ARTICLE
Journal of Cellular Biochemistry 112:3216–3226 (2011)

Journal of Cellular Biochemistry

PI3K/Akt Responses to Oxytocin Stimulation in Caco2BB Gut Cells

Benjamin Y. Klein,[1*] Hadassah Tamir,[1,2] and Martha G. Welch[1,3**]

[1] *Department of Pathology & Cell Biology, Columbia University College of Physicians and Surgeons, New York, New York*

[2] *Division of Molecular Imaging and Neuropathology, New York State Psychiatric Institute, Columbia University College of Physicians and Surgeons, New York, New York*

[3] *Department of Developmental Neuroscience, New York State Psychiatric Institute, Columbia University College of Physicians and Surgeons, New York, New York*

ABSTRACT

Recently, we discovered oxytocin receptor (OTR) expression in the developing gut villus epithelium that emerges in villus–crypt junctions after weaning. Oxytocin (OT) and OTR regulate many physiological functions in various tissues; however, their function in gut epithelium is unknown. We explored responses of PI3K and Akt phosphoisoforms to OT stimuli in the Caco2BB human gut cell line. In Caco2BB cells, PI3K and pAkt levels peaked at 62.5 nM OT. At higher concentrations, PI3K decreased more gradually than $pAkt^{S473}$ suggesting that the $pAkt^{S473}$ response is separate from PI3K. At $\leq$7.8 nM OT, $pAkt^{T308}$ increased while $pAkt^{S473}$ decreased. Using a specific OTR antagonist, we demonstrated that responses of $pAkt^{T308}$ to OT depend on OTR in contrast to the partial OTR-dependence of the $pAkt^{S473}$ response. Differential pAkt phosphoisoform responses included pAkt phosphoserine 473 persistently free of phosphothreonine 308. The reduction in PI3K after 62.5 nM OT for 30 min coincided with OTR internalization. The PI3K/Akt activation profile was somewhat different in other cell lines (MCF-7 breast cancer cells, HT29 gut cells), which have PI3K activating mutations, that were examined to establish experimental parameters. In Caco2BB cells, the divergent effects of OT upon pAkt phosphoisoforms suggests separate sub-pathways; $pAkt^{T308}$ activation depends on OTR via the PI3K pathway and $pAkt^{S473}$ presumably results from its specific kinase mTORC2 (mammalian target of rapamycin complex 2). Thus, OT may modulate gut cell functions downstream of mTOR complexes (e.g., translation control as suggested by others in uterine cells). We will next explore OT-stimulated kinase activities downstream of mTOR related to pAkt phosphoisoforms. J. Cell. Biochem. 112: 3216–3226, 2011.

KEY WORDS: OXYTOCIN RECEPTOR; SIGNALING; PI3K; HT-29; PTEN; Akt; Caco2BB

Oxytocin (OT) is a nonapeptide hormone well known for stimulation of uterine contractions during labor. OT stimulates cells in various tissues mainly through the oxytocin receptor (OTR), a Class I, G protein-coupled receptor encoded by a single copy gene [Kimura et al., 1992] that is widely distributed in the brain and body [Viero et al., 2010]. OT, the endogenous ligand, is synthesized in the hypothalamus and locally in extraneural tissues [Chibbar et al., 1993]. We have shown OT synthesis in the enteric nervous system [Welch et al., 2009]. Numerous physiological studies have demonstrated a plethora of functions for OT/OTR signaling beyond prominently ascribed roles in parturition[Mitchell et al., 1998] and lactation [Nishimori et al., 1996]. Two important intricacies of OTR function, however, have complicated the study of OT/OTR signaling. These include: (1) Dual, cofactor-modulated affinity states (cholesterol-mediated, high-affinity state, dissociation constant (Kd) of 1 nM OT; low-affinity state, Kd of 100 nM OT) [Gimpl et al., 2008]; (2) promiscuous coupling to at least two G proteins ($G\alpha_{q11}$ and/or $G\alpha_i$) [Reversi et al., 2005], which can elicit

Additional supplementary information may be found in the online version of this article.

Grant sponsor: Columbia Grant; Grant number: 15665; Grant sponsor: Einhorn Family Charitable Trust; Grant number: 644824.

*Correspondence to: Benjamin Y. Klein, MD, Department of Pathology & Cell Biology, Columbia University College of Physicians and Surgeons, 1051 Riverside Drive, Unit 40, New York, NY 10032. E-mail: benjamin.klein@gmail.com

**Correspondence to: Martha G. Welch, MD, New York State Psychiatric Institute, 1051 Riverside Drive, Unit 40, New York, NY 10032. E-mail: mgw13@columbia.edu

Received 12 May 2011; Accepted 23 June 2011 • DOI 10.1002/jcb.23243 • © 2011 Wiley Periodicals, Inc.
Published online 5 July 2011 in Wiley Online Library (wileyonlinelibrary.com).

opposing physiological effects after OT stimulation [Gravati et al., 2010]. Therefore, within each organ or cellular system, the physiological function of OT/OTR signaling and underlying molecular mechanisms must be carefully dissected both pharmacologically and molecularly.

Our group has characterized the expression of OTR in gut epithelium in newborn rats from day 1 to day 28 post-partum [Welch et al., 2009]. The prominent findings were strong OTR immunoreactivity in villus enterocytes that lasted until postnatal day 17 (i.e., until weaning and crypt formation). Subsequently OTR expression diminished in villus epithelium and appeared in the upper parts of newly formed crypt structures where it was also detected by electron microscopy in tight junctions between epithelial cells in the adult. The neonatal pattern of expression implies a role for OT in modulating the migration of newly generated enterocytes or in their function in the newborn gut. The adult expression pattern of the OTR is suggestive of a role of OT in modulating secretion and/or epithelial renewal processes (the crypt is the site of enterogenesis in the adult). Furthermore, epithelial tight junctions critically constitute a barrier within the gut epithelium to toxins and microorganisms and, OT may therefore be relevant in pathological states where the barrier function is compromised.

We were interested in characterizing the cellular signaling response to OT within enterocytes as one approach to defining OT function in this class of cells, which at present has not been studied. The bulk of research to date in other organ systems has linked OTR stimulation by OT with the activation of phospholipase C (PLC), phospholipase A2 (PLA2), protein kinase C (PKC), and mitogen activated protein kinase (MAPK). We became interested in OT-mediated signaling by way of PI3K/Akt pathways in the gut because of the important roles for this signaling pathway in modulating migration by OT in endothelial cells [Cattaneo et al., 2008] and, in the gut, in cell survival in pathological states [Zhang et al., 2004; Huang et al., 2011].However, there is little information about OT signaling via PI3K/Akt pathways in any system. The PI3K family largely controls the utilization of membrane phospholipids to derive phosphate moieties for signaling kinases and is regulated by active, non-phosphorylated phosphatase, and tensin homolog (PTEN) [Maehama and Dixon, 1999]. Importantly, Devost et al. [2005] showed that OT stimulation of OTR changes translation downstream of mTORC1. mTORC1 is indirectly stimulated by Akt [Foster and Fingar, 2010]. Consequently, we made Akt signaling the subject of our investigation. Also, we focused on understanding the temporal kinetics of PI3K/Akt activation, which is relevant in future dissection of downstream signaling pathways and function.

In the present work we first demonstrated a role for OT in activating the PI3k/Akt pathway in MCF-7 breast cancer cells (bona fide OTR expressing cells) and HT-29 cells (a gut cell line which has a PI3K activating mutation) and established key experimental conditions. Our main finding is from the study of the Caco2BB gut cell line in which we observed that the PI3K response to OT is at least in part separate from that of Akt and, the $pAkt^{S473/T308}$ response is partitioned from $pAkt^{S473}$. T308 phosphorylation is more likely to depend on OTR and on its coupling with $G\alpha_i$ at low OT concentrations, which is diametrically opposed to that under high OT concentrations. Under high OT there is a fraction of pAkt that appears as $pAkt^{S473}$ devoid of T308 phosphorylation. Akt phosphorylation is known to form $pAkt^{S473/T308}$ depending on both PI3K/PDK1 (PDK1 = T308 kinase) and on mTORC2 (S473 kinase). $pAkt^{S473/T308}$ also acts upstream of mTORC1, which places it between these two complexes. Therefore, defining the kinetics of PI3K/Akt under stimulation by OT is compelling.

These results set the stage for upcoming analysis of downstream pathways that respond to OT in relationship with Akt phosphoisoforms and dovetail with on-going functional analyses of OT-function in the gut.

MATERIALS AND METHODS

CELLS AND CULTURE REAGENTS

HT-29 and Caco2BB (C2BBe1 clone) colon carcinoma cell lines and the MCF-7 mammary carcinoma cell line, their specific media, and fetal calf serum (FCS) were purchased from the American Type Culture Collection (Manassas, VA). HT-29 cells were grown in McCoy's medium and Caco2BB cells were grown in Dulbecco modified essential medium (DMEM, 4.5 g glucose) fortified with bovine transferrin 10 ng/ml. MCF-7 cells were grown in Earle's Modified Eagle's minimum essential medium (MEM) with 10 ng/ml bovine insulin. All media were supplemented with standard penicillin and streptomycin, 2 mM glutamine, and 10% FCS. Cultures were propagated at 5% CO_2 and 37°C in a humid atmosphere.

REAGENTS

Human oxytocin (OT) was obtained from Phoenix Pharmaceuticals Inc. (Burlingame, CA) and Oxytocin Receptor antagonist (OTA) and desGly-NH_2-d(CH2)$_5$[D-Tyr^2,Thr^4]OVT (ST-11-61) were donated by Dr. Morris Manning, University of Toledo OH [Manning et al., 1995]. Pertussis toxin (salt-free) was from List Biological Laboratories Inc. (Campbell CA). Proteasome inhibitor MG-132 was from Calbiochem (Cat 474791; San Diego, CA). Reagents for siRNA silencing, Nedd4-1 siRNA sc-41079, control non-specific siRNA sc-37007, and siRNA transfection reagent sc-29528 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The siRNA transfection medium (Santa Cruz Biotechnology, Inc.; sc-36868) was prepared and utilized for transfection according to the reagent supplier.

ANTIBODIES

Rabbit polyclonal anti-OTR (#O4389) and mouse anti-β actin (mAb) were from Sigma-Aldrich (St Louis, MO). Mouse anti-PI3K p85 (mAb; sc-1637), mouse anti-PI3K p110β (mAb; sc-8010), goat anti-Nedd4-1 (sc-14428), three horseradish peroxidase (HRP) conjugates: Rabbit anti-goat IgG (sc-2922), goat anti-rabbit IgG (sc-2301), goat anti-mouse IgG were all from Santa Cruz Biotechnology. Mouse anti-$pAkt^{S473}$ (4051), rabbit anti-$pAkt^{S473}$ (mAb; 4060), rabbit anti-$pAkt^{T308}$ (9275), rabbit anti-Akt (9272), rabbit anti-PTEN (9552), mouse anti-phospho-PTEN (mAb; pPTEN; 9554), and rabbit anti-Nedd4-1 antibodies (2740) were all from Cell Signaling Technology (Danvers, MA), Laboratories, Inc. (West Grove, PA).

siRNA TRANSFECTION

HT-29 and Caco2BB cells, seeded 2×10^4 cells/cm^2 in their growth medium to 50% confluence, were placed antibiotic- and serum-free medium. Thirty two microliter Nedd4-1 siRNA (sc-41079) or control siRNA (sc-37007) was diluted in 400 μl of transfection medium (sc-36868). Transfection reagents (32 μl; sc-29528) was mixed with 400 μl of transfection medium and added to the siRNA mix, incubated 15 min, then mixed with plain 5.2 ml transfection medium and added to each cell culture (2 ml). After 8 h incubation, 2× concentrated growth medium was added. Proteins were extracted from the transfected cultures after 3 days of incubation.

OTR STIMULATION AND PROTEIN EXTRACTION

Stimulation experiments with OT were performed in cell cultures 24 h after seeding to25×10^4 cells/cm^2 and, antagonists or inhibitors were added 30 min before stimulation with OT. Continuous stimulation times ranged from 5 to 60 min, indicated with relevant results, were terminated by placing the cultures on ice. The cultures were quickly washed twice with ice cold phosphate-buffered saline (PBS) and, 0.1 ml of protein extraction cocktail was added for 5 min. The extraction cocktail contained protease inhibitors and phosphatase inhibitors (pre-added by the producer) and DTT (reducing agent), bromophenol blue (tracking dye), and 5% glycerol. The protein extracts were scraped, boiled for 5 min, cooled on ice for 5 min and spun at 10,000*g* for 10 min at 4°C. A sample of each extract was processed for protein determination and the extract was kept at −40°C until further use. Protein concentrations where measured by a paper spot protein assay against a bovine serum albumin (BSA) standard curve. Protein samples (4 μl) were applied to 3 mm filter paper, stained with Coomassie blue in 40% methanol and 10% acetic acid, washed with the same solution without dye and dried. Proteins were eluted with 3 ml of 2% SDS that was counted on an ELISA reader at 650 nm.

ELECTROPHORESIS AND WESTERN BLOTS

Protein samples (30 μl/well) were fractionated on 8, 10, or 12, or 7–15% gradient, discontinuous SDS polyacrylamide gels and blotted onto nitrocellulose filters that were Ponceau stained and scanned as a loading control. In most cases considering actin as a loading control was avoided since its abundance increased as a result of OT stimulation, possibly due to the participation of actin in endocytosis. Blots were blocked for 30 min in TBST (Tris-buffered saline 0.01%, Tween-20) and 3% BSA and then reacted with primary antibodies in blocking solution overnight and with secondary peroxidase-conjugated antibodies for 30 min. The blots were developed with the Luminol reagent (sc-2048; Santa Cruz Biotechnology, Inc.) on X-ray films that were scanned and saved as TIFF files using Photoshop software. Band density, at their dynamic-range, was measured by ImageJ software (Scion Corporation) and recorded in arbitrary units.

IMMUNOCYTOCHEMISTRY

Cells were seeded on microscope cover glasses and, after 24 h, exposed to 62.5 nM OT (or no OT) for 30 min. Cultures were fixed and permeabilized for 10 min in 10% cold methanol and subsequently placed in 0.5% glutaraldehyde for 10 min. The fixed cultures were washed twice with PBS and blocked overnight with TBST containing 3% BSA at 4°C. The fixed and blocked cultures were reacted at 4°C with mouse anti-pAkt^{S473} (1:1,000) and rabbit anti-OTR (1:1,000) for 5 h and subsequently washed twice with PBS. The dual-immunofluorescent labeling was developed by 2 h incubation with secondary antibodies (Molecular Probes, Carlsbad, CA) goat anti-mouse IgG (Alexa Fluor 488 nm) and donkey anti-rabbit IgG (Alexa Fluor 594), both at 1:1,000 dilution. Cells were washed with PBS (3×) and the cover glasses were mounted on slides and visualized by confocal fluorescent microscopy. Confocal images were collected on an LSM 510 Meta scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) using a 40×/0.6 NA objective. FITC was excited with a 488-nm laser and detected using a 500–550 nm bandpass emission filter. TRITC was excited with a 543-nm laser and detected using a 560 nm longpass emission filter. Pinholes were set to produce equal optical sections of 3 μm in each channel. Confocal slices through the middle plane of cells are presented to provide the clearest view.

STATISTICAL ANALYSIS

Band density differences at each time point or each reagent concentration were computed against controls using a paired Student's *t*-test (two-tailed; $\alpha = 0.05$). Data were collected from 3–8 replicates per condition and were analyzed using SPSS Base 9.0 (SPSS, Chicago, IL). All plots present mean ± standard error.

RESULTS

OT EFFECT ON PI3K/Akt IS DOSE-DEPENDENT

Given the important role of OT in milk letdown, we used a mammary cell line (MCF-7) as a bona fide OTR-expressing target cell [Bussolati et al., 2001] to test the effect of OT (serially diluted) on the endogenous stimulation of the PI3K/Akt pathway. After 30 min incubation in OT, the expression of PI3K p85 (Supplementary Fig. 1A), PI3K p110 (Supplementary Fig. 1B), and pAkt^{S473} (Supplementary Fig. 1C) were stable between 7.8 and 62.5 nM OT and their mean abundance at 30 min post-stimulation was not significantly different from untreated cultures. At higher concentrations (125–1,000 nM OT) there were significant reductions in PI3K p85, PI3K p110, and pAkt^{S473} compared with <62.5 nM OT cultures. The total amount of Akt did not significantly vary with high OT treatment. Note that higher concentrations of OT (125–1,000 nM OT) elicited significant reductions in the PI3K signaling components, perhaps by receptor desensitization, compared to cells treated with lower doses of OT (<62.5 nM OT).

These results indicate that using our experimental conditions, changes in the abundance of active PI3K signaling components are detectable in a cell line known to robustly express the OTR. The molar range of OT over which the phosphorylation of PI3K components is maintained was between the high- and low-affinity Kd values identified for the OTR [Gimpl and Fahrenholz, 2001]. These results also indicate that between 0 and 62.5 nM OT, the abundance of pAkt ^{S473} parallels that of the catalytic subunit of PI3K (p110) (Supplementary Fig. 1B). However, there is no stimulatory response of the PI3K pathway to lower doses of OT, perhaps due to the PI3K activating mutation [Vasudevan et al., 2009]. Thus, if PI3K/

Akt is a general target pathway of OTR stimulation it is reasonable to expect activation of PI3K/Akt proteins in other cell lines after stimulating the OTR at its low-affinity state (62.5 nM OT), a condition in which the receptor is approximately half-saturated.

OT ATTENUATES THE PI3K PATHWAY IN HT-29 CELLS

We examined the time course of PI3K signaling components in response to OT (62.5 nM) in HT-29 cells, an undifferentiated and highly proliferative colon cell line, with 20-fold less OTR [Bussolati et al., 2001]. The abundance of the PI3K p85 regulatory subunit (Fig. 1A) and PI3K p110 catalytic subunit (Fig. 1B) was significantly decreased after 5 and 10 min with OT and recovered by 20–30 min of OT stimulation. The levels of PI3K p85 were slower to recover compared with the PI3K p110 subunit. OTR stimulation was also followed by a decrease in pAkt^{S473} (Fig. 1C) that did not parallel the decrease in the fast migrating PTEN bands (active as PIP3 phosphatase) (Fig. 1D), implying that in HT-29 cells PTEN may be ineffective in regulating pAkt. There were no significant differences between slow migrating phospho-PTEN (inactive as phospholipid phosphatase) bands at all indicated time points.

These results indicate that PI3K and pAkt are temporally linked after OT stimulation, in distinction from slow PTEN, by virtue of both being membrane associated, whereas slow PTEN (pPTEN, inactive) is a membrane unassociated PTEN isoform [Tolkacheva et al., 2001]. OT stimulation may thus inhibit PI3K signaling in HT-29 cells by a process involving membranous proteins via OTR desensitization and turnover by endocytosis, as has been shown to occur in MCF-7cells and other cell lines [Kinsey et al., 2007]. Since HT-29 cells carry a PI3K activating mutation [Ihle et al., 2009] as observed in MCF-7 cells, changes in PI3K components may not support that the PI3K pathway is a direct OT-stimulated target, but rather may signify OTR desensitization.

PI3K SIGNALING ACTIVATION BY OT STIMULATION OF Caco2BB CELLS, A MODEL OF DIFFERENTIATED ENTEROCYTES

We examined the dose–response and time course of PI3K pathway activation in Caco2BB cells, a colon cell line, with no reported PI3K or Akt mutations, that may model differentiated enterocytes. In these cells we observed a different dose response. The regulatory PI3K p85 subunit was not significantly increased from baseline by

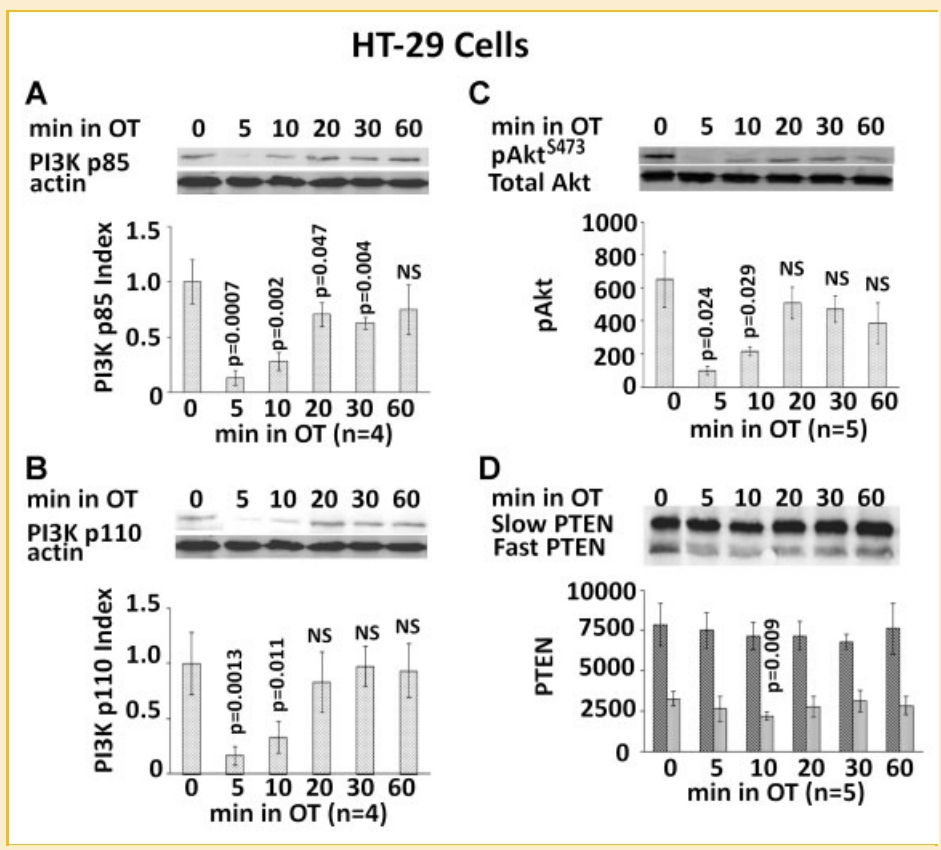

Fig. 1. OT inhibits PI3K, pAkt, and PTEN in HT-29 cells. Western blot analysis and quantification of HT-29 cell lysates stimulated with 62.5 nM OT for indicated time intervals is shown in each panel for (A) PI3K p85 (n = 4), (B) PI3K p110 (n = 4), (C) pAktS473 (n = 5), and (D) slow (pPTEN; dark gray bars) and fast PTEN (active PTEN; pale gray bars), (n = 5). Statistically significant differences were computed against the untreated (0 min) time point. In panel (D), no comparison was significant except for fast PTEN versus baseline with 10 min OT exposure. Note that slow PTEN did not significantly differ from baseline with any duration of OT treatment. Abbreviations: OT, oxytocin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog.

any of the OT serial dilutions; however, the increase with 62.5 nM OT was significant when compared with 31.2 or 125 nM OT (Fig. 2A). The catalytic PI3K p110 subunit was significantly elevated from baseline after 62.5 and 500 nM OT and, the elevation at 62.5 nM OT was significant compared with 31.2 or 125 nM OT (Fig. 2B). Similarly, pAkt^{S473} was significantly elevated after treatment with 31.2 and 62.5 nM OT and the elevation with 62.5 nM treatment was significant when compared with cells treated with 15.6 and 500 nM OT (Fig. 2C). Each of the activated PI3K proteins examined, as well as total Akt, demonstrated a peak abundance at 62.5 nM OT, as we found for MCF-7 cells (Fig. 2D). The reduction in pAkt at OT concentrations above 62.5 nM was parallel to the reduction in total Akt implying that at concentrations $\geq$125 nM OT most Akt molecules are unstable. This may reflect a state in which most of the Akt molecules are modified by phosphorylation, a phenomenon we observe with immuno-isoelectric focusing (data not shown).

To follow the time-dependent response of Caco2BB cells we selected the 62.5 nM OT concentration based on its associated pronounced elevation of PI3K and Akt levels. PI3K was not uniformly attenuated in Caco2BB cells as we observed in HT-29 cells and moreover, the time course of the PI3K response to OT was different from that observed in HT-29 cells. The expression of the PI3K p85 subunit did not significantly differ from baseline after 5–20 min with 62.5 nM OT, was significantly decreased after 30 min of stimulation and was equivalent to baseline by 60 min (Fig. 3A). The catalytic subunit was significantly elevated 20 min after OT, and did not change at 30 min but slightly, although insignificantly decreased at 60 min (Fig. 3B). pAkt^{S473} was elevated 30 and 60 min after OT (Fig. 3C) and pAkt^{T308} was significantly elevated only at 60 min after OT (Fig. 3D). The extramembranous protein pPTEN marker was elevated significantly 10–20 min after OT and remained relatively stable thereafter (Fig. 3E). Akt thus appears to have a slower response to OT stimulation than the regulatory and catalytic subunits of PI3K and PTEN. The delayed phosphorylation of pAkt can be appreciated in western blots that were sequentially processed to detect pAkt^{S473} followed by pAkt^{T308} (Fig. 3F). pAkt^{S473} appears to be a forerunner of pAkt^{T308} elevation and Akt phosphorylation at S473 appears more prominent than pAkt^{T308},

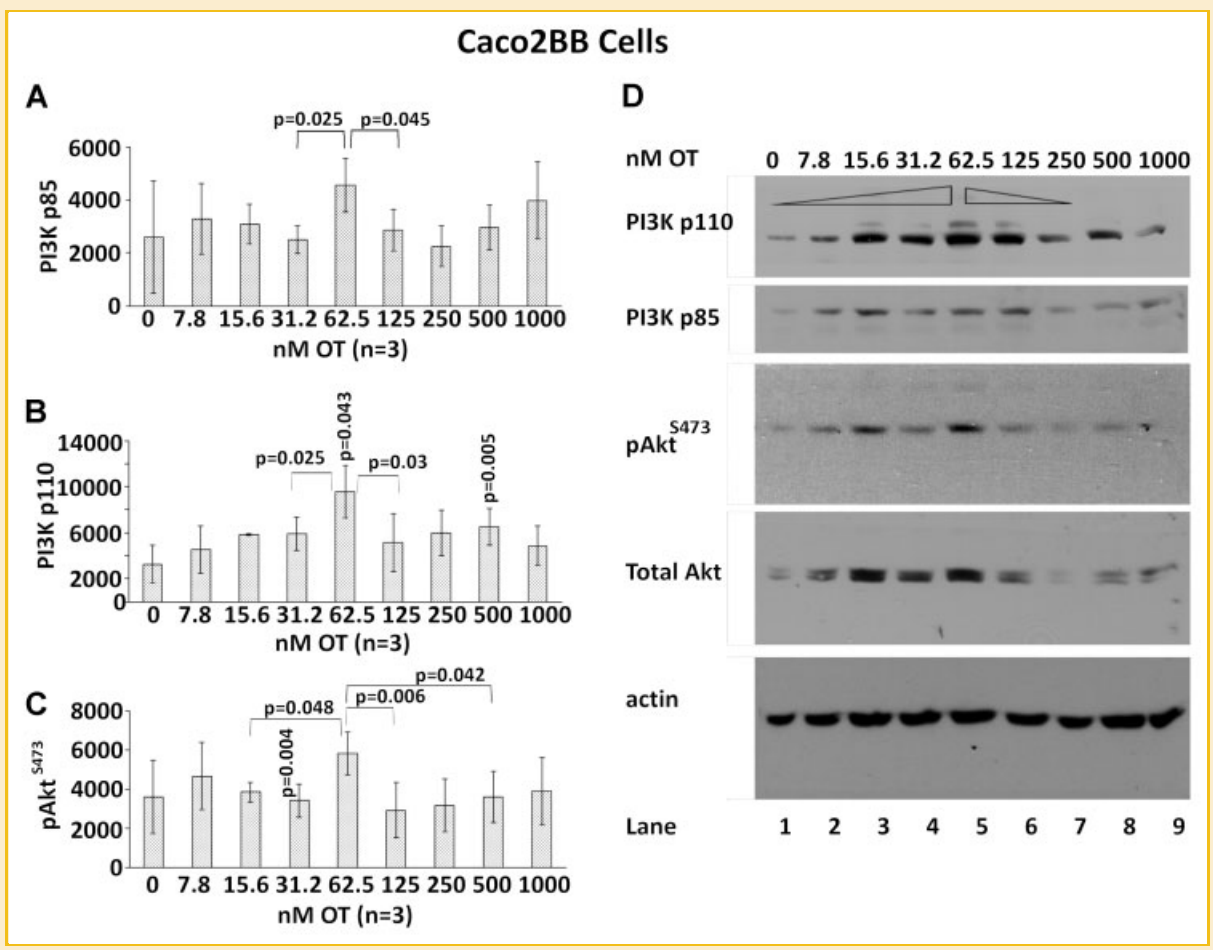

Fig. 2. Dose-dependent response of PI3K and Akt to OT in Caco2BB cells. Caco2BB cells were exposed to serial dilutions of OT prepared for 30 min and cell lysates were probed by western blotting. Plots show (A) PI3K p85 (n = 3), (B) PI3K p110 (n = 3), and (C) pAktS473 (n = 3). D: Representative western blot showing the response to serial dilutions of OT. Unbracketed comparisons are between OT and baseline. Bracketed comparisons demonstrate significant differences between OT-treated groups. All other comparisons between untreated or treated groups were not significant. Note most marked effects upon PI3K signaling with 62.5 nM OT (triangles in panel D). Abbreviations: OT, oxytocin; PI3K, phosphoinositide 3-kinase.

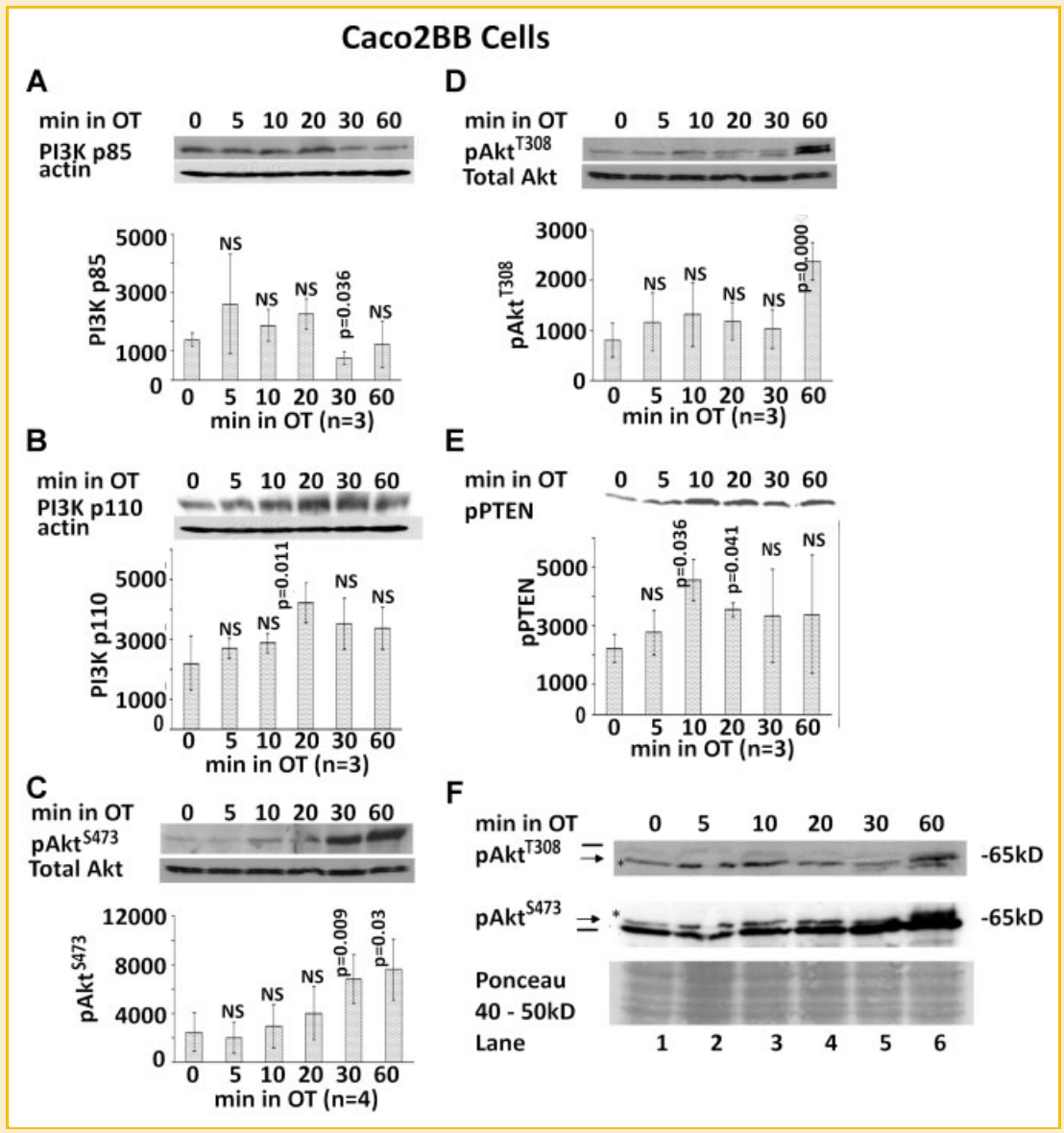

Fig. 3. OT activates PI3K p110, pAkt, and PTEN in Caco2BB cells. Western blot analysis and quantification of Caco2BB cells stimulated with 62.5 nM for indicated time intervals. Blots and plots show (A) PI3K p85 (n = 3), (B) PI3K p110 (n = 3), (C) pAktS473 (n = 4), (D) pAktT308 (n = 3), and (E) pPTEN (n = 3). Statistically significant differences were computed against the untreated (0 min) time point. Note the delay in pAktS473 accumulation (C) and the subsequent delay in pAktT308 accumulation (D). F: Representative western blot showing pAkt response to 62.5 nM OT. Blots were developed in tandem; development with mouse anti-pAktS473 (lower panel) was followed by processing with anti-pAktT308 (upper panel). Note the late pAkt surge represented by the band recognized exclusively by mouse anti-pAktS473 in lanes 5 and 6 (30 and 60 min) and the subsequent, delayed surge in the pAkt band that was recognized by both antibodies (asterisk), which represents doubly phosphorylated Akt at S473 and T308, in lane 6 (60 min). Abbreviations: OT, oxytocin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog.

this can be attributed to low efficiency of anti-phosphothreonine compared to anti-phosphoserine antibodies, thus requiring correction by radiographic exposure. These experiments indicate that OT stimulation activates or attenuates the PI3K pathway in Caco2BB cells depending on concentration and/or time, and with a different rate than in HT-29 cells. Significantly, the phosphoisoform of $pAkt^{S473}$ (Fig. 3F) is devoid of T308 phosphorylation at all stimulation intervals at 62.5 nM OT. At OT concentrations exceeding the low-affinity state (Fig. 2D), as determined in the past for OTR, the total abundance of pAkt diminishes in accord with that of total Akt. This implies degradation of Akt under very high OT concentrations under which most Akt may be pAkt and therefore, membrane associated and involved in endocytic internalization.

AKT PHOSPHORYLATION AFTER OTR STIMULATION REQUIRES $G\alpha_I$ AND A STRUCTURAL CHANGE

Based on what is known about G protein-coupled receptor responses to agonists, we expected that after OT stimulation of the OTR, a structural change in the receptor will allow for G protein interactions. We examined the ability of a 30 min pretreatment

with a specific allosteric antagonist of the OTR or a $G\alpha_i$ G protein inhibitor (pertussis toxin; PTX) to inhibit the phosphorylation of Akt after 30 min OT treatment (at 7.8 or 62.5 nM OT) in Caco2BB cells. Note that the Caco2 sub-line, Caco2BB, reacted to PTX after a relatively short incubation, between 30 and 60 min. The cells were exposed to plain medium, low, high, or very high doses of OT (0, 7.8, 62.5, and 1,000 nM, respectively). OTR antagonist (OTA) showed a very subtle effect on S473. $pAkt^{S473}$ was increased with control medium (0) (see discussion) and slightly at 62.5 nM OT (lanes 10 vs. 12 and 4 vs. 6) and reduced $pAkt^{S473}$ phosphorylation at both 7.8 and (slightly) at 1,000 nM concentrations of OT (lanes 7 vs. 5 and 1 vs. 3). PTX affected the abundance of $pAkt^{S473}$ similarly to, but even weaker than the OTA effect (Fig. 4). The OTA and PTX effects on the phosphorylation of T308 of Akt in OT-untreated cells was the opposite of that exhibited for S473 (compare lanes 10, 11, and 12 between both Akt panels). OTA and PTX show opposite effects that were more pronounced on the T308 than on the S473 Akt phosphoisoform, which is visible when 7.8 and 62.5 nM lanes are compared (4–5 and 6 vs. 7–8 and 9). Regardless of whether OT increases or decreases the Akt T308 phosphoisoform, both OTA and PTX oppose this OT effect. These responses to OTA indicate that the T308 Akt phosphoisoform occurs via the OTR whereas for the S473 isoform the OT effect is either indirect or occurs only partially via the OTR. These differences, in phosphorylation levels between T308 and S473, occur under high and low OT, while the concentration of OTA and PTX are kept constant, reflecting a functional duality of OTR states determined by the two different OT doses. The OT effect on both PI3K subunits is antagonized by OTA mainly at the low dose (7.8 nM) or the very high dose (1,000 nM) of OT. While large contrasting effects are seen for pAkt at high and low OT (lanes 4–9) no such changes are observed for pPTEN, which is used to mark signaling that is not associated with the plasma membrane, and thus implicates membrane-linked processes for the pAkt isoforms. The OTA and PTX induced changes under 1,000 nM OT may represent effects of very high doses on vasopressin receptor–OTR heteromers.

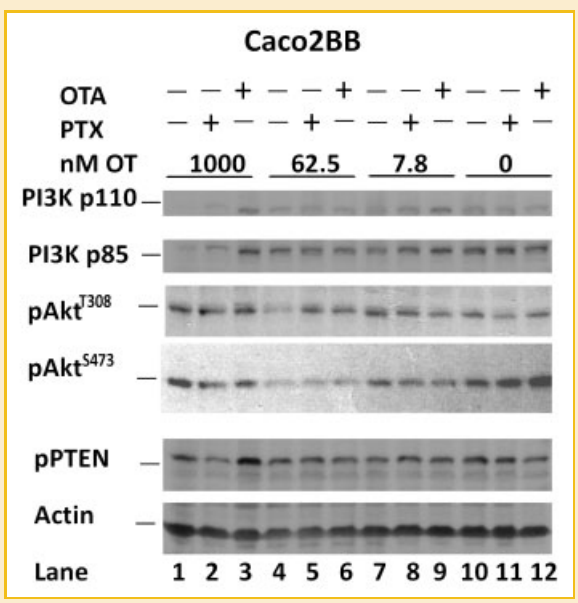

Fig. 4. Effects of Oxytocin antagonist (OTA) and pertussis toxin (PTX) on OT-stimulated PI3K/Akt proteins in Caco2BB cells. Western blot analysis of cultures that were exposed to OT for 30 min after prior exposure to OTA (4 nM) or to PTX (0.25 mg/L) for 30 min. Cultures were exposed to 1,000 nM OT (Lanes 1–3), 62.5 nM OT (Lanes 4–6), 7.8 nM OT (Lanes 7–9) or to plain growth medium (Lanes 10–12) and lysates were probed for the PI3K subunits, pAktT308, and pAktS473 phosphoisoforms, pPTEN and actin. The PTEN phosphoisoform is not associated with the plasma membrane. Note the differential effect of OTA (OT antagonist) and of PTX, an inhibitor of Gαi, upon pAktT308 compared with pAktS473 levels at 62.5 nM OT. Abbreviations: OT, oxytocin; OTA, oxytocin antagonist; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; PTX, pertussis toxin.

Nedd4-1 CONTRIBUTES TO pAkt TURNOVER AFTER OT STIMULATION

We tested whether Nedd4-1, a protein involved in ubiquitination necessary for trafficking, internalization and degradation of G protein-coupled receptors, contributes to alterations in pAkt stability observed with OT stimulation. We used siRNA silencing in HT-29 and Caco2BB cells to follow alterations in pAkt abundance. In HT-29 cells, Nedd4-1 was significantly reduced, by 54%, by siRNA treatment (Fig. 5A and C) and, after 10 min stimulation with 62.5 nM OT, $pAkt^{T308}$ was significantly elevated compared with control (Fig. 5B). We had to use anti-$pAkt^{T308}$ antibodies in HT-29 cell as $pAkt^{S473}$ was undetectable in HT-29 cells under these conditions (data not shown). Nedd4 siRNA-treated cells showed a twofold increase in $pAkt^{T308}$ relative to total Akt compared with OT-stimulated control siRNA transfectants (Fig. 5C). Nedd4-1 was reduced, by 40%, in siRNA-treated Caco2BB cells (Fig. 6A and D), $pAkt^{S473}$ significantly increased threefold (Fig. 6B and D) and PTEN significantly decreased by 38% (Fig. 6C and D), which is consistent with increased pAkt. The results obtained by silencing indicate that Nedd4-1 participates in the turnover of the OT-stimulated OTR, possibly by ubiquitination of the receptor internalization complex or a protein involved in PI3K/Akt pathway turnover.

OT STIMULATION OF Caco2BB CELLS INDUCES OTR INTERNALIZATION AND TRAFFICKING

We co-labeled OT-stimulated cells for $pAkt^{S473}$ and OTR immunoreactivities to examine the effect of 62.5 nM OT on OTR intracellular localization 30 min post-stimulation, a time when pAkt peaks. In control cells, $pAkt^{S473}$ and OTR immunofluorescences were co-localized at the cell membrane (Fig. 7A–C). After OT, $pAkt^{S473}$ immunofluorescence was increased and we observed a more prominent concentration of OTR fluorescence intracellularly compared with control cells (Fig. 7 compare E to B and C to F). OTR internalization may initially attenuate pAkt, and only later (at 30 min) permit robust Akt activation, in line with our western blot results (Fig. 3).

DISCUSSION

In summary, our main experimental result is that OT activates the PI3K signaling pathway in gut cell lines. It should be stressed that MCF-7 and HT-29 cells served to calibrate OT concentrations so that doses for desensitization and OTR internalization could be derived.

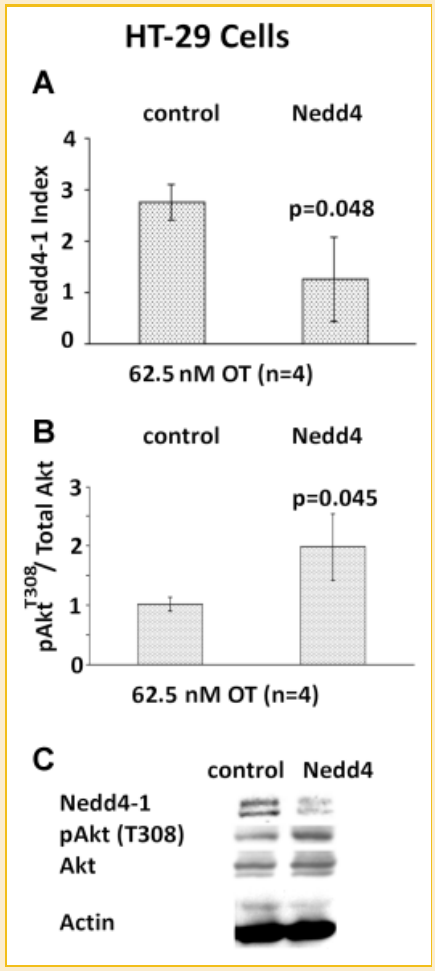

Fig. 5. Nedd4-1 knockdown increases pAkt in HT-29 cells. Western blot analysis and quantification of HT-29 cell lysates from cells that were stimulated with OT 72 h after transfection with Nedd4 siRNA or control siRNA. Cultures were stimulated with 62.5 nM OT for 10 min. Western blots were probed with antibodies against (A,C) Nedd4-1, (n = 4) (B,C) pAktT308 (n = 4), or (C) total Akt. Statistical significance denotes difference between control and Nedd4 siRNA treated cultures. Abbreviations: Nedd4-1, neural precursor cell expressed, developmentally down-regulated 4; OT, oxytocin.

However, the stimulation pattern of Akt phosphorylation in gut cells cannot be deduced from these cell lines due to the activating mutation of PI3K. Caco2BB cells were used to understand the Akt response of the gut to OT. Caco2BB cells exhibit differential pAkt phosphoisoform levels in response to OT and a differential inhibition pattern by OTA and PTX. These results have implications for the mechanisms by which OT modulates other signaling proteins downstream of pAkt.

The stability of PI3K subunits and pAkt that we observed in MCF-7 cells, between 0 and 62.5 nM OT, is presumably due to an activating mutation in PI3K p110 [Bachman et al., 2004; Saal et al., 2005]. Therefore, the steep drop in these membrane-associated proteins was probably an indirect effect of OTR desensitization at >125 nM OT. HT-29 cells in the time course assay were exposed to OT at approximately half-OTR-saturation (62.5 nM for MCF-7) and showed a steep drop in these same membrane-associated PI3K/Akt proteins with 5 and 10 min exposures, presumably due to desensitizing endocytosis with a later partial recovery. HT-29 carries the same PI3K mutation as in MCF-7, which makes stimulation via PI3K in both cell lines undetectable within the range of normal PI3K responsiveness. PTEN did not rise to oppose PI3K, which is typical for PI3K activating mutations [Ihle et al., 2009]. HT-29 has 20-fold less surface OTR than MCF-7 [Bussolati et al., 2001], thus not requiring more than 62.5 nM OT for desensitization. Although the PI3K mutation did not allow us to study pAkt changes in a proper PI3K range of stimulation (≤62.5 nM), it still enabled us to determine that 62.5 nM OT is either close to, or on the brink of causing OTR desensitization. Conveniently, in Caco2BB gut cells PI3K and Akt have not been reported to harbor functional mutations. They are thus fit to display changes in pAkt in response to a lower OT dose range (≤62.5 nM). The gradual increase in PI3K p110 from 0 to 62.5 nM OT, and its subsequent gradual decrease, is in contrast to the steep drop in $pAkt^{S473}$, indicating that the effect on PI3K and $pAkt^{S473}$ may proceed independently (Fig. 2). The accompanying fall in total Akt at ≥125 nM OT is consistent with most of the Akt being S473-phosphorylated, and therefore membrane associated and prone to endocytosis. The relatively low exposure time of the total Akt autoradiography may contribute to this perception. The response of $pAkt^{S473}$ also seems to be partitioned from that of $pAkt^{T308}$ and, to emphasize this point we increased autoradiograph exposure (Fig. 4) to compensate for low efficiency of anti-phosphothreonine antibodies in contrast to exposures performed for Figure 3. Despite differences between anti-phosphothreonine and anti-phosphoserine antibody efficiencies, it is clear that OT affects $pAkt^{S473}$ differentially from $pAkt^{T308}$, which is an important finding of the present work. It implies that OT can interfere with homeostasis as determined by PI3K/Akt sub-pathways and by the balance between the two mTOR complexes and their downstream signals (see review on mTOR [Foster and Fingar, 2010]). Note that in Figure 4 pAkt was seen in samples devoid of OT, and at 1,000 nM OT, in response to OTA and PTX; we ascribe this phenomenon to "functional selectivity" of G protein-coupled receptors to drugs [Kenakin and Miller, 2010], also known as the "biased agonist" effect [Reversi et al., 2005].

S473 in Akt is phosphorylated by the mammalian target of rapamycin complex-2 (mTORC2), which facilitates additional phosphorylation on T308 by PDK1 [Sarbassov et al., 2005]. PDK1 ablation strongly down regulates $pAkt^{T308}$ but not $pAkt^{S473}$, and control of FoxO1/3A remains intact [Hashimoto et al., 2006]. The solitary $pAkt^{S473}$ phosphoisoform is thus sufficient to target the forkhead transcription factor FoxO1/3A. This may imply that one function of the exclusively S473-phosphorylated phosphoisoform that we observe in OT-treated Caco2BB cells is down-regulation of FoxO1/3A. If varying OT levels can modulate $pAkt^{S473}$ levels, cell survival may be regulated through phosphorylation of FoxO1/3A, which keeps it out of the nucleus (i.e., out of its site of activity) [Brunet et al., 1999]. mTORC1, on the other hand, is activated indirectly by fully active pAkt (phosphorylated on both serine and threonine) that results in phosphorylation and activation of the kinase p70S6K1 [Romanelli et al., 2002] that in its turn phosphorylates S6RP (S6 ribosomal protein) and translation factors

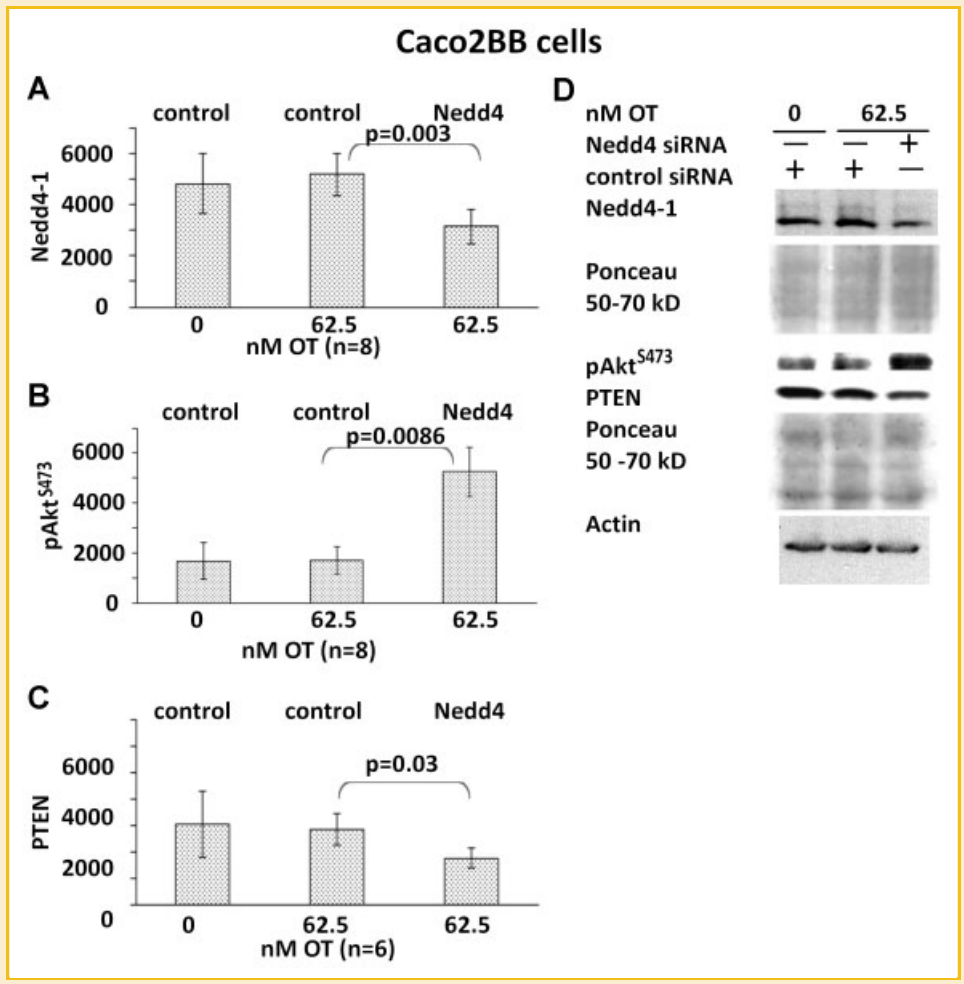

Fig. 6. Nedd4-1 knockdown inverts the ratio between pAkt and PTEN in OT stimulated Caco2BB cells. Western blot analysis and quantification of Caco2BB cell lysates from cultures stimulated with OT 72 h after transfection with Nedd4 siRNA or control siRNA. Cultures were stimulated with 62.5 nM OT or plain medium for 10 min. Western blots were probed with antibodies against (A) Nedd4-1 ($n = 8$), (B) pAktS473 ($n = 8$), or (C) PTEN ($n = 6$). D: Blot bands representative of pooled experiments shown in (A–C). The statistical significance of differences computed between Nedd4 siRNA and control siRNA are presented for cultures of OT stimulated samples. Abbreviations: Nedd4-1, neural precursor cell expressed, developmentally down-regulated 4; OT, oxytocin; PTEN, phosphatase and tensin homolog.

[Ruvinsky and Meyuhas, 2006]. S6RP, S6K1, mTORC1, and mTORC2 control translation factors and cell survival, which influence protein synthesis and cell growth [Ma and Blenis, 2009], cell size [Partovian et al., 2008; Skurk et al., 2005], and nutrient sensing [Byfield et al., 2005; Nobukuni et al., 2005]. P70S6K1 has been shown recently to down regulate mTORC2 by phosphorylation of RICTOR (rapamycin insensitive component of TOR) [Julien et al., 2010] creating a negative feedback loop between the two mTOR complexes, which according to our results may reflect the partition between both pAkt phosphoisoforms. Since in the present work OT concentration and time course determine pAkt phosphoisoform abundance in a partitioned manner, there may be a propensity for OT to modulate the balance between mTORC1 and mTORC2 (i.e., to influence the regulation of cell survival, growth, cell size, and nutrient sensing normally maintained by both mTOR complexes). This hypothesis derives from the different pAkt responses to low versus high OT in Caco2 cells. An additional issue to consider is the effect of high OT on the reduced abundance of PI3K/pAkt proteins, known to be membrane associated, which we suspected to reflect OTR endocytosis, leading to receptor desensitization. This process has been shown to occur in G protein-coupled receptors under continuous stimulation where interaction with E3 ubiquitin ligases resulted in receptor trafficking [Wolfe and Trejo, 2007; Hanyaloglu and von Zastrow, 2008]. One of the E3 ligases involved in regulating the β_2AR (adrenergic receptor) is Nedd4-1 [Shenoy et al., 2008]. We show that Nedd4-1 is involved in diminishing the abundance of pAkt 10 min after exposure to high oxytocin, which in Caco2BB cells is in accord with higher PTEN levels. We have not determined the direct targets for Nedd4-1, it may be only indirectly related to the receptor internalization machinery. Thus, the significance of Nedd4-1 involvement is yet to be revealed. Others have studied the internalization process of OTR in recycling by early endosomes [Conti et al., 2009] and involvement of arrestin, dynamin, and components of clathrin coated pits [Smith et al., 2006] and trafficking to the nucleus [Kinsey et al., 2007]. Our contribution is in recording the PI3K/pAkt changes during OTR trafficking, for which

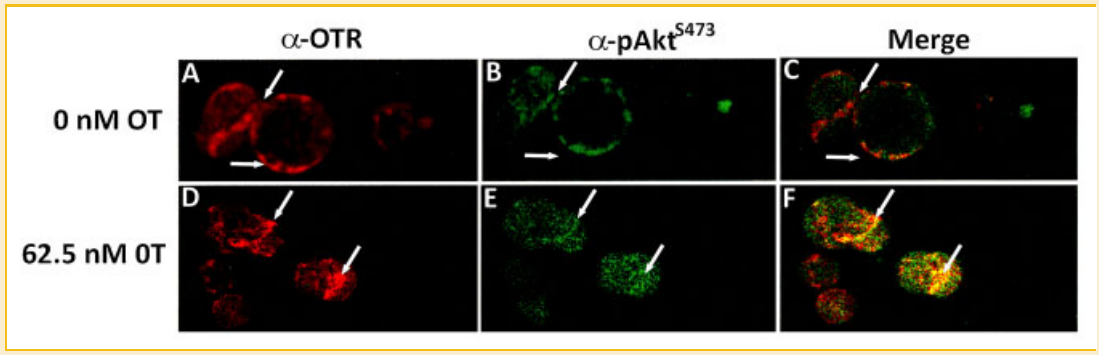

Fig. 7. Dual-label immunocytochemistry showing redistribution of the OTR intracellularly with OT stimulation of Caco2BB cells in concert with pAktS473 elevation. Cells were unstimulated (A–C) or stimulated with 62.5 nM OT (D–F) for 30 min, fixed, washed, and dual-immunolabeled for anti-OTR immunoreactivity (red; A,D) and anti-pAktS473immunoreactivity (green; B,E). Merged fluorescent images are shown in yellow (C,F). In control cells there was a higher density of OTR immunostaining at the cell periphery (arrows in A) compared with OT stimulated cells (D). Note the migrated density of pAktS473 immunofluorescence after OT stimulation from the periphery to the inner cell compartment (B vs. E). The merged fluorescence shows a yellow periphery of the OTR (arrows) co-localized with pAktS473 in unstimluated cells (C) versus merged, yellow immunofluorescence more evenly distributed away from the cell membrane with OT treatment (F), which leaves a greener rim at peripherally for each cell. The section shown is a confocal slice at the middle plane of the cells. Abbreviations: OT, oxytocin; OTR, oxytocin receptor.

we demonstrated the internalization itself by confocal immunocytochemistry 30 min after high OT.

Finally, this study was motivated by our recent finding of OTR expression in newborn rat gut epithelium, including tight junctions [Welch et al., 2009] that constitute a barrier against gut bacterial penetration into internal organs. We were puzzled by the significance of this finding, but the results here hold some functional implications. Recently a model of gut ischemia, which increases bacterial penetration across the epithelial barrier, was used to examine whether tight junction malfunction is implicated in epithelial apoptosis [Huang et al., 2011]. These authors have shown that by glucose instillation, the ischemia-induced intestinal barrier damage can be alleviated via activation of the sodium/glucose transporter1 (SGLT1). The exposure to glucose was accompanied by increased pAkt, phosphorylated mTOR and phosphorylated FoxO1/3A. This finding, together with our discovery that OT can modulate the balance between the two pAkt isoforms may be a relevant clue to the importance of OTR in epithelial junctions. Thus, it will be interesting to test the ability of the OT/OTR stimulation to prevent epithelial apoptosis in such a model. If so, this would provide vital evidence of a role for OT in the prevention of acute necrotizing enterocolitis in newborns, especially in preterm neonates where it is highly prevalent.

ACKNOWLEDGMENTS

The authors thank Robert J. Ludwig and Sara Glickstein, PhD for their assistance. This work was supported by a Columbia Grant #15665, and Einhorn Family Charitable Trust.

REFERENCES

Bachman KE, Argani P, Samuels Y, Silliman N, Ptak J, Szabo S, Konishi H, Karakas B, Blair BG, Lin C, Peters BA, Velculescu VE, Park BH. 2004. The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther 3:772–775.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96:857–868.

Bussolati G, Chinol M, Chini B, Nacca A, Cassoni P, Paganelli G. 2001. 111In-labeled 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid-lys(8)-vasotocin: a new powerful radioligand for oxytocin receptor-expressing tumors. Cancer Res 61:4393–4397.

Byfield MP, Murray JT, Backer JM. 2005. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. J Biol Chem 280:33076–33082.

Cattaneo MG, Chini B, Vicentini LM. 2008. Oxytocin stimulates migration and invasion in human endothelial cells. Br J Pharmacol 153:728–736.

Chibbar R, Miller FD, Mitchell BF. 1993. Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. J Clin Invest 91:185–192.

Conti F, Sertic S, Reversi A, Chini B. 2009. Intracellular trafficking of the human oxytocin receptor: Evidence of receptor recycling via a Rab4/Rab5 "short cycle". Am J Physiol Endocrinol Metab 296:E532–E542.

Devost D, Girotti M, Carrier ME, Russo C, Zingg HH. 2005. Oxytocin induces dephosphorylation of eukaryotic elongation factor 2 in human myometrial cells. Endocrinology 146:2265–2270.

Foster KG, Fingar DC. 2010. Mammalian target of rapamycin (mTOR): Conducting the cellular signaling symphony. J Biol Chem 285:14071–14077.

Gimpl G, Fahrenholz F. 2001. The oxytocin receptor system: Structure, function, and regulation. Physiol Rev 81:629–683.

Gimpl G, Reitz J, Brauer S, Trossen C. 2008. Oxytocin receptors: Ligand binding, signalling and cholesterol dependence. Prog Brain Res 170:193–204.

Gravati M, Busnelli M, Bulgheroni E, Reversi A, Spaiardi P, Parenti M, Toselli M, Chini B. 2010. Dual modulation of inward rectifier potassium currents in olfactory neuronal cells by promiscuous G protein coupling of the oxytocin receptor. J Neurochem 114:1424–1435.

Hanyaloglu AC, von Zastrow M. 2008. Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu Rev Pharmacol Toxicol 48:537–568.

Hashimoto N, Kido Y, Uchida T, Asahara S, Shigeyama Y, Matsuda T, Takeda A, Tsuchihashi D, Nishizawa A, Ogawa W, Fujimoto Y, Okamura H, Arden KC, Herrera PL, Noda T, Kasuga M. 2006. Ablation of PDK1 in pancreatic beta

cells induces diabetes as a result of loss of beta cell mass. Nat Genet 38:589–593.

Huang CY, Hsiao JK, Lu YZ, Lee TC, Yu LC. 2011. Anti-apoptotic PI3K/Akt signaling by sodium/glucose transporter 1 reduces epithelial barrier damage and bacterial translocation in intestinal ischemia. Lab Investig J Tech Method Pathol 91:294–309.

Ihle NT, Lemos R Jr, Wipf P, Yacoub A, Mitchell C, Siwak D, Mills GB, Dent P, Kirkpatrick DL, Powis G. 2009. Mutations in the phosphatidylinositol-3-kinase pathway predict for antitumor activity of the inhibitor PX-866 whereas oncogenic Ras is a dominant predictor for resistance. Cancer Res 69:143–150.

Julien LA, Carriere A, Moreau J, Roux PP. 2010. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. Mol Cell Biol 30:908–921.

Kenakin T, Miller LJ. 2010. Seven transmembrane receptors as shapeshifting proteins: The impact of allosteric modulation and functional selectivity on new drug discovery. Pharmacol Rev 62:265–304.

Kimura T, Tanizawa O, Mori K, Brownstein MJ, Okayama H. 1992. Structure and expression of a human oxytocin receptor. Nature 356:526–529.

Kinsey CG, Bussolati G, Bosco M, Kimura T, Pizzorno MC, Chernin MI, Cassoni P, Novak JF. 2007. Constitutive and ligand-induced nuclear localization of oxytocin receptor. J Cell Mol Med 11:96–110.

Ma XM, Blenis J. 2009. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol 10:307–318.

Maehama T, Dixon JE. 1999. PTEN: A tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol 9:125–128.

Manning M, Miteva K, Pancheva S, Stoev S, Wo NC, Chan WY. 1995. Design and synthesis of highly selective in vitro and in vivo uterine receptor antagonists of oxytocin: Comparisons with Atosiban. Int J Pept Protein Res 46:244–252.

Mitchell BF, Fang X, Wong S. 1998. Role of carboxy-extended forms of oxytocin in the rat uterus in the process of parturition. Biol Reprod 59:1321–1327.

Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, Matzuk MM. 1996. Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci USA 93:11699–1704.

Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkruis FJ, Thomas G. 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci USA 102:14238–14243.

Partovian C, Ju R, Zhuang ZW, Martin KA, Simons M. 2008. Syndecan-4 regulates subcellular localization of mTOR Complex2 and Akt activation in a PKCalpha-dependent manner in endothelial cells. Mol Cell 32:140–149.

Reversi A, Rimoldi V, Marrocco T, Cassoni P, Bussolati G, Parenti M, Chini B. 2005. The oxytocin receptor antagonist atosiban inhibits cell growth via a "biased agonist" mechanism. J Biol Chem 280:16311–16318.

Romanelli A, Dreisbach VC, Blenis J. 2002. Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. J Biol Chem 277:40281–40289.

Ruvinsky I, Meyuhas O. 2006. Ribosomal protein S6 phosphorylation: From protein synthesis to cell size. Trends Biochem Sci 31:342–348.

Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, Yu JS, Malmstrom PO, Mansukhani M, Enoksson J, Hibshoosh H, Borg A, Parsons R. 2005. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 65:2554–2559.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098–1101.

Shenoy SK, Xiao K, Venkataramanan V, Snyder PM, Freedman NJ, Weissman AM. 2008. Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the beta2-adrenergic receptor. J Biol Chem 283:22166–22176.

Skurk C, Izumiya Y, Maatz H, Razeghi P, Shiojima I, Sandri M, Sato K, Zeng L, Schiekofer S, Pimentel D, Lecker S, Taeqtmeyer H, Goldberg AL, Walsh K. 2005. The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. J Biol Chem 280:20814–20823.

Smith MP, Ayad VJ, Mundell SJ, McArdle CA, Kelly E, Lopez Bernal A. 2006. Internalization and desensitization of the oxytocin receptor is inhibited by Dynamin and clathrin mutants in human embryonic kidney 293cells. Mol Endocrinol 20:379–388.

Tolkacheva T, Boddapati M, Sanfiz A, Tsuchida K, Kimmelman AC, Chan AM. 2001. Regulation of PTEN binding to MAGI-2 by two putative phosphorylation sites at threonine 382 and 383. Cancer Res 61:4985–4989.

Vasudevan KM, Barbie DA, Davies MA, Rabinovsky R, McNear CJ, Kim JJ, Hennessy BT, Tseng H, Pochanard P, Kim SY, Dunn IF, Schinzel AC, Sandy P, Hoersch S, Sheng Q, Gupta PB, Boehm JS, Reiling JH, Silver S, Lu Y, Stemke-Hale K, Dutta B, Joy C, Sahin AA, Gonzalez-Angulo AM, Lluch A, Rameh LE, Jacks T, Root DE, Lander ES, Mills GB, Hahn WC, Sellers WR, Garraway LA. 2009. AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. Cancer Cell 16:21–32.

Viero C, Shibuya I, Kitamura N, Verkhratsky A, Fujihara H, Katoh A, Ueta Y, Zingg HH, Chvatal A, Sykova E, Dayanithi G. 2010. REVIEW: Oxytocin: Crossing the bridge between basic science and pharmacotherapy. CNS Neurosci Ther 16:e138–e156.

Welch MG, Tamir H, Gross KJ, Chen J, Anwar M, Gershon MD. 2009. Expression and developmental regulation of oxytocin (OT) and oxytocin receptors (OTR) in the enteric nervous system (ENS) and intestinal epithelium. J Comp Neurol 512:256–270.

Wolfe BL, Trejo J. 2007. Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. Traffic 8:462–470.

Zhang HM, Rao JN, Guo X, Liu L, Zou T, Turner DJ, Wang JY. 2004. Akt kinase activation blocks apoptosis in intestinal epithelial cells by inhibiting caspase-3 after polyamine depletion. J Biol Chem 279:22539–22547.