

Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells

Ian M. Dick^{a,b,c,*}, Jie Liu^a, Paul Glendenning^{a,c}, Richard L. Prince^{a,b,c}

^a School of Medicine and Pharmacology, University of Western Australia, Nedlands, WA 6009, Australia

^b The Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, WA 6009, Australia

^c The Western Australian Institute of Medical Research, Nedlands, WA 6009, Australia

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Abstract

The ATP dependent plasma membrane calcium pump (PMCA) is a regulator of renal calcium reabsorption. The effect of estrogen and dihydrotestosterone to increase the activity of the PMCA in membrane vesicle preparations from a distal tubule cell line was investigated. 17β Estradiol (10^{-10} M) increased PMCA activity (1.5 ± 0.2 -fold increase compared to control) with 24 h, but not 1 or 5 h, of exposure, an effect that was blocked by the addition of the estrogen antagonist ICI 164384. α Estradiol did not increase PMCA activity. Dihydrotestosterone (10^{-11} M) resulted in a dose dependent increase in PMCA activity (1.5 ± 0.1 -fold increase compared to control) with 24 h, but not 1 or 5 h, of exposure, an effect that was blocked by the androgen receptor agonist flutamide. Testosterone (10^{-5} M) also increased PMCA activity (1.9 ± 0.3 -fold increase compared to control). Neither estrogen nor dihydrotestosterone increased PMCA protein expression in MDBK cells, indicating that these hormones increase PMCA activity by regulating PMCA activity rather than PMCA expression. These results demonstrate receptor dependent stimulatory effects of both estrogen and dihydrotestosterone to increase PMCA activity, and have significance for our understanding of estrogen and androgen deficient states on calcium transport.

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1. Introduction

The plasma membrane calcium pump (PMCA) is a high calcium affinity, low capacity, ATP dependent calcium transporter that is encoded by four separate genes in both the human and the rat, resulting in the designation of PMCA1 through PMCA4 (Carafoli, 1994). The various isoforms of the PMCA have important effects on calcium transport in the majority of tissues in the body, particularly the neuromuscular system, intestine and kidney (Carafoli, 1991). PMCA is expressed on the basolateral membrane of renal distal tubule cells in both humans (Borke et al., 1988) and rats (Borke et al., 1989). As a consequence of its localization and its ability to transport calcium against a concentration gradient, PMCA probably has an important function in renal calcium reabsorption.

PMCA is regulated by 1,25-dihydroxyvitamin D (calcitriol) in chick (Wasserman et al., 1992) and rat (Zelinski

et al., 1991; Armbrecht et al., 1994) intestine where it is known to increase calcium absorption. Vitamin D depletion has been shown to result in a decrease in both apical and basolateral membrane calcium transport in the rabbit renal distal tubule (Bouhtiauy et al., 1993). Both estrogen (Nordin et al., 1991; Prince et al., 1991; Adami et al., 1992) and androgens (Mauras et al., 1999) are known to regulate calcium transport in the human kidney. This regulatory effect of estrogen and androgens on calcium transport may partly account for the negative calcium balance that results from a deficiency of these hormones. There is at present no information regarding estrogen and androgen regulation of PMCA activity. The presence of both estrogen (Davidoff et al., 1980; Hagenfeldt and Eriksson, 1988) and androgen (Stefani et al., 1994; Ouar et al., 1998) receptors in kidney cells, however, indicate that PMCA regulation by these hormones in the kidney is possible.

The aim of this study was to examine the effect of estrogen, dihydrotestosterone and testosterone on PMCA activity in the MDBK renal distal tubular cell line. This cell line was chosen for this study as it phenotypically resembles distal

* Corresponding author. Fax: +61-8-93462816.

E-mail address: iand@cyllene.uwa.edu.au (I.M. Dick).

tubular cells (Gagnon et al., 1994) and expresses estrogen receptors (Miller et al., 1994) and 1,25-dihydroxyvitamin D₃ (calcitriol) receptors (Gagnon et al., 1994). We have demonstrated that the MDBK cell line expresses the PMCA1b isoform, the sodium calcium exchanger and calbindin D28k (Glendenning et al., 2000), three calcium transport proteins found in the renal distal tubule. Calcitriol upregulates PMCA activity, protein expression, mRNA expression and mRNA stability in this cell line (Glendenning et al., 2000).

2. Material and method

The MDBK (NBL-1) cell line was obtained from the American Type Culture Collection (Manassas, VA). The cell line was used between passages 114 and 135 and seeded at a density of approximately 1×10^5 cells per 75 cm² flask. This represented a split ratio of 1/13 from a confluent 75 cm² flask. In some experiments, cells were grown in 175 cm² flasks after seeding at a 1/7 split ratio in order to obtain more material for substrate concentration experiments. The cell line was routinely tested to confirm the absence of mycoplasma contamination. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM Gibco BRL 23700-040, Gaithersburg, MD) supplemented with 5% (v/v) foetal bovine serum, 1% (v/v) non essential amino acids, and 50 µg/ml gentamicin (Gibco). They were kept at 37 °C in 5% CO₂ atmosphere. After reaching confluence at approximately day four the media was changed and the cells incubated for a further 24 h. At the time the estrogen, testosterone, dihydrotestosterone or control treatments (vehicle only) were added, the media was changed to serum free, phenol red free DMEM (Gibco BRL 13000-021) containing 0.25% albumin and 1% (v/v) non essential amino acids. β Estradiol, α estradiol, dihydrotestosterone and testosterone (Sigma, St. Louis, MO) and ICI 164384 (A gift of Dr Allen Wakeling ICI Pharmaceuticals Macclesfield, UK) solutions (100×) were made up from an ethanol stock into DMEM containing 0.25% albumin so as to contain equivalent amounts of ethanol and added for the last 24 h of the incubation. The estrogen and dihydrotestosterone concentrations used are described in the legend of each experiment. The cells were washed with PBS and 5 ml of trypsin added to remove the cells from the surface of the flask and membrane vesicles prepared as previously described for the LLC-PK1 cell line (Parys et al., 1986) and reported by us for the MDBK cell line (Glendenning et al., 2000). Trypsinized cells were resuspended in 5 ml of DMEM containing 1 mg/ml egg white trypsin inhibitor (Boehringer Mannheim, Mannheim Germany) and centrifuged at $300 \times g$ for 20 min. After removal of supernatant, the cells were resuspend in a 10 mM triethanolamine, 250 mM sucrose buffer pH 7.5 containing 1 mg/ml soybean trypsin inhibitor (Boehringer Mannheim) and 0.1 mM phenylmethylsulfonylfluoride (PMSF, Sigma). The cells were disrupted by 4×20 s sonication cycles using

a Branson B15 cell disruptor at setting 3 using a 50% duty cycle. The cell homogenates were centrifuged for 15 min at $2400 \times g$ at 4 °C and the pellet resuspended in 20 mM Hepes, 250 mM sucrose intravesicular buffer pH 7.5 containing 0.1 mM PMSF and stored at –80 °C.

2.1. Vesicular calcium uptake

Membrane calcium transport was determined by a modified method previously described (Parys et al., 1986). The assay was performed in a buffer containing 20 mM Hepes, 40 mM phosphorus, 1 mM MgCl₂, 1 mM EGTA, 2 mg/ml valbumin pH 7.5. ATP dependent calcium uptake was determined by comparing calcium transport with and without 2 mM ATP added to the buffer and is described hereafter as PMCA activity. CaCl₂ was added so as to obtain a free calcium concentration of 0.56 µM, unless stated otherwise. The amount of CaCl₂ added to obtain the free concentration of calcium required was calculated using the EqCal computer program (Biosoft, Ferguson, MO). ⁴⁵CaCl was added at an activity of approximately 1.7 MBq/µmol total calcium. The reaction was started by adding approximately 50 µg protein in 10–190 µl of incubation buffer in a 96 well assay plate. After 5 min, 150 µl of the samples were filtered through a 96 well filtration plate containing a 0.45 µm pore size filter (Millipore Multiscreen MAHV N45, Bedford, MA) to remove membrane vesicles. The membranes were washed with 750 µl of 20 mM Tris–HCl pH 7.5 stop buffer containing 150 mM KCl and 2 mM EGTA and punched out into scintillation vials for counting of beta radiation in the membrane vesicles. PMCA activity measured by this method was inhibited by vanadate, an inhibitor of PMCA and not significantly inhibited by thapsigargin, an inhibitor of sarcoplasmic reticulum calcium, indicating that no significant microsomal contamination was present in the vesicle preparations. A 20% inhibition of calcium ATPase activity in the preparations was observed by the addition of oligomycin, indicating mitochondrial contamination. The addition of 5 mM sodium azide did not result in any further reduction of calcium ATPase activity compared to oligomycin alone, indicating that complete abolition of the effect of mitochondrial contamination on calcium ATPase activity was obtained with the addition of oligomycin alone. Therefore, oligomycin (1 µg/ml Sigma) was added to inhibit mitochondrial activity. Protein was measured using a bovine serum albumin standard by a modified Lowry method (DC protein assay, Bio-Rad, Hercules CA). PMCA activity was calculated per mg of protein.

2.2. Western blots

Estrogen and dihydrotestosterone effects on protein expression of PMCA were determined by Western Blotting. MDBK cells were cultured and treated with estrogen or dihydrotestosterone as described above for the calcium transport studies. Membrane vesicles were prepared as described for the calcium transport studies, and were

subsequently solubilized in 20 mM Tris buffer pH 8.0 containing 135 mM NaCl, 1 mM EDTA 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 250 μ g/ml Pefabloc. The samples (100 μ g protein per well) were loaded onto precast 7.5% polyacrylamide gels (Bio-Rad) and electrophoresed at 25 mA for 2 h followed by overnight electrophoretic transfer to nitrocellulose membrane (0.45 μ m, Bio-Rad) at 15 mA using a Bio-Rad transblot apparatus. The membranes were then blocked in 5% skim milk made up in TBS buffer containing 0.2% Tween-20 (TBS-T). The membranes were incubated overnight in a 1:200 dilution of 5F10 anti-PMCA monoclonal antibody (Sigma) in 0.2% skim milk in TBS-T. Blots were visualized using the Bio-Rad Immun-Star chemiluminescent detection system after a 2 h incubation with a 1:3000 dilution of alkaline phosphatase conjugated goat anti-mouse IgG followed by exposure to Kodak X-Omat film (Rochester, NY). The film was scanned on a transmissive flatbed scanner and bands were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.3. Statistical analysis

Results are expressed as mean \pm standard error and were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparison test using the SPSS for Windows statistical computer program version 10.

3. Results

3.1. Estrogen effects on PMCA activity in MDBK cells

The effect of 17β estradiol on PMCA activity in membrane vesicle preparations from MDBK renal distal tubule cells is shown in Fig. 1A. A dose of 10^{-10} M 17β estradiol resulted in a 1.5 fold stimulation of PMCA activity, with no further increase in activity being observed at a dose of 10^{-7} M 17β estradiol. The time course of estrogen stimulation of PMCA activity (Fig. 1B) indicated that an incubation period of 24 h with 17β estradiol was required in order to observe a stimulatory effect of 17β estradiol on PMCA activity. No effect of 1 or 5 h incubation with 17β estradiol on PMCA activity in MDBK cells was observed. In contrast to 17β estradiol, incubation with 17α estradiol did not result in a significant increase in PMCA activity (Fig. 2). Coincubation of 17β estradiol with the pure estrogen antagonist ICI 164384 resulted in the abolition of PMCA stimulation by 17β estradiol (Fig. 2).

3.2. Androgen effects on PMCA activity in MDBK cells

The effect of dihydrotestosterone on PMCA activity in membrane vesicle preparations from MDBK renal distal tubule cells is shown in Fig. 3A. A dose of 10^{-11} M dihydrotestosterone resulted in a 1.5-fold stimulation of PMCA

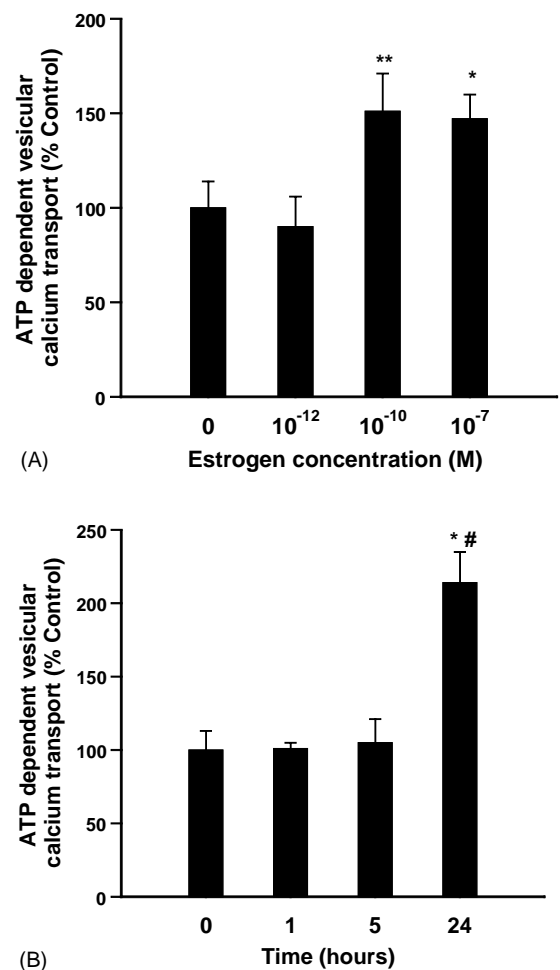


Fig. 1. The effect of estrogen on PMCA activity: (A) confluent MDBK cells were incubated for 24 h with the indicated concentration of 17β estradiol ($n = 12$ replicate experiments per dose). (B) Confluent MDBK cells were incubated for the times indicated with 10^{-7} M 17β estradiol ($n = 5$ replicate experiments per time point). Results are mean \pm S.E.M. of PMCA activity compared to the no estrogen added control for each experiment. (A) * $P < 0.05$, ** $P < 0.02$ compared to 0 and 10^{-12} M estrogen; (B) * $P < 0.05$ compared to 5 h, # $P < 0.002$ compared to 0 and 1 h exposure.

activity, increasing to a 1.8-fold stimulation of PMCA activity at a dose of 10^{-9} M dihydrotestosterone. The time course of the dihydrotestosterone stimulation of PMCA activity (Fig. 3B) indicated that an incubation period of 24 h with dihydrotestosterone was required in order to observe a significant stimulatory effect of dihydrotestosterone on PMCA activity. No significant effect of 1 or 5 h incubation with dihydrotestosterone on PMCA activity in MDBK cells was observed. Coincubation with the androgen receptor agonist flutamide resulted in the abolition of the stimulatory effect of dihydrotestosterone on PMCA activity in MDBK cells (Fig. 4).

Testosterone also stimulated PMCA activity, with a significant 1.9-fold stimulation compared to control at 10^{-5} M testosterone (Fig. 5). Similar to dihydrotestosterone, the

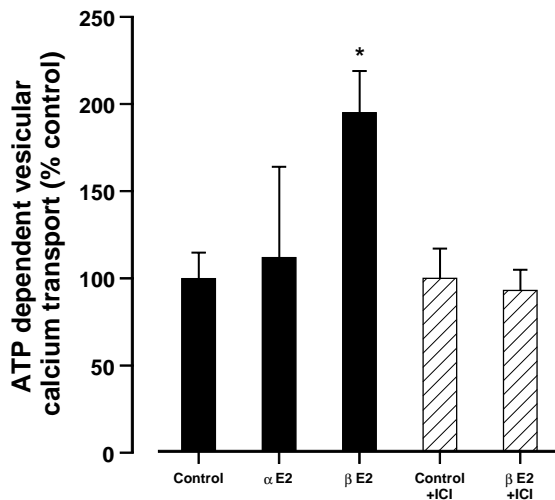


Fig. 2. Comparison of α estradiol (10^{-10} M) with β estradiol (10^{-10} M) on PMCA activity ($n = 5$ replicate experiments) and the effect of co-addition of the pure estrogen antagonist ICI 164384 (10^{-7} M) with 17β estradiol (10^{-10} M) on PMCA activity ($n = 4$ replicate experiments). Confluent MDBK cells were incubated for 24 h with estradiol or ICI 164384. Results are mean \pm S.E.M. of PMCA activity compared to the no estrogen added controls. * $P < 0.05$ compared to control.

stimulatory effect of testosterone on PMCA activity was inhibited by the co-incubation of flutamide (Fig. 4).

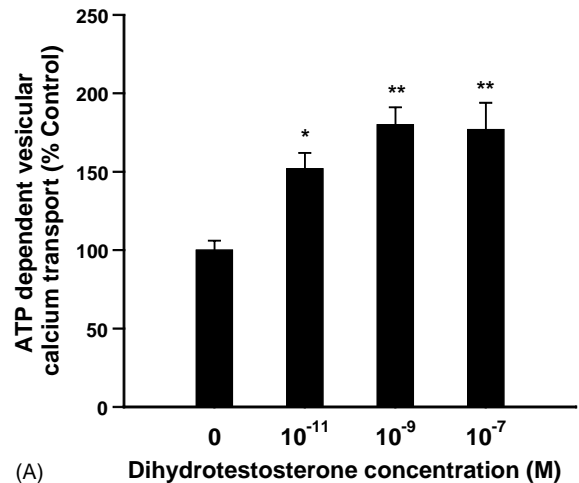
3.3. The effect of estrogen and dihydrotestosterone on PMCA protein expression

Estrogen and dihydrotestosterone effects on the protein expression of PMCA were determined by Western blotting and are shown in Fig. 6. Two adjacent bands corresponding approximately to the 140 kDa band previously described in human kidney using this antiserum (Borke et al., 1988) were observed. Quantification of these bands indicated that neither estrogen nor dihydrotestosterone administration for 24 h to MDBK cells resulted in a change in PMCA protein expression.

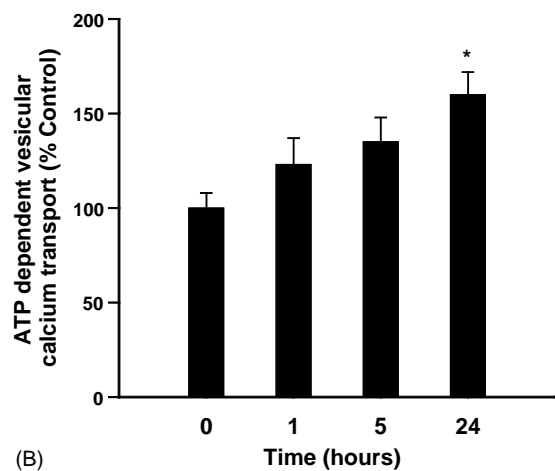
4. Discussion

An understanding of the regulation of PMCA is important to the understanding of cellular calcium physiology. PMCA has a major role in calcium transport in the kidney as a result of its action to transport calcium out of the cell against a calcium gradient. It is highly likely that hormonal regulation of distal tubule calcium reabsorption involves the regulation of PMCA activity.

In this study, an effect of estrogen and dihydrotestosterone, at physiological concentrations, were shown to stimulate PMCA activity. The effect of 24 h of estrogen, dihydrotestosterone and testosterone exposure to increase PMCA activity was of a similar magnitude to that of calcitriol, a known regulator of calcium transport (Glendenning et al.,



(A)



(B)

Fig. 3. The effect of dihydrotestosterone (DHT) on PMCA activity: (A) confluent MDBK cells were incubated for 24 h with the indicated concentration of dihydrotestosterone ($n = 6$ replicate experiments per dose). (B) Confluent MDBK cells were incubated for the times indicated with 10^{-11} M dihydrotestosterone ($n = 6$ replicate experiments per time point). Results are mean \pm S.E.M. of PMCA activity compared to the no dihydrotestosterone added control for each experiment. * $P < 0.01$, ** $P < 0.001$ compared to 0 M dihydrotestosterone; (B) * $P < 0.01$ compared to 0, 1 and 5 h.

2000). Unlike calcitriol, which stimulates PMCA protein expression in MDBK cells (Glendenning et al., 2000), neither estrogen nor dihydrotestosterone increased PMCA protein expression in this study. This suggests that both estrogen and dihydrotestosterone regulate PMCA calcium transport by increasing PMCA activity, rather than by increasing PMCA abundance. Alternatively, it is possible that estrogen may modify the isoform and splice variant profile of PMCA, which would not be detected by the 5F10 antibody used in this study as the 5F10 antibody recognizes all PMCA isoforms (Caride et al., 1996). If this is true, it would indicate that alternative isoforms or splice variants had different calcium pumping activity in distal tubule cells. It is possible that estrogen stimulates PMCA activity by phosphorylation of the carboxyl terminus of the PMCA molecule by protein

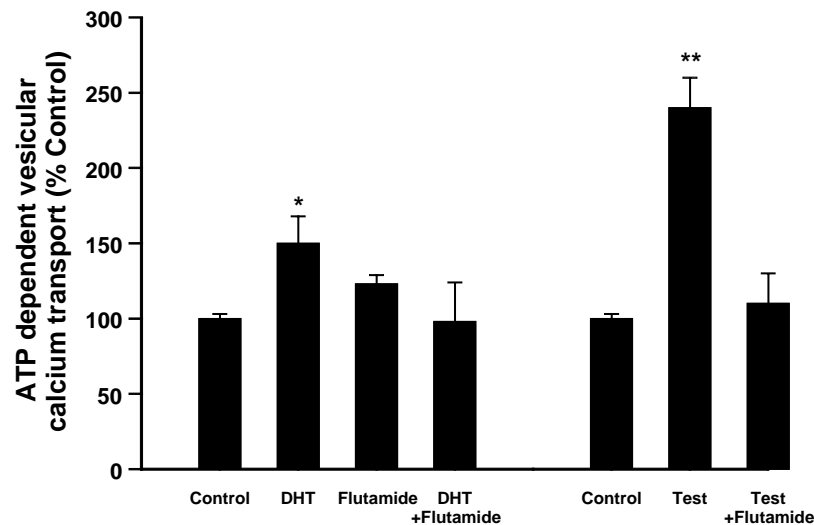


Fig. 4. (Left) Comparison of dihydrotestosterone (DHT 10^{-11} M) with control and control plus flutamide (10^{-6} M) with dihydrotestosterone plus flutamide ($n = 6$ replicate experiments per group). (Right) Comparison of testosterone (Test 10^{-5} M) with control and testosterone plus flutamide. Confluent MDBK cells were incubated for 24 h with vehicle alone, flutamide alone, dihydrotestosterone, dihydrotestosterone and flutamide, testosterone or testosterone plus flutamide ($n = 6$ replicate experiments per group). Results are mean \pm S.E.M. of PMCA activity compared to no dihydrotestosterone control or no testosterone control as appropriate. * $P < 0.05$, ** $P < 0.001$ compared to control.

kinase C (PKC), as has been demonstrated in the purified protein (Wang et al., 1991), as estrogen has been clearly shown to stimulate PKC activity in primary renal tubule cells (Han et al., 2000), although no information is available concerning dihydrotestosterone effects on PKC activity in the kidney. This, mechanism, however, would be expected to result in a more rapid increase in PMCA activity than was observed in the present study. It is also possible that estrogen and dihydrotestosterone regulate production of

some other protein that resulted in the stimulation of PMCA activity. One such protein is calbindin D28k, for which we have previously demonstrated increased mRNA in response to estrogen (Criddle et al., 1997) and which has been shown to stimulate PMCA activity (Morgan et al., 1986; Timmermans et al., 1995). Regardless of the mechanism, the stimulation of PMCA activity by estrogen is likely to be estrogen receptor mediated. This is supported by two observations. Co-incubation of the estrogen antagonist ICI 164384 abolished the estrogen induced stimulation of PMCA activity with 24 h of incubation, indicating that the estrogen effect could be inhibited by competition for the estrogen receptor. Consistent with the reported lower affinity of α estradiol for both estrogen receptor α and estrogen receptor β (Kuiper et al., 1997), α estradiol did not have a significant effect on PMCA activity. Comparable with estrogen, dihydrotestosterone stimulated PMCA activity in MDBK cells. The androgen receptor antagonist flutamide completely inhibited the stimulatory effect of dihydrotestosterone on PMCA activity, indicating that dihydrotestosterone is likely to be stimulating PMCA activity through the androgen receptor. Testosterone also stimulated PMCA activity, but at a 4-fold higher concentration than the concentration of dihydrotestosterone that was required for maximal stimulation of PMCA activity. This suggests that testosterone is metabolized to dihydrotestosterone in the kidney as a result of five alpha reductase activity that has been reported in the kidney (Martel et al., 1994).

The demonstration of regulation of PMCA activity by estrogen and androgens in the kidney may have implications for the role of these hormones in transcellular calcium transport or regulation of cellular calcium concentration. An apical membrane calcium influx channel (ECaC) has been

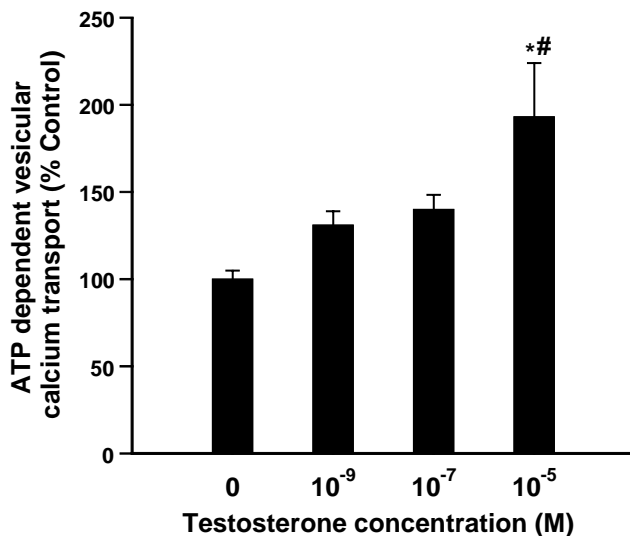


Fig. 5. The effect of testosterone on PMCA activity. Confluent MDBK cells were incubated for 24 h with the indicated concentration of testosterone ($n = 6$ replicate experiments per dose). Results are mean \pm S.E.M. of PMCA activity compared to the no testosterone added control. * $P < 0.005$ compared to control, # $P < 0.05$ compared to 10^{-9} M, 10^{-7} M testosterone.

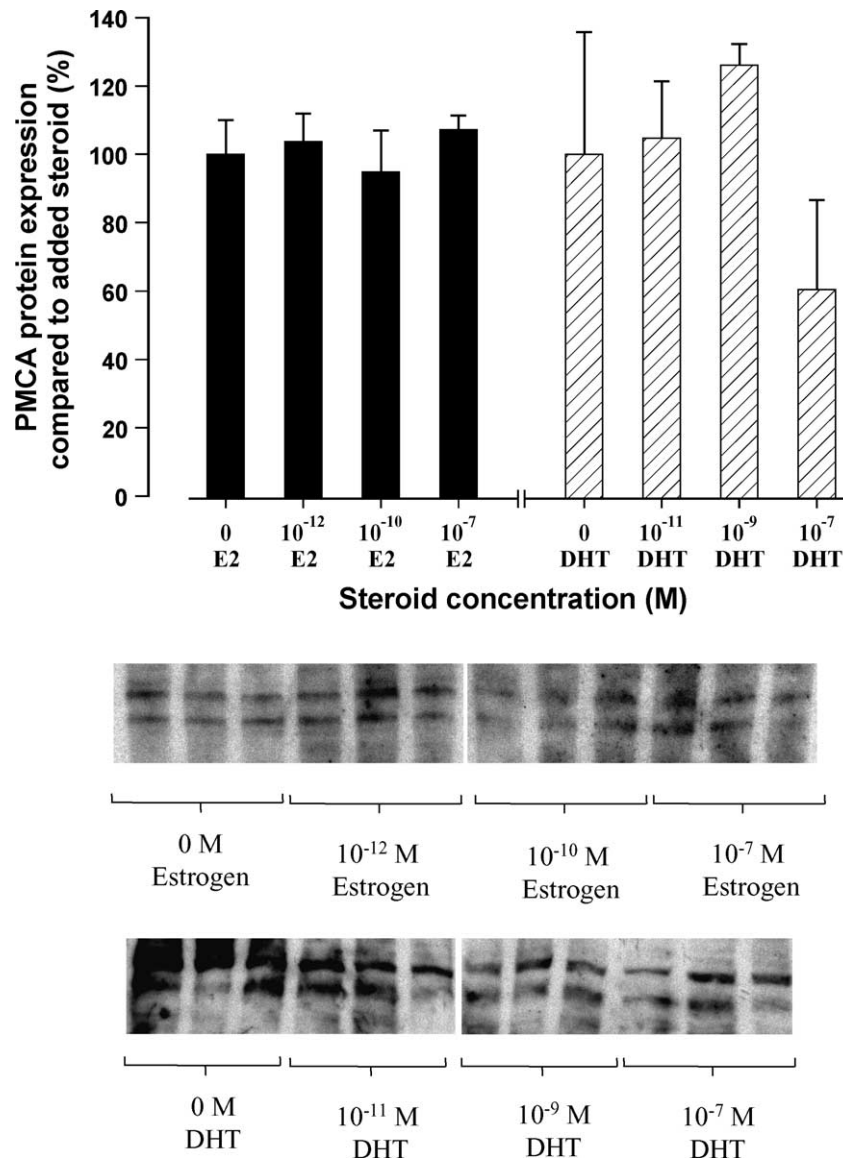


Fig. 6. Dose response of estrogen and dihydrotestosterone (DHT) on PMCA protein expression. Confluent MDBK cells were incubated for 24 h with the indicated concentrations of steroid ($n = 3$ replicate experiments per dose). Western blotting was performed as described in the text. Results are mean \pm S.E.M. of protein expression compared to control.

identified that is present exclusively in epithelial tissues associated with transcellular calcium transport in the rabbit, including duodenum and renal distal tubule (Hoenderop et al., 1999). In the kidney, this calcium channel is only present in distal tubule and collecting duct cells that also express the PMCA, the sodium calcium exchanger (NCX) and the calcium binding protein calbindin D28k (Hoenderop et al., 2000). The mRNA and protein abundance for ECAC1 has been shown to be upregulated in the kidney of the ovariectomized rat by the action of 17β estradiol (Van Abel et al., 2002). In this same study, significant increases in mRNA expression for calbindin D28k, PMCA1b and the sodium calcium exchanger were observed with 17β estradiol administration, although the effect on protein expression of these calcium transport proteins was not reported (Van Abel

et al., 2002). In the 25-hydroxyvitamin D_3 - 1α -hydroxylase deficient mouse, a similar effect of 17β estradiol to increase mRNA expression of ECAC1 was observed, with no effect being observed on calbindin D28k, PMCA1b or NCX mRNA expression. In addition, an effect of 17β estradiol to increase circulating ionized calcium concentration was observed. This suggests that the estrogen effect on the ECAC1 was independent of calcitriol, whereas the effect on calbindin D28k, PMCA1 and NCX was dependent on calcitriol regulation (Van Abel et al., 2002). This observation is compatible with the results from the present study demonstrating no effect of estrogen on PMCA protein expression in MBDK cells, as these distal tubular cells were growing in calcitriol deficient media and do not have 25-hydroxyvitamin D_3 - 1α -hydroxylase activity. Transcellular calcium transport

in the kidney tubule cell requires the coordination of apical membrane calcium entry with basolateral membrane calcium extrusion (Raber et al., 1997). Therefore, the observation that 17β estradiol increased serum ionized calcium concentration in the 25-hydroxyvitamin D_3 - 1α -hydroxylase deficient mouse, despite a lack of effect on basolateral calcium transporting protein synthesis (Van Abel et al., 2002), strongly suggests that a regulatory effect on the activity of the PMCA, the NCX or both must have been present. There is also evidence that calcium uptake in rabbit primary kidney tubule cells is stimulated by estrogen via a non-genomic mechanism (Han et al., 2000).

The in vitro effect of 17β estradiol exposure to increase PMCA activity demonstrated in this study is consistent with the effect of estrogen supplementation that has been observed in vivo to increase renal calcium reabsorption (Selby et al., 1985; Nordin et al., 1991; Prince et al., 1991; Adami et al., 1992; McKane et al., 1995), and the effect of estrogen deficiency in the postmenopausal woman to decrease calcium reabsorption (Nordin et al., 1991; Adami et al., 1992; Prince et al., 1995). Similarly, the observation of this study that dihydrotestosterone stimulates PMCA activity in kidney cells is consistent with observations from in vivo studies. Hypogonadism is associated with deleterious changes in calcium balance in men similar to estrogen deficiency in women (Mauras et al., 1999). The administration of dihydrotestosterone to ovariectomized rats was shown to prevent changes to both bone turnover and renal calcium handling (Mason and Morris, 1997) and the administration of flutamide to the female rat was shown to result in bone loss, indicating the importance of androgens in maintaining a positive calcium balance in the female rat (Goulding and Gold, 1993). Administration of estrogen to hypogonadal male rats was shown to prevent bone loss as effectively as dihydrotestosterone or testosterone (Vanderschueren et al., 1992), indicating the importance of androgens and estrogens in maintaining a positive calcium balance in both male and female rats.

5. Conclusion

In conclusion, the results of this study indicate that both estrogen and androgens regulate membrane calcium transport in renal distal tubule like cells, via a possible receptor mediated mechanism and is not the result of an effect on increasing PMCA protein expression. These observations supports in vivo evidence for regulation by these hormones of renal tubular calcium reabsorption and suggests that decreased renal calcium reabsorption may be important in bone loss associated with the deficiency of these hormones.

Acknowledgements

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