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OXYTOCIN INHIBITS THE PROLIFERATION OF MDA-MB231 HUMAN BREAST-CANCER CELLS *VIA* CYCLIC ADENOSINE MONOPHOSPHATE AND PROTEIN KINASE A

Paola CASSONI[1], Anna SAPINO[1], Nicoletta FORTUNATI[2], Luca MUNARON[3], Bice CHINI[4] and Gianni BUSSOLATI[1]*

[1]*Department of Biomedical Sciences and Oncology, University of Turin, Turin, Italy*
[2]*Laboratory of Endocrinology, IInd Division of Internal Medicine, University of Turin, Turin, Italy*
[3]*Department of Animal Biology, Laboratory of General Physiology, University of Turin, Turin, Italy*
[4]*Department of Pharmacology, University of Milan, Milan, Italy*

Oxytocin (OT) inhibits the proliferation of breast-cancer cells *in vitro via* a specific G-coupled receptor. To elucidate the intracellular mechanism involved in this biological effect, different G-coupled receptor mediators have been investigated in untreated and OT-treated MDA-MB231 breast-carcinoma cells. In these cells, after OT treatment, a significant cAMP increase was observed using a radioimmunoassay procedure, whereas the Ca^{2+} (determined with the fluorescent probe fura-2) and the inositol phosphate (determined after cell labeling with myo(2-^{3}H)-inositol) concentrations were not modified, contrary to what has been observed in myometrial and myo-epithelial cells. The PKA inhibitor PKI (6-22) amide reverted the effect of OT, indicating that the anti-proliferative effect of the peptide is strictly related to the cAMP–PKA pathway. OT treatment did not modify tyrosine phosphorylation either. Our results indicate that in breast epithelial cells devoid of contractile activity, cAMP is the intracellular mediator of OT action, whereas the Ca^{2+}-phosphoinositide system is not involved. *Int. J. Cancer* 72:340–344, 1997.
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The biological effect of oxytocin (OT) is traditionally associated with the promoting action of contraction in myometrial and myo-epithelial cells (Maggi *et al.,* 1994). However, OT induces specific responses in target cells devoid of contractile activity. In endometrial cells, it regulates the secretion of prostaglandin $F_{2\alpha}$ and luteolysis (Fuchs, 1987; Fuchs *et al.*, 1981; Roberts *et al.*, 1976). In breast-cancer cells, it inhibits cell proliferation both *in vitro* (Cassoni *et al.,* 1994) and *in vivo* (Cassoni *et al.,* 1996). This inhibition is due to specific G protein–linked cell surface receptors (OTR) (Cassoni *et al.,* 1994, 1996) that we demonstrated in breast-cancer cell lines and in primary breast carcinomas using reverse-transcription (RT)-PCR and immunocytochemical procedures (Bussolati *et al.,* 1996).

The mechanism of signal transduction activated during cell contraction is well known, but the same is not true for other responses, such as the inhibition of breast-cancer cell proliferation.

In myo-epithelial cells, OT produces a transient increase of intracellular calcium ions (Ca^{2+}), which participate in the phosphorylation of the contractile protein myosin through the soluble mediator inositol triphosphate (Olins and Bremel, 1984). However, in the same cells Olins and Bremel (1984) observed that a second signal-transduction mechanism is activated, through the increase of cAMP. This increase of cAMP is not directly related to myosin phosphorylation but may play a role in the regulation of other metabolic pathways (Olins and Bremel, 1984).

In epithelial cells, particularly breast-cancer cells, cAMP and cAMP analogues have been reported to reduce cell proliferation (Tagliaferri *et al.,* 1988; Cho-Chung and Gullino, 1974; Cho-Chung, 1980). In this study, we hypothesized that cAMP participates in the signal transduction that inhibits breast-cancer cell proliferation after OT treatment. To test this hypothesis, we first measured the intracytoplasmic concentrations of cAMP, Ca^{2+} and phosphoinositol in OT-treated cells. In addition, we studied the effect of OT in the presence of PKI (6-22) amide, an inhibitor of protein kinase A (PKA) that is the exclusive mediator for the entire cAMP effect. Finally, considering that signaling through G protein–coupled receptors and through receptor tyrosine kinases appears to be tightly interconnected, we measured tyrosine phosphorylation as a function of tyrosine kinase receptor activity.

MATERIAL AND METHODS

Material

MDA-MB231 and MCF7 cells (respectively, human hormone-independent and hormone-dependent breast-cancer cells) were purchased from the ATCC (Rockville, MD). Penicillin, streptomycin, FCS and trypsin/EDTA were obtained from GIBCO BRL (Gaithersburg, MD). Culture medium RPMI 1640, Kreb's solution, Tyrode solution and ionomycin were purchased from Sigma (St. Louis, MO). OT and arginine vasopressin (AVP) were a kind gift of Dr. P. Melin (Ferring, Malmö, Sweden). The PKA inhibitor PKI (6-22) amide was obtained from GIBCO BRL. The anti-phosphotyrosine monoclonal antibody (4G10 anti-phosphotyrosine MAb, UBI, Lake Placid, NY) was a kind gift of Dr. De Bortoli (Torino, Italy). The ECL system for Western blotting was purchased from Amersham (Aylesbury, UK). The cAMP radioimmunological kit was purchased from Incstar (Stillwater, MN). The fluorescent probe fura-2 was purchased from Calbiochem (La Jolla, CA).

Cell cultures

MDA-MB231 and MCF7 cells were routinely cultured in 25-cm^2 flasks at 37°C, 5% CO_2 and 95% humidified atmosphere in RPMI 1640 medium added with 100 IU/ml penicillin and 100 µg/ml streptomycin, supplemented with 10% FCS. When a subconfluent state was reached, cells were detached from the flasks with trypsin/EDTA.

For the different experiments, cells were seeded at a density ranging from 10,000 to 30,000 cells/ml in 200-ml T-flasks or in 24- and 6-multiwell plates.

Measurement of intracellular cAMP

In MDA-MB231 cells plated in 200-ml T-flasks, a dose-response curve was determined and cAMP percent increment was calculated after 30-min incubation in the presence of OT, 1 nM, 10 nM, 100 nM and 1 µM.

The medium was removed from flasks, and cells were washed with 2 ml of sterile PBS and mechanically detached. Cell number was determined by counting with a hemocytometer. Cells were then briefly centrifuged and resuspended in 1 ml of ethanol-HCl and sonicated at 30 W for 3×15 sec. Extraction was accomplished overnight at −20°C. Cell extracts were then dry-evaporated and recovered with 1.0 ml of the acetate buffer provided with the kit.

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*Correspondence to: Department of Biomedical Science and Oncology, Via Santena 7, Turin, Italy. Fax: 39-11-6635267.

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Samples were diluted 1:50 and used for radioimmunoassay determination of cAMP, following the manufacturer's instructions. Values of cAMP were given as pmol/10^6 cells. Following the same procedure, to establish a time course, MDA-MB231 cells were treated with the addition of 100 nM OT in the culture medium for 15, 30, 45, 60 and 90 min after a 24-hr rest in serum-free medium. The concentration of intracytoplasmic cAMP in untreated cells was used as baseline control.

Measurement of intracellular calcium

Intracellular calcium concentration (Ca^{2+}) was measured with the fluorescent probe fura-2 as described by Grynkievics *et al.* (1985). MDA-MB231 cells, grown to sub-confluence on glass coverslips in 6-multiwell plates, were loaded with 3 μM fura-2 pentacetoxymethylesther (fura-2/AM) by 30-min incubation at 37°C. The medium was then replaced with standard Tyrode solution, and the coverslips were placed on an inverted Zeiss microscope with a fuorescence objective (Nikon 100×). Diaphragms were used to observe single cells or groups of cells (5–10 cells). Fluorescence records were taken at excitation wavelengths of 350 nm and 380 nm and emission of 520 nm using a spectrophotometer (Cairn, Newnham, UK) in untreated cells and cells treated with OT 1–100 nM or ionomycin 1 μM (as positive control). Temperature was 22°–24°C. Calibration was done according to Almers and Neher (1985).

Measurement of intracellular inositol phosphates

Intracellular accumulation of inositol phosphates was performed as described by Berridge *et al.* (1982). Briefly, MDA-MB231 cells were grown in 12-well plates until they reached 80% confluence and were labeled for 24 hr with myo(2-^{3}H)-inositol at a final concentration of 2 μCi/ml. Cells were washed twice with Kreb's saline solution and incubated for 10 min in Kreb's solution supplemented with 10 mM LiCL. Finally, cells were stimulated for 20 min with a concentration of agonist ranging from 3.16×10^{-6} M to 1 nM. Maximal accumulation of inositol phosphates was determined by stimulating cells with a concentration of AlF_4 of 10 μM, while that of basal inositol phosphates was determined by adding Kreb's solution. After stopping the reactions with cold PCA, a fraction called total inositol phosphates (total InsP) and containing IP3, IP2 and IP1 phosphoinositols was extracted, recovered and counted.

PKI (6-22) amide treatment

MDA-MB231 cells were treated with OT in the presence of the PKA inhibitor PKI (6-22) amide. Cells were seeded in 24-multiwell plates. Twenty-four hours after plating, cells were incubated in the presence of OT 100 nM, PKI 100 nM or PKI 100 nM + OT 100 nM. Media were changed every 24 hr. Cells were detached with trypsin after 48 and 96 hr of treatment and counted in a hemocytometer. Each experiment was done in triplicate and repeated 3 times.

In a second series of experiments, a dose-dependent curve for PKA inhibitor was obtained. PKI (6-22) amide ranging from 25 to 200 nM was added to medium containing OT 100 nM. Treatment lasted 24 hr. Untreated cells and cells treated with OT or PKI (6-22) amide alone were used as control.

Tyrosine phosphorylation assay

Phosphorylation of tyrosine residues was evaluated on MDA-MB231 cells treated with 100 nM OT for 1, 3, 6 and 10 min. MCF7 cells treated with 10 nM EGF were chosen as positive control (Antoniotti *et al.,* 1994) because of the clear-cut kinase induction exerted by EGF on these cells. Cells were lysed on ice in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 50 mM EGTA, 1% Triton X-100, 150 nM NaCl, 3 mM PMSF, 8 mg/ml aprotinin, 4 mg/ml pepstatin, 1 mM Na orthovanadate, 20 nM Na vanadate, 20 mM theuryl arsine oxide and 20 mM NaF. Cell extracts were centrifuged at 2,000 *g* at 4°C for 15 min. Supernatants were then boiled at 95°C for 5 min in Laemmli sample buffer. Denaturated proteins (20 μg/well) were resolved on an 8% polyacrylamide gel and gel-blotted onto a nitrocellulose filter (BioRad, Richmond, CA). Filters were incubated with 1:2000 4G10 anti-phosphotyrosine MAb at room temperature for 4 hr. The ECL system, using as secondary antibody the anti-mouse IgG provided with the kit, was used for signal detection following the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± SD calculated from a minimum of 3 experiments. Statistical analysis was carried out using ANOVA followed by Bonferroni's correction for statistical comparison among groups or Student's *t* test.

RESULTS

Intracellular cAMP determination

The dose-response curve showed increasing intracellular cAMP levels in the presence of OT, from 1 nM to 100 nM. Analogously to the highest anti-proliferative effect (Cassoni *et al.,* 1994), the highest intracellular cAMP concentration was reached using 100 nM OT. OT 1 μM determined a lower cAMP increase, probably due to a receptor desensitization mechanism (Fig. 1*a*).

A progressive and significant increase in intracellular cAMP was observed when MDA-MB231 cells were incubated from 20 to 60 min with OT 100 nM in serum-free medium. After 10 and 90 min of treatment, the concentrations of intracellular cAMP were similar to those observed in basal conditions (Fig. 1*b*).

Intracellular calcium

MDA-MB231 cells showed a basal calcium concentration of 80–100 nM (50 cells), which was substantially stable and without spontaneous oscillations.

Generally, in more than 80% of the cells this concentration was not altered by OT (Fig. 2). In some experiments only, a very small and rapid transient was observed.

In all experiments, subsequent treatment with 1 μM ionomycin, an ionophore that induces calcium influx from the extracellular medium, led to a significant Ca^{2+} increase (Fig. 2).

Inositol phosphate assay

Neither OT nor AVP was able to increase basal production of total InsP in MDA-MB231 cells at any concentration used. In the same experiments, total InsP concentrations were increased by a factor of 2 after stimulation with AlF_4 (not shown).

Effect of PKI (6-22) amide on cell proliferation

PKI (6-22) amide at a concentration of 100 nM did not affect the proliferation of MDA-MB231 cells in basal growth conditions. However, at the same concentration, PKI (6-22) amide inhibited the anti-proliferative effect of OT (Fig. 3). Moreover, the effect of PKI (6-22) amide on cell growth was dose-dependent (Fig. 4).

Tyrosine phosphorylation

OT did not stimulate tyrosine phosphorylation in MDA-MB231 cells at any point in the time course. On the contrary, EGF 10 nM induced tyrosine phosphorylation in MCF7 cells (positive control) (Fig. 5).

DISCUSSION

We have shown here that cAMP participates in the signal-transduction mechanism that inhibits proliferation of breast-cancer cells after OT treatment, whereas the inositol–Ca^{2+} pathway is not activated.

We previously described the presence of OTR in different breast-cancer cell lines and in primary and metastatic carcinomas of the breast (Bussolati *et al.,* 1996), providing supporting evidence for a possible role of the peptide in the biology of breast cancer. Using a MAb directed against the amino-terminal sequence of the OTR, we observed all over the cell membrane of MDA-MB231 cells a well-marked immunofluorescence staining (Bussolati *et al.,* 1996), confirming our previous RT-PCR results (Cassoni *et al.,*

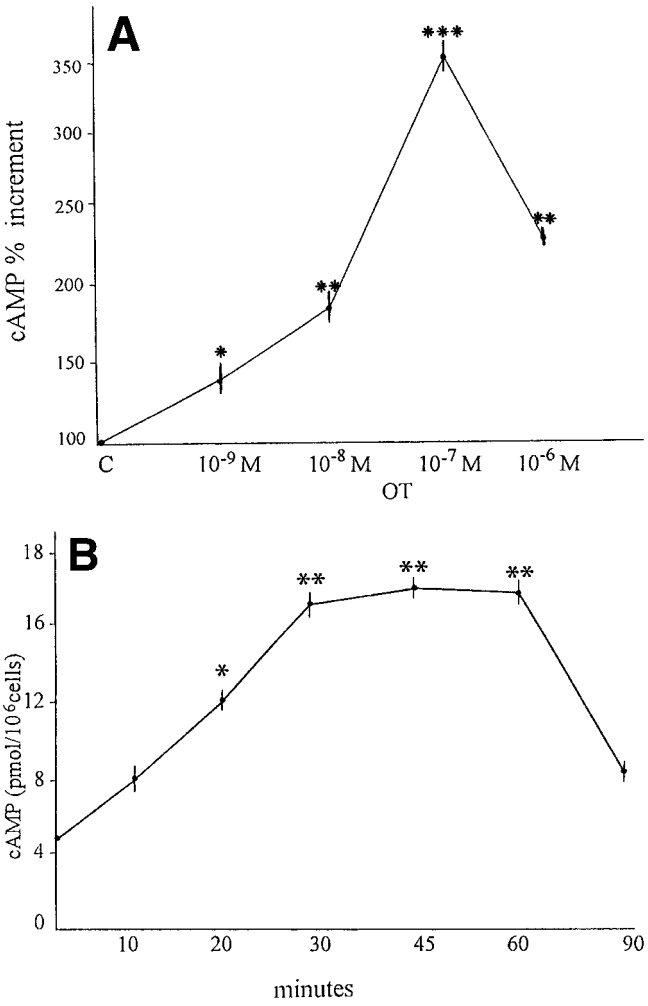

FIGURE 1 – Effect of OT on cAMP intracellular levels: dose-response curve (*a*) and time course (*b*). (*a*) Intracellular cAMP concentration was modified after 30-min OT treatment in a dose-dependent manner. OT 100 nM, which best inhibited the MDA-MB231 cell growth in our previous experiments, determined the maximum cAMP increase. The other OT concentrations used determined intracellular cAMP variations parallel to the values of growth inhibition previously observed. Results are expressed as mean ± SD (n = 3) (test *vs.* control, $*p = 0.01$, $**p = 0.001$ and $***p = 0.001$). (*b*) Addition of OT 100 nM to culture medium determined a significant increase in intracellular cAMP, as observed at 20, 30, 45 and 60 min ($*p = 0.01$ and $**p = 0.001$ *vs.* basal). Results are mean ± SD (n = 3).

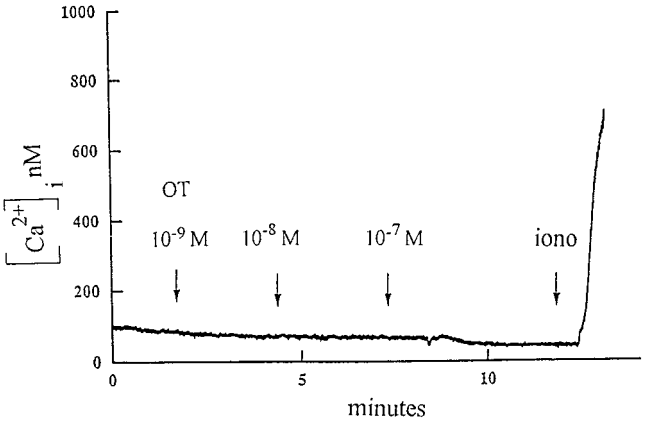

FIGURE 2 – Intracellular Ca^{2+} levels after OT treatment in MDA-MB231 cells. Stimulation with increasing concentrations of OT in single-cell fura-2 experiments did not increase intracellular Ca^{2+}; subsequent application of 1 µM ionomycin induced a rapid increase of Ca^{2+}.

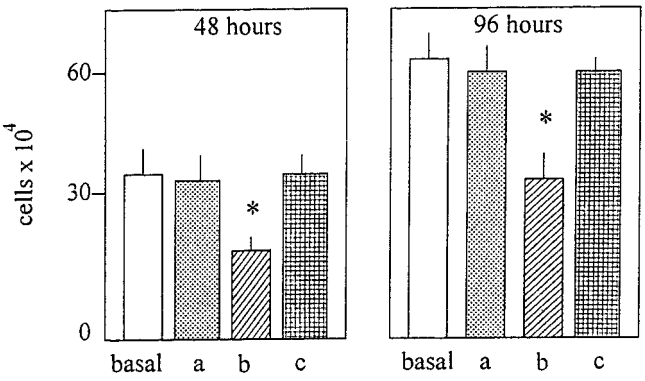

FIGURE 3 – Effect of PKI (6-22) amide on MDA-MB231 cell growth. Cell growth was determined by cell counting at 48 and 96 hr under basal growth conditions and in the presence of (a) PKI (6-22) amide 100 nM, (b) OT 100 nM, (c) PKI (6-22) amide 100 nM + OT 100 nM. OT alone determined a significant reduction of cell number, which was completely reverted by the addition of PKI ($*p = 0.0001$ *vs.* control, PKI alone and OT + PKI) Values are mean ± SD (n = 3).

1994). The specificity of this MAb was demonstrated by staining myo-epithelial cells in tissue sections and by immunoprecipitation techniques (Bussolati *et al.*, 1996). Others have demonstrated the presence of OTR in the MCF7 breast-cancer cell line using binding procedures (Taylor *et al.*, 1990), as well as in primary breast carcinomas using MAb (Ito *et al.*, 1996).

We suggest that OTR might mediate a spectrum of different cellular responses, through different signal-transduction systems, in cells with different phenotypes.

In myometrial cells, which express only the smooth muscle phenotype, OTR activates the phosphoinositide–Ca^{2+} system, causing myosin phosphorylation and contraction (Anwer *et al.*, 1989). In addition, in cultured myometrial cells, Ohmichi *et al.* (1995) reported a mild tyrosine phosphorylation within 1 min of exposure to OT, followed by a quick decrease.

In myo-epithelial cells, in which the muscle phenotype co-exists with the epithelial one, the signal transduction induced by OT involves 2 different and independent pathways. Olins and Bremel (1984) reported that the signal transduction inducing myosin phosphorylation depends on the Ca^{2+}–inositol pathway. However, they indicated that a second pathway is activated through an increase in intracellular cAMP concentration. Interestingly, the increase in cAMP levels in response to OT is not directly involved in the regulation of myosin phosphorylation. On the contrary, the cyclic nucleotide could play a role in the regulation of other metabolic pathways independent of cell contraction and possibly related to proliferative and differentiative processes.

In the present study, we used a ''fully epithelial'' system, devoid of contractile activity, and neither intracellular Ca^{2+} nor phosphoinositol concentration nor tyrosine phosphorylation was modified by OT treatment. On the contrary, we could observe a 4-fold increase in intracellular cAMP 20–60 min after 100 nM OT treatment. This effect was dose-dependent and related to the dose-dependent inhibition of cell proliferation (Cassoni *et al.*, 1994). The 100 nM OT concentration, which determined the highest inhibition of cell growth, also induced the highest intracel-

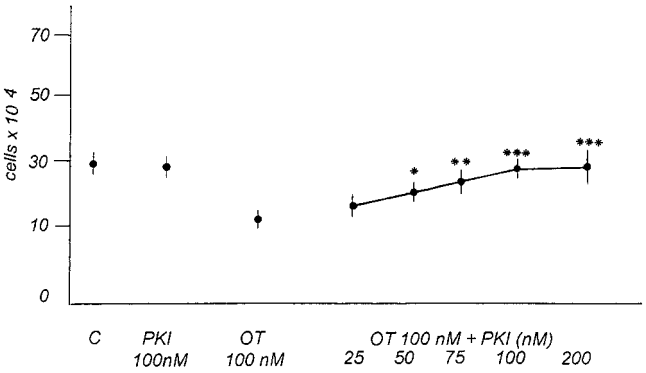

FIGURE 4 – Effect of PKI (6-22) amide on MDA-MB231 cell growth, dose-dependent curve. Cell number was determined 48 hr after cell seeding in basal conditions (C) and in the presence of PKI (6-22) amide 100 nM, OT 100 nM, OT 100 nM + increasing doses of PKI (6-22) amide. Data are expressed as mean ± SD. (*p = 0.05, **p = 0.01, ***p = 0.001 *vs.* OT alone, n = 3).

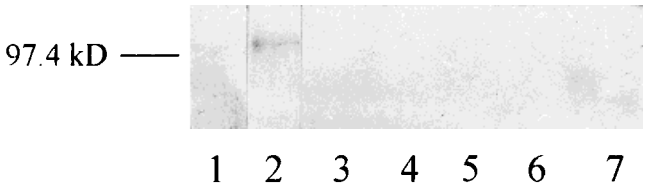

FIGURE 5 – Western blot of tyrosine phosphorylation after OT treatment in MDA-MB231 cells. Cell extracts of MCF7 (untreated and EGF 10 nM–treated) and MDA-MB231 cells (untreated and OT 100 nM–treated at 1, 3, 6 and 10 min) were electrophoresed, transferred to filters and probed with specific MAb against phosphotyrosine. Lane 1, untreated MCF7 cells; 2, MCF7 after EGF 10 nM (positive control); 3, untreated MDA-MB231 cells; MDA-MB231 cells treated with OT 100 nM for 1 min (lane 4), 3 min (lane 5), 6 min (lane 6) and 10 min (lane 7). OT did not induce tyrosine phosphorylation.

lular cAMP concentrations. Higher concentrations of OT (1 µM) induced a lower intracellular cAMP accumulation, probably due to a receptor-desensitization mechanism. Moreover, the anti-proliferative effect of the peptide was abolished by a simultaneous treatment with PKI (6-22) amide, a PKA inhibitor. Taken together, our data indicate that the anti-proliferative effect of OT is strictly related to the cAMP–PKA pathway.

The effect of cAMP on cell proliferation has been widely investigated. cAMP can act as an inhibitor or a promoter of cell growth depending on cell type, cAMP concentration and specific gene induction (Sewen and Pouyssequz, 1992; Coffino *et al.*, 1975; Starzec *et al.*, 1994).

It has been shown that cAMP inhibits cell proliferation *via* blocking the signals transmitted from *ras* to the protein product of another oncogene, *raf*-1, and from there to a series of mitogen-activated protein kinases. Increased concentration of cAMP results in PKA-mediated phosphorylation of Raf, thereby inhibiting its activation and blocking the mitogenic cascade (Cook and McCormick, 1993).

It also has been described that the cAMP-induced growth inhibition parallels the process of cell differentiation (Tortora *et al.*, 1988). Accordingly, we have previously observed that the OT-induced inhibition of breast-cancer cell proliferation is accompanied by a re-arrangement of cytoskeletal filaments and the expression of a novel acquired cell differentiation (Cassoni *et al.*, 1994). Moreover, in developing mouse mammary gland, OT treatment determines a reduction of the undifferentiated (null) cells and a parallel differentiation toward the myo-epithelial phenotype (Sapino *et al.*, 1993).

This concomitant anti-proliferative–differentiative effect indeed suggests that cAMP is the intracellular mediator of OT in breast-carcinoma cells.

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