

Effect of estrogen on calcium and sodium transport by the nephron luminal membranes

M G Brunette and M Leclerc

Maisonneuve-Rosemont Hospital, Research Center and University of Montreal, Montreal, Quebec, Canada

(Requests for offprints should be addressed to M G Brunette, Maisonneuve-Rosemont Hospital, Research Center, 5415, Boulevard de l'Assomption, Montréal, Québec H1T 2 M4, Canada; Email: visionfrsq@hmr.qc.ca)

Abstract

Estrogens are widely used for contraception and osteoporosis prevention. The aim of the present study was to investigate the effect of 17 β -estradiol on calcium (Ca²⁺) transport by the nephron luminal membranes, independently of any other Ca²⁺-regulating hormones. Proximal and distal tubules of rabbit kidneys were incubated with 17 β -estradiol or the carrier for various periods of time, and the luminal membranes of these tubules were purified and vesiculated. Ca²⁺ uptake by membrane vesicles was measured using the Millipore filtration technique. Incubation of proximal tubules with the hormone did not influence Ca²⁺ uptake by the luminal membranes. In contrast, incubation of distal tubules with 10⁻⁸ M 17 β -estradiol for 30 min decreased the initial uptake of 0.5 mM Ca²⁺ from 0.34 \pm 0.04 (S.E.M.) to 0.17 \pm 0.04 pmol/ μ g per 5 s (P < 0.05). In the presence of 100 mM Na⁺, 0.5 mM Ca²⁺ uptake was strongly diminished and the effect of 17 β -estradiol disappeared (0.17 \pm 0.01 and

0.21 \pm 0.07 pmol/ μ g per 5 s in vesicles from the control and treated tubules). Direct incubation of the membranes with 17 β -estradiol, however, failed to show any influence of the hormone on Ca²⁺ transport. The action of 17 β -estradiol was dose-dependent, with a half-maximal effect at approximately 10⁻⁹ M. Ca²⁺ uptake by the distal tubule membranes presents dual kinetics. 17 β -Estradiol decreased the V_{max} value of the high-affinity component from 0.42 \pm 0.02 to 0.31 \pm 0.03 pmol/ μ g per 10 s (P < 0.02). In contrast with the effect of the hormone on Ca²⁺ transport, estradiol increased Na⁺ uptake by both the proximal and distal tubule luminal membranes. In conclusion, incubation of proximal and distal tubules with estrogen decreases Ca²⁺ reabsorption by the high-affinity Ca²⁺ channels of the distal luminal membranes, and enhances Na⁺ transport by the membranes from proximal and distal nephrons.

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Introduction

The two major indications for estrogen administration are contraception and menopause. For many years now, clinical observations and clearance studies have clearly established an association between estrogen status and sodium (Na⁺) retention in normal dogs (Johnson & Davis 1976), in women during the estrogenic cycle (Thorn *et al.* 1938, Preedy & Aitken 1956) and in postmenopausal women injected with 17 β -estradiol (Dignam *et al.* 1956). This effect appears to be independent of mineralocorticoid secretion. Indeed, De Vries *et al.* (1972) observed a 35% decrease in urinary excretion of Na⁺ when 17 β -estradiol was administered to adrenalectomized male rats in doses of 40 μ g/day. A similar conclusion was reported more recently in cultured mouse distal tubules (Tran *et al.* 1998) and distal tubules of ovariectomized rats (Verlander *et al.* 1998a). In contrast to these studies reporting an antinatriuretic action of estrogen, the literature concerning the effects on renal calcium (Ca²⁺) handling is scanty and inconclusive. In postmenopausal women and particularly

in women with osteoporosis, Nordin *et al.* (1991, 1994) observed a relatively high Ca²⁺ excretion which could not be explained by the slight rise of filtered Ca²⁺, therefore suggesting that estrogens promote tubular reabsorption of this cation. Similar findings were reported by McKane *et al.* (1995) who proposed an inhibition of bone resorption and as a consequence, an increase in serum parathyroid hormone (PTH) as the principal mechanisms involved in estrogen action.

However, different conclusions have resulted from animal experimentation. According to Dick *et al.* (1996) and Dick & Prince (1997) ovariectomy in rats results in a transitory increase in the 24 h urine Ca²⁺ excretion. But this hypercalciuria was mainly attributed to an increase in ultrafiltrable Ca²⁺ and therefore in the filtered load of Ca²⁺ rather than a change in renal Ca²⁺ handling. Aside from ovarian hormones, the remaining calcitropic hormonal status was not modified by the intervention. However, when the filtered load was corrected by EDTA infusion, administration of 17 β -estradiol to these ovariectomized animals significantly increased Ca²⁺ and,

unexpectedly, Na^{+} excretion, thus suggesting that estrogen curtailed the tubular reabsorption of the two cations. Creighton *et al.* (1999) proposed similar conclusions in women. These authors measured Ca^{2+} excretion in 71 postmenopausal women who presented or not Ca^{2+} nephrolithiasis. In both groups, urinary excretion of Ca^{2+} was significantly higher in patients receiving estrogen than in those who were not. The authors concluded that estrogen increases urinary Ca^{2+} , and then favors stone formation. Most of the confusion about the effect of estrogen on Ca^{2+} excretion probably results from the fact that the clearance experiments provide data which result from a complex hormonal interrelationship.

Another source of controversy concerns the localization of estrogen receptors in the kidney. Pantic *et al.* (1974) studied the ability of kidney cells to incorporate tritiated estradiol in hamsters previously treated for 6 months with estrogen implants. The hormone was detected in kidney slices by autoradiography and was essentially localized in the proximal tubules, although the concentration was not uniform from one cell to the other. The distal convoluted tubule cells were labeled to a much smaller extent. In the study of Dick & Prince (1997), the correlation between Na^{+} and Ca^{2+} excretion following estrogen administration to ovariectomized rats also incited the authors to conclude that the hormone is acting in the proximal tubule. In contrast, Verlander *et al.* (1998b) recently presented an immunohistochemistry study showing the presence of these receptors predominantly in the distal and cortical collecting tubules.

The purpose of the present experiments was to detect the direct action of estrogen on Ca^{2+} reabsorption by the proximal and cortical distal tubules, independently of any other parameters, hormonal or not. Results indicated that incubation of distal tubules with 17β -estradiol not only increased Na^{+} transport by the proximal and distal luminal membranes, but, unexpectedly, significantly decreased Ca^{2+} uptake by the distal membranes exclusively.

Materials and Methods

Tubule preparation

Ten to 12 rabbit kidneys, directly obtained from the slaughter house, were used for each experiment. The procedure for the purification of the proximal and distal tubules was similar to that previously described (Brunette *et al.* 1992). Slices of cortex were incubated for 20 min at 37°C in a modified Krebs–Henseleit (KH) buffer, containing 1 mg/ml collagenase type IV and 0.5 mg/ml BSA. The digested tissue was filtered through a stainless steel mesh and the filtrate was centrifuged at 200 g for 20 s. The tubule-containing pellets were washed three times in KH solution supplemented with 0.5 mg/ml BSA, suspended in 40% Percoll (final concentration) previously equilibrated with 95% O_2 and 5% CO_2 and centrifuged at 28 000 g at

4°C for 20 min. Three bands were clearly identified: glomeruli, proximal and distal tubules. The tubules were collected separately, washed in KH and BSA, and incubated for various periods of time in a cell culture medium containing 2% fetal bovine serum, 0.1 μM phenylmethylsulfonylfluoride and 17β -estradiol at the indicated concentrations. Incubation was stopped by centrifugation, tubules were washed in KH, suspended in 10 mM mannitol, 2 mM Tris–Hepes pH 7.4 and frozen at -80°C until the day of experiment.

Luminal membrane purification

The luminal membranes were isolated using the MgCl_2 precipitation technique. The proximal and distal tubule suspensions were thawed and homogenized with ten strokes of a Potter homogenizer. Following the addition of MgCl_2 (12 mM final concentration), the suspensions were stirred in ice for 20 min (proximal tubules) or 10 min (distal tubules) and centrifuged at 3000 g for 20 min at 4°C . The supernatants were collected and centrifuged again at 28 000 g for 30 min at 4°C . The sedimented membranes were washed twice in 280 mM mannitol, 20 mM Tris–Hepes pH 7.4 and allowed to vesiculate at 4°C for 1 h.

Enzyme marker measurements

The purity of the membranes was monitored by the measurement of the activities of specific enzyme markers (Table 1). Alkaline phosphatase activity was determined by the technique of Kelly & Hamilton (1970) and Na-K ATPase by the technique of Post & Sen (1967). Previous experiments showed negligible contamination with mitochondrial or endoplasmic reticulum membranes (Brunette *et al.* 1992).

$^{45}\text{Ca}^{2+}$ uptake by the membrane vesicles

$^{45}\text{Ca}^{2+}$ uptake was measured by the rapid Millipore filtration technique. The uptake was initiated by adding 25 μl incubation medium at 35°C to 5 μl membrane suspension (approximately 20–25 μg protein). Uptake was stopped by addition of 1 ml of ice-cold stop-solution. The suspension was immediately filtered through Millipore filters (HAWP 0.45 μm pore size; Fisher Scientific, Nepeau, Ontario, Canada). The filters were rinsed with an additional 5 ml stop-solution and the trapped radioactivity was counted. The incubation medium contained either 120 mM NaCl and 20 mM choline chloride or 140 mM choline chloride, with 20 mM Tris–Hepes pH 7.4 and, unless otherwise mentioned, 0.5 mM $^{45}\text{CaCl}_2$. Stop-solution contained 150 mM KCl, 20 mM Tris–Hepes pH 7.4 and 2 mM EGTA (or more if high concentrations of Ca^{2+} were used).

Table 1 Enzyme activities in the various preparations

	Alkaline phosphatase (nmol/μg protein per 15 min)	Na-K ATPase (nmol/μg protein per 20 min)
Total cortex	2.31 ± 0.10	4.51 ± 0.33
PT homogenates	1.53 ± 0.11	2.90 ± 0.35
PT luminal membranes	11.0 ± 0.99 (4.76 ×)	2.03 ± 0.18 (0.45 ×)
DT homogenates	0.51 ± 0.06	0.75 ± 0.099
DT luminal membranes	2.26 ± 0.31 (0.96 ×)	0.63 ± 0.08 (0.14 ×)

PT: proximal tubules; DT: distal tubules; ×: enzyme enrichments compared with total cortex values.

In the few experiments where ^{22}Na uptake was measured, the incubation medium contained 1 mM $^{22}\text{NaCl}$, 278 mM mannitol, 20 mM Tris-Hepes pH 7.4.

Statistics and calculations

All measurements of electrolyte uptake were carried out in duplicates, and the data presented in the figures and tables reflect the mean values ± s.e.m. of three to five experiments as indicated. The kinetic values of Ca^{2+} uptake were calculated using the non-linear regression analysis as described by Huntson (1975). Significancies of results were estimated according to the unpaired Student's *t*-test.

Results

Effect of 17β-estradiol on Ca^{2+} uptake by proximal and distal tubule luminal membranes

The data in Fig. 1 show the time-course of 0.5 μM Ca^{2+} uptake by membranes isolated from proximal and distal

tubules incubated with 10^{-8} M 17β-estradiol. The incubation medium contained 140 mM choline chloride, 20 mM Tris-Hepes pH 7.4 and 0.5 mM $^{45}\text{CaCl}$. A 30 min treatment of the tubules with the hormone decreased the initial Ca^{2+} uptake from 0.34 ± 0.04 to 0.17 ± 0.04 pmol/μg per 5 s ($P < 0.05$, $n = 4$) and from 0.63 ± 0.065 to 0.33 ± 0.04 pmol/μg per 10 s ($P < 0.001$, $n = 4$) by the proximal and distal tubule membranes respectively. At equilibrium, however, the intravesicular Ca^{2+} was similar under the two experimental conditions. In contrast, the hormone did not affect Ca^{2+} transport by the proximal tubule membranes (0.27 ± 0.05 vs 0.30 ± 0.09 pmol/μg; NS, $n = 3$).

The effect of 17β-estradiol disappears in the presence of Na^+

The experiments presented in Fig. 1 were performed in the absence of Na^+ . We previously reported that Ca^{2+} uptake by the distal luminal membranes was strongly

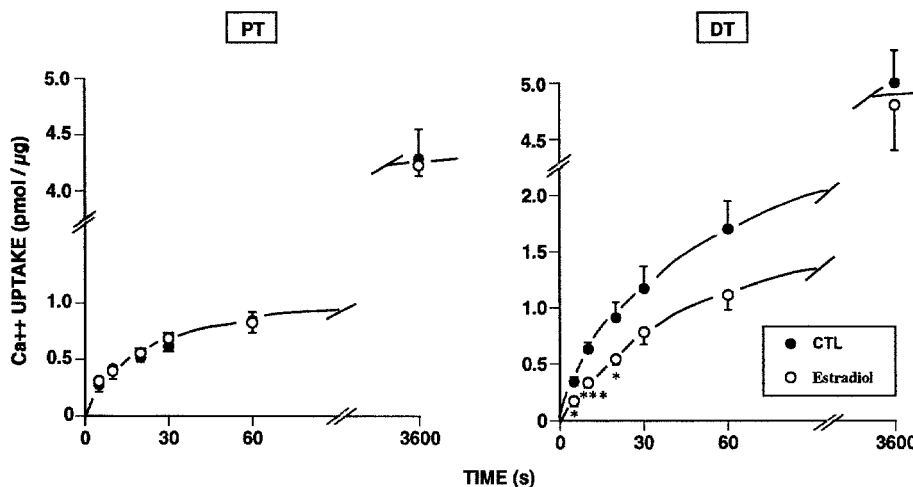


Figure 1 Effect of proximal (PT) and distal (DT) tubule incubation with 10^{-8} M 17β-estradiol on 0.5 mM Ca^{2+} uptake by the luminal membranes in the absence of Na^+ . Incubation medium contained 140 mM choline chloride, 20 mM Tris-Hepes and 0.5 mM $^{45}\text{CaCl}_2$, $n = 3$ (PT) or $n = 4$ (DT). Data are means ± s.e.m. * $P < 0.05$, *** $P < 0.001$, $n = 4$. (CTL: control.)

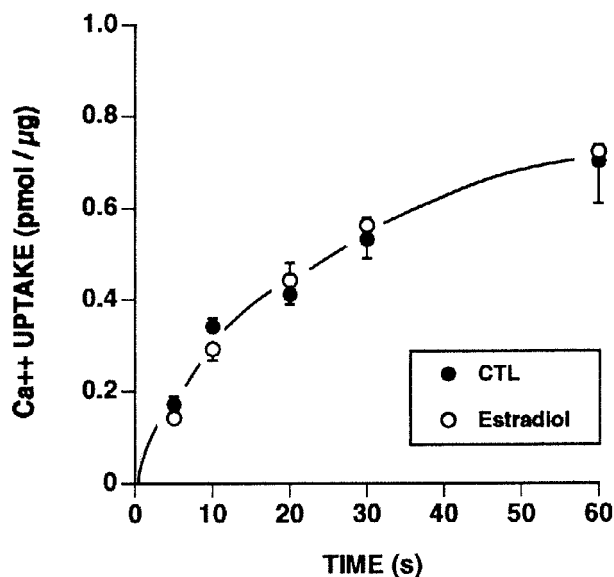


Figure 2 Effect of incubation of distal tubules with 10^{-8} M 17β -estradiol on Ca^{2+} uptake by the luminal membranes in the presence of Na^+ . Incubation medium contained 120 mM NaCl, 20 mM choline chloride and 0.5 mM $^{45}\text{CaCl}_2$, $n=3$. Data are means \pm S.E.M. (CTL: control.)

decreased when Na^+ was present in the medium (Brunette *et al.* 1992). Then we were interested to investigate whether the influence of 17β -estradiol persisted despite the replacement of choline chloride by NaCl in the incubation medium. As shown in Fig. 2, the presence of 100 mM NaCl not only decreased Ca^{2+}

uptake, but it completely abolished the effect of the hormone. The cation uptakes were 0.17 ± 0.01 and 0.21 ± 0.07 pmol/ μg per 5 s by vesicles from control and treated tubules respectively (NS, $n=3$).

Effect of the duration of incubation

17β -Estradiol affected the Ca^{2+} transport by the distal luminal membrane within a relatively short period of time. As shown in Fig. 3, the uptake was affected after only a 15 min incubation of the tubules. Although not statistically significant, a tendency to decrease was observed within the few initial minutes.

Dose-response of the effect of 17β -estradiol on Ca^{2+} uptake

The response of Ca^{2+} uptake to 17β -estradiol was dose-dependent. Figure 4 shows the profile curve of this dependence. Incubation of distal tubules with 10^{-8} M 17β -estradiol decreased Ca^{2+} transport to $73.9 \pm 3.6\%$ of the control values ($P < 0.05$). However, a slight but not significant decrease of uptake was already present at 10^{-10} M, i.e. within the physiological range. For comparison, a dose-response curve of the effect of the hormone on Na^+ uptake is presented in the inset of the figure.

Does estrogen act directly on the luminal membranes?

It has been previously reported that estrogen acutely applied to human cardiac myocytes was decreasing the L-type current within a few seconds (Meyer *et al.* 1998),

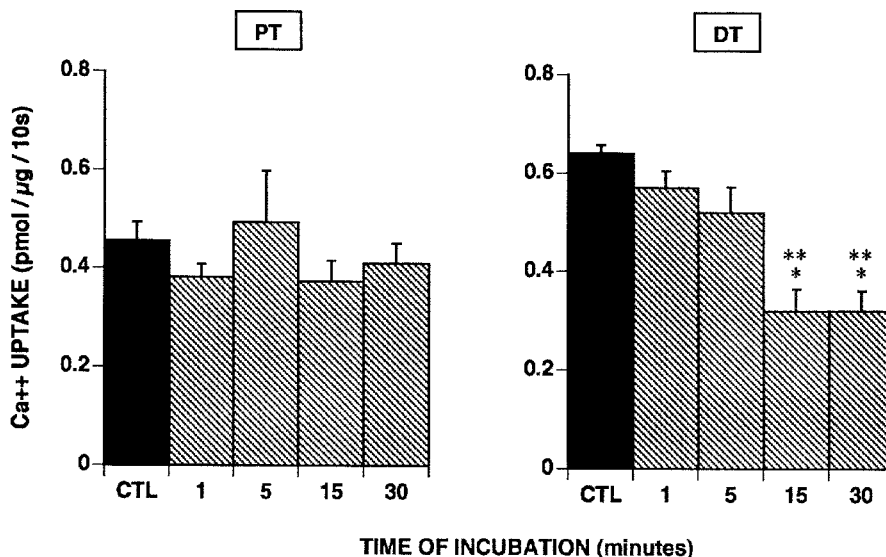


Figure 3 Effect of incubation of proximal (PT) and distal (DT) tubules with 10^{-8} M 17β -estradiol on 0.5 mM $^{45}\text{Ca}^{2+}$ uptake by the luminal membranes: variation with incubation time. Data are means \pm S.E.M. *** $P < 0.005$, $n=3$.

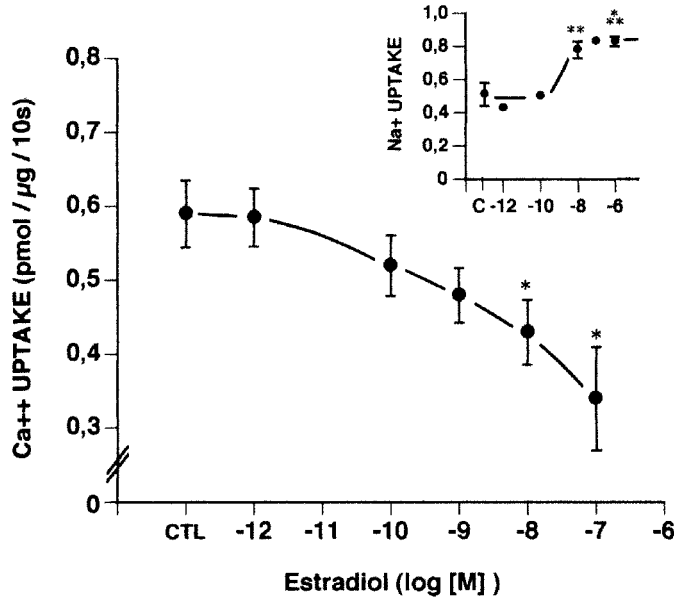


Figure 4 Dose–response curve of the effect of 17β-estradiol on 0.5 mM $^{45}\text{Ca}^{2+}$ uptake by distal tubule luminal membranes. * $P < 0.05$, $n = 4$. Inset: dose–response curve of the effect of the hormone on 1 mM Na^+ uptake. ** $P < 0.01$, *** $P < 0.001$. Data are means \pm S.E.M.

suggesting a direct effect on the cell membranes. We investigated whether the hormone, which is filtered by the glomeruli, was also able to directly influence Ca^{2+} influx by the luminal membranes. Neither a 1 min incubation of the vesicles with 10^{-8} M 17β-estradiol (therefore in CIS position) nor addition of the hormone to the incubation medium did influence Ca^{2+} uptake (Fig. 5).

On which component of Ca^{2+} uptake by the distal luminal membrane is estrogen acting?

Ca^{2+} transport by the distal luminal membranes presents dual kinetics (Brunette *et al.* 1992). To determine which component is involved in estrogen action, Ca^{2+} uptake was measured in incubation media containing from 75 μM

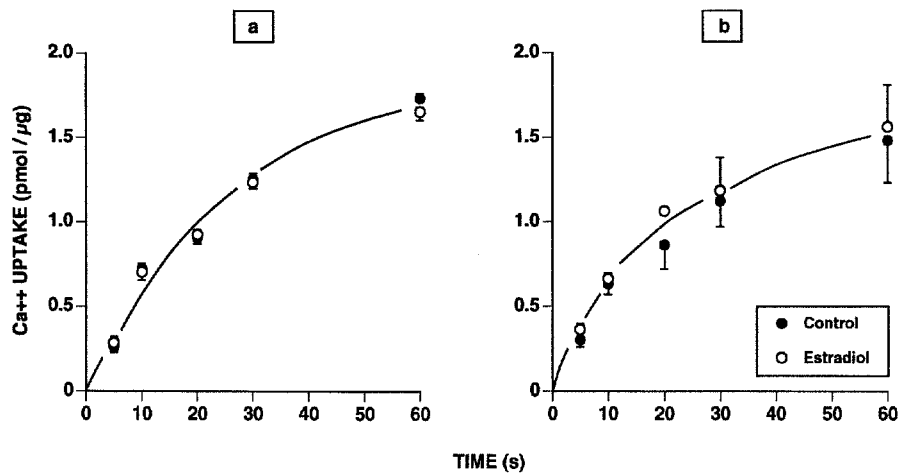


Figure 5 Effect of a 1 min incubation of distal tubule membrane vesicles with 10^{-8} M 17β-estradiol in the vesicle suspension (a) or in the incubation medium (b) on 0.5 mM $^{45}\text{Ca}^{2+}$ uptake. Data are means \pm S.E.M.

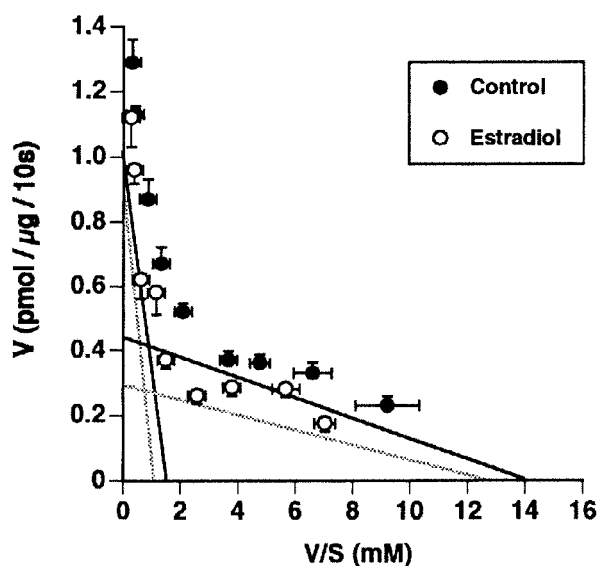


Figure 6 Eadie-Hofstee plot of Ca²⁺ uptake by membranes from tubules incubated with 10⁻⁸ M 17β-estradiol or the carrier. Data are means ± S.E.M.

to 4 mM Ca²⁺. The corresponding Eadie-Hofstee plot is presented in Fig. 6. The presence of 10⁻⁸ M 17β-estradiol influenced very specifically the high-affinity component, decreasing the V_{max} from 0.42 ± 0.02 to 0.31 ± 0.03 pmol/μg per 10 s (*P* < 0.02, *n* = 5) (Table 2).

Effect of 17β-estradiol on Na⁺ transport by the luminal membranes

Because of the interaction of Ca²⁺ and Na⁺ in the distal luminal membranes, we tested whether estrogen influences Na⁺ transport by these membranes. Results were compared with those obtained in proximal tubules. A 15 min incubation of proximal and distal tubules with 10⁻⁸ M 17β-estradiol resulted in a strong increase in 1 mM Na⁺ uptake from 0.55 ± 0.03 to 0.97 ± 0.12 (*P* < 0.05) in proximal tubules and from 0.51 ± 0.07 to 0.78 ± 0.05 pmol/μg per 10 s (*P* < 0.02, *n* = 3) in the distal segments.

Then in a new series of experiments we investigated first whether aldosterone influences Na⁺ transport in our distal tubule membranes, and if so, whether actions of estrogen and aldosterone on Na⁺ transport were additive, and secondly, whether aldosterone, as estrogen, has any effect on Ca²⁺ uptake by our distal luminal membranes. Results are presented in Fig. 7. A 30 min incubation with both 17β-estradiol and aldosterone increased Na⁺ uptake, and their combined effects were significantly higher than those of each of them (0.84 ± 0.04 vs 0.89 ± 0.05 vs 1.00 ± 0.06 pmol/μg per 10 s by membranes from tubules incubated with 17β-estradiol, or aldosterone, or both respectively). The *P* values were < 0.02 for Na⁺ uptakes obtained in combined conditions, vs either estrogen or aldosterone conditions (*n* = 4). However, Ca²⁺ transport by membranes from distal tubules treated with the carrier (0.66 ± 0.03) or estrogen alone (0.55 ± 0.02), or aldosterone alone (0.69 ± 0.03) or both (0.68 ± 0.07 pmol/μg per 10 s), again showed a significant effect of estrogen (*P* < 0.05) but a complete lack of influence of the other two experimental conditions (aldosterone alone, or aldosterone and 17β-estradiol).

Discussion

Estrogen decreases tubular reabsorption of Ca²⁺ in the distal tubule

As previously mentioned, *in vivo* experiments investigating the effect of estrogen on the renal excretion of Ca²⁺ have been the source of controversy. In women, menopause has been shown to be accompanied by an increase in calciuria (Nordin *et al.* 1991, 1994, Adami *et al.* 1992, McKane *et al.* 1995, Heshemati *et al.* 1998) therefore suggesting that estrogen enhances Ca²⁺ reabsorption. On the contrary, a decrease in Ca²⁺ excretion was reported by Dick *et al.* (1996), Dick & Prince (1997) and Creighton *et al.* (1999) following estrogen administration, an observation which is compatible with the opposite hypothesis. Several factors are probably at the origin of these different conclusions. Plasma ultrafiltrable Ca²⁺ rises at menopause, and falls following administration of estrogen. Therefore, in clinical situations, estrogen deficiency might induce

Table 2 Effect of 10⁻⁸ M estradiol on the kinetics parameters of Ca²⁺ transport by the distal luminal membranes

	High-affinity component		Low-affinity component	
	K _m (mM Ca ²⁺)	V _{max} (pmol/μg per 10 s)	K _m (mM Ca ²⁺)	V _{max} (pmol/μg per 10 s)
Controls	0.03 ± 0.006	0.43 ± 0.02	0.68 ± 0.11	1.03 ± 0.06
17β-Estradiol	0.02 ± 0.004	0.31 ± 0.03*	0.72 ± 0.15	0.88 ± 0.04

**P* < 0.02 compared with control values (unpaired *t*-test); *n* = 5.

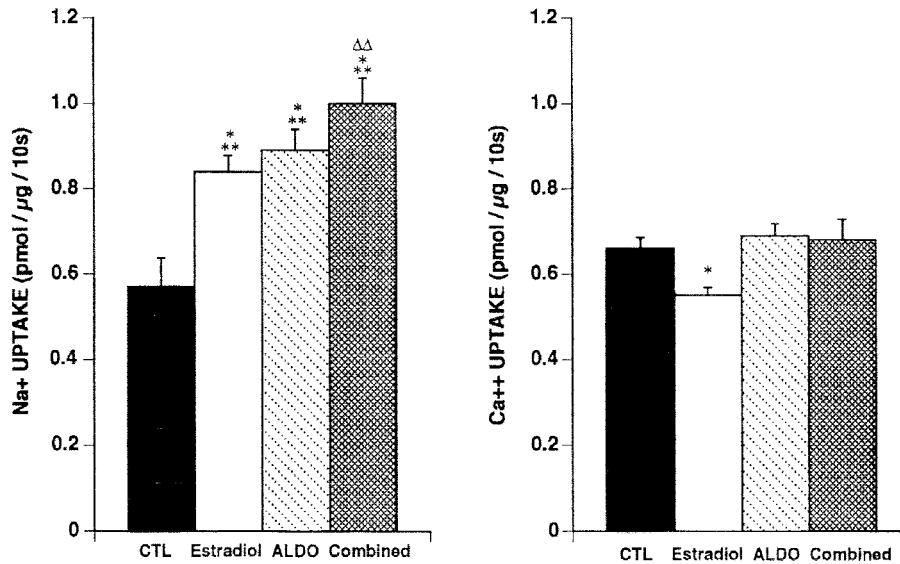


Figure 7 Separate and combined effects of 10^{-6} M 17β -estradiol and 10^{-6} M aldosterone on 1 mM Na^+ and 0.5 mM Ca^{2+} uptakes by distal luminal membranes. * $P < 0.05$ and *** $P < 0.01$ compared with the control values; $\Delta\Delta$ $P < 0.02$ compared with the uptakes by membranes from tubules treated with either 17β -estradiol or aldosterone alone. $n = 3$ (Na^+ uptake), $n = 4$ (Ca^{2+} uptake). Data are means \pm S.E.M.

calciuria by increasing the filtered load of Ca^{2+} (Dick *et al.* 1996). Moreover, *in vivo* regulation of calciuria is complex. In addition to its effect on bone, estrogen has been shown to interfere with the PTH-dependent cAMP synthesis in opossum kidney cells (Stock *et al.* 1992) and to stimulate both the vitamin D active metabolite $1,25(\text{OH})_2\text{D}_3$ (Tanaka *et al.* 1978, Cheema *et al.* 1989, Castillo *et al.* 1977) and calbindin 28K (Gill & Christakos 1995) expression. Not only does $1,25(\text{OH})_2\text{D}_3$ increase Ca^{2+} intestinal absorption but it also directly regulates PTH secretion by the gland (Chertow *et al.* 1980, Russel *et al.* 1986, Silver *et al.* 1986), which is a site of receptors for this hormone (Brumbaugh *et al.* 1975). Therefore, because of this complex hormonal interrelationship, the reported clearance studies do not necessarily reflect the direct action of estrogen on Ca^{2+} reabsorption capacity of the nephron. The purpose of our study was to investigate such an action, independently of any other hormone influence. The results clearly indicated that estrogen by itself regulates electrolyte reabsorption, increasing Na^+ and decreasing Ca^{2+} uptake by the distal tubule luminal membrane.

Estrogen receptors are present in distal as in proximal nephron segments

The site of estrogen receptors has not been clearly established. Autoradiographic studies following long-term estrogen implants in the hamster have localized the hormone

binding predominantly in the proximal tubule although some binding was also observed in individual cells of distal tubules (Pantic *et al.* 1974). Similar results were reported in the rat (Davidoff *et al.* 1980). In primary cultures of rabbit kidney proximal tubule cells, immunoprecipitation studies also detected receptors to α and β estradiol (Han *et al.* 1999). But suggesting also a distal site of action are the observations by Tran *et al.* (1998) and Verlander *et al.* (1998a). Verlander *et al.* (1998a) reported that replacement 17β -estradiol administered *in vivo* to ovariectomized rats enhances the density of immunoreactive thiazide-sensitive NaCl transporter in the apical membrane of distal tubules. More precisely using immunoblot analysis and ELISA assay, Tran *et al.* (1998) showed in *in vitro* studies that the hormone increases both the NaCl cotransport and Na-K ATPase proteins in cultured distal tubule cells.

Our experiments confirmed the presence of receptors at both sites, since the hormone increased Na^+ reabsorption in proximal as in distal tubules. However, only the distal Ca^{2+} transport was affected by estradiol. Therefore, the hormonal regulation of Ca^{2+} reabsorption occurs essentially if not exclusively in the distal convoluted tubule.

Two types of estrogen receptors have been described by *in situ* hybridization and immunochemistry. α -Receptors have been detected in uterus (Kuiper *et al.* 1996) whereas β -receptors are rather present in prostate, testis and ovary (Enmark *et al.* 1997). Recently Verlander (1998b), using immunocytochemistry techniques, showed α -receptors in

glomeruli and distal tubules of intact rats, as well as in proximal tubules in ovariectomized animals.

Estrogen decreases the high-affinity component of Ca^{2+} transport by the distal tubule

We previously reported that Ca^{2+} uptake by distal tubule luminal membranes presents dual kinetics. The low-affinity component is activated by calcitonin (Zuo *et al.* 1997) whereas the high-affinity component is influenced by PTH (Lajeunesse *et al.* 1994) and calbindin 28K (Bouhtiauy *et al.* 1994). Microdissection studies precisely localized the PTH-sensitive segment in the rabbit distal tubule, showing a granular appearance under microscopic examination (Morel *et al.* 1976, 1982). This high-affinity channel is strongly inhibited by Na^+ (Brunette *et al.* 1992). We now report that estrogen interacts with this high-affinity component, decreasing Ca^{2+} while increasing Na^+ transport. Several studies showed a tight interrelationship between estrogen and thiazide receptor. Chen *et al.* (1994) studied the influence of gender on these thiazide receptors in the rat. They reported that ovariectomy decreases metolazone binding by more than 20%, suggesting that sex hormones regulate the density of the NaCl cotransport system in the luminal membrane, and therefore electrolyte reabsorption at this site. Confirming this hypothesis, Verlander *et al.* (1998a) raised rabbit antibodies against the NaCl cotransporter, and measured this transporter density in ovariectomized rats receiving or not 17β -estradiol. The authors also concluded that estrogen enhances the density of the NaCl cotransporter in the distal convoluted tubule. It is therefore probable that estrogen increases Na^+ and decreases Ca^{2+} uptake at the same site where thiazide, PTH and calbindin 28K on the contrary decrease Na^+ and enhance Ca^{2+} reabsorption, i.e. in the early 'granular' segment of the distal tubule.

Why does the presence of Na^+ curtail the action of estrogen on Ca^{2+} uptake?

The Ca^{2+} -regulating hormones also influences Na^+ transport by the distal luminal membranes; PTH increases Ca^{2+} (Lajeunesse *et al.* 1994) and decreases Na^+ (authors' unpublished observations) uptakes, as does calcitonin (Zuo *et al.* 1997) and calbindin 28K (Bouhtiauy *et al.* 1994). Ca^{2+} and Na^+ reabsorption are tightly related. Na^+ decreases Ca^{2+} (Brunette *et al.* 1992) and conversely Ca^{2+} decreases Na^+ transports. Indeed, in a recent study, we observed that 2 mM Ca^{2+} decreases 1 mM Na^+ uptake from 1.22 ± 0.14 to 0.70 ± 0.10 pmol/ μg per 10 s ($P < 0.05$) by the distal luminal membranes.

This mutual interrelationship suggests that the Ca^{2+} channels in these membranes are also permeable to Na^+ , and therefore, that these cations are in some way in a competitive situation. However, this competition favors Ca^{2+} since relatively high concentrations of Na^+ (35 mM)

are necessary to cut by half 0.5 mM Ca^{2+} uptake (Brunette *et al.* 1992). Hypothetically, the effect of estrogen on Ca^{2+} transport disappears in the presence of 100 mM Na^+ because this transport is already maximally inhibited.

The actions of estrogen and aldosterone on Na^+ transport are additive

Also because the site of action of estrogen is in the early part of the distal nephron, the additivity of this hormone and aldosterone effects is logical. Indeed as clearly demonstrated in the binding to the microdissected tubule experiments (Doucet & Katz 1981, Farman *et al.* 1982, Farman & Bonvalet 1983), aldosterone action is essentially localized in the late segments of the distal nephron, i.e. in the connecting and collecting tubules. However, the reason why in our experiments aldosterone, which alone did not influence Ca^{2+} uptake, prevented the effect of 17β -estradiol on this uptake is obscure.

What messengers are involved in the estrogen actions?

The action of estrogen occurs through complex mechanisms probably involving genomic and non-genomic mechanisms. Indeed, several data indicate that the estrogen receptor, in addition to acting as a transcription factor, is involved in non-genomic mechanisms implicating cAMP release, increase in intracellular Ca^{2+} and activation of MAP kinase. The relatively rapid response of Ca^{2+} uptake by the luminal membranes of tubules incubated with the hormone for only 15 min, argues for such mechanisms in the kidney as well as in other cells. In *in vitro* studies, estrogenic hormones have been found to increase cAMP in breast cancer and uterine cell cultures (Aronica *et al.* 1994) and in intestinal cells isolated from female rat duodenum (Picotto *et al.* 1996). In the kidney, however, the action of estrogen on adenylate cyclase is less well defined; Imanaka *et al.* (1998) reported that estrogen administration to elderly women enhances cAMP excretion induced by PTH loading. In contrast, 17β -estradiol has been shown to inhibit the PTH-dependent cAMP release in opossum kidney cells (Stock *et al.* 1992). In the renal distal tubule, we previously showed that either cAMP alone, or the combined cAMP and protein kinase C activation, increases rather than decreases Ca^{2+} transport by the luminal membrane (Hilal *et al.* 1997). Confirming that cAMP is not involved in the action of estrogen on Ca^{2+} transport, we measured the messenger concentration in tubules treated with either the hormone or the carrier. cAMP contents were identical in the two experimental conditions (11.51 ± 0.55 vs 11.99 ± 0.26 pmol/mg protein). It is therefore possible that in the present experiments, estrogen acts through an inhibition of protein kinase C or through activation of a MAP kinase. Further experiments should investigate the possible implication of

an eventual role of MAP kinase in the action of estrogen on Ca^{2+} transport by the distal tubule luminal membranes.

In conclusion, incubation of rabbit distal tubules with estrogen decreased Ca^{2+} and increased Na^+ reabsorption by the distal luminal membranes. This effect occurred following a relatively short period of time, to become highly significant after a 15 min incubation, suggesting a non-genomic mechanism. It involved the high-affinity Ca^{2+} channel, i.e. the channel which is strongly influenced by Na^+ and PTH.

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References

- Adami S, Gatti D, Bertoldo F, Rossini M, Fratta-Pasini A, Zamberian N, Facci E & Lo-Cascio V 1992 The effects of menopause and estrogen replacement therapy on the renal handling of calcium. *Osteoporosis International* **2** 180–185.
- Aronica SM, Kraus WL & Katzenellenbogen BS 1994 Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *PNAS* **91** 8517–8521.
- Bouhthiauy I, Lajeunesse D, Christakos S & Brunette MG 1994 The two vitamin D_3 dependent calcium binding proteins (CaBP) increase calcium reabsorption by different mechanisms. Effect of CaBP 28K. *Kidney International* **45** 461–468.
- Brumbaugh PF, Hughes MR & Haussler MR 1975 Cytoplasmic and nuclear binding components for 1,25-dihydroxyvitamin D_3 in chick parathyroid glands. *PNAS* **72** 4871–4875.
- Brunette MG, Mailloux J & Lajeunesse D 1992 Calcium transport through the luminal membrane of the distal tubule. I. Interrelationship with sodium. *Kidney International* **41** 282–288.
- Castillo L, Tanaka Y, DeLuca HF & Sunde ML 1977 The stimulation of 25-hydroxyvitamin D_3 1 α -hydroxylase by estrogen. *Archives of Biochemistry and Biophysics* **179** 211–217.
- Cheema C, Grant BF & Marcus R 1989 Effects of estrogen on circulating free and total 1,25-dihydroxyvitamin D and on the parathyroid-vitamin D axis in post-menopausal women. *Journal of Clinical Investigation* **83** 537–542.
- Chen Z, Vaughn DA & Fanestil DD 1994 Influence of gender on renal thiazide diuretic receptor density and response. *Journal of the American Society of Nephrology* **5** 1112–1119.
- Chertow BS, Baker GR, Henry HL & Norman AW 1980 Effect of vitamin D on bovine parathyroid hormone release *in vitro*. *Clinical Journal of Physiology* **238** 384–388.
- Creighton A, Dey A, Romani W, James C, Cole FE, Hamm L & Lindberg AS 1999 The role of estrogen (E_2) on citrate and calcium excretion in postmenopausal women. *American Society of Nephrology Annual Meeting, Miami* (Abstract A3077).
- Davidoff M, Caffier H & Schiebler TH 1980 Steroid hormone binding receptors in the rat kidneys. *Histochemistry* **69** 39–48.
- De Vries J, Ludens J & Fanestil DD 1972 Estradiol renal receptor molecules and estradiol-dependent antinatriuresis. *Kidney International* **2** 95–100.
- Dick IM & Prince RL 1997 Estrogen effects on the renal handling of calcium in the ovariectomized perfused rat. *Kidney International* **51** 1719–1728.
- Dick IM, St-John A, Heal S & Prince RL 1996 The effect of estrogen deficiency on bone mineral density, renal calcium and phosphorus handling and calciotropic hormones in the rat. *Calcified Tissue International* **59** 174–178.
- Dignam WS, Voskian J & Assali NA 1956 Effects of estrogens on renal hemodynamics and excretion of electrolytes in human subjects. *Journal of Clinical Endocrinology and Metabolism* **16** 1032–1042.
- Doucet A & Katz A 1981 Mineralocorticoid receptors along the nephrons: ^3H aldosterone binding in rabbit tubules. *American Journal of Physiology* **241** 605–611.
- Enmark E, Peltö-Huikko M, Gradien K, Lagercrantz S, Lagercrantz J, Nokdenskjöld M & Gustafsson JA 1997 Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism* **89** 4258–4265.
- Farman N & Bonvalet JP 1983 Aldosterone binding in isolated tubules. III autoradiography along the rat nephron. *American Journal of Physiology* **245** F606–F614.
- Farman N, Vandewalle A & Bonvalet JP 1982 Autoradiographic study of aldosterone and dexamethasone binding in isolated glomeruli of rabbit kidney. *American Journal of Physiology* **243** F235–F242.
- Gill RK & Christakos S 1995 Regulation by estrogen through the 5' flanking region of the mouse calbindin D 28K gene. *Molecular Endocrinology* **9** 319–326.
- Han HJ, Jung JC & Taub M 1999 Response of primary rabbit kidney proximal tubule cells to estrogens. *Journal of Cellular Physiology* **178** 35–48.
- Heshemati HM, Khosla S, Burritt MF, O'Fallon WM & Riggs BL 1998 A defect in renal calcium conservation may contribute to the pathogenesis of postmenopausal osteoporosis. *Journal of Clinical Endocrinology and Metabolism* **83** 1916–1920.
- Hilal G, Claveau D, Leclerc M & Brunette MG 1997 Ca^{2+} transport by the luminal membrane of the distal nephron: action and interaction of protein kinases A and C. *Biochemical Journal* **328** 371–375.
- Huntson D 1975 Two techniques for evaluating small molecule–macromolecule binding in complex system. *Analytical Biochemistry* **63** 99–109.
- Imanaka S, Morimoto S, Onishi T, Takamoto S, Fukuo K, Koh E, Itoh K, Hironaka T, Shiraishi T, Morita R *et al.* 1998 Effect of estrogens on renal responsiveness to parathyroid hormone in elderly female subjects. *Endocrinologia Japonica* **35** 593–599.
- Johnson JA & Davis JO 1976 The effect of estrogens on renal sodium excretion in the dog. *Perspectives in Nephrology and Hypertension* **5** 239–248.
- Kelly A & Hamilton JR 1970 A microtechnique for the assay of intestinal alkaline phosphatase. Results in normal children and in children with celiac disease. *Clinical Biochemistry* **3** 33–43.
- Kuiper GGJM, Enmark E, Peltö-Huikko M, Nilsson S & Gustafsson JA 1996 Cloning a novel estrogen receptor expressed in rat prostate and ovary. *PNAS* **93** 5925–5930.
- Lajeunesse D, Bouhthiauy I & Brunette MG 1994 Parathyroid and hydrochlorothiazide increase calcium transport by the luminal membrane of rabbit distal nephron segments through different pathways. *Endocrinology* **134** 35–41.
- McKane WR, Khosla S, Burritt MF, Kao PC, Wilson DM, Ory SJ & Riggs BL 1995 Mechanism of renal calcium conservation with estrogen replacement therapy in women in early post menopause. A clinical research center study. *Journal of Clinical Endocrinology and Metabolism* **80** 3458–3464.
- Meyer R, Linz KW, Surges R, Meinardus S, Veas J, Hoffmann A, Windholz O & Grohé C 1998 Rapid modulation of L-type calcium

- current by acutely applied oestrogens in isolated cardiac myocytes from human, guinea-pig and rat. *Experimental Physiology* **83** 305–321.
- Morel F, Chabardes D, Imbert-Teboul M, le Bouffaut F, Hus-Cetharel A & Montegut M 1976 Functional segmentation of the rabbit distal tubule by microdetermination of hormone-dependent adenylate cyclase activity. *Kidney International* **9** 264–277.
- Morel F, Chabardes D, Imbert-Teboul M, le Bouffaut F, Hus-Cetharel A & Montegut M 1982 Multiple hormonal control of adenylate cyclase in distal segments of the rat kidney. *Kidney International* **21** S55–S62.
- Nordin BE, Need AG, Morris HA, Horowitz M & Robertson WA 1991 Evidence for a renal calcium leak in postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* **72** 401–407.
- Nordin BE, Horowitz M, Need A & Morris HA 1994 Renal leak of calcium in post-menopausal osteoporosis. *Clinical Endocrinology* **41** 41–45.
- Pantic V, Li J & Villed C 1974 Autoradiographic studies of the kidneys of hamsters bearing long term oestrogen implants. *Cytobiologia* **9** 89–99.
- Picotto G, Massheimer V & Boland R 1996 Acute stimulation of intestinal cell calcium influx induced by 17β-estradiol via the cAMP messenger system. *Molecular and Cellular Endocrinology* **119** 129–134.
- Post R & Sen A 1967 Sodium and potassium ATPase. *Methods in Enzymology* **10** 762–768.
- Preedy JRJ & Aitken EH 1956 The effect of estrogen on water and electrolyte metabolism. I. The normal. *Journal of Clinical Investigation* **35** 423–429.
- Russel J, Lettieri D & Sherwood LM 1986 Suppression by 1,25(OH)₂D₃ of transcription of the pre-proparathyroid hormone. *Endocrinology* **119** 2864–2866.
- Silver J, Naveh-Manly T, Mayer M, Schmelzer HJ & Popovtzer MM 1986 Regulation by vitamin D metabolites of parathyroid hormone gene transcription *in vivo* on the rat. *Journal of Clinical Investigation* **78** 1296–1301.
- Stock JL, Coderre JA, Burke EM, Danner DB, Chipman SD & Shapiro JR 1992 Identification of estrogen receptor mRNA and the estrogen modulation of parathyroid hormone-stimulated cyclic AMP accumulation in opossum kidney cells. *Journal of Cellular Physiology* **150** 517–525.
- Tanaka T, Castillo L, Wineland MJ & De Luca HF 1978 Synergistic effect of progesterone, testosterone and estradiol in the stimulation of chick renal 25 hydroxyvitamin D₃ 1α hydroxylase. *Endocrinology* **103** 2035–2039.
- Thorn GW, Nelson KR & Thorn DW 1938 A study of the mechanism of edema associated with menstruation. *Endocrinology* **22** 155–163.
- Tran TM, Zhang L & Verlander JW 1998 17β-Estradiol *in vitro* enhances expression of sodium transport protein in cultured mouse distal convoluted tubule (DCT) cells. *American Society of Nephrology Annual meeting, Philadelphia* (Abstract A0245).
- Verlander JW, Tran TM, Zhang L, Kaplan MR & Hebert SC 1998a Estradiol enhances thiazide-sensitive NaCl cotransporter density in the apical plasma membrane of the distal convoluted tubule of ovariectomized rats. *Journal of Clinical Investigation* **101** 1661–1669.
- Verlander JW, Wilber W & Zhang L 1998b Immunolocalization of estrogen receptor-α (ERα) in rat renal cortex. *American Society of Nephrology Annual Meeting, Philadelphia* (Abstract A2083).
- Zuo Q, Claveau D, Hilal G, Leclerc M & Brunette MG 1997 Effect of calcitonin on calcium transport by the luminal and basolateral membranes of the rabbit nephron. *Kidney International* **51** 1991–1999.

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