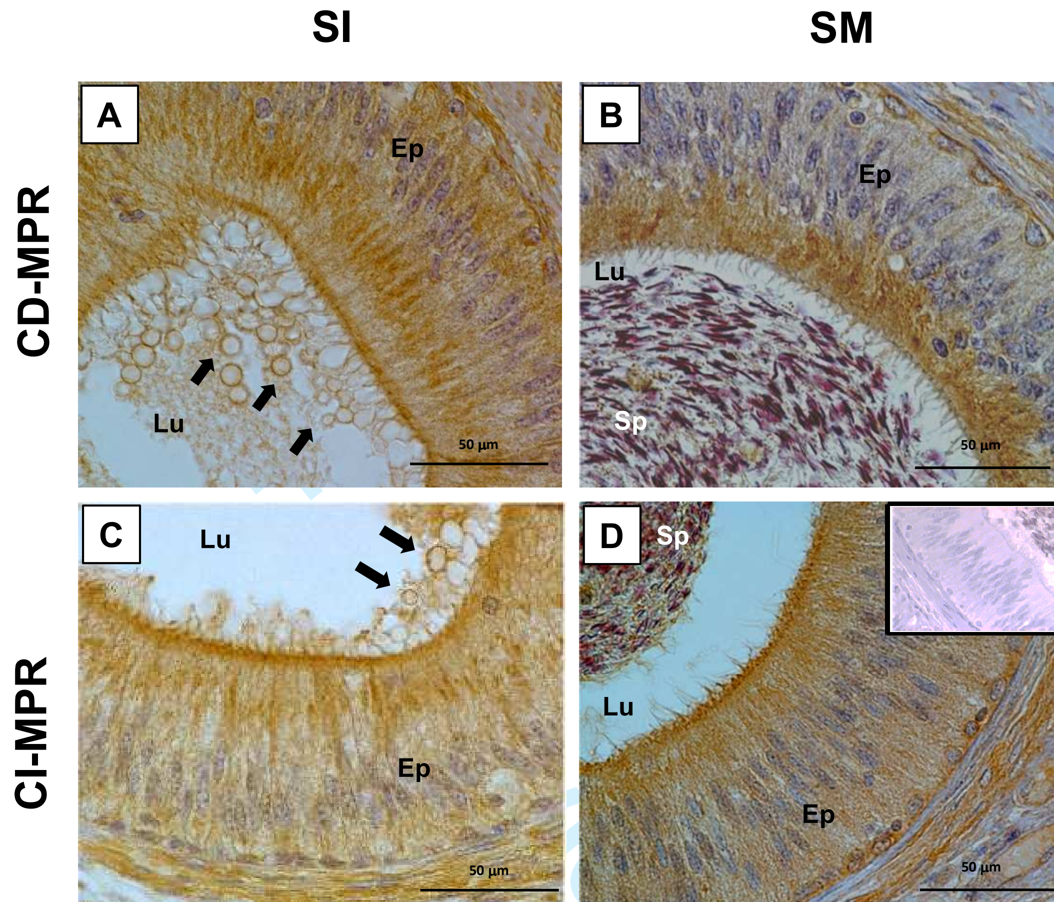

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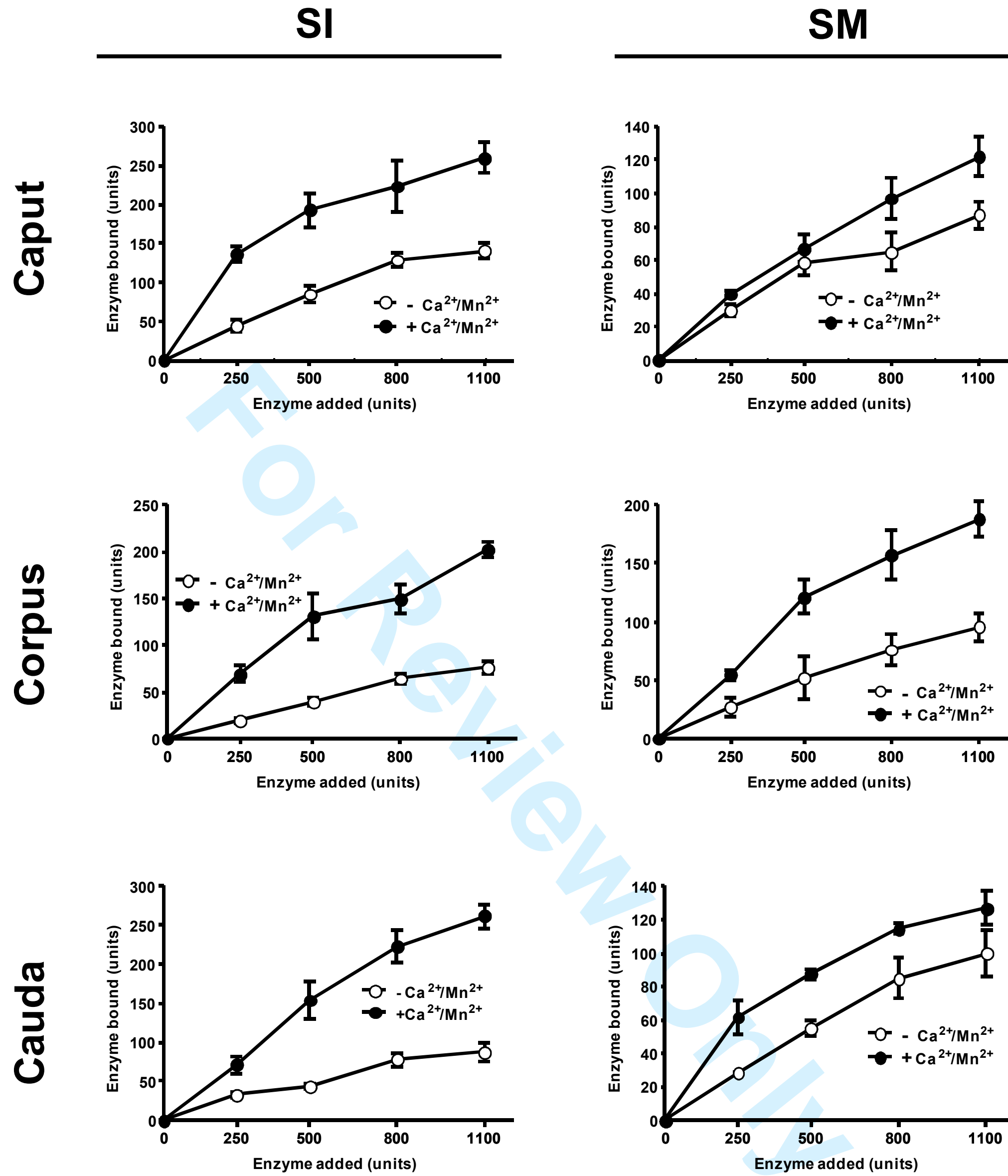

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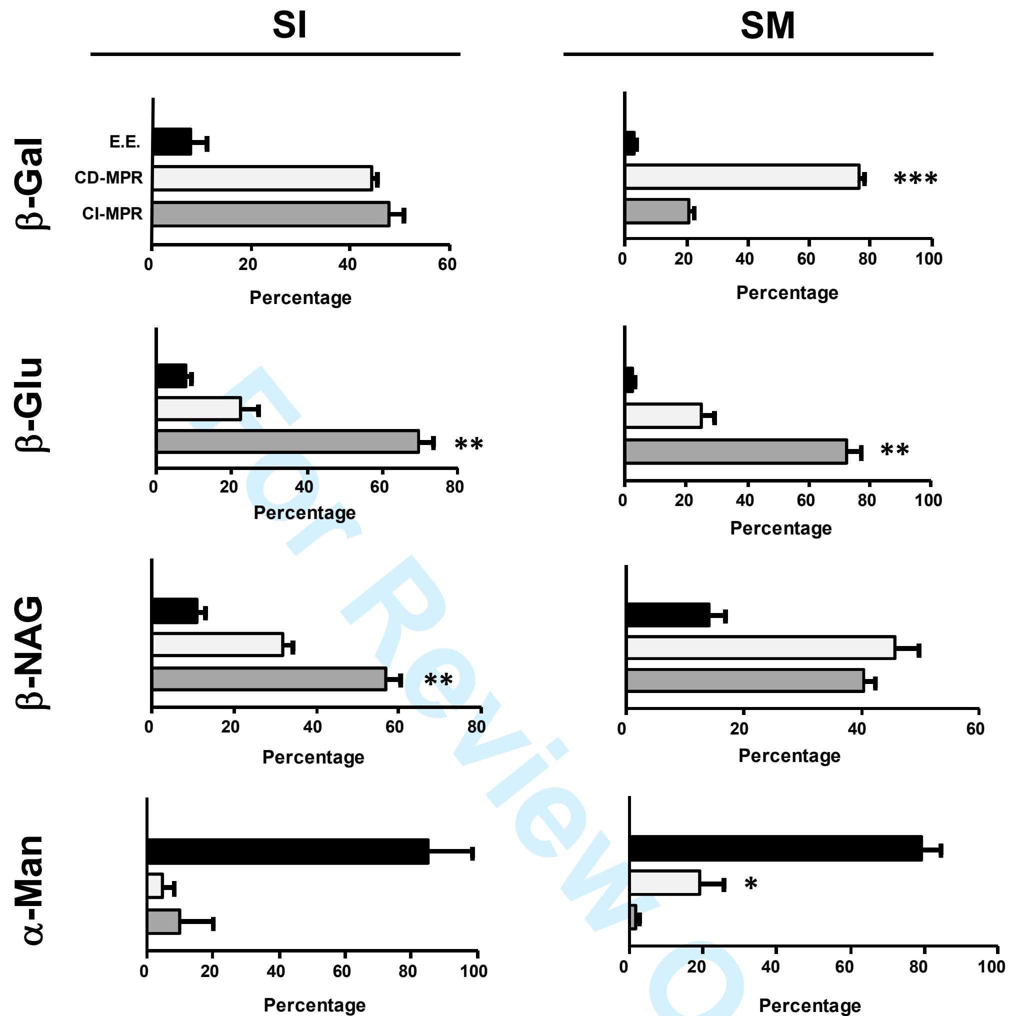

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Table 1. Values of B_{max} and K_D for CI-MPR and CD-MPR in the different areas of bull epididymis obtained from either SI or SM

			Caput	Corpus	Cauda
SI	CI-MPR	B_{max} (pmol/mg protein)	30.2	15.5	17.5
		K_D (nM)	2.8	6.23	4.4
	CD-MPR	B_{max} (pmol/mg protein)	22.5	23.7	42.5
		K_D (nM)	0.2	0.66	2.2
SM	CI-MPR	B_{max} (pmol/mg protein)	17.5	17.5	23.1
		K_D (nM)	10.2	3.9	5.1
	CD-MPR	B_{max} (pmol/mg protein)	10.1	22.5	6.25
		K_D (nM)	1.4	1.66	0.82

Data calculated from the binding curves. B_{max} are expressed as pmol/mg protein

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