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Growth-promoting Activity of Desmopressin in Murine Leukemia Cells Treated *in Vitro*[1]

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ABSTRACT

The synthetic vasopressin analogue, desmopressin (dDAVP), has been shown to influence membrane transport of melphalan in murine L5178Y lymphoblasts. Accordingly, the effect of dDAVP on the cytocidal activity of melphalan in L5178Y cells was evaluated. dDAVP did not affect the cytocidal activity of melphalan in these cells, but significantly affected the cloning efficiency of stationary phase or slowly dividing L5178Y cells over a range of concentrations. In particular, stationary phase cells showed an increase in cloning efficiency from 4.3 ± 0.5% in control cells to 7.0 ± 0.3% in cells treated with 25 nM dDAVP ($P < 0.001$), whereas cells doubling every 26 h showed an increase from 10.8 ± 1.2% in control cells to 21.0 ± 2.0% in cells treated with 150 nM dDAVP ($P < 0.001$). This phenomenon was associated with significant elevations of 1,2[^{3}H] diacylglycerol after incubation with dDAVP for 9 min ($P < 0.01$) and of total [^{3}H]diacylglycerols after incubation for both 3 min ($P < 0.05$) and 9 min ($P < 0.02$). Within 10 s of treatment with 100 nM dDAVP, there was a marked decrease in the levels of inositol 1,4,5-trisphosphate and inositol 1-phosphate, but subsequently no change was observed for up to 9 min after treatment. We postulate that the increase of diacylglycerol content produced by dDAVP might be primarily from a phosphatidylcholine source and that the growth-promoting activity of desmopressin may be a consequence of activation of protein kinase C.

INTRODUCTION

The synthetic vasopressin analogue, dDAVP[4] or desmopressin, has been shown to influence a wide range of cell membrane-related events. Recently, Goldenberg *et al.* reported on modulation of membrane transport of alkylating agents and amino acids by dDAVP (1). dDAVP stimulated melphalan uptake, inhibited uptake of nitrogen mustard, and had no effect on the uptake of cyclophosphamide. dDAVP is also recognized as the drug of choice in the treatment of diabetes insipidus and in other situations where an antidiuretic effect is useful, and in the treatment of bleeding disorders (2).

Vasopressin, the native counterpart of dDAVP, has also been shown to be a potent mitogen for quiescent cultures of Swiss 3T3 cells (3). Vasopressin induces phosphatidylinositol breakdown (4) in several cell lines such as smooth muscle A-10 and in rat hepatocytes (5, 6) and also stimulates the efflux of calcium from an intracellular pool (7). The action of vasopressin and other growth factors, *e.g.*, platelet-derived growth factor and bombesin, on Swiss 3T3 cells results in an increased formation of DAG (8) and IP_3 (4). The latter is likely to function as a second messenger to mediate the calcium-mobilizing action of growth factors. The hydrolysis of PIP_2 induced by growth factors is known to result in the activation of two signal pathways which may regulate key ionic events responsible for cell proliferation. Changes in pH may be controlled through DAG, whereas calcium levels are regulated by IP_3.

The present study was undertaken to determine if the effect of dDAVP on transport could be exploited therapeutically to enhance the cytocidal activity of melphalan against L5178Y lymphoblasts. dDAVP treatment was found to increase cloning efficiency of these cells, so the investigation was expanded to elucidate the mechanism of this apparent growth-promoting activity.

MATERIALS AND METHODS

Drugs and Chemicals. Melphalan, *p*-di(2-chloroethyl)amino-L-phenylalanine, was provided by Dr. J. R. MacDougall, Burroughs Wellcome and Co. (Canada), Ltd., Montreal, Quebec. dDAVP was obtained from Richmond Pharmaceuticals, Inc., Richmond Hill, Ontario, Canada. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid in ethanol (94.5 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Unlabeled arachidonic acid (5,8,11,14-eicosatetraenoate), 1,2- and 1,3-diacyl-*sn*-glycerol, and pig liver triglycerides were obtained from Serdary Research Laboratories, Inc., London, Ontario, Canada. 2-*myo*-[^{3}H]Inositol (specific activity, 15 Ci/mmol) was purchased from American Radiolabelled Chemicals, Inc., St. Louis, MO.

Cytotoxic Activity of Melphalan in the Presence and Absence of dDAVP. The sensitivity of murine L5178Y lymphoblasts to melphalan in the presence and absence of dDAVP was determined using the clonogenic assay of Chu and Fisher (9). Exponential phase cells at a concentration of 2 to 3 × 10^5 cells/ml were treated with melphalan for 15 min at 37°C in DPBS after treatment with either 100 nM dDAVP or 0.85% NaCl solution as control for 10 min. The cloning efficiency of treated cells was determined at each drug concentration, and the surviving cell fraction was calculated. Linear regression analysis of the dose-survival curves was performed, the regression equation being in the form, $\log_e y = mx + b$, where y is the surviving cell fraction, x is the dose of drug, m is the slope of the regression line, and b is the y-intercept. D_0, the dose of drug reducing survival to $1/e$, *i.e.*, 37% of the initial cell population, was derived from the negative reciprocal of the slope of the regression line as described previously (10). Statistical analysis of the dose-survival curves and intercepts was performed by a t test comparing the significance of the difference of slopes.

Assay of Growth-promoting Activity. The growth-promoting effect of dDAVP on murine L5178Y lymphoblasts was determined in Fisher's medium containing 15 to 20% horse serum using the clonogenic assay of Chu and Fisher (9). Stationary phase cells and cells with doubling times ranging from 12 to 26 h, each at a concentration of 2.5 to 3.5 × 10^5 cells/ml, were treated with dDAVP or 0.85% NaCl solution as control for 10 min at 37°C in DPBS, and cloning efficiency was determined. The growth-promoting effect of dDAVP on exponential phase P388 leukemia cells was also determined. Statistical analysis of the difference in cloning efficiency of control and dDAVP-treated cells was determined using unpaired t tests.

DAG Assay. [^{3}H]Arachidonic acid was added to exponentially multiplying L5178Y cells at 2 × 10^5 cells/ml in Fisher's medium containing 10% horse serum. After incubation for 44 h the cells, which were doubling approximately every 27 h, were centrifuged at 1000 rpm for 5 min, washed, and resuspended in DPBS at 3.0 to 3.5 × 10^5 cells/ml. Cell suspensions of 18.2 ml were treated with either 100 nM dDAVP

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[4] The abbreviations used are: dDAVP, 1-deamino-8-D-arginine vasopressin; IP_3, inositol 1,4,5-trisphosphate; IP_2, inositol 1,4-bisphosphate; IP, inositol 1-phosphate; DPBS, Dulbecco's phosphate-buffered saline; DAG, diacylglycerol; TLC, thin-layer chromatography; PIP_2, phosphatidylinositol 4,5-bisphosphate.

or 0.85% NaCl solution as control for 10 min at 37°C. Incubation was terminated by addition of 30 ml of ice-cold DPBS and centrifugation at 1000 rpm, and the supernatant was discarded. The cell pellet was resuspended in 3 ml of ethyl acetate, agitated for 15 s, and centrifuged, and the supernatant was collected for TLC (11). One additional extraction was performed with 3 ml of ethyl acetate, and two with 3 ml of ethyl acetate after addition of 20 μl of 1 N HCl. The solvent was evaporated under N_2 at 37°C; 5 μl of ethyl acetate were added to the cell extracts; and unlabeled arachidonic acid, 1,2- and 1,3-DAG, and pig liver triglycerides were added as standards. The cell extracts and standards were applied to a "Baker" 7000-4 Si 250 TLC plate (J. B. Baker Co.) which had been activated at 100°C for 60 min. Lipid migration took place in a solvent system of benzene:diethyl ether:ethanol:acetic acid (50:40:2:0.2) (12). After drying, the lipids were localized by brief exposure to iodine vapor, and their mobility was compared with that of standards on the same plate. Radioactivity was counted using a System 200 imaging scanner (Bioscan, Inc.) coupled to an IBM-PC XT.

Assay of Inositol Phosphates. Murine L5178Y lymphocytes were usually cultured in Fisher's medium containing 15% horse serum; after transfer to Fisher's medium containing 7.5% horse serum, the doubling time of these cells was prolonged from 12 to approximately 22 h. Cells were incubated in this suboptimal medium for 32 h, at which point *myo*-[^{3}H]inositol (0.4 μCi/ml) was added, and the cells were incubated for an additional 16 h without changing medium. At the end of 48-h incubation, cells were harvested, washed, and suspended in DPBS.

To study the effect of dDAVP on inositol phosphates, cells were incubated at 37°C with either 100 nM dDAVP or 0.85% NaCl solution as control. Aliquots of 500 μl of incubation mixture were removed at 0 and 10 s and at 3 and 9 min and immediately placed in 1.88 ml of chloroform:methanol:HCl (100:200:2). To this mixture, 0.6 ml of chloroform were added, followed by 0.6 ml of water. Tubes were centrifuged briefly at 1000 rpm, and the upper aqueous layer was collected and applied on a 2.0-ml column of Bio-Rex (formate form). The radioactive phosphate esters were eluted sequentially by using 5 mM disodium tetraborate/60 mM sodium formate (for glycophosphoinositol), 0.1 M formic acid/0.2 M ammonium formate (for IP), 0.1 M formic acid/0.4 M ammonium formate (for IP_2), and 0.1 M formic acid/1.0 M ammonium formate (for IP_3) as described by Berridge *et al.*. (13).

RESULTS

Dose-Survival Curves of Murine L5178Y Lymphoblasts Treated with Melphalan in the Presence or Absence of dDAVP. Dose-survival curves of murine L5178Y lymphoblasts treated with melphalan in the presence or absence of dDAVP were obtained. The D_0 (mean ± SE) for L5178Y cells treated with melphalan alone was 0.75 ± 0.04 μM, and that for cells treated with melphalan and dDAVP was 0.73 ± 0.02 μM; there was no significant difference between the slopes or intercepts of these curves.

Effect of dDAVP and Vasopressin on the Cloning Efficiency of Murine L5178Y Lymphoblasts and P388 Leukemia Cells. The effect of dDAVP on the cloning efficiency of L5178Y cells with varying proliferative rates is shown in Table 1. dDAVP at a concentration range of 25 to 225 nM significantly augmented the cloning efficiency of stationary phase cells or cells dividing every 26 h, whereas exponential phase cells and those with a doubling time of 16 h showed significant augmentation of cloning efficiency only after treatment with 150 nM dDAVP. Vasopressin also enhanced the cloning efficiency of stationary phase L5178Y cells at a concentration range of 5 to 100 nM. The maximum effect of vasopressin was noted at 100 nM, at which concentration the cloning efficiency of treated cells was 22.92 ± 2.9% compared to 15.98 ± 0.7% for untreated control cells.

The effect of dDAVP on the cloning efficiency of exponential phase P388 leukemia cells was also examined at a dDAVP concentration range of 5 to 150 nM. A dose-response effect was observed with maximal augmentation occurring at a dDAVP concentration of 100 nM, at which the cloning efficiency of treated cells was 1.84-fold greater than that of untreated control cells, and this difference was highly significant ($P < 0.001$). This observation indicated that the growth-promoting activity of dDAVP was not restricted to one cell type.

Effect of dDAVP on 1,2- and 1,3-DAG Levels in Murine L5178Y Lymphoblasts. The effect of 100 nM dDAVP desmopressin on profiles of ^{3}H-labeled 1,2- and 1,3-DAG in L5178Y cells 3 min after treatment is shown in Fig. 1. Markedly higher peaks for both tritiated 1,2- and 1,3-DAG suggest increased synthesis of these products after incubation with dDAVP. A time course of 1,2-[^{3}H]DAG and total ^{3}H-labeled diacylglycerols in control and dDAVP-treated cells is shown in Fig. 2. In three separate experiments, dDAVP treatment was associated with increases in tritiated diacylglycerols at 3 and 9 min. Despite considerable interexperimental variability, randomized block design analysis of variance showed an interaction between time and dDAVP effect, and comparisons of tritiated diacylglycerols were made between control and dDAVP-treated cells at each time point. Treatment with dDAVP resulted in a significant increase of 1,2[^{3}H]DAG at 9 min ($P < 0.01$) and of total tritiated diacylglycerols at both 3 min ($P < 0.05$) and 9 min ($P < 0.02$) (Fig. 3).

Effect of dDAVP on Inositol Phosphate Levels. The level of radioactivity in IP, IP_2, and IP_3 of cells treated with [^{3}H]inositol was determined in slow growing murine L5178Y cells at 10 s and after 3 and 9 min after the addition of 100 nM dDAVP (Fig. 4). Within 10 s of treatment with dDAVP there was a marked decrease ($P < 0.001$) in the level of IP_3, but subsequently no change was observed for up to 9 min (Fig. 4*A*). Whereas in untreated control cells, IP_3 content decreased gradually from zero to 9 min, and this decline, as evaluated by repeated measures analysis of variance, was statistically significant ($P < 0.001$). There was a significant reduction ($P < 0.02$) of IP_2 in both control and dDAVP-treated cells with time; however, no difference was noted between control and dDAVP-treated cells (Fig. 4*B*). The response of IP to dDAVP treatment was similar to that of IP_3, with an initial significant drop ($P < 0.01$) being followed by no change for up to 9 min, whereas in control cells, a significant decline ($P < 0.001$) of IP was observed over 9 min (Fig. 4*C*).

DISCUSSION

Vasopressin receptors have been found in the kidney, liver, brain, pituitary, aortic smooth muscle, and on platelets (2), and two subtypes have been identified. V_1 receptors mediate pressor activity and are associated with increased phosphatidylinositol turnover and formation of IP_3 and DAG (14–16). V_2 receptors mediate antidiuretic activity and are associated with activation of adenylate cyclase to raise cyclic AMP levels (17–20). Rozengurt and workers have found vasopressin to be a potent mitogen for murine Swiss 3T3 cells, acting synergistically with insulin and several other growth-promoting factors (3); to modulate epidermal growth factor affinity (21); and to induce desensitization to its mitogenic effect in 3T3 cells after prolonged incubation (22). Additional evidence was provided suggesting that the growth-promoting activity in 3T3 cells is mediated by a pressor-type (V_1) receptor (22).

dDAVP has a relative affinity for V_1 receptors of only 0.009 the relative affinity of vasopressin, while having 1.6-fold the

Table 1 *Cloning efficiency of murine L5187Y lymphoblasts treated with dDAVP*

Cells were treated with dDAVP for 10 min as described in the text. In separate experiments, the growth-promoting effect of dDAVP was determined using stationary phase cells and cells doubling at different rates as described in the text. The cloning efficiency of cells treated with desmopressin is expressed as a percentage of the cloning efficiency of untreated control cells.

Doubling time (h)	Control	25 nM dDAVP	75 nM dDAVP	100 nM dDAVP	150 nM dDAVP	225 nM dDAVP	375 nM dDAVP
Stationary	4.3 ± 0.5[a]	7.0 ± 0.3[b]	6.7 ± 0.4[c]	7.2 ± 0.5[c]	6.0 ± 0.4[d]	ND[e]	ND
26	10.8 ± 1.2	7.6 ± 0.6	17.1 ± 0.9[b]	19.5 ± 2.6[f]	21.0 ± 2.0[b]	19.3 ± 2.7[d]	ND
24	22.3 ± 2.7	27.1 ± 1.4	19.6 ± 2.4	21.3 ± 2.9	29.6 ± 2.5	ND	23.0 ± 2.5
16	38.3 ± 3.1	27.8 ± 2.8	36.8 ± 2.9	36.0 ± 0.8	58.0 ± 0.7[c]	ND	39.3 ± 0.6
12	40.9 ± 3.5	39.1 ± 3.6	51.2 ± 2.7	43.8 ± 3.4	54.9 ± 3.6[d]	ND	41.8 ± 4.2
12	78.0 ± 4.7	69.9 ± 1.2	74.2 ± 3.9	79.7 ± 3.0	75.4 ± 3.6	ND	81.0 ± 2.8

[a] Mean ± SE of 4 to 12 determinations.
[b] $P < 0.001$ (two-tailed t test).
[c] $P < 0.0025$ (two-tailed t test).
[d] $P < 0.01$ (one-tailed t test).
[e] ND, not determined.
[f] $P < 0.01$ (two-tailed t test).

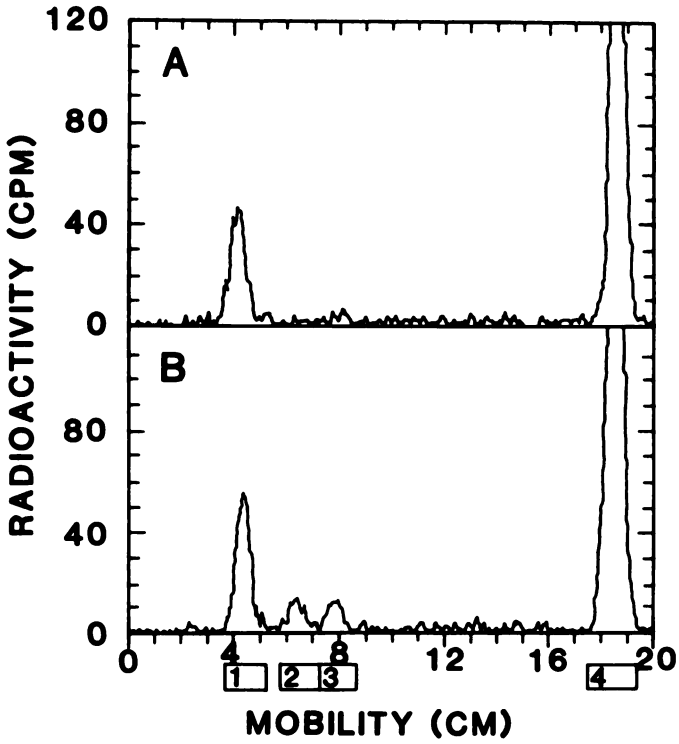

Fig. 1. Tritiated triglycerides, 1,2-[^{3}H]- and 1,3-DAG, and ^{3}H-labeled phospholipids in L5178Y cells in the absence (*A*) and presence (*B*) of dDAVP. Cells were preincubated for 44 h with [^{3}H]arachidonic acid and then treated for 3 min with either 0.85% NaCl solution as control (*A*) or 100 nM dDAVP (*B*). A profile, based on radioactivity, of lipid migration on a TLC plate was produced by an imaging scanner. After exposure of the TLC plate to iodine vapor for visualization of unlabeled standards, the plate was compared to the radioactivity profile, and the following radioactive fractions were identified: triglyceride (*Peak 1*); 1,3-DAG (*Peak 2*); 1,2-DAG (*Peak 3*); and phospholipid (*Peak 4*), as illustrated in the *boxes* along the *abscissa*. In subsequent computer analysis, the radioactivity of each peak, expressed as a percentage of the total radioactivity, was determined.

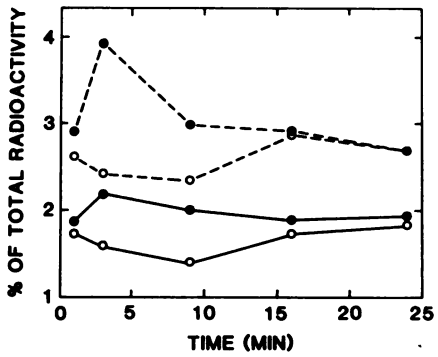

Fig. 2. A time course of 1,2-[^{3}H]DAG (——) and total ^{3}H-labeled diacylglycerols (– – – –) in control L5178Y cells (○) and in cells treated with 100 nM dDAVP (●) after 44-h preincubation with [^{3}H]arachidonic acid. Total [^{3}H]DAG includes tritiated 1,2- and 1,3-DAG, and the data are expressed as a percentage of the total radioactivity as described in the legend to Fig. 1. Incubations were terminated by addition of ice-cold DPBS, and centrifugation was at 1000 rpm for 5 min as described in the text.

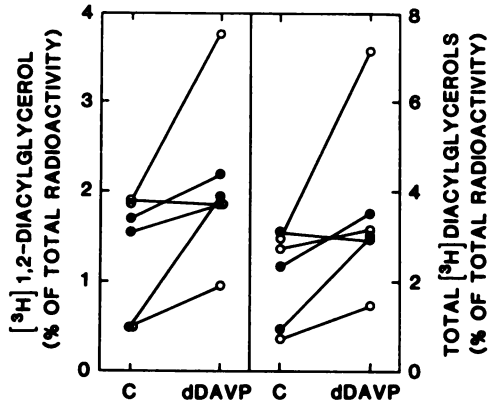

Fig. 3. 1,2-[^{3}H]DAG and total ^{3}H-labeled diacylglycerols in control L5178Y cells and in cells treated with 100 nM dDAVP for 3 min (○) and for 9 min (●). Total [^{3}H]DAG includes tritiated 1,2- and 1,3-DAG, and the data are expressed as a percentage of the total radioactivity as described in the legend to Fig. 1. Each control point (*C*) represents one or two determinations, and each treatment point (*dDAVP*) represents the mean of two determinations. Analysis of variance showed a significant difference between control and dDAVP-treated cells for 1,2-[^{3}H]DAG at 9 min ($P < 0.01$) and for total ^{3}H-labeled diacylglycerols at 3 min ($P < 0.05$) and 9 min ($P < 0.02$).

relative affinity of vasopressin for V_2 receptors (23). These differences are the result of molecular modifications of hemicystine at position 1 and substitution of the D-isomer of arginine for L-arginine at position 8 (2). Correspondingly, Rozengurt *et al.* found that 10 μg/ml of dDAVP induced the same maximal stimulation of DNA synthesis as 100 ng/ml of vasopressin in 3T3 cells (21).

In this study, dDAVP did not affect the cytocidal activity of melphalan in murine L5178Y lymphoblasts, despite an increased influx of melphalan, but significantly augmented the cloning efficiency of stationary phase or slowly dividing L5178Y cells and exponential phase murine P388 leukemia cells. In addition, there were significant differences between control and dDAVP-treated cells for tritiated diacylglycerols. 1,2-[^{3}H]DAG production increased after 9-min incubation with dDAVP, whereas total [^{3}H]DAG was increased after 3 and 9 min, providing evidence that the growth-promoting activity of dDAVP may be mediated through V_1 receptors. Using the TLC methods which provided for excellent separation of the 1,2- and 1,3-DAG isomers, we found that production of both iso-

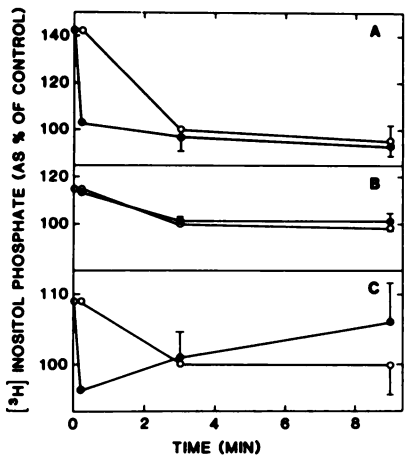

Fig. 4. Effect of dDAVP on the level of inositol phosphates in L5178Y cells: IP_3 (*A*); IP_2 (*B*); and IP (*C*). Cells were prelabeled with [^{3}H]inositol and incubated with either 100 nM dDAVP in saline (●) or saline alone (○) for various times. All the values are normalized with respect to the control value at 3 min, which was set at 100%. Each *point* is the mean of at least 6 independent determinations.

mers was significantly increased in murine L5178Y lymphoblasts after 3- and 9-min incubation with desmopressin.

A series of studies by Nishizuka and coworkers has provided evidence that DAG, a product of the phospholipase C-mediated hydrolysis of PIP_2, initiates the activation of protein kinase C, resulting in cell proliferation (24). Diacylglycerols containing a 1,2-*sn* configuration are capable of activating protein kinase C in rat brain, while two other stereoisomers, 2,3-DAG and 1,3-DAG, neither activate nor inhibit the enzyme (25). The importance of increased 1,3-DAG production in this setting is unclear, and this finding requires further investigation. The generation of DAG as a result of receptor-ligand interaction is classically considered the result of phosphatidylinositol hydrolysis by phospholipase C (26, 27). Recently investigators have reported that DAG may also be produced by hydrolysis of phosphatidylcholine by a phosphatidylcholine preferring phospholipase C (28–30). Grillone *et al.* (30) have shown that, in smooth muscle cells A-10, vasopressin induces V_1 receptors to activate both phosphatidylinositol- and phosphatidylcholine-specific phospholipase C, resulting in the release of arachidonic acid and the formation of DAG from both sources.

In the present study an apparently paradoxical decrease in the level of IP_3 and IP has been observed as a result of treatment of L5178Y cells with dDAVP. This reduction in the levels of inositol phosphates suggests that the increase of DAG content produced by dDAVP might be primarily from the phosphatidylcholine source. In addition, increased DAG levels might also be involved in the breakdown of inositol phosphates by activating phosphatases through the protein kinase C pathway (31, 32). After an initial rapid drop, the level of IP_3 in dDAVP-treated cells did not decline further, suggesting that, during this time, there may be an increased synthesis of IP_3 or possibly curtailment of phosphatase activation in these cells.

This study does not provide direct evidence for the mechanism of action of dDAVP; however, the increased cloning efficiency may represent interaction of dDAVP with V_1 receptors triggering hydrolysis of both PIP_2 and phosphatidylcholine with increased production of DAG. Increased production of DAG may in turn initiate activation of protein kinase C, thereby resulting in cell proliferation. The mitogenic activity of dDAVP, a nonpeptide of low molecular weight, is particularly intriguing since most of the known growth factors are much larger compounds (33). This and other structural analogues of vasopressin may be used to study the regulation of cell proliferation and to characterize further receptor-ligand interactions.

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