Ap_4A induces apoptosis in human cultured cells

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Abstract Diadenosine oligophosphates (Ap_nA) have been proposed as intracellular and extracellular signaling molecules in animal cells. The ratio of diadenosine 5′,5‴-P^1,P^3-triphosphate to diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_3A/Ap_4A) is sensitive to the cellular status and alters when cultured cells undergo differentiation or are treated with interferons. In cells undergoing apoptosis induced by DNA topoisomerase II inhibitor VP16, the concentration of Ap_3A decreases significantly while that of Ap_4A increases. Here, we have examined the effects of exogenously added Ap_3A and Ap_4A on apoptosis and morphological differentiation. Penetration of Ap_nA into cells was achieved by cold shock. Ap_4A at 10 μM induced programmed cell death in human HL60, U937 and Jurkat cells and mouse VMRO cells and this effect appeared to require Ap_4A breakdown as hydrolysis-resistant analogues of Ap_4A were inactive. On its own, Ap_3A induced neither apoptosis nor cell differentiation but did display strong synergism with the protein kinase C activators 12-deoxyphorbol-13-*O*-phenylacetate and 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate in inducing differentiation of HL60 cells. We propose that Ap_4A and Ap_3A are physiological antagonists in determination of the cellular status: Ap_4A induces apoptosis whereas Ap_3A is a co-inductor of differentiation. In both cases, the mechanism of signal transduction remains unknown.

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Key words: HL60; Differentiation; Apoptosis; Diadenosine 5′,5‴-P^1P^3-triphosphate; Diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Human cell culture; Cold shock; Diadenosine oligophosphate analogue

1. Introduction

The study of signal transduction pathways has become one of the most rapidly developing areas of modern molecular cell biology. Among the most widespread and universal signaling mechanisms are those which employ the phosphorylation-dephosphorylation reactions catalyzed by a variety of protein kinases and phosphatases (reviewed in [1–6]) and guanine nucleotide binding regulatory proteins (see [7,8]). These and other pathways are involved in directing the cell toward normal or malignant proliferation, differentiation or apoptosis. Recently, diadenosine oligophosphates (Ap_nA) (for review see [9]) have emerged as putative extra- and intracellular signaling molecules implicated in the maintenance and regulation of vital cellular functions and may be considered as a new class of signal transducers. A wide variety of physiological effects in bacteria and mammalian cells are associated with alterations in intracellular Ap_nA levels, e.g. metabolic responses to heat, oxidative stress and glucose, and the regulation of proliferation and the growth cycle, while extracellularly, they act as neurotransmitters and potent vasoactive agents [10–13]. Recently, we showed that interferons (IFNs) and phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) cause the accumulation of diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A), but not diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) in cultured human cells [14]. In contrast, in cells undergoing apoptosis induced by the DNA topoisomerase II inhibitor epipodophylotoxin (VP16), the concentration of Ap_3A decreased markedly while the concentration of Ap_4A increased [15]. Since TPA-activated and IFN-activated pathways can apparently converge to produce similar long-term effects on cells, such as growth inhibition, it is possible that differences in cellular status may be associated with varying intracellular levels of Ap_4A and Ap_3A.

The discovery of Ap_3A binding and hydrolytic activity of Fhit, the protein encoded by the putative *FHIT* tumor suppressor gene [16], has provided insight into the possible function(s) of Ap_3A in vivo. Since Fhit is a close structural analogue of an inhibitor of protein kinase C (PKCI) [17], it is possible that binding of Ap_3A to PKCI may turn off the kinase inhibitor. It has been suggested that sustained activation of PKC is necessary for cell differentiation [18]. In order to determine whether Ap_3A and Ap_4A can directly influence the cellular status, we have examined the effect of Ap_4A on apoptosis in various permeabilized human and mouse cell lines. We have also studied the effect of Ap_3A on the differentiation of HL60 cells in culture.

2. Materials and methods

2.1. Reagents

Ap_3A and Ap_4A were purchased from Sigma. TPA, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate (D1), 12-deoxyphorbol-13-*O*-acetate (D2), 12-deoxyphorbol-13-*O*-phenylacetate (D3), diacylglycerol (DAG), 10-oleoyl-2-acetylglycerol (OAG), *N*-palmitoylsphingosine (PS) and sapintoxin (ST) were from Calbiochem. [^{3}H]Thymidine (TdR) (925 TBq/mol) was from Amersham International, VP16 was

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Abbreviations: Ap_3A, diadenosine 5′,5‴-P^1P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; $ApCh_2ppA$, diadenosine 5′,5‴-(P^1,P^2-methylene)-P^1,P^3-triphosphate; $AppCF_2ppA$, diadenosine 5′,5‴-(P^2,P^3-difluoromethylene)-P^1,P^4-tetraphosphate; $ApCH_2ppCH_2pA$, diadenosine 5′,5‴-P^1,P^4-(P^1,P^2-methylene-P^3,P^4-methylene)-tetraphosphate; VP16, epipodophylotoxin; IFN, interferon; PKC, protein kinase C; TdR, thymidine; TCA, trichloroacetic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; OAG, 10-oleoyl-2-acetylglycerol; PS, *N*-palmitoyl-sphingozine; ST, sapintoxin; D1, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate; D2, 12-deoxyphorbol-13-*O*-acetate; D3, 12-deoxyphorbol-13-*O*-phenylacetate; DAG, diacylglycerol

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a kind gift from U. Armato, [^{32}P]Ap_3A and [^{32}P]Ap_4A were kindly provided by Yu. Skoblov. Diadenosine 5′5‴-(P^1,P^3-difluoromethylene)-P^1,P^4-tetraphosphate ($AppCF_2ppA$) and diadenosine 5′,5‴-P^1,P^4-(P^1,P^2-methylene-P^3,P^4-methylene)-tetraphosphate ($ApCH_2ppCH_2pA$) were kindly provided by A. Tshepitsin. Diadenosine 5′,5‴-(P^1,P^2-methylene)-P^1,P^3-triphosphate ($ApCH_2ppA$) was a gift from S. Kochetkova. The structure of Ap_nA analogues was confirmed by UV, ^{1}H and ^{31}P NMR spectroscopy. All other reagents were of analytical grade.

2.2. Cell growth and permeabilization

The human promyelocytic leukemia cell line HL60 was grown in suspension at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were permeabilized by cold shock [19]. Cells in the logarithmic phase were harvested by centrifugation at $1000\times g$ for 10 min at 4°C, washed once with PBS, suspended in permeabilizing buffer composed of 0.01 M EDTA, 30 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 0.25 M sucrose and 0.01 M Tris-HCl, pH 7.8, and incubated for 30 min at 4°C with Ap_nA. To reseal, cells were centrifuged, washed twice with PBS and seeded in complete RPMI medium at 37°C in an atmosphere of 5% CO_2. Cellular viability was determined by trypan blue exclusion.

2.3. [^{3}H]TdR incorporation into DNA

Aliquots (2 ml) containing 1×10^6 permeabilized cells and 80 kBq/ml of [^{3}H]TdR were incubated for 2 h at 37°C, centrifuged, then 2 ml of ice-cold trichloroacetic acid (TCA) was added to the pellet and shaken vigorously. The samples were filtered through Whatman GF/C glass filters, washed five times with 5% TCA and once with ethanol. The filters were dried and counted in a toluene scintillation fluid. For radioautography, [^{3}H]TdR (925 TBq/mol, 29.6 kBq/ml) was added to the culture medium immediately after cold shock. After 24 h, the cells were fixed using ice-cold ethanol and acetic acid (3:1 v/v). The cell preparations were washed with water, covered with photographic emulsion, exposed for 3 days, then developed and stained with Giemsa stain (Merck). The percentage of ^{3}H-labelled cell nuclei was determined microscopically.

2.4. Analysis of DNA and cell morphology

The HL60 cells were subjected to cold shock in the presence of Ap_4A for 30 min at 4°C as described above, centrifuged, washed with PBS and seeded in six well plates in complete RPMI 1640 medium. For examination of morphology, the cells were washed twice with cold serum-free RPMI 1640 and cell smears were prepared and stained with 1 μg/ml of Hoechst 33258 (Sigma) in PBS, embedded in glycerol. Morphological assessment was performed with a Leitz fluorescent microscope ($\times$60 objective). The type of cell death in Ap_4A-treated cells was determined based on morphological features characteristic of apoptosis (cell shrinkage, nuclear condensation and extensive formation of membrane blebs and apoptotic bodies). DNA was extracted 8 h after cell seeding using a DNA purification kit (Fermentas). Electrophoresis was carried out on a 1% agarose gel containing 0.5 μg/ml ethidium bromide.

2.5. Cell differentiation

2×10^5 cells were cold-shocked in the presence of Ap_nA, centrifuged, washed with PBS and seeded in 24 well plates in complete RPMI 1640 medium and grown under 5% CO_2 for 48 h at 37°C in the presence of PKC activators, TPA, OAG, PS, ST, D1, D2 or D3. The differentiated cells exhibited intense adherence to the plastic dish with prominent pseudopodia formation. To follow cell differentiation, cells were washed with cold RPMI 1640, cell smears were prepared and stained with nitroblue tetrazolium. The percentage of positive cells was counted.

3. Results

3.1. Cell permeabilization

The primary source of intracellular Ap_nA is synthesis in the cytoplasm by aminoacyl-tRNA synthetases [9,10]. To change intracellular Ap_nA experimentally, cells were permeabilized by cold shock [19] in the presence of Ap_nA. Cold-shocked HL60 cells were morphologically intact and more than 95% of the treated cells remained viable as revealed by trypan blue exclusion. Uptake of Ap_3A was monitored with ^{32}P-labelled or fluorescently labelled Ap_3A. The Ap_3A fluorescence was evenly distributed in the cell population and located mostly in the cytosol. Uptake of Ap_3A at different concentrations in the cold shock buffer was studied. Routinely, we used cold shock buffer with 10 μM Ap_3A which corresponded to an intracellular concentration of 0.36 μM.

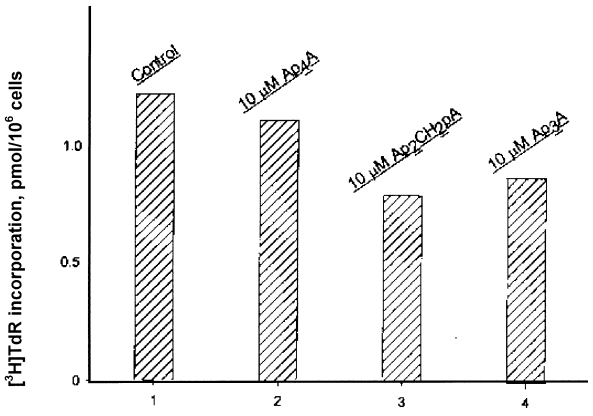

Fig. 1. DNA synthesis in permeabilized HL60 cells in the presence of Ap_nA. DNA synthesis was measured by [^{3}H]TdR incorporation into DNA as described in Section 2.

3.2. DNA synthesis in permeabilized cells

Permeabilized cells retain their ability to actively incorporate [^{3}H]TdR into DNA after a short lag period of 1 h (Fig. 1). This DNA synthetic ability was only slightly reduced ($8\pm3\%$) by the presence of 10 μM Ap_4A in the cold shock buffer. Addition of 10 μM Ap_3A or a non-hydrolyzable analogue of Ap_3A, $ApCH_2ppA$, resulted in a more significant inhibition of DNA synthesis ($24\pm3\%$ and $26\pm3\%$, respectively). We also followed [^{3}H]TdR incorporation into DNA of Ap_nA-treated cells by autoradiography of fixed cells on emulsion-covered slides. The number of cells actively synthesizing DNA 24 h after cold shock with 10 μM Ap_3A did not change significantly compared to control permeabilized cells. Addition of $ApCH_2ppA$ resulted in a slight ($7\pm2\%$) decrease in the number of cells actively synthesizing DNA.

3.3. Ap_4A-induced apoptosis in different mammalian cell lines

In the HL60 cells permeabilized in the presence of Ap_4A, the appearance of cytoarchitectural features typical of programmed cell death were observed (Fig. 2A). The proportion of apoptotic cells reached $24\pm2\%$ of the total cell number after incubation for 24 h with Ap_4A. The number of cells in the S-phase was $18\pm2\%$. DNA extracted from permeabilized HL60 cells treated with Ap_4A under cold shock conditions showed a characteristic fragment size distribution after agarose gel electrophoresis. DNA fragments of about 200 bp appeared after 8 h of incubation with Ap_4A (Fig. 2B). Ap_4A also induced apoptotic DNA fragmentation in human U937 monocytes, human Jurkat T-cells and in mouse adenocarcinoma VMRO cells but not in human H299 lung cancer cells, human teratocarcinoma NT2 cells or mouse Swiss 3T3 fibroblasts (Table 1). Interestingly, the hydrolysis-resistant Ap_4A analogues $AppCF_2ppA$ and $ApCH_2ppCH_2pA$ failed to induce apoptosis in any of the cell lines tested (e.g. Fig. 3),

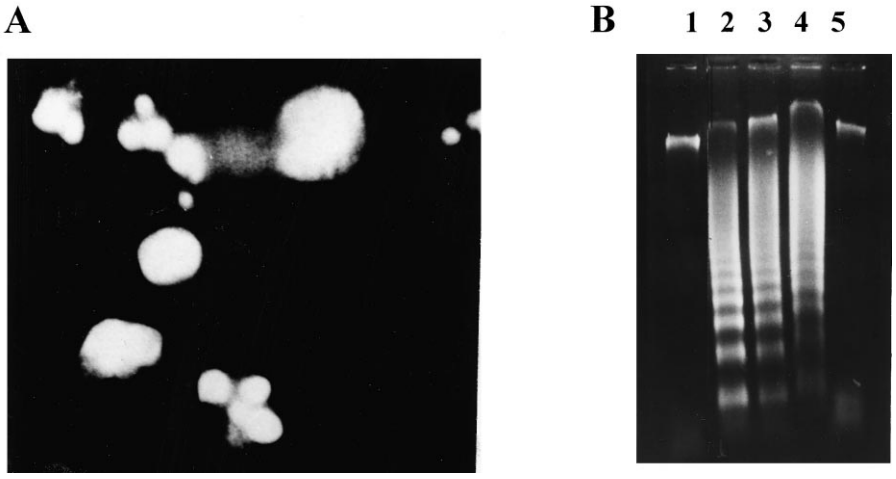

Fig. 2. Ap_nA-induced apoptosis in HL60 cells. A: Apoptotic morphology of HL60 cells treated with 10 μM Ap_4A. The formation of membrane blebs and apoptotic bodies is visible 24 h after treatment. B: Apoptotic DNA degradation in cold-shocked cells. DNA was extracted from the cells 8 h after treatment with VP16 or Ap_nA and electrophoresed in a 1% agarose gel. Lane 1, no additions; lane 2, 20 μg/ml of VP16; lane 3, 1 μM Ap_4A; lane 4, 10 μM Ap_4A; lane 5, 10 μM Ap_3A.

suggesting that hydrolysis of Ap_4A by its intracellular target may be required for its action.

3.4. Ap_nA and cell differentiation

The HL60 cells cold-shocked in the presence of 1 or 10 μM Ap_3A yielded a few cells with a slightly altered morphology and occasional apoptotic cells. However, no typical macrophage-like differentiated cells were observed after 48 h of incubation in complete RPMI 1640 medium. No changes were observed using the hydrolysis-resistant Ap_3A analogue $AppCH_2pA$ either. Next, we tested known differentiation-inducing agents such as PKC-activating compounds for their potential ability to cooperate with Ap_3A in inducing differentiation of HL60 cells into macrophage-like cells (Table 2). When cells were treated with 10 μM Ap_3A in combination with PKC activators D1 or D3 at 20 nM, a concentration which by itself did not induce differentiation (Table 2), 24 ± 2% and 20 ± 2% of the cells, respectively, differentiated into macrophage-like cells after 48 h of incubation in complete RPMI 1640 medium (Fig. 4). The PKC activators OAG, PS, ST and D2 were unable to cooperate with Ap_3A in induction of differentiation. Consequently, Ap_3A manifests a synergistic effect with certain PKC activators in the differentiation of HL60 cells.

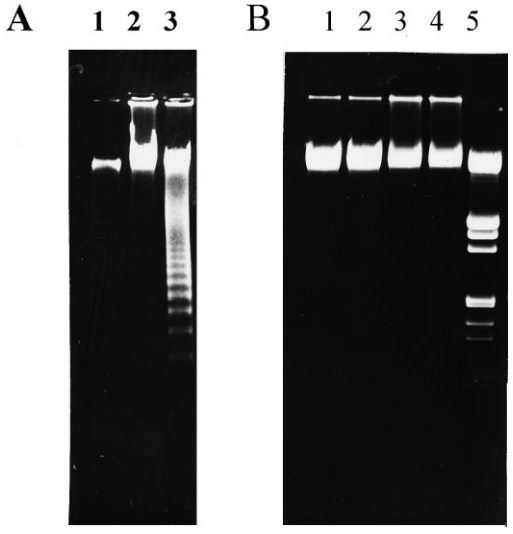

Fig. 3. Induction of apoptosis by hydrolysis-resistant Ap_4A analogues in mouse adenocarcinoma VMRO cells. A: DNA from control cells. DNA was extracted from the cells and electrophoresed in a 1% agarose gel. Lane 1, DNA from non-permeabilized cells treated with Ap_4A; lane 2, DNA from permeabilized cells; lane 3, DNA from cells permeabilized in the presence of Ap_4A. B: Effect of Ap_4A analogues on the induction of apoptosis in VMRO cells. Lane 1, 1 μM $AppCF_2ppA$; lane 2, 10 μM $AppCF_2ppA$; lane 3, 1 μM $ApCH_2ppCH_2pA$; lane 4, 10 μM $ApCH_2ppCH_2pA$; lane 5, DNA from λ *Hin*d digest.

4. Discussion

A variety of conditions and agents have been identified

Table 1
Ap_4A effect on induction of apoptotic DNA fragmentation in mammalian cells

Cells	Ap_4A, μM	Induction of DNA fragmentation[a]
HL60	1	+
	10	+
U937	1	+
	10	+
Jurkat	1	+
	10	+
H299	1	−
	10	−
	50	−
NT2	1	−
	10	−
	50	−
VMRO	1	+
	10	+
Swiss 3T3	1	−
	10	−

(+), DNA ladder typical for apoptosis; (−), no DNA fragmentation.
[a]DNA was isolated from human and mouse cells after 18 h of incubation with Ap_4A.

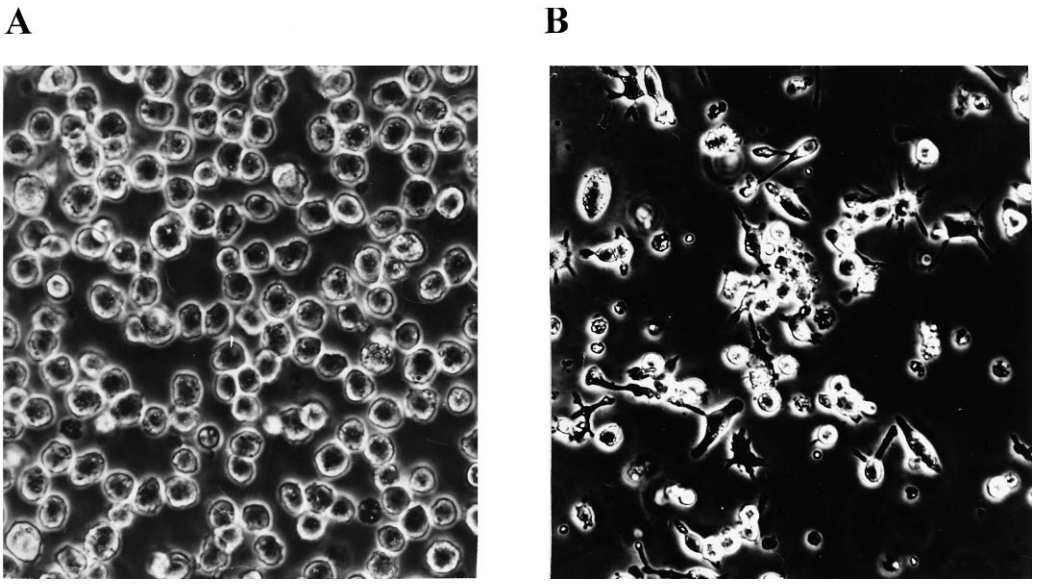

Fig. 4. Adherence of HL60 cells induced by PKC activator D1 in combination with Ap_3A. Cells were incubated for 48 h in complete RPMI 1640 medium after cold shock. A: No additions. Cell differentiation was not observed in the presence of Ap_3A or D1 added separately and the picture was identical to that shown here. B: 10 μM Ap_3A+20 nM D1. Differentiated HL60 cells are clumped and adhere to the bottom of the flask. Similar results were obtained with 20 nM D3+10 μM Ap_3A.

during the last decade as inducers of programmed cell death or differentiation (for reviews see [20–24]). To this list can now diadenosine oligophosphates be added. Previously, we observed that the intracellular concentration of Ap_3A and Ap_4A in cultured cells undergoing apoptosis changed in opposite directions: the level of Ap_4A became elevated whereas the level of Ap_3A diminished [15]. We suggested that apoptosis may be intimately associated with elevated levels of Ap_4A or an increased Ap_4A/Ap_3A ratio. Here, we have confirmed that in cells made permeable to Ap_4A, an increase in intracellular concentration of Ap_4A induces a large proportion of cells (about one quarter of the total number) to undergo apoptosis. This effect of Ap_4A is specific since (i) Ap_3A, a close analogue of Ap_4A, failed to induce apoptosis (Fig. 2) in agreement with our earlier observation showing that Ap_3A decreases in apoptotic cells [15] and (ii) hydrolysis-resistant analogues of Ap_4A were also inactive as apoptotic inducers. A further specific aspect of the action of Ap_4A is the sensitivity of only certain cell types.

Table 2
Cooperation between Ap_3A and PKC activators in differentiation of HL60 cells

Drugs	Concentration, M	Adherence	
		Without Ap_3A	With Ap_3A
TPA	1.3×10^{-8}	+[a]	+
	6×10^{-9}	+	+
	1.3×10^{-9}	+	+
OAG	2×10^{-5}	–[b]	–
	5×10^{-5}	–	–
	1×10^{-4}	–	–
PS	2×10^{-6}	–	–
	2×10^{-5}	–	–
	1×10^{-4}	–	–
ST	1.2×10^{-8}	+	+
	1.2×10^{-9}	–	–
D1	2×10^{-8}	–	+
	2×10^{-6}	+	+
D2	2.5×10^{-8}	–	–
	2.5×10^{-9}	–	–
D3	2.2×10^{-8}	–	+
	2.2×10^{-7}	+	+

[a](+), more than 10% of the cells were differentiated.
[b](–), no differentiated cells were found.

The inability of two different Ap_4A analogues to induce apoptosis is interesting given their structures and sensitivities to hydrolysis. The isosteric $ApCH_2ppCH_2pA$ is highly resistant to asymmetrical hydrolysis by the eukaryotic Ap_4A hydrolase but is recognized by this enzyme as a competitive inhibitor, suggesting a structure similar to Ap_4A [25]. It is also totally resistant to symmetrical hydrolysis [25]. Thus, its inability to induce apoptosis may indicate that hydrolysis of Ap_4A is required for manifestation of its activity. The isopolar $AppCF_2ppA$ is an excellent substrate for the eukaryotic Ap_4A hydrolase but strongly resists symmetrical hydrolysis [25]. Hence, the combined ineffectiveness of these analogues implies that symmetrical hydrolysis by their intracellular target may be an essential factor in the mode of action of Ap_4A. What might this target be? It was suggested some years ago that the Ap_4A binding protein associated with DNA polymerase-α possessed a symmetrical Ap_4A hydrolyzing activity [26]. If DNA polymerase-α is the target, Ap_4A would provide another intriguing connection between apoptosis and the processes of DNA replication, repair and the cell cycle, with which DNA polymerase-α is intimately linked [27].

An alternative target for apoptotic action of Ap_4A might be the Fhit Ap_3A hydrolase, which also binds and hydrolyzes Ap_4A [28]. However, in this case, Ap_3A might be expected to behave similarly to Ap_4A but from our data, this seems not to be the case (Fig. 2). Also, although the ability of $AppCF_2ppA$ and $ApCH_2ppCH_2pA$ to be recognized or hydrolyzed by the Fhit hydrolase has not been measured directly, mechanistic studies of the Fhit reaction would suggest that $AppCF_2ppA$ should be an as effective substrate for Fhit as Ap_4A [29]. Clearly, further studies are required to define the genuine target more precisely for the apoptotic action of Ap_4A.

Our main conclusion that Ap_4A is associated with the induction of apoptosis in certain cell types is consistent with a number of earlier observations. The action of apoptosis-inducing agents, such as γ-irradiation, bleomycin and other DNA damaging treatments that cause the accumulation of strand breaks, is accompanied by an increase in the intracellular levels of Ap_4A [30,31]. Other agents known to cause an increase in the percentage of apoptotic cells, such as ethanol, arsenite, cadmium and heat shock, are also accompanied by an elevation in the level of Ap_4A [31].

It is known that many apoptosis-inducing agents block cell proliferation at the G1/G0 phase (reviewed in [32–34]). The G1 arrest is required for DNA repair [35]. To distinguish whether Ap_4A may be involved in cell cycle arrest or in apoptotic DNA cleavage, we studied the effect of Ap_4A on thymidine incorporation into DNA. The DNA-synthesizing activity of cells permeabilized in the presence of Ap_4A was slightly reduced (8–10%) when [^{3}H]TdR incorporation was measured by TCA precipitation. However, when the percentage of ^{3}H-labelled cell nuclei was determined microscopically, the number of Ap_4A-treated cells actively synthesizing DNA was found to have diminished by 18–20% compared to control cells (43%), suggesting that the principal effect of Ap_4A is probably on cell cycle arrest.

Recently, it was shown in *Escherichia coli* that Ap_4A can act as a signal coupling DNA replication and initiation of cell division. The elevation of the Ap_4A concentration induces cell division and produces many small cells, each with a single nucleoid [36]. If the same was true in mammalian cells, then, the apoptotic-inducing activity of Ap_4A may somehow be related to uncoupling of the timing of mitotic activation and completion of DNA replication ('aberrant mitosis').

In addition to physiological regulators of cell death, many environmental stresses also cause apoptosis and an elevation of Ap_4A [31,37]. Recent studies have implicated stress-activated protein kinases (SAPKs) in the response to environmental stress [38]. SAPKs induce apoptosis via the phosphorylation of factors regulating transcription (c-Jun, Elk-1, ATF-2) [39] as well as by direct or indirect activation of the caspase cascade [40]. Since activation of SAPKs is frequently observed in apoptotic cells, cooperation may occur between Ap_4A and SAPKs.

Since the level of Ap_3A increases during differentiation [15], we tested Ap_3A for its potential ability to cooperate with other differentiation-inducing agents. TPA, a well-known inducer of HL60 myeloid cell differentiation to macrophage-like, exerts many of its effects through PKC activation [41]. We have screened a number of PKC-activating compounds in order to induce HL60 differentiation in combination with Ap_3A (Table 2). When cells were treated with D1 of D3 together with Ap_3A, 20–22% of cells were differentiated into macrophages (Fig. 4). No cell differentiation was induced by either Ap_3A alone or D1/D3 alone at the concentrations used. Consequently, Ap_3A manifests a synergistic effect with some PKC activators. The activation of PKC by TPA or its analogues results in the phosphorylation of specific substrate proteins. Some observations suggest, however, that activation of PKC cannot by itself account for this macrophage-like differentiation [42]. For example, OAG binds to and activates PKC and induces the rapid phosphorylation of HL60 cellular proteins but does not induce HL60 differentiation [43]. An effect of TPA other than or in addition to activation of PKC might account for the macrophage-like differentiation of HL60 [44].

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