Rapid sequence-independent cellular response to oligodeoxynucleotides

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Abstract The presence of receptors for oligodeoxynucleotides (OdN) on the surface of L929 cells has previously been described. To study the possible coupling of the receptor to cellular signal transducing systems, the effect of phosphodiester OdN of different sequences on cellular phospholipase C and protein kinase C (PKC) activities in L929 fibroblasts was studied. Treatment of cells with OdN induced an increase in ^{32}P labeling of phosphatidic acid which was accompanied by a gradual decrease in diacylglycerol. These effects seem to be independent of the OdN sequence. PKC activity in membranes isolated from OdN-treated cells was found to be lower than that in membranes of control cells. SDS-PAGE of the ^{32}P-labeled cellular proteins revealed that OdN treatment caused a decrease in phosphorylation of the 26 and 73 kDa cellular proteins in the cells.

Key words: Oligodeoxynucleotide; Receptor; Phospholipase C; Protein kinase C

1. Introduction

The existence of cell surface receptors for extracellular oligonucleotides has previously been demonstrated (reviewed in [1]). It is well known that extracellular nucleotides and nucleosides have their own receptors on the cell surface and through such receptors can influence many biological processes. The biological effects of nucleotides and nucleosides on cells are mediated by signal-transducing systems, in particular that of phospholipase C. For example, the addition of ATP to cells results in receptor-coupled activation of phosphatidylinositol-specific phospholipase C and elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) [2,3]. Other nucleotides, such as GTP and UTP, can also elicit cellular response by stimulation of phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ [4,5]. A recently described third type of A-receptors, sensitive to adenosine and AMP, was found to be coupled to the phospholipase C signaling system [6].

The aim of this study was to examine the possible coupling of the OdN receptor to the phospholipase C (PLC)-protein kinase C (PKC) signal-transducing pathway. The investigation was performed with the murine fibroblast L929 cell line, which was selected for experiments as these cells have been shown to contain a surface 79 kDa oligonucleotide-binding protein (putative receptor) and the protein is present in greater amounts as compared with other cell lines tested [7,8].

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Abbreviations: DAG, diacylglycerol; $d(pN_{16})$, pTCACCCTCTTCCCATC; $d(pN'_{16})$, pATACCCTGCTTTTGCT; $d(pT_{10})$, pTTTTTTTTTT; OdN, oligodeoxynucleotide(s); PKC, protein kinase C; PLC phospholipase C; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine.

2. Materials and methods

2.1. Materials

$^{32}P_i$ (5000 Ci/mmol), [γ-^{32}P]ATP, was from 'Biosan' (Novosibirsk, Russia) and sodium [^{14}C]acetate (50 mCi/mmol) from 'Isotope' (St. Petersburg, Russia). The oligodeoxynucleotides $d(pN_{16})$ [pTCACCCTCTTCCCATC] and $d(pN'_{16})$ [pATACCCTGCTTTTGCT] complementary to vernal encephalitis virus and influenza virus mRNA, respectively, and $d(pT_{10})$, were synthesized using the triester method in solution in the Novosibirsk Institute of Bioorganic Chemistry as described in [9], purified by reverse-phase HPLC, precipitated with lithium perchlorate in acetone, washed with acetone and kept dry. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and Hank's solution were from 'Vector' (Novosibirsk, Russia). Phosphatidic acid and 1,2-dioleoyl-*sn*-glycerol used as standards were from Sigma (St. Louis, USA).

2.2. Cell culture

L929 murine fibroblasts were grown in DMEM supplemented with 6% FCS, 2 mM glutamine and 40 μg/ml gentamycin in a humidified atmosphere of 5% CO_2. 2 days after plating, the medium was replaced with serum-free medium. Cells which had rested in serum-free medium for 1–2 days were used as quiescent cells. Cells were treated with oligonucleotides in serum-free medium.

2.3. Labeling of cellular lipids with [^{14}C]acetate (or $^{32}P_i$) and lipid analysis

During the serum deprivation period, confluent L929 cells were incubated with sodium [^{14}C]acetate (250 μCi/ml). $^{32}P_i$ was added to cells (80 μCi/ml) simultaneously with OdN or as described in the text. Cellular monolayers were extracted as described [10]. Lipids were extracted according to Bligh and Dyer [11]. Extracted lipids were analyzed by TLC on silica gel 60 coated plates (0.2 mm, E. Merck, Darmstadt, Germany). To separate neutral lipids from phospholipids, samples were first developed to the middle of the plate in diethyl ether. After removing the top half of the plate containing neutral lipids, the plates were finally developed in solvent comprising chloroform/methanol/acetone/acetic acid/H_2O (10:2:4:2:1, v/v) to separate phospholipids. Neutral lipids were separated using a solvent consisting of hexane/diethyl ether/acetic acid (60:40:1, v/v). Lipid spots were detected by autoradiography, identified by comparison with known standards and scraped off. Radioactivity of lipids was measured by liquid scintillation or autoradiograms were scanned with an Ultrascan XL laser densitometer (LKB).

2.4. SDS-PAGE

Cells preincubated in the presence of oligonucleotides and $^{32}P_i$ (100 μCi/ml) were boiled with Laemmli sample buffer for 5 min and electrophoresis was performed on 12% acrylamide gels [12]. Dried gels were submitted to autoradiography.

2.5. Membrane-associated protein kinase C activity

Cellular membrane fractions were obtained as described [13]. Protein concentration was determined according to Bradford, PKC activity in membranes being assayed by measuring the phosphorylation of histone H1 and determined as the difference in the amount of incorporated ^{32}P radioactivity in the presence and absence of activators (Ca^{2+} and phosphatidylserine) [14].

3. Results

Accumulation of radiolabeled phosphatidic acid (PtdOH) in

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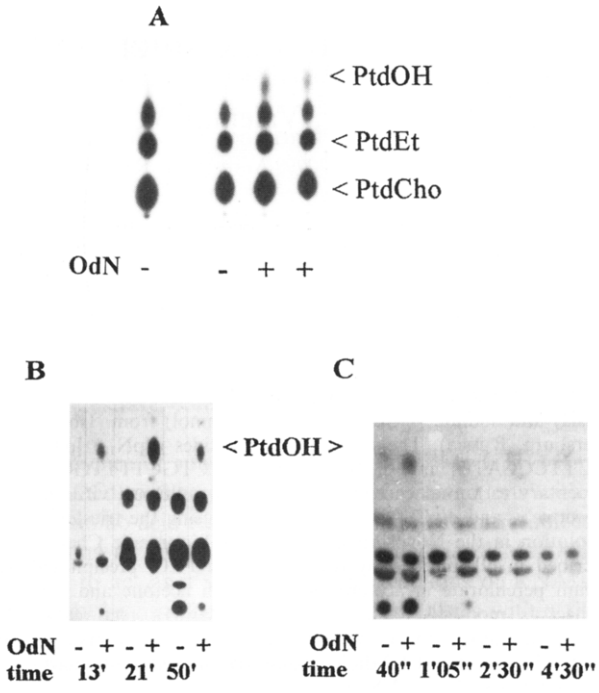

Fig. 1. Sequence-independent effect of OdN on ^{32}P turnover of PtdOH. (A) L929 cells were incubated in the presence of 1 μM $d(pT_{10})$ (lanes 3,4; replicates) and $^{32}P_i$ (80 μCi/ml) for 2 h or in the absence of OdN (lanes 1,2; replicates). Lipids were extracted and separated by TLC as described in Section 2. (B) Time course of ^{32}P labeling of cellular phospholipids in the presence or absence of $d(pN_{16})$ (1 μM). $^{32}P_i$ was added to cells as described in (A). (C) The same as in (B) except that $d(pN_{16})$ were added to fibroblasts prelabeled with $^{32}P_i$ for 30 min. PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; PtdEt, phosphatidylethanolamine.

cells stimulated with agonists in the presence of $^{32}P_i$ is considered as an indication that agonists cause DAG liberation in cells due to phospholipase C (PLC) activation [15]. To assess whether oligonucleotides could activate PLC, we first examined ^{32}P incorporation into phospholipids of L929 cells. Decathymidylate ($d(pT_{10})$) was added to cells simultaneously with $^{32}P_i$ to 10^{-6} M final concentration. Fig. 1 shows that addition of OdN to quiescent cells had no effect on phosphatidylcholine (PtdCho) and phosphatidylethanolamine labeling (PtdEt). In contrast, significant induction of PtdOH radiolabeling was found in OdN-treated cells. Addition of another OdN, $d(pN_{16})$, gave similar results, a near 2-fold increase in PtdOH radiolabeling after 90 min incubation being observed (data not shown).

A characteristic feature of receptor-stimulated activation of phospholipase C is the rapidity of the process, leading to the formation of DAG within the first 30 s after ligand addition [16]. In contrast, penetration of the compound by endocytosis is rather slow process. To determine whether increased radiolabeling of PtdOH is the result of OdN interaction with the cell surface or its penetration into cells, the time course of OdN-induced changes in phospholipid radiolabeling was studied. Fig. 1C shows that stimulated PtdOH turnover was observed as fast as 40 s after OdN addition.

These results prompted us to investigate whether the rise in PtdOH radiolabeling was due to increased DAG production. Fig. 2 illustrates that addition of $d(pN_{16})$ causes a gradual decrease in the [^{14}C]DAG level in cells. Similarly, treatment of quiescent L929 cells with an OdN having a different sequence $d(pN'_{16})$ (5×10^{-7}M) for 90 min results in an ~25% decrease in [^{14}C]DAG (9980 ± 2300 and 7600 ± 1700 cpm/10^5 cells before and after OdN addition, respectively).

The next series of experiments were performed in order to examine the relationship between ^{32}P radiolabeling of PtdOH, [^{14}C]DAG level and concentration of added OdN. Quiescent fibroblasts were first prelabeled with [^{14}C]acetate and then treated with various concentrations of $d(pN_{16})$ in the presence of $^{32}P_i$. It was found that, in cells, OdN resulted in a dose-dependent increase in [^{32}P]PtdOH radiolabeling (~200% maximal effect) and a slight decrease in [^{14}C]DAG level (~20%) (Fig. 2B). Despite the fact that the decrease in DAG was found to be slight, it was reproducible.

When the effect of OdN on cellular protein phosphorylation was studied, it was found that the pattern of cellular protein phosphorylation was altered in OdN-treated cells. Fig. 3A illustrates the effect of OdN on ^{32}P incorporation into cellular proteins. It was observed that radiolabeling of some cellular proteins, namely, those of 26 and 73 kDa, was reduced in OdN-treated fibroblasts (lanes 3,4) compared to control (lanes

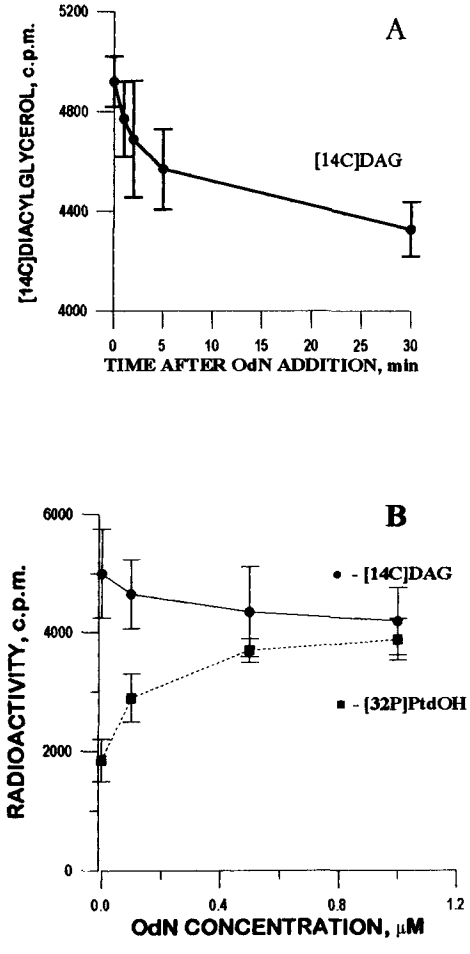

Fig. 2. Effect of OdN on cellular [^{14}C]DAG. (A) Changes in the amount of [^{14}C]DAG in L929 fibroblasts following OdN treatment. Cells were labeled for 24 h with [^{14}C]acetate and then treated with $d(pN_{16})$ (1 μM) for various times. (B) Effect of different concentrations of OdN on changes in [^{14}C]DAG level and [^{32}P]PtdOH labeling. Cells prelabeled with [^{14}C]acetate were treated with different concentrations of $d(pN_{16})$ for 2 h. $^{32}P_i$ (100 μCi/ml) was added to cells simultaneously with OdN. Lipids were extracted, separated by TLC and ^{14}C radioactivity of DAG (●) and ^{32}P radioactivity of PtdOH (■) were measured.

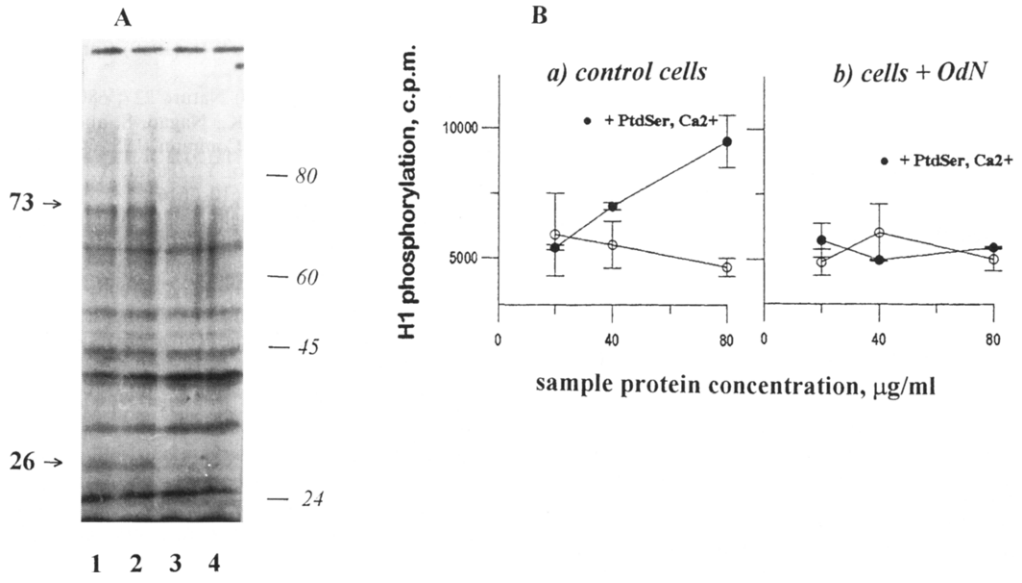

Fig. 3. Effect of OdN on cellular protein kinase activity. (A) ^{32}P incorporation into cellular proteins. L929 fibroblasts (1.1×10^5 cells) were pre-incubated for 90 min with $p(N_{16})$ (1 μM) (lanes 3,4) or in its absence (lanes 1,2). The cells were then incubated with $^{32}P_i$ (100 μCi/ml) for a further 30 min and cellular proteins were then submitted to 12% SDS-PAGE. (B) Fibroblasts (1.1×10^5 cells) were incubated in the presence of $d(pT_{10})$ (1 μM) for 30 min, homogenized and membrane fractions were obtained. PKC activity in membranes of control (a) and oligonucleotide-treated (b) cells was assayed by measuring phosphorylation of histone H1 and determined as the difference in the amount of ^{32}P incorporated into histone H1 (400 μg/ml) in the presence of activators (●—●): 0.5 mM Ca^{2+} and 20 μg/ml phosphatidylserine and in their absence (○—○).

1,2). This finding could reflect the inhibition of some cellular protein kinase(s) in cells treated with OdN.

Previously, it was found that the addition of OdN to cells inhibited cellular protein-kinase C-dependent processes in a sequence-independent manner [17]. Activation of PKC results from at least three processes: translocation of the enzyme from the cytosol to membranes, formation of the complexes with phosphatidylserine in the membranes and activation of the complex by DAG [18]. Since the DAG level was found to decrease in OdN-treated cells, a lower level of PKC activity could be expected in membranes of these cells. PKC activity in membranes of control and oligonucleotide-treated L929 cells was assayed by measuring Ca^{2+}-, phosphatidylserine-dependent phosphorylation of histone H1 and indeed, the administration of OdN to cells induced a decrease in membrane-associated protein kinase C activity (Fig. 3A).

4. Discussion

The results of our study demonstrate that (1) ^{32}P turnover of PtdOH is stimulated in oligonucleotide-treated L929 cells. The rate of change induced in lipid metabolism suggests the possible participation of an OdN receptor. (2) The DAG level is decreased. (3) Protein kinase C activity in membranes of OdN-treated cells is diminished as well as the degree of phosphorylation of some cellular proteins. (4) The observed oligonucleotide effects are sequence-independent.

^{32}P turnover of phosphatidylinositol and its derivatives (PtdIns, PtdInsP and $PtdInsP_2$) as well as phosphatidic acid is stimulated when cells are incubated with ligands specific for a cellular receptor, providing that the receptor is coupled to the phospholipase C signal-transducing system [15]. The turnover of inositol lipids is increased due to stimulated hydrolysis of one of them, $PtdInsP_2$, by PLC, whereas an increase in PtdOH radiolabeling is the result of DAG formation and its subsequent phosphorylation by DAG kinase, one of the enzymes which regulate cellular level of DAG [16]. In our experiments, in cells, the addition of oligonucleotides to L929 fibroblasts causes rapid, increased ^{32}P incorporation into PtdOH (Figs. 1 and 2), suggesting the possible activation of phospholipase C by oligonucleotides and DAG liberation. However, measurements of the amount of DAG in [^{14}C]acetate-prelabeled cells have shown that the addition of OdN did not induce DAG production. On the contrary, the DAG level was reduced in OdN-treated cells (Fig. 2A). The reason for this is not well understood.

Addition of OdN to cells was found to result in a lower degree of phosphorylation of cellular 26 and 73 kDa proteins (Fig. 3A) and a loss of PKC activity in cellular membranes (Fig. 3B). Thus, it appears likely that the effect of OdN on phosphorylation of cellular proteins can be explained by the inhibition of cellular protein kinase C, possibly, via the decrease in cellular DAG level.

Our study describes sequence-independent effects of oligodeoxynucleotides on cellular lipids and PKC. Protein kinase C is known to regulate the expression of certain oncogenes. In view of our results, it seems plausible to consider that expression of PKC-regulated oncogenes can be inhibited by OdN treatment in a sequence-independent manner. Some additional sequence-independent effects of OdN are known [19]. For example, treatment of cells with phosphothioate analogs of oligonucleotides and to a lesser extent with phosphodiester oligonucleotides resulted in sequence-independent inhibition of endocytosis, a PKC-dependent process in HL60 cells [17]. Moreover, it has been reported that OdN added to cells can induce Sp1 transcription factor, also in a sequence-independent manner [20]. A number of oligonucleotide-protein interactions capable of affecting different cellular processes have

been detected recently [21]. In addition, in view of the use of antisense oligodeoxynucleotides to inhibit specifically gene expression, the question of understanding OdN action may be of importance.

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