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A novel vasopressin-induced transcript promotes MAP kinase activation and ENaC downregulation

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In the principal cell of the renal collecting duct, vasopressin regulates the expression of a gene network responsible for sodium and water reabsorption through the regulation of the water channel and the epithelial sodium channel (ENaC). We have recently identified a novel vasopressin-induced transcript (VIT32) that encodes for a 142 amino acid vasopressin-induced protein (VIP32), which has no homology with any protein of known function. The *Xenopus* oocyte expression system revealed two functions: (i) when injected alone, VIT32 cRNA rapidly induces oocyte meiotic maturation through the activation of the maturation promoting factor, the amphibian homolog of the universal M phase trigger Cdc2/cyclin; and (ii) when co-injected with the ENaC, VIT32 cRNA selectively downregulates channel activity, but not channel cell surface expression. In the kidney principal cell, VIP32 may be involved in the downregulation of transepithelial sodium transport observed within a few hours after vasopressin treatment. VIP32 belongs to a novel gene family ubiquitously expressed in oocyte and somatic cells that may be involved in G to M transition and cell cycling.

Keywords: cell cycle/ERK/meiotic maturation/progesterone/sodium transport

Introduction

Vasopressin and aldosterone actions on the kidney are crucial for the maintenance of water and sodium balance, and for the control of extracellular fluid, blood volume and blood pressure (for reviews, see Schafer, 1994; Verrey *et al*., 2000; Schrier *et al*., 2001). Vasopressin main renal target cell is the principal cell of collecting ducts (CD), where it controls water and sodium reabsorption through the activation of water channels (aquaporin 2) and epithelial sodium channels (ENaC), respectively. Both actions of vasopressin are mediated by the occupancy of a vasopressin receptor (V2R), a GPCR located in the basolateral membrane, coupled to adenylate cyclase (for a review, see Bankir, 2001). The effect of vasopressin (or antidiuretic hormones) on transepithelial sodium transport is synergistic with that of aldosterone; this synergism was demonstrated in amphibian epithelia (Girardet *et al*., 1986) and in isolated perfused rat cortical collecting duct (CCD; Tomita *et al*., 1985; Reif *et al*., 1986; Chen *et al*., 1990), indicating that this dual regulation is highly conserved throughout evolution. In both experimental systems, the effect on sodium transport is rapid (within 5 min), peaks within 60–120 min and then declines progressively to reach a new steady state higher than that of an unstimulated epithelium (Girardet *et al*., 1986; Djelidi *et al*., 1997). The short-term effect of vasopressin is actinomycin D-independent, indicating a non-genomic regulation of sodium reabsorption through the activation of a V2R, leading to a rapid increase in intracellular cAMP. However, up to 50% of the sodium transport response becomes actinomycin D-sensitive after longer hormonal exposure (2–4 h), suggesting that the peptide hormone mediates part of its effect through transcription (Girardet *et al*., 1986; Djelidi *et al*., 1997). Thus, vasopressin also participates to the long-term genomic regulation of renal sodium and water reabsorption through a cAMP-dependent transcriptional activation of a gene network. This network includes the water channel aquaporin 2 (Promeneur *et al*., 2000) and the ENaC (Ecelbarger *et al*., 2000). The analysis of the transcriptome of principal cells of the mouse renal CCD by serial analysis of gene expression (SAGE) has recently revealed that the vasopressin-regulated gene network may encompass over 50 different mouse genes: 48 vasopressin-induced transcripts (mVIT) and 11 vasopressin-repressed transcripts (mVRT; Robert-Nicoud *et al*., 2001). One of them, mVIT32, corresponded to a cluster of orphan expression sequence tags (ESTs). Northern blot hybridization confirmed the vasopressin-induced expression of a mRNA corresponding to the selected SAGE library tag (Robert-Nicoud *et al*., 2001). The induction was present at 4 h and, unlike other VITs, was maintained at 24 h, suggesting a role in either the downregulation and/or the maintenance of a new steady state in sodium transport. Since the sequence of mVIT32 did not reveal any homology with known functional protein domains, the aim of this study was to define its possible functions on the assumption that, as a vasopressin-induced transcript, mVIT32 could up- or downregulate the activity of ENaC in the principal cell of the renal CCD. We tested this possibility using the *Xenopus* oocyte heterologous expression system, which has proven to be sensitive and specific for the detection of functional interactions between ENaC subunits (Canessa *et al*., 1994), ENaC repression by the ubiquitin ligase Nedd-4 (Kamynina *et al*., 2001) and ENaC activation by the channel activating serine protease CAP-1 (Vallet *et al*., 1997) or by the serum- and glucocorticoid-

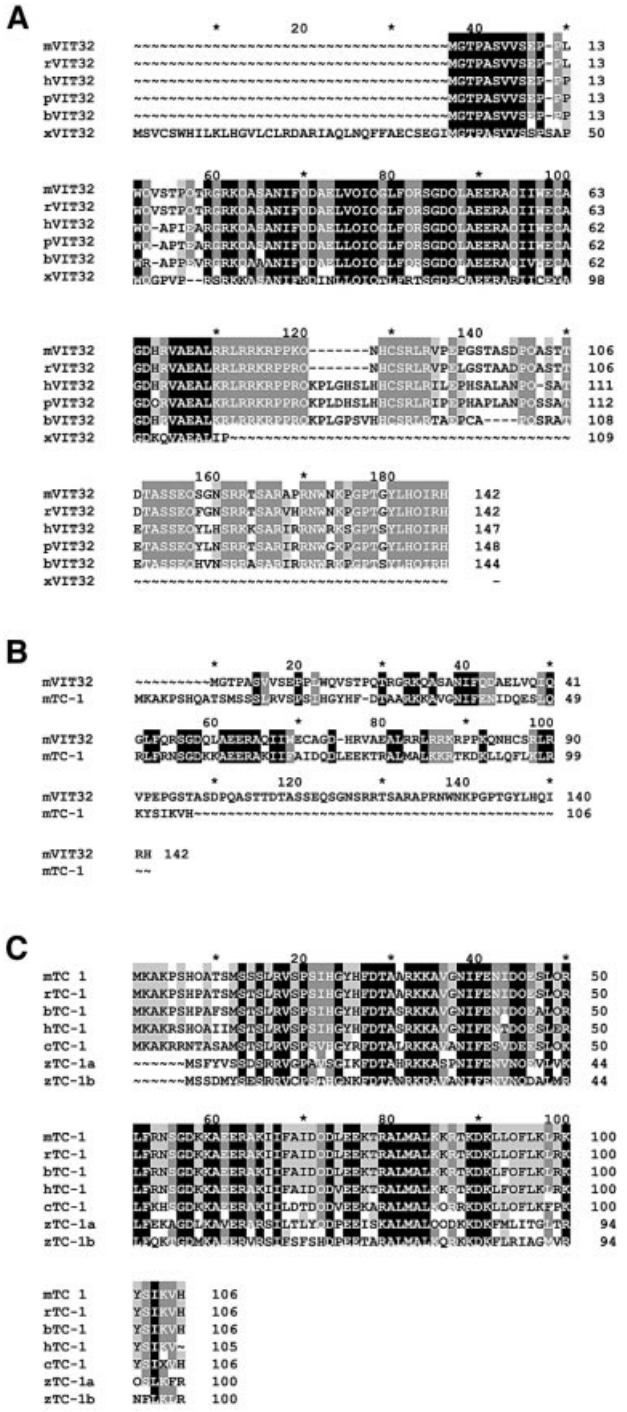

Fig. 1. (**A**) Alignment of ortholog VIT32 proteins from mouse, rat, human, pig, bovine and frog (*Xenopus laevis*). Mouse (m), rat (r) and human (h) VIT32 amino acid sequences are available at the DDBJ/EMBL/GenBank databases as hypothetical proteins with unknown function (accession Nos: BAB26163, NP_599200 and NP_068378, respectively). Pig (p), bovine (b) and *Xenopus laevis* (x) VIT32 sequences were obtained by alignment of multiple ESTs. (**B**) Comparison of amino acid sequences of mVIT32 and mTC-1. The mTC-1 amino acid sequence is available at the DDBJ/EMBL/GenBank databases under accession No. BAB25041. (**C**) Alignment of ortholog TC-1 proteins from mouse, rat, bovine, chicken, human and zebra fish. The hTC-1 amino acid sequence is available at the DDBJ/EMBL/GenBank databases under accession No. NP_064515. Rat (r), bovine (b), chicken (c) and zebra fish (z) TC-1 sequences were obtained by alignment of multiple ESTs.

regulated kinase Sgk1(Chen *et al*., 1999). To our surprise, we observed that mVIT32 cRNA injected alone was able to induce rapidly and efficiently oocyte maturation, suggesting the activation of the MAP kinase cascade. We find that mVIT32 is a potent inducer of amphibian oocyte maturation and mimics the effect of progesterone. In addition, mVIT32 cRNA, when co-injected with ENaC, selectively downregulates its activity without changing its cell surface expression.

Results

mVIT32 is a member of a novel gene family

A 0.85 kb cDNA was obtained and sequenced (see Materials and methods). The deduced protein sequence of mVIT32 (Figure 1A) predicts a chain of 142 amino acids, probably located in the cytoplasm. Ortholog proteins from rat, human, pig, bovine and frog were identified in EST databases sharing a high degree of identity (Figure 1A).

As shown in Figure 2, vasopressin led to a time- and dose-dependent increase of mVIT32 transcripts in the mpk CCD cell line, which was used to establish the SAGE library. Vasopressin increased VIT32 mRNA abundance as early as 1 h after vasopressin addition and reached a maximum at 4 h, but remained elevated at 24 h (Figure 2A). The dose–response curve (Figure 2B) shows a $K_{1/2}$ at ~10 pM, within the physiological range of hormone action. The effect of vasopressin was actinomycin D-sensitive and cycloheximide-resistant, suggesting that it is transcriptionally mediated (data not shown).

A human paralog, sharing 25% identity with mVIT32 (Figure 1B), has been previously identified by differential

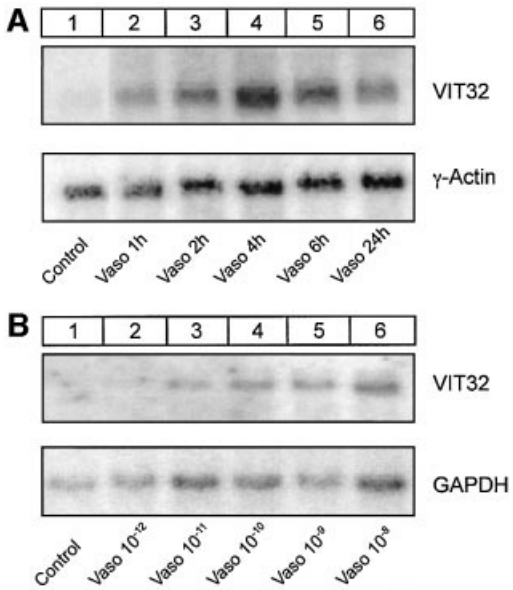

Fig. 2. Vasopressin regulates mVIT32 mRNA abundance in mpkCCD cells. (**A**) Time course. Northern blot analysis with mVIT32 probe was performed on mRNAs extracted from untreated mpkCCD cells (control) or vasopressin (10^{-8} M)-stimulated mpkCCD cells after indicated period of time. (**B**) Dose response. Northern blot analysis was performed on mRNAs extracted from untreated mpkCCD cells (control) or mpkCCD cells stimulated for 4 h with vasopressin at different concentrations.

hybridization as one of the transcripts upregulated in human thyroid cancer (hTC-1; Chua *et al*., 2000). TC-1 ortholog proteins from mouse, rat, bovine, chicken and zebra fish (two proteins) were also identified in EST databases (Figure 1C). According to genomic information presently available, mVIT32 and hTC-1 belong to a novel small gene family found only in vertebrates.

Northern blot analysis of various mouse tissues revealed an ubiquitous expression of mVIT32 transcripts of one size (1 kb) in brain, heart, liver, skeletal muscle, spleen and testis, and of two sizes (1 and 1.4 kb) in lung and kidney. mTC-1 transcripts of two sizes (1.8 and 1.45 kb) were detected in the same tissues with the exception of brain and testis (Figure 3).

mVIT32 mimics progesterone-induced maturation of Xenopus oocytes

When mVIT32 cRNA was injected alone in *Xenopus* oocytes, meiotic maturation was consistently observed a few hours after injection. In the *Xenopus laevis*, oocyte maturation can be conveniently monitored and quantitated by scoring the appearance of a white spot resulting from rearrangement of cortical pigment granules at the oocyte animal pole, indicating that germinal vesicular breakdown (GVBD) has occurred (Ferrell, 1999). Injection of 5 ng of mVIT32 cRNA induced maturation in 100% of oocytes within a median time of 9.1 ± 0.9 h (mean ± SD; Figure 4C, I and O), while progesterone induced oocyte maturation within 7.7 ± 0.6 h (Figure 4E, K and Q). Control (water injected) oocytes did not show any GVBD when observed for as long as 21 h post injection (Figure 4A, G and M). mVIT32 appears to fully mimic with a 2-h delay progesterone-induced maturation.

mVIT32 activates the MAP kinase signaling cascade upstream of MPF

The effect of mVIT32 cRNA is rapid if one takes into account the time necessary for the cRNA to be translated and enough of the protein accumulated within the oocyte. This suggests that mVIT32 could act early in the progesterone-dependent signaling cascade. It implies three main possible steps (Ferrell, 1999; Maller, 2001): (i) binding to a as yet uncharacterized progesterone receptor; (ii) inhibition of adenylate cyclase with a drop in cAMP concentration; and (iii) activation of MAP kinase cascade involving Mos (a MAPKKK), MEK1 (a MAPKK) and ERK2 (the p42MAPK), which activates maturation promoting factor (MPF). MPF consists of a complex of Cdc2 and cyclin B. Upon progesterone maturation, MPF kinase activity can be conveniently monitored by an *in vitro* phosphorylation assay, using histone H1 as a

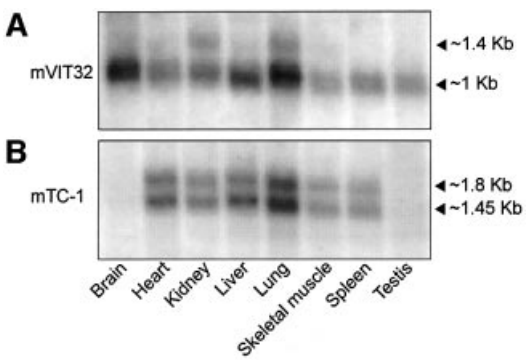

Fig. 3. (**A**) Northern blot analysis of mVIT32 mRNA expression in mouse tissues. In the majority of tested tissues (brain, heart, liver, skeletal muscle, spleen, testis), a single mVIT32 transcript of 1 kb long is present. In kidney and lung, an additional minor transcript of 1.4 kb long is expressed. (**B**) Northern blot analysis of mTC-1 mRNA expression in mouse tissues. In most of the tested tissues (heart, kidney, liver, lung, skeletal muscle and spleen), two mTC-1 transcripts of 1.45 and 1.8 kb long are present. In brain and testis, no TC-1 expression was detected.

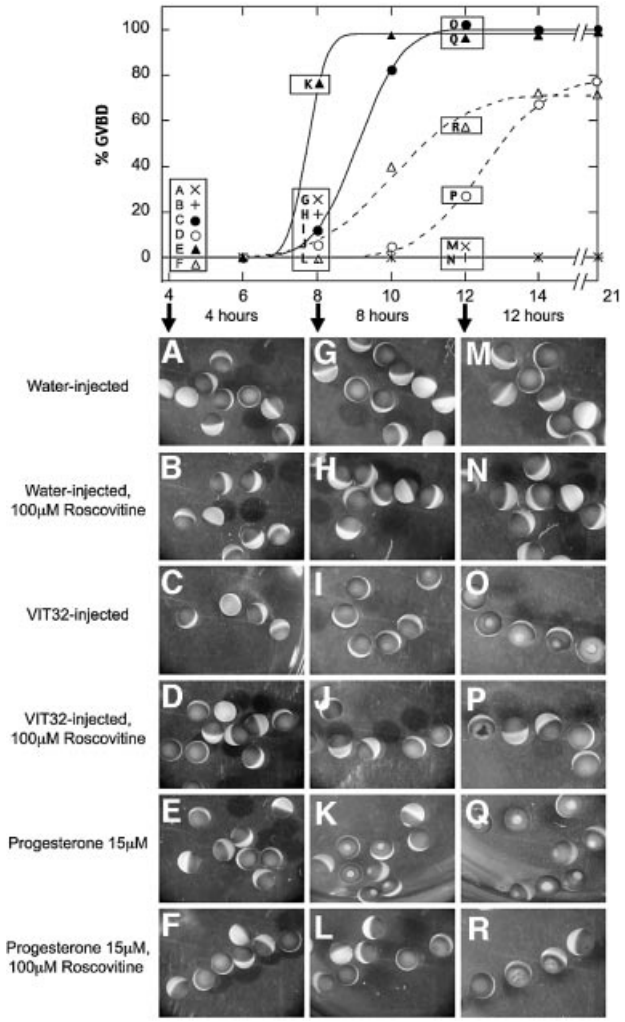

Fig. 4. Induction of oocyte maturation by mVIT32. GVBD is scored by the appearance of a white spot on the oocyte animal pole reflecting maturation. Water-injected oocytes incubated with DMSO (0.5%) (x on the top panel; **A**, **G** and **M**) or with roscovitine (100 μM) (+ on the top panel; **B**, **H** and **N**) show no maturation. GVBD was delayed by 2 h for oocytes injected with mVIT32 cRNA (filled circles on the top panel; **C**, **I** and **O**), compared with progesterone (15 μM)-incubated oocytes (filled triangles on the top panel; **E**, **K** and **Q**). In presence of roscovitine (100 μM), mVIT32- (open circle on the top panel; **D**, **J** and **P**) and progesterone-induced (open triangle on the top panel; **F**, **L** and **R**) maturation was delayed by 4 h. At 12 h, mVIT32- and progesterone-induced maturation had apparently different morphologic features, depending on the presence of roscovitine. The percentage of oocytes with GVBD as a function of time after treatment was evaluated in groups of 38–40 oocytes. The best fitting cumulative distribution functions are shown as lines. The parameters of these functions are indicated in the text.

substrate (Rempel *et al*., 1995). Using this assay, we can show that progesterone induces the MPF kinase activity after 13 h of exposure [Figure 5A, lane 2 (progesterone) versus lane 1 (control)]. mVIT32-injected oocytes (Figure 5A, lane 4 versus 3) showed a dramatic increase in kinase activity. The time-dependent effect of progesterone and mVIT32 on the histone kinase activity is shown in Figure 5C. These effects precisely parallel the appearance of GVBD (Figure 4).

To examine whether mVIT32 acts downstream or upstream of MPF, we used the specific MPF inhibitor roscovitine (Meijer *et al*., 1997). As shown in Figure 4D, J and P, roscovitine fully prevented the appearance of the characteristic white spot (Figure 4Q) at the animal pole, indicative of a physiological GVBD. Instead, an abnormal rearrangement of pigments (in some instances as a brown spot, Figure 4P) was observed in ~70–80% of the oocytes with a lag period of 2–3 h longer (median time: 12.5 ± 0.9 versus 10.0 ± 1.3 h) than that observed with progesterone or mVIT32 alone. Roscovitine *per se* did not induce meiotic maturation (Figure 4B, H and N). As expected, roscovitine was able to fully inhibit histone phosphoryl-

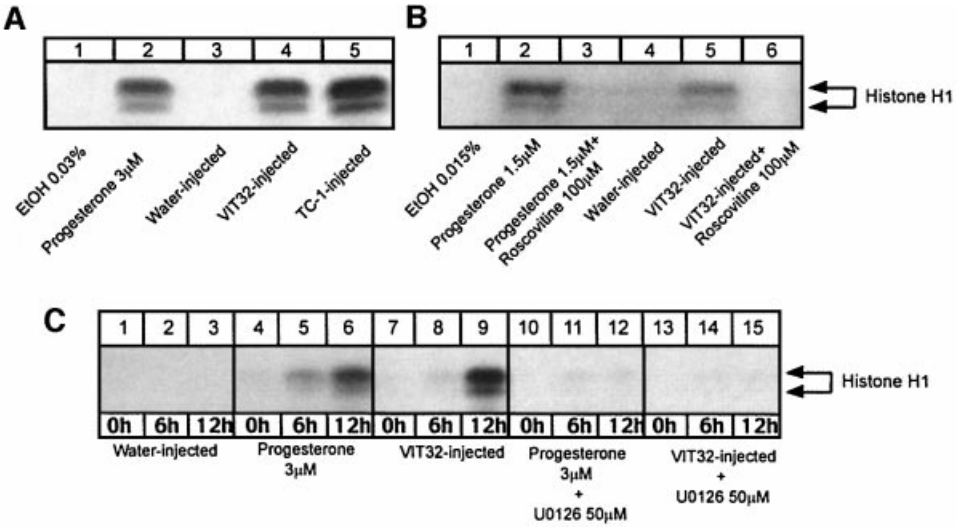

Fig. 5. Kinase activity of Cdc2–cyclin B complexes. (**A**) mVIT32 and mTC-1 cRNAs injection induce Cdc2 activity in *Xenopus* oocytes. Cdc2 activity in total *Xenopus* oocytes protein extracts was tested by histone H1 phosphorylation assay (see Materials and methods). Oocyte protein extracts were prepared either from oocytes treated for 13 h with EtOH (0.03%) or progesterone (3 µM) or from oocytes incubated for 13 h after water or mVIT32 cRNA injection. Protein extracts prepared from EtOH-treated (lane 1) and water-injected oocytes (lane 3) show no phosphorylation of histone H1. Progesterone treatment (lane 2) as well as mVIT32 (lane 4) or mTC-1 (lane 5) cRNA injection leads to induction of histone H1 phosphorylation. This experiment was repeated on three independent batches of oocytes with similar results. (**B**) Roscovitine inhibits the mVIT32-induced Cdc2 activity. Roscovitine (100 µM) was added to oocytes simultaneously with progesterone or immediately after cRNA injection. The kinase assay was performed 13 h after progesterone addition or mVIT32 cRNA injection. Ethanol-treated (lane 1) and water-injected oocytes (lane 4) show no phosphorylation of histone H1. Progesterone-induced kinase activity (lane 2) is fully inhibited by roscovitine (lane 3). mVIT32-induced kinase activity (lane 5) is fully inhibited by roscovitine (lane 6). (**C**) UO126 inhibits the mVIT32- and progesterone-induced kinase activity. Oocytes were incubated with or without UO126 (50 µM) 12 h before progesterone stimulation or cRNA injection. UO126 (50 µM) was also present in the oocyte incubating medium after progesterone stimulation or cRNA induction. The kinase activity was measured 0, 6 and 12 h after progesterone stimulation or cRNA injection. Water-injected oocytes (lanes 1–3) show no phosphorylation of histone H1. Progesterone (lanes 4–6) induces kinase activity as early as 6 h after treatment. mVIT32- (lanes 7–9) induced histone H1 phosphorylation is delayed and occurs after 12 h (lane 9). UO126 fully inhibits progesterone (lanes 10–12), and mVIT32 (lanes 13–15) induced kinase activity.

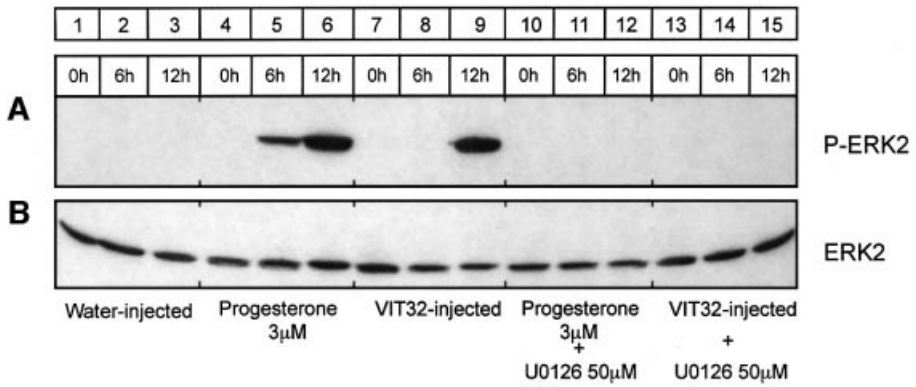

Fig. 6. mVIT32 injection and progesterone treatment induce ERK2 phosphorylation. Immunoblotting with antibodies directed against ERK1/2 and phospho-ERK1/2 was performed on the same oocyte protein extracts as those used for detection of Cdc2 kinase activity (Figure 5C). Water-injected oocytes (lanes 1–3) show no phosphorylation of ERK2. Progesterone- (lanes 4–6) and mVIT32- (lanes 7–9) induced ERK2 phosphorylation was fully inhibited by UO126 (lanes 10–15). mVIT32-induced ERK2 phosphorylation is delayed compared to the progesterone effect.

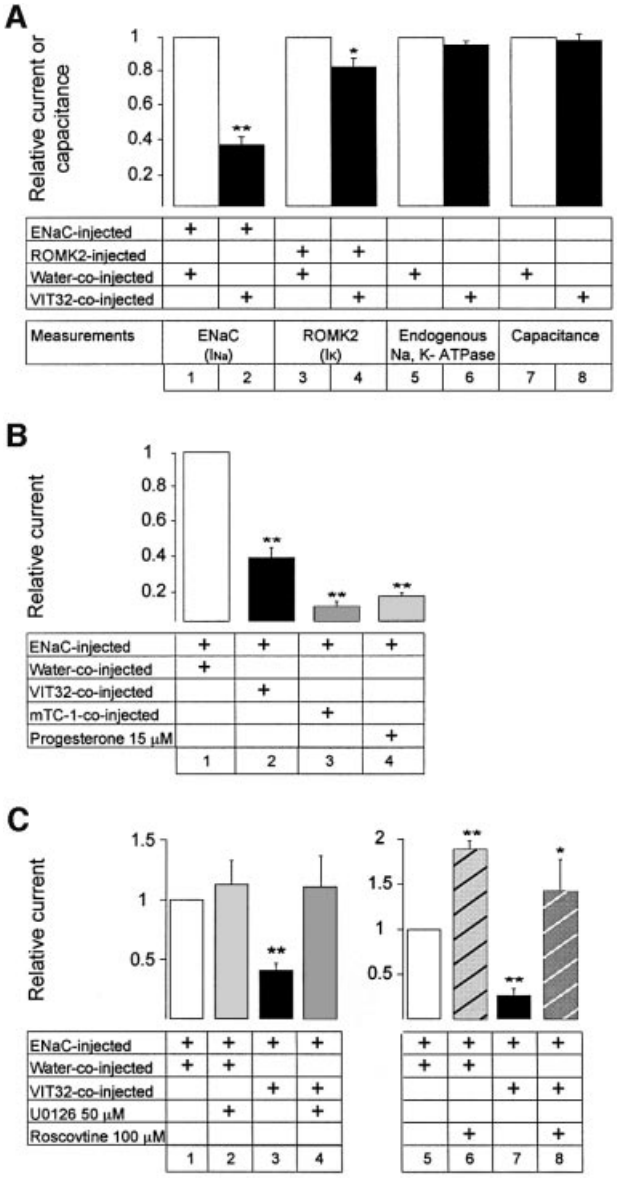

Fig. 7. For all conditions, 4–8 experiments were performed with at least five oocytes measured per condition. Oocytes were injected with 1 ng of cRNAs of each α, β and γ subunit of ENaC or with 0.2 ng of ROMK2 cRNA. About 12 h later, oocytes were co-injected with water, mVIT32 or mTC-1 cRNA, or treated with progesterone. Five hours later, electrophysiological measurements were made to test ion channel macroscopic currents (I_{Na} and I_K), endogenous Na,K-ATPase activity and membrane capacitance. (**A**) Effect of mVIT32 on ENaC and ROMK2 currents, on oocyte capacitance and on endogenous Na,K-ATPase activity. The absolute values for ENaC, ROMK2 and the endogenous Na,K-ATPase currents was 917 ± 297 nA, 475 ± 43 nA and 65 ± 10 nA, respectively. The absolute value of oocyte capacitance was 274 ± 21 nF. mVIT32 significantly decreases I_{Na} (lane 1 versus 2) with little effect on I_K (lane 3 versus 4). mVIT32 has no effect on endogenous Na,K-ATPase activity (lane 5 versus 6) and oocyte membrane capacitance (lane 7 versus 8). (**B**) Effect of mVIT32, mTC-1 and progesterone on ENaC current. mVIT32 (lane 2), mTC-1 (lane 3) and progesterone (lane 4) downregulate ENaC activity (lane 1). (**C**) U0126 and roscovitine inhibit the effect of mVIT32 on ENaC current. UO126 (lane 4) and roscovitine (lane 8) restore the sodium current downregulated by mVIT32 (lanes 3 and 7). *$p < 0.05$; **$p < 0.001$ (Student's *t*-test).

ation induced by progesterone (Figure 5B, lane 3 versus 2), as well as the phosphorylation induced by mVIT32 (Figure 5B, lane 6 versus 5). Diluent- (lane 1) or water-injected oocytes (lane 4) were negative for background phosphorylation. These data indicate that mVIT32 acts upstream of MPF. To test this possibility further, we used UO126, an inhibitor of MEK1 (a MAPKK). As shown in Figure 5C (lanes 10–15), both the effects of progesterone and mVIT32 were fully inhibited by UO126.

Since mVIT32 activates MEK1, we tested the phosphorylation of the extracellular regulated kinase 2 (ERK2), a known substrate for MEK1. This signaling pathway operates in the oocyte, in the principal cell of CD during branching morphogenesis (Fisher *et al*., 2001) and in adult kidney (W.Tian *et al*., 2000). As shown in Figure 6A, progesterone induces a time-dependent increase in the phosphorylation of ERK2 (lanes 4–6). mVIT32 had a similar effect at 12 h (lane 9), but not at 6 h (lane 8). As expected, both effects were fully inhibited by UO126 (lanes 10–15). The total biochemical pool of ERK2 was measured by an antibody recognizing both phosphorylated and non-phosphorylated ERK2 (see Materials and methods). As shown in Figure 6B, the total biochemical pool was unchanged.

The mouse homolog of the hTC-1 was cloned (mTC-1) and mTC-1 cRNA was injected into oocytes. The effect of mTC-1 cRNA on oocyte maturation was compared with that of mVIT32 cRNA. The two cRNAs were equally potent in promoting oocyte maturation (data not shown) and histone phosphorylation (Figure 5A).

mVIT32 downregulates ENaC activity before any change occurs in membrane capacitance

GVBD is accompanied by a major endocytic process, whereby up to 50% of the plasma membrane with its microvilli is internalized. This process leads to a dramatic decrease in membrane capacitance. The expression of endogenously expressed plasma membrane protein (Na,K-ATPase; Vasilets *et al*., 1990; Pralong Zamofing *et al*., 1992) or exogenously expressed channels is greatly diminished during oocyte maturation (Bruggemann *et al*., 1997; Shcherbatko *et al*., 2001). mVIT32 is expressed in the principal cell of the renal CCD where it could interact synergistically or antagonistically with the amiloride-sensitive sodium channel. We therefore examined the effect of mVIT32 on ENaC activity during the first 5 h after mVIT32 cRNA injection at a time where there is neither GVBD nor induced changes in membrane capacitance. As shown in Figure 7A, mVIT32 selectively downregulated the activity of exogenously expressed ENaC by 70% (lane 2 versus 1, $p < 0.001$) with little effect (–18%, lane 3 versus 4, $p < 0.05$) on an exogenously expressed potassium channel (ROMK-2), a channel that co-localizes with ENaC at the apical membrane of the principal cell. mVIT32 has no significant effect on endogenously expressed Na,K-ATPase (Figure 7A, lane 5 versus 6) or membrane capacitance (lane 7 versus 8). These data indicate a selective and early effect of mVIT32 on the sodium channel. Likewise (Figure 7B), progesterone (lane 4) and mTC-1 (lane 3), downregulated ENaC activity (lane 1). The specific effect of mVIT32 on ENaC activity (Figure 7C) can be efficiently blocked by UO126 (lanes 1–4) and by roscovitine (lanes 5–8). Our data

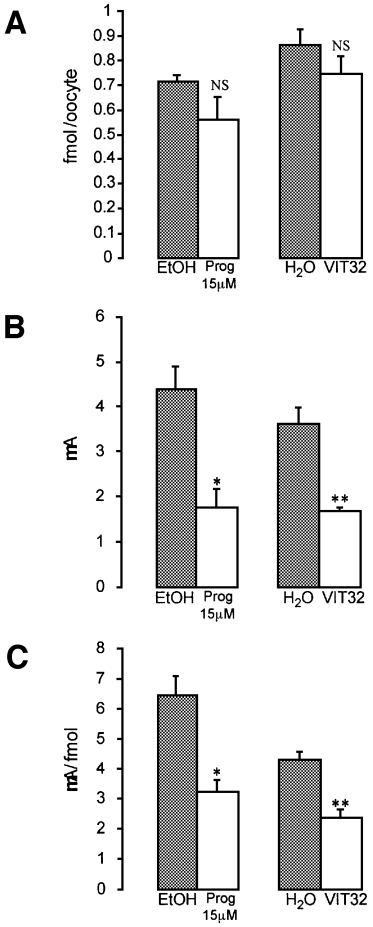

Fig. 8. Effect of mVIT32 and progesterone on ENaC current and ENaC cell surface expression. Oocytes were injected with 2 ng of each α, β and γ subunit of ENaC cRNA. Twelve hours later, these oocytes were injected with 3 ng of mVIT32 cRNA or with the same volume of water, or treated with either 15 μM progesterone or ethanol (0.15%). Eight hours later, cell surface expression of ENaC was measured. I_{Na} was measured on the same oocytes 1 h after measurement of ENaC cell surface expression. In each group, ENaC cell surface expression and I_{Na} were measured on 30–36 oocytes. (**A**) Cell surface expression of ENaC. Neither progesterone nor mVIT32 affect cell surface expression of ENaC. (**B**) ENaC current. I_{Na} was significantly decreased under progesterone and mVIT32 effects. (**C**) I_{Na}:ENaC cell surface expression ratio. This ratio is proportional to the total channel open probability and is significantly decreased by progesterone and mVIT32. NS, not significant; $*p < 0.05$; $**p < 0.001$ (Student's *t*-test).

suggest that mVIT32 acts upstream of MPF activation (roscovitine experiment) and upstream of MEK1 (UO126 experiment). It is worthwhile noting that roscovitine *per se* significantly increased ENaC activity (Figure 7C, lane 6 versus 5), suggesting that MPF may exert a repressor effect on basal ENaC activity in the *Xenopus* oocyte expression system. The downregulation of ENaC activity could be due to an effect on its open probability and/or its cell surface expression (Firsov *et al*., 1996, 1997). To distinguish between these two possibilities, we used an assay that measures, in the same oocyte, the binding of ^{125}I radio-iodinated monoclonal antibody against a FLAG epitope inserted in the ectodomain of each ENaC subunit. The sodium transport was measured in the same oocytes by two-electrode voltage clamp (Firsov *et al*., 1996, 1997). As shown in Figure 8A, neither progesterone nor mVIT32 induced any significant change in cell surface expression of ENaC whereas I_{Na} was significantly decreased (Figure 8B). The I_{Na}:binding ratio, which is directly proportional to the average open probability (Po) of all channels expressed at the cell surface of the oocyte (Firsov *et al*., 1996, 1997), is significantly decreased by progesterone and mVIT32 (Figure 8C).

Discussion

We previously reported the primary structure of the ENaC (Canessa *et al*., 1994), which is tightly controlled by aldosterone and vasopressin to achieve sodium balance. To dissect the signaling cascades involved in the fine regulation of ENaC, we have undertaken the analysis of the transcriptome of principal cells of the renal CCDs, stimulated by vasopressin or aldosterone. Using SAGE, we have identified 48 VITs and selected mVIT32 for further functional characterization. With this kind of screen, we were confronted with the challenging task of identifying the function of an induced transcript, which encodes a protein without any homology with a known protein or protein domain. As a new example of serendipity, we were helped by the fact that mVIT32 induces meiotic maturation in the heterologous expression system (*Xenopus* oocyte) we routinely use to look at possible interactions between ENaC and candidate genes regulating sodium transport. Our data provide evidence for two functions that might be related: (i) the activation of the MAP kinase cascade; and (ii) the downregulation of ENaC activity. We would like to discuss our data along three lines: (i) possible role of VIT32 in the fine control of sodium transport in the principal cell of the CD; (ii) possible role of VIT32 in cell replication during embryonic kidney development or in adult kidney; and (iii) a more general role of VIT32 (since it is ubiquitously expressed) in the meiotic maturation of the oocyte and in the cell cycle of somatic cells.

Possible role of VIT32 in the fine control of sodium transport in the principal cell of the CD

Vasopressin and aldosterone play a major role in controlling water and sodium balance by regulating the activity of their two main effectors, i.e. aquaporin 2 and the ENaC, respectively. ENaC activity must be finely regulated in order to achieve sodium and osmotic balance within a small range, despite large variations in salt and water intake. Aldosterone and vasopressin act synergistically to upregulate sodium transport in the principal cell of the CCD. Vasopressin has been recently shown to upregulate the expression of aquaporin 2 and ENaC mRNA *in vivo* (Ecelbarger *et al*., 2000; Promeneur *et al*., 2000). In the toad bladder system *in vitro*, it was shown that the long-term effect (24 h) of antidiuretic hormone on the amiloride-sensitive electrogenic sodium transport was always lower than the peak effect, but higher than the control unstimulated preparations (Girardet *et al*., 1986). In cultured CCD cells, a similar observation was made. For vasopressin, this downregulation appears to be independent of receptor desensitization (Dublineau *et al*., 1989). One can postulate that such fine tuning requires a balance between factors that up and downregulate ENaC activity. We provide evidence for a novel signaling pathway by

which a vasopressin-induced protein (VIP) could activate a MAP kinase cascade, leading to a marked down regulation of ENaC at the plasma membrane. ENaC activity is primarily regulated by factors controlling the number (N) of channels at the plasma membrane and/or its Po. During the rapid and non-genomic phase of hormone action in kidney cells, it has been proposed that vasopressin (or cAMP) could control N (Morris and Schafer, 2002) and Po (Marunaka and Eaton, 1991). We show here that downregulation of ENaC activity takes place without a significant change in cell surface expression (Figure 8). Our data are best interpreted by a decrease in Po, as discussed previously (Firsov *et al*., 1997), for a loss-of-function mutation of the β subunit of ENaC (βG37S) causing pseudohypoaldosteronism type 1 (PHA-1; Grunder *et al*., 1997). We propose that this effect could be related to the downregulation of sodium transport observed after long-term exposure to vasopressin that leads to a new steady state. This new steady state is caused by long-term genomic effects of vasopressin that may imply a new balance between an increase in N, mediated by increased transcription of ENaC mRNA (Ecelbarger *et al*., 2000) and a decrease in Po, mediated by the MAP kinase cascade (present study). The molecular mechanisms by which mVIT32 (or progesterone) mediates its effect on Po is not understood. A recent paper reports the effect of ERK2 on ENaC phosphorylation and activity (Shi *et al*., 2002). Using a number of different approaches, Shi and co-workers were able to demonstrate that a protein kinase acting on the phosphorylation of two threonine residues, respectively located on the C-terminus of β or γ ENaC (βT613 and γT623), is indeed ERK2, a member of the ERK gene family. The authors suggested that a ERK-mediated phosphorylation of βT613 and γT623 down-regulates ENaC activity by facilitating its interaction with Nedd-4, implying a decreased cell surface expression of ENaC. Our data suggest an additional mechanism by which Po is also downregulated by the MAP kinase cascade. Our present working hypothesis is that such a signaling pathway also exists in the kidney and the data reviewed above are compatible with this proposal. It is now important to test this novel regulatory cascade in kidney cells experimentally, either *in vitro* or *in vivo*.

Possible role of VIT32 in cell replication during embryonic kidney development or in adult kidney

The role of MAPK signaling in the kidney has been recently reviewed extensively (W.Tian *et al*., 2000). It is clear that a very large number of factors (or agonists) could regulate MAPK, both during development or in adult kidney. In the developing mouse kidney, Fisher and co-workers recently reported that the MEK–ERK kinase pathway is normally active in the ureteric bud, the developing urinary CD of the metanephric kidney, and that its inhibition reversibly inhibited branching morphogenesis (Fisher *et al*., 2001).

Does VIT32 *per se* trigger, or contribute to trigger, the cell cycle in mpkCCD cells? A simple explanation would be that this cell line is de-differentiated and responds to a vasopressin-dependent signaling cascade that does not take place under physiological circumstances, since only a few percent of principal cells normally replicate *in vivo*. mpkCCD has been shown, however, to be a highly differentiated epithelial cell line and to express an aldosterone- and vasopressin-regulated sodium transport (Bens *et al*., 1999; Vandewalle *et al*., 1999). Thus, alternative explanations can be discussed. VIT32 would be able to trigger cell replication in the kidney *in vivo* under physiological or pathophysiological circumstances. A prolonged increase in the sodium concentration within renal epithelial cells caused by chronic salt and water deprivation *in vivo* has been shown to cause dramatic hypertrophy and hyperplasia *in vivo*, suggesting increased cell replication (Stanton and Kaissling, 1989). Hypertonicity and hypotonicity have been shown to activate the ERK pathway in a variety of cultured cells, including MDCK and inner medulary collecting duct (IMCD; for a review, see W.Tian *et al*., 2000). Cell-cycle delay and apoptosis are induced by high salt and urea in cultured renal inner medullary cells (mIMCD3; Michea *et al*., 2000), cells that are expected to be exposed to hypertonicity under water restriction, a potent stimulus for vasopressin secretion. The effect of vasopressin in mIMCD3 on the cell cycle was, however, not studied. It will be interesting to study the cell cycle and specifically G to M transition in mpkCCD cell lines, originating from the renal cortex, which is physiologically not exposed to hyperosmolar stresses. The responses to osmotic and/or vasopressin might be quite different in the two cell lines.

Another intriguing possibility for a physiological role of VIT32 in the kidney is its possible involvement in the progesterone signaling pathway in the principal cell itself. Progesterone *in vivo* is natriuretic by its well known antagonist effect on aldosterone-dependent sodium transport (Wambach and Higgins, 1978; Geller *et al*., 2000). Progesterone is a potent competitive antagonist for aldosterone on the mineralocorticoid receptor (Wambach and Higgins, 1978). The natriuretic effect of progesterone has been assumed to be a classical genomic effect on the aldosterone signaling cascade. In the *Xenopus* oocyte, a classical progesterone receptor has recently been cloned and proposed to mediate maturation through a non-genomic mechanism (Bayaa *et al*., 2000; J.D.Tian *et al*., 2000). Whether such a mechanism exists in somatic kidney cells can now be tested experimentally.

More general role of VIT32 in the meiotic maturation of the oocyte and in the cell cycle of somatic cells

Stage VI oocytes are arrested at the G_2 to M transition of the first meiotic division. Progesterone can overcome the block, inducing GVBD, spindle formation and meiosis I. The mature oocyte is then again arrested at the metaphase of meiosis II until fertilization. This experimental system has been instrumental in identifying components of the MAP kinase cascade, MPF and cell cycle regulatory proteins (for a review, see Ferrell, 1999). The early steps in oocyte maturation are far from being understood. Recently, a product of novel *Xenopus* genes (Speedy or Spy1; Lenormand *et al*., 1999), was shown to induce rapid oocyte maturation, resulting in GVBD and MPF activation through the activation of MAPK pathway. VIT32 shares these properties with Spy, but differs in an important aspect, namely that while Spy1 leads to the premature activation of Cdc2 (Ferrell, 1999), i.e. before any detectable effect of progesterone, VIT32 does not (Figure 6).

The difference may be species specific: VIT32 is a mouse gene, whereas Spy1 was cloned from a *Xenopus* ovary cDNA library (Lenormand *et al*., 1999). We have recently cloned the *Xenopus* ortholog of VIT32 (Figure 1A), which is characterized by a different and longer N-terminus. It will now allow us to study its functional expression in *Xenopus* oocytes.

During oocyte maturation, a number of ionic conductances may be regulated. For example, it has been shown that MPF suppresses the functional expression of a cloned delayed rectifier potassium channel (Bruggemann *et al*., 1997). The mechanism for this change is proposed to be downstream of MPF (Pardo *et al*., 1998). It has also been shown that progesterone treatment abolishes exogenously expressed ionic currents in *Xenopus* oocytes (Shcherbatko *et al*., 2001). Thus, voltage-gated potassium and voltage-gated sodium channels were downregulated, but this was a relatively late effect occurring only at the time of GVBD, indicating that it was taking place downstream of MPF (Shcherbatko *et al*., 2001). The effects of MPF on exogenously expressed ionic conductances are therefore distinct from the rapid effect VIT32 that impacts on the G_2 to M phase transition during meiotic maturation of *Xenopus* oocytes. According to the physiological, biochemical and pharmacological evidences presented here, we propose that it functions upstream of MPF activation. It is interesting to observe that TC-1, which shares only 25% identity, appears to have a similar function in the oocyte. It suggests that this novel family of proteins could have an important role, not only in oocyte maturation, but in the cell cycle of somatic cells as well.

Materials and methods

Cloning of mVIT32 and mTC-1

The expression sequence tags AA624381 and AA245504 containing full coding sequences for mVIT32 and mTC-1, respectively, were obtained from I.M.A.G.E Consortium (clones 1006644 and 699426, respectively). mVIT32 and mTC-1 were subcloned in pSD5easy vector for *in vitro* transcription. For *Xenopus* oocyte expression, the 5′ UTR of corresponding cDNAs were replaced by a modified Kozak sequence (5′-ggaagtgacc-ATG-3′).

Northern blot analysis

Time-course and dose-dependence of mVIT32 stimulation by vasopressin (Figure 2) was studied on northern blots prepared with 2 µg of poly(A) mRNA extracted from mpkCCD cells grown on filter. Conditions of cell culture for mpkCCD cells have been described previously (Robert-Nicoud *et al*., 2001). The mouse multi-tissue poly(A) blot (Figure 3) was obtained from Stratagene.

Detection of Cdc2 activity

Kinase activity using histone H1 as substrate was measured as described previously (Rempel *et al*., 1995). Stage VI *Xenopus* oocytes were treated with progesterone (3 µM) or ethanol (0.03%) as control or microinjected with 0.1 µl of water or mVIT32 cRNA at 30 ng/µl. Ten oocytes per condition were lysed in 200 µl extraction buffer (0.1 M NaCl, 0.5% digitonin, 20 mM Tris–HCl pH 7.6, 1 mM PMSF and protease inhibitors). Cdc2 was immunoprecipitated from 40 µl of this oocyte lysate, using anti-Cdc2 antibody (Santa Cruz Biotechnology). Immunoprecipitated Cdc2 was incubated at 25°C for 20 min into 40 µl of kinase buffer (40 mM HEPES pH 7.5, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 µM ATP, 15 µCi [γ-^{32}P]ATP and 0.5 mg/ml histone H1). The reaction was stopped by the addition of 40 µl of sample buffer and analyzed by 10% SDS–PAGE.

Immunoblotting

Stage VI *Xenopus* oocytes were treated with progesterone (3 µM) or ethanol (0.03%) as control or microinjected with 0.1 µl of water or mVIT32 cRNA at 30 ng/µl. Two oocyte lysates (40 µl) for each condition, mixed with 40 µl of sample buffer, were resolved by 10% SDS–PAGE. Immunoblotting was performed with anti-MAP kinase (ERK1 + ERK2) antibody (Zymed) and anti-phospho-p44/42 MAPK antibody (Cell Signaling).

Electrophysiological measurements

Ion channel macroscopic currents. Oocytes were injected with 3 ng of $\alpha\beta\gamma$ rat ENaC or 0.2 ng of ROMK2. The following day, the oocytes were reinjected with 3–5 ng of mVIT32, mTC-1 or with water. Five hours later, electrophysiological measurements of current were made, using a two-electrode voltage-clamp method, as described for ENaC (Firsov *et al*., 1996) and ROMK2 (Zhou *et al*., 1994), for which the Ba^{2+}-sensitive K^+ current was measured in a 5 mM K^+ bath solution at –100 mV. In experiments using MAP kinase inhibitors, the oocytes were incubated with the inhibitor for 1 h before mVIT32 injection and for 5 h after injection. Controls were incubated for 5 h in 0.5% DMSO.

Sodium pump currents. For the measurement of endogenous Na,K-ATPase activity, the oocytes were injected with water or 3 ng mVIT32 and Na^+-loaded in a K^+ free solution for 5 h. The pump current was measured as the current activated by 10 mM K^+ at –50 mV (Horisberger *et al*., 1991).

Membrane capacitance. The capacitance of oocytes injected with either water or mVIT32 was estimated from the integral of the capacitive transient, measured by stepping the membrane potential from –50 to –60 mV.

Cell surface expression of ENaC. Oocytes were injected with 2 ng of each α, β and γ subunits of ENaC. Twelve hours later, oocytes were treated with 15 µM progesterone or co-injected with 3 ng of VIT32. After 8 h, cell surface expression of ENaC was measured according to the binding method described previously (Firsov *et al*., 1996, 1997). One hour after the binding analysis, the same oocytes were used for sodium current measurement.

Chemicals. Roscovitine was purchased from Calbiochem. U0126 was obtained from Promega. Progesterone was obtained from Sigma. Roscovtine and U0126 were dissolved in DMSO. Progesterone was dissolved in ethanol. The final concentration in the oocyte incubation solution of DMSO or ethanol was 0.5 and 0.015%, respectively.

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