Biochemical Pharmacology, Vol. 38, No. 18, pp. 3111–3114, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

SHORT COMMUNICATIONS

1,25 Dihydroxyvitamin D3 modulates adenylate cyclase activity of the human breast cancer cell line T47D through increased synthesis of Gs

(*Received* 4 *January* 1989, *accepted* 21 *March* 1989)

In addition to its classic effects on mineral metabolism, 1, 25 dihydroxyvitamin D3 (1, 25 (OH)2 D3) has been shown to modulate the growth and differentiation of malignant cells in culture [1–4]. These effects have been linked to its binding to specific intracellular receptors [5, 6], but the exact mechanisms by which 1,25 (OH)2 D3 exerts its action on cell growth are still unknown.

In a previous study, we showed that 1, 25 (OH)2 D3 in a time and concentration dependent manner markedly inhibited proliferation in liquid culture and cloning efficiency of the human breast cancer cell line T47D. These effects were accompanied by the increased capacity of the treated cells to synthesize and accumulate cyclic AMP upon stimulation by the adenylate cyclase effectors forskolin and prostaglandin E1 (PGE1) [7]. Since we and others had found that agents which increased intracellular concentrations of cyclic AMP inhibited mammary cancer growth, our results could explain the inhibitory effect of 1, 25 (OH)2 D3 upon *in vitro* and *in vivo* replication of human breast cancer cells [8, 9]. An increased effector-stimulated cyclic AMP production in intact cells, measured in the presence of a phosphodiesterase inhibitor, could be due to either a quantitative or qualitative difference in the plasma membrane receptors of adenylate cyclase effectors, or to a modification of the interaction between the catalytic subunit of the cyclase and its regulatory proteins Gs and Gi [10]. To explore the effect of 1, 25 (OH)2 D3 on these parameters, we have cultured T47D cells for two days in the absence or presence of the hormone and measured the following in both types of cells: (a) the plasma membrane adenylate cyclase activity in the basal state and after stimulation by forksolin, which stimulates the known components of adenylate cyclase (regulatory proteins and catalytic subunit) and can directly activate the catalytic subunit of the enzyme in the absence of the regulatory proteins [11], or by vasoactive intestinal peptide (VIP) which stimulates the cyclase activity after binding to specific plasma membrane receptors present in T47D cells [12]; (b) the number of VIP binding sites and their dissociation constant: and (c) the extent of cholera toxin ADP ribosylation of the α subunit of Gs [13, 14].

In the present report, we show that the adenylate cyclase of 1, 25 (OH)2 D3-treated T47D cells has a higher sensitivity to stimulation by forskolin and VIP, and that this is due essentially to increased synthesis of the stimulatory protein Gs.

Materials and methods

Cell line. The human breast cancer cell line T47D was derived from a metastatic pleural effusion [15]. Cells were cultured in Dulbecco's minimum essential medium (DMEM, Gibco Lab., France) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (0.3 mg/ml) and 10% fetal calf serum (FCS, Boehringer, France). Cells were passaged weekly by trypsinization (trypsin-EDTA, Gibco, France). To study the 1, 25 (OH)2 D3 effect, cells were grown in DMEM supplemented with 2% FCS in order to minimize binding of 1, 25 (OH)2 D3 by the serum vitamin D binding protein.

Adenylate cyclase assay. Control and 1, 25 (OH)2 D3 treated cells were washed twice in 50 mM Tris–maleate, 10% sucrose (w/v), pH 7.2 and harvested using a rubber policeman (10^7 cells in 1.5 ml buffer). The cell suspension was homogenized at 4° by 5 strokes of a Teflon pestle in a Potter-Elvehjem glass homogenizer. Protein concentration was determined by the method of Bradford [16]. The enzymatic reaction was initiated by the addition of 20 μl of homogenates (10–25 μg proteins) to 40 μl of incubation medium (50 mM Tris–Maleate, 0.5 mM $MgSO_4$, 0.05 mM ATP, 20 mM phosphocreatinine, 0.2 mg/ml creatine kinase 1, 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), 0.1 mM GTP, 0.1 mM papaverine, 1 μCi α^{32}P ATP, 0.002 μCi ^{3}H cyclic AMP, pH:7.2). The mixture was incubated at 30° for 10 min and the reaction was stopped by addition of 100 μl of a medium containing sodium dodecyl sulfate (1% SDS (w/v), 5 mM ATP, 2 mM cyclic AMP, 50 mM Tris–HCl, pH:7.2). The α^{32}P cyclic AMP synthesized during incubation was fractionated using Dowex–alumina chromatography according to Salomon *et al.* [17]. All determinations were performed in triplicate and the internal variability was always less than 5% of the obtained values.

Membrane preparation and NAD labeling. Control, 1, 25 (OH)2 D3 and vitamin D3 (D3) treated cells for 48 hr were harvested in PBS containing 2.5 mM EDTA. Cell membranes were prepared according to the method of Ross *et al.* [18]. Membranes were washed in 50 mM potassium phosphate, pH:7.3, and then suspended in 50 mM potassium phosphate containing 20 mM thymidine, 5 mM ADP ribose, 20 mM arginine, pH:7.3 [19]. 100 μl of membrane extracts in this buffer were incubated with 100 μM GTP and 10 μM α^{32}P NAD in the presence or absence of 10 μg/ml cholera toxin (Sigma, St Louis, MO), which had been previously activated with 20 mM dithiothreitol at 30° for 10 min. The mixture was incubated for 30 min at 30° and the reaction was stopped by the addition of 1 ml of ice cold potassium phosphate buffer. Membranes were centrifuged at 35,000 *g* for 15 min and solubilized by addition of Lubrol 12A9 to a final concentration of 0.7% in 2.5 mM Hepes, 0.25 mM $MgCl_2$, 0.125 mM EDTA, pH:8.0. The solution was allowed to stand in ice for 30 min with periodic agitation.

SDS-polyacrylamide gel electrophoresis. Lubrol extracts were prepared for SDS-gel electrophoresis by adding SDS and 2β mercaptoethanol to a final concentration of 1% (w/v) and 5% (v/v) respectively. SDS gel electrophoresis was performed according to Laemmli [20] using 10% polyacrylamide gel. The gels were stained by Coomassie blue, dried and exposed to X-ray film (Kodak X-Omat) for 12 hr at −80°.

VIP binding. Conditions of apparent equilibrium for ^{125}I VIP binding were obtained at 37° for 45 min in 0.2 ml of the binding assay buffer containing: 35 mM Tris–HCl (pH = 7.5), 1.2% BSA, 50 mM NaCl, 0.6 mg/ml bacitracin and 5×10^{-11} M ^{125}I VIP as previously described [12]. Cell associated ^{125}I VIP was separated by centrifugations of triplicate 160 μl aliquots of the incubation mixture layered over 1 ml of ice cold 35 mM Tris–HCl, 2% BSA, 50 mM NaCl (pH:7.5). Centrifugations were performed in plastic microtest tubes (Eppendorf, F.R.G.) with a Beckman Model B microfuge for 3 min. After removal of the supernatant, cell

pellets were washed twice with cold Tris–HCl buffer. The bottom of the microfuge tubes was cut off and counted in a LKB autogamma counter. Competition of ^{125}I VIP binding was determined in the presence of 10^{-11} to 10^{-6} M unlabeled VIP.

Materials. 1, 25 (OH)2 D3 and vitamin D3 were a generous gift of Mr Meier and Dr Muller (Hoffman-LaRoche, Basel, Switzerland). Radioactive ATP ($\alpha^{32}P$ ATP, 30–40 Ci/mmol.), cyclic AMP (^3H cAMP, 25 Ci/mmol) and NAD^+ (^{32}P NAD, 30 Ci/mmol) were obtained from New England Nuclear (Boston, MA). ^{125}I VIP (2000 Ci/mmol) was from Amersham (U.K.). Creatinine phosphate, creatine kinase and GTP were from Boehringer Mannheim (F.R.G); VIP was from Peninsula (Sweden); cholera toxin and all other reagents were purchased from Sigma (St Louis, MO).

Results

Table 1 shows the results of adenylate cyclase measurements in membranes of control and 5×10^{-7} M 1, 25 (OH)2 D3 treated cells for 48 hr. In the absence of stimulation, control and 1, 25 (OH)2 D3 treated cells had similar enzymatic activity. In the presence of forskolin and VIP, there was a marked activation of adenylate cyclase in control T47D membranes (35- and 26-fold respectively). This activation is significantly higher in 1, 25 (OH)2 D3-treated cell membranes (52- and 40-fold respectively), leading to a 1.87- and 1.92-fold increase in stimulated adenylate cyclase activity in 1, 25 (OH)2 D3 treated cells.

The data presented in Table 2 show that 1, 25 (OH)2 D3 treatment of T47D cells did not change either the absolute number of VIP receptors (10,000 sites per cell) or their affinity for the ligand ($K_d = 5 \times 10^{-10}$ M).

In an attempt to study the effect of 1, 25 (OH)2 D3 on the α subunit of the regulatory protein Gs and its influence on adenylate cyclase activation, we studied the ADP ribosylation of control and 1, 25 (OH)2 D3-treated cells mediated by cholera toxin. Membranes from T47D cells exposed to cholera toxin had two ADP-ribosylable bands at M_r 41 and 46 kilodaltons (kDa) (Fig. 1).

Treatment of T47D cells with 1, 25 (OH)2 D3 for 48 hr increased the relative amount of $\alpha^{32}P$ incorporated in both bands. The effect was more pronounced for the 46 kDa band as measured by laser densitometry (2202 Ultroscan, LKB) (Fig. 1, lane 2 and Fig. 2, lane 1). Figure 1 also shows that non-hydroxylated D3, which had no effect on T47D growth (not shown), did not increase cholera toxin induced ADP-ribosylation of these two bands.

In order to determine whether the increase in cholera toxin induced ADP-ribosylation of Gsα in 1, 25 (OH)2 D3 treated cells was a consequence of protein synthesis, we cultured T47D cells first for 24 hr with 1, 25 (OH)2 D3 and then for another 17 hr in the presence of 1, 25 (OH)2 D3 and cycloheximide (10 μg/ml). Figure 2 shows that, under these conditions, ADP ribosylation of the two bands strongly decreased, predominantly in the 46 kDa band.

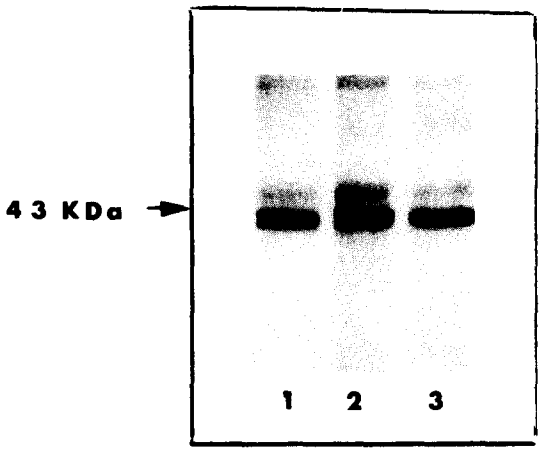

Fig. 1. Autoradiography of SDS polyacrylamide gel (SDS-PAGE) of ($\alpha^{32}P$) labeled GTP binding proteins resulting from cholera toxin-ADP-ribosylation of membranes of control (1), 1, 25 (OH)2 D3 treated T47D cells (2) and D3 treated T47D cells (3) for 48 hr as described in Materials and Methods. Aliquots of T47D membranes containing approximately 50 μg protein per lane were separated by 10% SDS-PAGE. Only the region of the gel between 35 and 55 KDa is shown. Apparent molecular weights of proteins labeled with $\alpha^{32}P$ ADP ribosyl moiety were 41 and 46 KDa, respectively. These experiments were performed four times with similar results.

When T47D cells were first treated by 1, 25 (OH)2 D3 for 48 hr and then by cholera toxin (1 μg/ml) for 5 hr, ADP ribosylable bands were no longer present in membranes prepared from those cells and incubated *in vitro* for 30 min in the presence of cholera toxin and $\alpha^{32}P$ NAD.

Discussion

In a previous study, we showed that 1, 25 (OH)2 D3 (5×10^{-7} M) inhibited cell proliferation and induced an increase in effector-stimulated cyclic AMP production in the human breast cancer cell line T47D [7]. In the present

Table 1. Effect of 1, 25 (OH)2 D3 on basal and stimulated adenylate cyclase activity in T47D cells (pmol cyclic AMP/mn/mg protein)

Effector	Control cells	1, 25 (OH)2 D3-treated cells	P
none	1.13 ± 0.28	1.63 ± 0.66	NS
forskolin	42.93 ± 5.15	84.86 ± 4.56	<0.005
VIP	32.16 ± 3.61	63.33 ± 1.67	<0.005

T47D cells were incubated for 48 hr in the presence or absence of 1, 25 (OH)2 D3 (5×10^{-7} M). Membranes were incubated and assayed for adenylate cyclase activity as described in Materials and Methods with no effector (none), forskolin (10^{-5} M) or Vasoactive intestinal peptide (VIP: 10^{-4} M). Values represent the mean ± SE of three different experiments done in triplicate. Statistical analysis was done using Mann and Whitney tests.

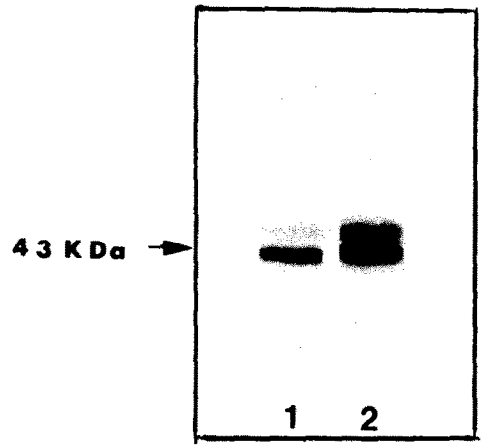

Fig. 2. Autoradiography of SDS polyacrylamide gel of (α^{32}P) labeled GTP binding proteins resulting from cholera toxin ADP ribosylation of membranes of T47D cells treated first for 24 hr with 1, 25 (OH)2 D3 and then for another 17 hr in the presence of 1, 25 (OH)2 D3(1) or 1, 25 (OH)2 D3 and cycloheximide (10 μg/ml) as described in Materials and Methods

Table 2. Effect of 1, 25 (OH)2 D3 on the binding of ^{125}I VIP by T47D human breast cancer cells

	Control	1, 25 (OH)2 D3
K_d ($\times 10^{-10}$ M)	5.37 ± 2.29	5.35 ± 2.24
Receptor number	10065 ± 3760	9472 ± 2441

The dissociation constant and receptor number were determined by Scatchard analysis as described in Materials and Methods. Data represent mean ± SE of four separate experiments performed in triplicate.

paper we show that 1, 25 (OH)2 D3 treatment induces an increase in effector-stimulated adenylate cyclase activity in T47D membranes.

Our results are consistent with the observation that perfused bones from vitamin D deficient rats showed reduced levels of both basal and parathromone-stimulated cAMP, and are restored in vitamin D treated rats, which suggests that vitamin D might play a key role in cAMP response to PHT in the bones [21].

The increased adenylate cyclase activity may be due to either a quantitative or qualitative change in the effector plasma membrane receptor, or to a modulation of the interaction between the catalytic subunit of adenylate cyclase and its regulatory proteins.

Using the polypeptide VIP, which stimulates cyclic AMP production in T47D cells through binding to specific receptors [12], we demonstrate that this increase is not related to a quantitative or qualitative difference in plasma membrane VIP receptors, since there were no differences in VIP receptor affinity and site numbers in control and 1, 25 (OH)2 D3 treated cells.

In addition to the effector receptor, the adenylate cyclase complex consists of two components, the catalytic subunit and regulatory G proteins. There are two G proteins: stimulatory (Gs) and inhibitory (Gi), and both are heterotrimeric proteins with α, β and γ subunits. Gsα is ADP-ribosylated and is activated by cholera toxin, whereas Giα is ADP-ribosylated by pertussis toxin [14, 22]. Modifications in these components could also lead to an increase in enzyme activity.

The lack of a difference in stimulation of adenylate cyclase activity by VIP and forskolin indicates that an increase in the catalytic subunit activity itself cannot be involved. Moreover, no modification in adenylate cyclase activity in the presence of Mn^{2+} or Mg^{2+} [11] was observed in 1, 25 (OH)2 D3 treated cells (not shown). On the other hand, cholera toxin-induced ADP-ribosylation experiments showed that 1, 25 (OH)2 D3 treated T47D cells had a higher amount of Gsα, than untreated cells, and that this increase corresponded to *de novo* protein synthesis. To our knowledge, this is the first report of an effect of 1, 25 (OH)2 D3 upon modulation of Gsα synthesis in human mammary cancer cells.

Different tissues and cells have been shown to contain varying amounts of two forms of Gsα [23]. Here, in T47D cells, cholera toxin-ADP ribosylated two proteins with apparent M_r of 41 and 46 kDa; the relative amount of the 41 kDa band was consistently greater than that of the 46 kDa band. No functional specificity has yet been ascribed to either of these bands. However, it has been shown that maturation of rat reticulocytes into erythrocytes is accompanied by a decrease in the relative amount of the Gsα subunit of higher molecular weight which might explain in part the lower adenylate cyclase activity of erythrocytes [24]. Moreover, Sternweis *et al.* found that rabbit hepatic fractions enriched in the higher M_r subunit of Gsα appeared to reconstitute a more efficient coupling between hormone receptor and the catalytic subunit of adenylate cyclase in cyc-S49 cell membranes [25]. Our results with 1, 25 (OH)2 D3-treated T47D cells, showing both increased ADP-ribosylation of the 46 kDa band and adenylate cyclase activity, are in agreement with these findings.

In a previous study, we had shown that 1, 25 (OH)2 D3 treatment inhibited the proliferation of T47D cells in a medium containing 2% fetal calf serum [7]. This effect could be related to a direct action of the hormone after binding to its nuclear receptor [5, 6]. It may also be related to an indirect effect, through higher cyclic AMP production upon adenylate cyclase stimulation by effectors present in serum. Indeed, we and others had previously found that agents which increased intracellular cyclic AMP concentration inhibited mammary cancer growth [8, 9, 26].

Cyclic AMP may act either by a direct effect upon gene expression [26] or by modulation of growth factors receptors [27]. Koga *et al.* recently showed that the epidermal growth factor receptor is down regulated in 1, 25 (OH)2 D3-treated human breast cancer cell lines T47D and MCF7 [28]. This and our data indicate that the antiproliferative effect of 1, 25 (OH)2 D3 in human breast cancer cells could imply interactive mechanisms.

In summary, we showed that 1, 25 (OH)2 D3 induced an increase in stimulated adenylate cyclase activity in membranes of T47D human breast cancer cells. We demonstrated that this increase is related neither to a modification in receptor effector nor to activation of the adenylate cyclase catalytic subunit, but is due essentially to increased synthesis of the stimulatory protein Gs.

Acknowledgements—This work was supported by grants from the Ministère de la Recherche et de l'Enseignement Superieur (MRES) and the Association pour la Recherche contre le cancer (ARC).

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