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Suramin Alters Phosphoinositide Synthesis and Inhibits Growth Factor Receptor Binding in HT-29 Cells[1]

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ABSTRACT

Initiation of cell growth frequently involves activation of growth factor receptor-coupled tyrosine kinases and stimulation of the phosphoinositide second messenger system. The antitrypanosomal and antifiliarial drug suramin has been shown to exert antiproliferative activities by inhibition of growth factor receptor binding. We therefore investigated the effect of suramin on epidermal growth factor receptor-binding characteristics and, additionally, searched for effects on basal or cholinergically stimulated phospholipid metabolism in HT-29 cells.

Suramin caused a dose-dependent and noncompetitive inhibition of ^{125}I-epidermal growth factor binding (concentration producing 50% inhibition, 44.2 μg/ml) but did not alter muscarinic receptor binding. Suramin did not affect the basal ^{32}P incorporation into phosphoinositides at concentrations of <200 μg/ml suramin. In contrast, the carbachol-stimulated enhancement of ^{32}P incorporation into phosphatidic acid, phosphatidylinositol, and polyphosphoinositides was reduced by 48–95% in the presence of 100 μg/ml suramin. Thus, phosphoinositide and diacylglycerol kinases involved in basal and receptor-stimulated phosphoinositide metabolism may be localized in different subcellular compartments, which can be dissociated by the use of suramin. Direct measurements of phosphatidylinositol kinase and diacylglycerol kinase activities showed a potent inhibition when treated with suramin. Suramin did not affect the stimulation of phospholipase C by carbachol, determined by release of [^{3}H]inositol phosphates in [^{3}H]myoinositol-prelabeled cells.

Our data indicate that suramin potently inhibits phosphoinositide resynthesis under stimulated conditions. Additionally, we confirm the inhibitory effects of suramin on epidermal growth factor receptor binding in a human intestinal cell line. The inhibitory effects of suramin on phospholipid metabolism may play a role in the antiproliferative actions of this drug.

INTRODUCTION

Regulation of proliferation seems to be mediated by two different signaling pathways (1). Binding of growth factors, such as EGF,[3] transforming growth factor α, insulin-like growth factor 1, or insulin, to specific receptors leads to activation of tyrosine-specific protein kinases, an intrinsic property of this receptor family (2). Other growth factors, such as PDGF, bombesin, or thrombin, additionally mediate their mitogenic signals by activating the phosphoinositide system. This results in a specific receptor-coupled hydrolysis of the membrane phospholipid PIP_2 into the metabolically active second messengers inositol trisphosphate and DAG (3, 4). Inositol trisphosphate and its metabolites cause an increase of intracellular calcium levels and a stimulation of calcium/calmodulin kinases. Diacylglycerol activates protein kinase C, a family of Ca^{2+}-sensitive and phospholipid-dependent isoenzymes, known to phosphorylate regulatory proteins and to elevate cytosolic pH levels (5, 6). Activation of protein kinase C by phorbol esters and elevation of intracellular calcium levels by calcium ionophores have been shown to be mitogenically active cofactors during the initiation of DNA synthesis (7–10).

HT-29 colon carcinoma cells have recently been shown to produce EGF/transforming growth factor α and insulin-like growth factor 1-like activities (11), indicating a possible autocrine proliferative effect of these growth factors. Whether other signal transduction pathways are involved during the transmission of mitogenic signals to the nucleus is presently unknown. Previous experiments in our laboratory demonstrated that the phosphoinositide system of HT-29 cells shows a rapid carbachol-induced stimulation of phosphatidylinositol metabolism via muscarinic M_3 receptors, as measured by an enhanced release of inositol phosphates (12).

The antitrypanosomal and antifiliarial drug suramin was reported to selectively dissociate growth factors (EGF, heparin-binding growth factor 2, PDGF, and TGF β) from their receptors and to produce antiproliferative effects in lymphocytes and fibroblasts (13–18). These effects were proposed to occur by inhibition of growth factor receptor binding, direct binding of suramin to the PDGF, modified interaction of growth factor receptor and autosecreted oncogene products, or inhibition of specific DNA polymerases. Reports available at present did not investigate a possible interference of suramin with the phosphoinositide system.

Therefore, we investigated the effect of suramin on EGF receptor binding and searched for a possible involvement of phosphoinositide metabolism in unstimulated and carbachol-treated cells.

We report that suramin inhibited ^{125}I-EGF binding to HT-29 colon carcinoma cells, causing a complete disappearance of the high affinity-binding site and a reduction of binding capacity of the low affinity site. Additionally, suramin potently inhibited phosphoinositide synthesis and generation of phosphatidic acid after cholinergic stimulation, most likely due to reduction of phosphoinositide and diacylglycerol kinase activities. Suramin did not affect the activation of phospholipase C when incubated with carbachol.

MATERIALS AND METHODS

Materials. All reagents were of analytical grade and purchased from Merck (Darmstadt, West Germany) unless indicated otherwise. *N*-(2-(hydroxymethyl)piperazine-*N*′-2-ethanesulfonic acid was from Serva (Heidelberg, West Germany). Dulbecco's modified Eagle's medium,, HBSS, and fetal calf serum were from Gibco (Karlsruhe, West Germany). DE-52 was purchased from Whatman (Milestone, United Kingdom). Phosphatidylinositol and sn-1,2-diacylglycerol were from Sigma (Taufkirchen, West Germany). [^{3}H]NMS, [^{3}H]myoinositol, and [^{32}P]P_i were from Amersham Buchler (Dreieich, West Germany) and suramin (Germanin) from Bayer (Leverkusen, West Germany).

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[3] The abbreviations used are: EGF, epidermal growth factor; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; HBSS, Hank's balanced salt solution; NMS, *N*-methylscopolamine; PDGF: platelet-derived growth factor; DAG, diacylglycerol; ED_{50}, 50% effective dose.

Cell Culture. Cells were obtained from the American Type Culture Collection (Rockville, MD), subcultured with 0.05% trypsin/1 mM EDTA in Dulbecco's modified Eagle's medium,, containing 6% fetal calf serum, 1 mM pyruvic acid, 2 mM glutamine, 100 units/ml penicillin, 50 units/ml streptomycin, and 200 IU/ml nystatin in 75-cm^2 disposable tissue culture flasks in a humidified atmosphere of 5% CO_2/95% air. For the experiments, cells were seeded at a density of 2×10^5 cells in 24-well flat bottom tissue culture plates (Falcon, Heidelberg, West Germany) and grown until subconfluency ($0.7–1.0 \times 10^6$ cells/well).

Determination of Inositol Phosphate Accumulation. For measurements of inositol phosphate release, cells were preincubated with [^{3}H] myoinositol (2 μCi/ml) for 48 h, until complete isotopic equilibrium was achieved. Accumulation of inositol phosphates was measured in the presence of LiCl (10 mM), known to inhibit the degradation of inositol monophosphates (19). Cells were stimulated for 30 min and inositol phosphate release was determined as described previously (20) using the Dowex ion exchange technique (21). Inositol phosphate release was linear during incubations up to 30 min.

Phospholipid Turnover. For investigations of phospholipid turnover, cells were cultured in phosphate-free HBSS [containing 1 mM pyruvic acid, 2 mM glutamine, 20 mM *N*-(2-(hydroxymethyl)piperazine-*N'*-2-ethanesulfonic acid, and 3.7 g/liter $NaHCO_3$] for 60 min prior to the experiment and then incubated under nonequilibrium conditions in HBSS (PO_4-free) with [^{32}P]P_i (10 μCi/ml) and simultaneously with indicated substances at 36.5°C for 30–60 min in a shaking water bath. Incubations were terminated by aspirating the incubation medium, followed by two rapid washes with ice-cold HBSS and addition of ice-cold methanol. Cells were scraped off into polypropylene tubes and phospholipids were extracted as described before (20). Separation of lipids was performed by thin-layer chromatography according to the system described by Jolles *et al.* (22) after pretreatment of silica gel plates (Merck DC 60) with 50% ethanol, 1% potassium oxalate, and 2 mM EDTA (23). Phospholipids were stained with iodine vapor, exposed to autoradiography film (Amersham Hyperfilm) for 48–76 h, and identified by comigration with lipid standards (Sigma). Identity of separated phospholipids was controlled by means of two-dimensional thin-layer chromatography, using the above described solvent in the first dimension and $CHCl_3/CH_3OH/NH_4OH$ (45/35/10, v/v/v) in the second dimension.

PI and DAG Kinase Activity. PI kinase activity was determined according to the method described by Tuazon and Traugh (24). Briefly, cells were sonicated (3 × 30 s) in buffer A containing 20 mM Tris-HCl, 1 mM EDTA, 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 10 mM 2-mercaptoethanol, 10 μM cyclic AMP, and 0.15% Triton X-100 (pH 7.5). PI and DAG kinases were partially purified by DEAE chromatography (DE 52, 200-μl packed volume) in microtubes, and kinases were eluted with buffer A containing 300 mM NaCl (500 μl). PI and DAG, used as substrates, were stored in chloroform solutions, dried under an N_2 stream, redissolved in ethanol (final 0.1%) and buffer B (40 mM Tris-HCl, 0.375% Triton X-100, 75 mM 2-mercaptoethanol, and 0.25 mM sodium vanadate), and sonicated. The assay was performed at 30°C in a volume of 50 μl and started by the addition of $MgCl_2$ and [γ-^{32}P]ATP [final assay concentrations: 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.1 mM sodium vanadate, 30 mM 2-mercaptoethanol, 0.15% Triton X-100, 0.20 mM [γ-^{32}P]ATP (specific activity, 100–200 cpm/pmol)]. The assay was stopped after 15 min with EDTA (final concentration, 20 mM) and nonradioactive ATP (final concentration, 7 mM). After the addition of 1 N HCl (200 μl), phospholipids were extracted with 400 μl of $CHCl_3$/MeOH (2/1, v/v) and the lower phase was transferred to new tubes. The lipid phase was reextracted with 400 μl $CHCl_3$. Pooled lipid phases were evaporated to dryness by N_2 and separation of phospholipids and determination of incorporated radioactivity was performed as described above.

Binding Studies. The cells were incubated in HBSS (0.5 ml) in the presence of ^{125}I-labeled EGF (specific activity, 80–150 μCi/μg) for 60 min at room temperature. Cells were then rinsed twice with cold HBSS and dissolved in 0.5 M NaOH, and specifically bound radioactivity was measured in a Beckman gamma counter. Similar results were obtained in experiments at 4°C, performed to prevent internalization of receptors. For determination of cholinergic receptors, cells were incubated with 0.25 nM [^{3}H]NMS (specific activity, 72 Ci/mmol) for 60 min in HBSS at room temperature. Total bound radioactivity was measured by liquid scintillation spectrophotometry. Specific receptor binding varied between 10 and 30% (5–10,000 cpm/well added) for EGF receptor binding and 3–5% (6,000 cpm/well added) for [^{3}H]NMS binding.

Murine EGF (Collaborative Research) was iodinated as described previously (25) and specific activity was estimated by the self-displacement technique (26).

Statistical Methods. Data are reported as means ± SE from three or more independent experiments. SEM was <10% if not indicated otherwise. For statistical calculations Student's *t* test for paired samples was used. The data were analyzed using the computerized nonlinear least-squares regression programs LIGAND and ALLFIT (26, 27).

RESULTS

Effect of Suramin on EGF Binding. The effect of suramin on EGF binding was assessed by means of radioreceptor assays with ^{125}I-labeled EGF. ^{125}I-EGF binding in untreated HT-29 cells was best fitted using a model of two binding sites, with a K_d high of 7.8 pM and a K_d low of 250 pM, corresponding to 1,400 and 35,000 binding sites/single cell. Scatchard analysis after pretreatment with 50 μg/ml suramin for 60 min revealed a complete disappearance of the high affinity-binding sites and

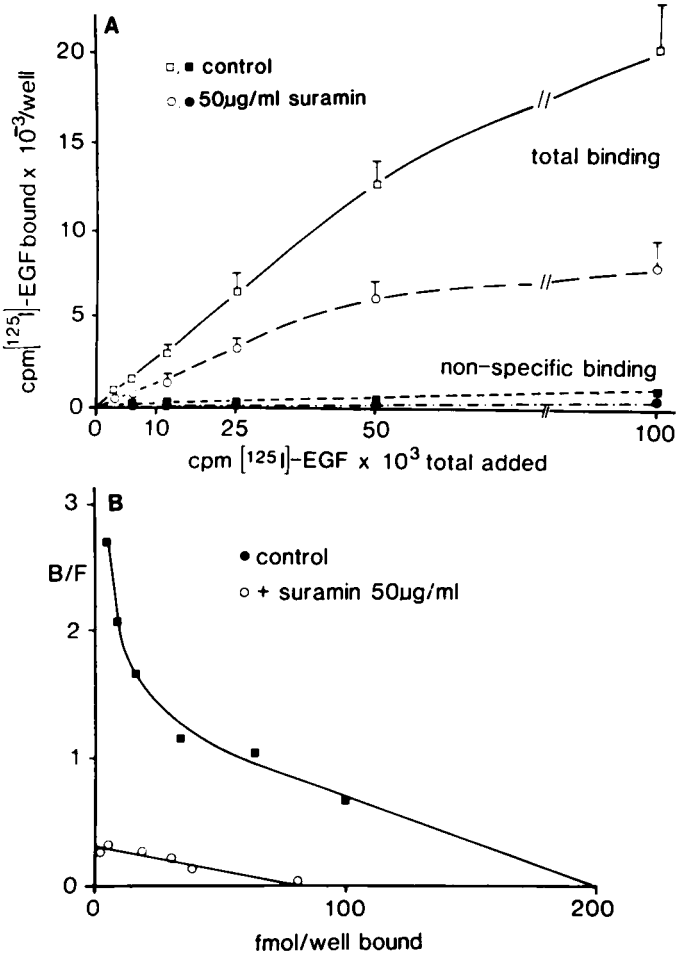


Fig. 1. Effect of suramin on ^{125}I-EGF binding. Cells were labeled with ^{125}I-EGF for 60 min at room temperature with or without suramin. ^{125}I-EGF was added 10 min prior to the addition of suramin. Data show cpm/well of totally bound ligand of one experiment performed in triplicate (*A*). Nonspecific binding was determined by adding unlabeled EGF (100 ng/ml) to the incubation medium. Two further experiments revealed identical results. *B*, data plotted according to Scatchard. Results were fitted with the LIGAND program (27).

a 54% reduction in receptor capacity from 106 fmol to 49 fmol/10^6 cells ($P < 0.01$), indicating a noncompetitive mode of action (Fig. 1).

Investigation of EGF receptor binding in the presence of increasing concentrations of suramin showed a dose-dependent decrease of specifically bound ^{125}I-EGF with a concentration producing 50% inhibition of 44.2 ± 22.0 μg/ml suramin (Fig. 2). The effect of suramin on EGF receptor binding was >80% after an incubation time of <30 min and persisted during long-term treatment with suramin (100 μg/ml) for at least 24 h (not shown).

Effect of Suramin on Basal Phospholipid Metabolism. The receptor-activated breakdown of phospholipids is closely linked to the subsequent resynthesis of those phospholipids involved in the hydrolysis by phospholipase C. To investigate the effect of suramin on phospholipid metabolism in HT-29 cells, ^{32}P incorporation in untreated and cholinergically stimulated cells was determined.

Pretreatment of the cells with suramin for 60 min (Fig. 3) showed no changes in the basal ^{32}P labeling of phosphoinositides and phosphatidic acid at concentrations up to 200 μg/ml. At concentrations >200 μg/ml suramin caused a reproducible 1.5-fold increase in labeling of [^{32}P]phosphatidylinositol (ED_{50}, 307 ± 89 μg/ml), suggesting either an increased activity of specific kinases or an increase in the total amount of the lipid.

Effect of Suramin on Carbachol-activated Phospholipid Metabolism. To investigate the effect of suramin on stimulated phospholipid turnover, the cells were incubated with the cholinergic agonist carbachol (0.2 mM), either alone or in the presence of suramin. Carbachol significantly increased the incorporation (60-min incubation) of ^{32}P into phosphatidic acid (1.5-fold) and phosphatidylinositol (4.3-fold) (Fig. 4; Table 1). ^{32}P contents of PIP and PIP_2 were increased to a lesser extent (30–50%) after 60 min of cholinergic stimulation, since the metabolism of these phospholipids is very rapid. The turnover of other major lipids, such as phosphatidylcholine and phosphatidylethanolamine, was not markedly affected (Table 1).

In the presence of suramin (100 μg/ml, 60-min pretreatment) ^{32}P incorporation of phospholipids was significantly decreased into phosphatidylinositol (48% inhibition, $P < 0.01$), phosphatidic acid (90%, $P < 0.01$), phosphatidylinositol-4-phosphate (95%, $P < 0.05$), and phosphatidylinositol-4,5-bisphosphate (95% inhibition, $P < 0.05$), during a 30-min incubation period, as shown in Fig. 5. These experiments indicated an effect of suramin on stimulated phosphoinositide synthesis. The inhibitory effects of suramin on carbachol-stimulated synthesis of PA, PI, PIP, and PIP_2 were constantly observed during time course experiments for up to 60 min. Suramin did not have major effects on ^{32}P labeling of phosphatidylcholine under basal conditions or in the presence of carbachol (data not shown).

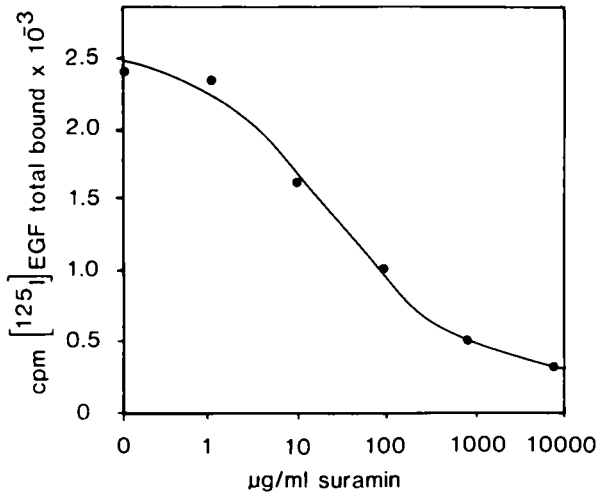


Fig. 2. Dose-response curve of ^{125}I-EGF binding to HT-29 cells in the presence of suramin. Data show means ± SEM of three independent experiments performed in triplicate. ^{125}I-EGF was added 10 min prior to the addition of various concentrations of suramin. The curve was analyzed with the ALLFIT program.

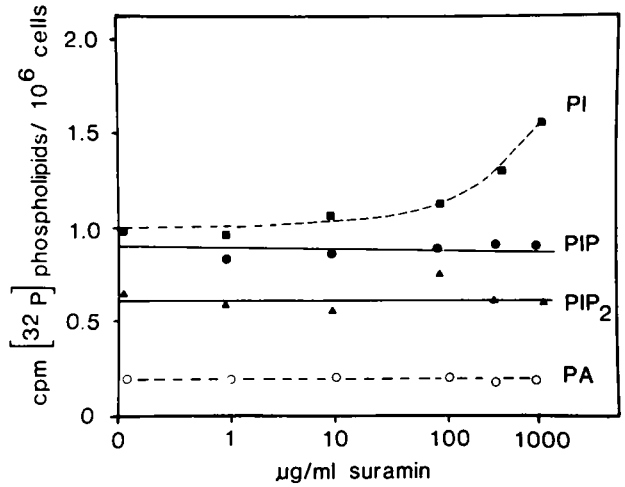


Fig. 3. Basal ^{32}P incorporation into phospholipids in the presence of suramin. Cells were incubated with phosphate-free HBSS (60 min) and various concentrations of suramin 2 min prior to the addition of 10 μCi/ml [^{32}P]P_i. After 60 min of incubation lipid extraction was performed as described in "Materials and Methods." Phospholipids were separated by thin-layer chromatography and exposed to autoradiography film, and radioactivity was determined by Cerenkov counting. Data show means ± SEM of three experiments, each performed in triplicate.

Determination of PI Kinase and DAG Kinase Activities. The experiments performed with whole cells showed a potent reduction of ^{32}P incorporation into phosphoinositides and a decrease in phosphatidic acid synthesis. To get a more precise estimate of the target of suramin action we determined PI and DAG kinase activities. As shown in Fig. 6 suramin treatment potently inhibited both PI kinase and DAG kinase activities with half-maximal inhibitory effects achieved at concentrations of 14.2 ± 2.7 μg/ml (PI kinase) and 20.4 ± 5.7 μg/ml (DAG kinase). Furthermore, the addition of a crude preparation of PIP as substrate for PIP kinase indicated a similar inhibition of PIP kinase activity in the presence of suramin (95% inhibition using 200 μg/ml suramin) (not shown).

Effect of Suramin on Inositol Phosphate Accumulation. To assess effects on phospholipase C activity, cells were prelabeled with [^{3}H]myoinositol for 24 h and then stimulated with increasing concentrations of carbachol alone or in the presence of 500 μg/ml suramin. As shown in Fig. 7, incubations with suramin for 60 min and 24 h (not shown) did not affect cholinergically stimulated release of inositol phosphates [control: ED_{50}, 15 μM; suramin (500 μg/ml): ED_{50}, 17 μM], indicating that suramin did not alter receptor-activated hydrolysis of polyphosphoinositides by phospholipase C.

Estimation of Total Phospholipid Mass after Suramin Treatment. Suramin may cause changes in the total amount of phosphoinositides and thereby reduce stimulated metabolism due to a decrease in substrate. Therefore, we measured the incorporation of [^{3}H] myoinositol into phosphoinositides after 48 h of labeling, when isotopic equilibrium was achieved. Separation of PI, PIP, and PIP_2 by thin-layer chromatography did

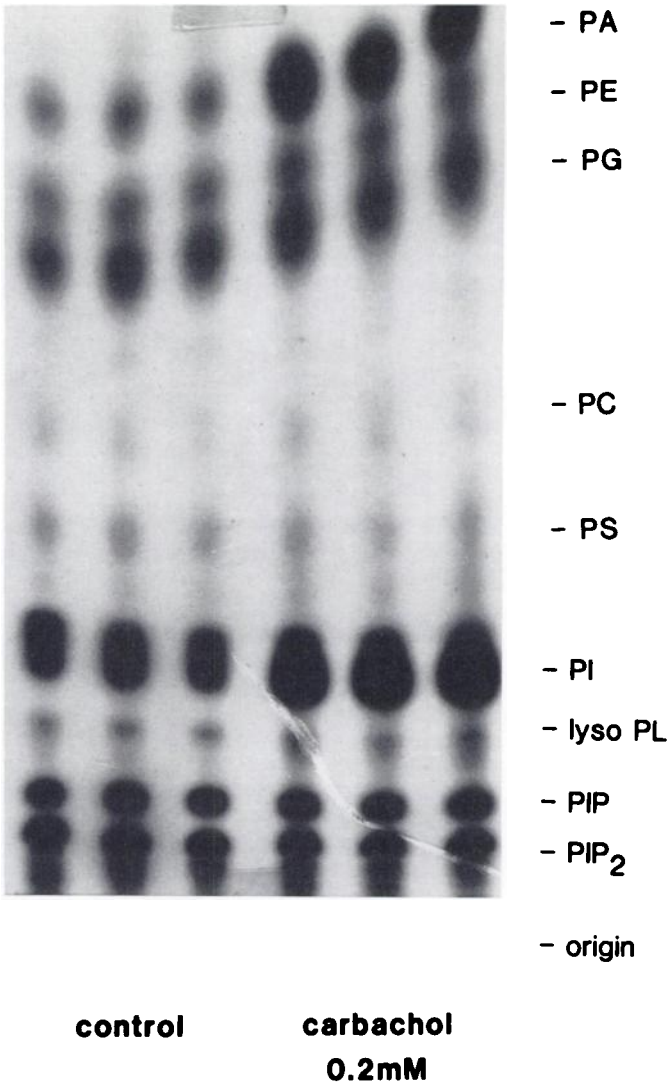


Fig. 4. [^{32}P]P_i incorporation into phospholipids. Autoradiography shows ^{32}P labeling of phospholipids into untreated HT-29 cells (control) and after incubation with carbachol (0.2 mM, 60 min). Phospholipids were extracted and separated as described in "Materials and Methods." Autoradiography films were exposed for 48 h at room temperature. RF values were as follows: PIP_2, 0.10; PIP, 0.15; PI, 0.31; phosphatidylcholine (PC), 0.51; phosphatidylglycerol (PG), 0.71; phosphatidylethanolamine (PE), 0.82; PA, 0.90.

not reveal any significant change of the [^{3}H]inositol content of each investigated phospholipid (not shown), after incubations with suramin at concentrations up to 1 mg/ml, indicating that changes of stimulated phospholipid turnover seem to be more likely due to alterations of phospholipid kinase activities.

Binding to Muscarinic Receptors in the Presence of Suramin. Another possibility to explain the reduction of carbachol-stimulated phospholipid metabolism by suramin would be an interaction of the drug with muscarinic receptors on HT-29 cells. Results in Fig. 8 show that muscarinic receptor binding, as measured by incubations with [^{3}H]NMS, was not affected by suramin at concentrations up to 1.0 mg/ml, after an incubation time of 60 min or 24 h (not shown) and therefore did not account for the alterations of cholinergically stimulated phospholipid turnover.

DISCUSSION

The antiprotozoal drug suramin potently inhibited stimulated phosphoinositide metabolism in HT-29 cells most likely because of a direct interference with kinase activities involved in phospholipid synthesis. Suramin caused a dose-dependent inhibition of carbachol-stimulated phosphoinositide synthesis and generation of phosphatidic acid, without affecting phospholipase C activity or basal phosphoinositide metabolism at concentrations <200 μg/ml suramin. In addition, investigation of EGF receptor characteristic in the presence of suramin confirmed the previously reported inhibitory effect of suramin on growth factor receptor binding.

Since several reports have shown that initiation of cell growth involves activation of the phosphoinositide system (1, 28), our data indicate that antiproliferative effects of suramin could also be produced by changes in the phospholipid-dependent second messenger release and therefore may need a critical reevaluation. The known properties of suramin on growth factor receptor binding, its inhibitory activities on retroviral reverse transcriptase (17, 18), and the effects on phosphoinositide metabolism reported in this study may indicate a potential anticancer activity of this compound.

In HT-29 cells suramin showed a noncompetitive inhibition of ^{125}I-EGF binding, as indicated by Scatchard analysis. This is in agreement with the noncompetitive effects of suramin on DNA polymerases found in lymphoid cells (15) and inhibition of GTPase activities in neuroblastoma cells (29) but is in contrast to reports of Betsholtz *et al.* (30) and Williams *et al.* (31), who found a competitive inhibition of PDGF receptor binding and growth factor-induced mitogenic activity by suramin. The reported inhibitory effect on EGF binding seems to include tyrosine kinase-coupled receptors but, remarkably, did not affect muscarinic receptors, which belong to the family of the G-protein-coupled receptors, such as the β-adrenergic receptors and the rhodopsins.

Table 1 *Effect of carbachol (0.2 mM) on ^{32}P incorporation into phospholipids in HT-29 cells*

Results of ^{32}P labeling into phospholipids (60 min) of untreated and carbachol-stimulated HT-29 cells. ^{32}P incorporation was performed under nonequilibrium conditions. Lipids were extracted and separated as described in "Materials and Methods." Data represent two independent experiments, each performed in triplicate.

	Control (100%) (cpm ^{32}P incorporation)		Carbachol [cpm ^{32}P incorporation (%)]	
Phospholipid	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Phosphatidic acid	682	823	1053	1201 (150)[a]
Phosphatidylinositol	955	955	4060	4296 (438)[a]
Phosphatidylinositol-4-phosphate	745	418	1013	734 (155)[b]
Phosphatidylinositol-4,5-bisphosphate	1346	466	1565	745 (138)
Phosphatidylethanolamine	416	328	443	365 (111)
Phosphatidylcholine	623	655	679	699 (108)

[a] $P < 0.01$.
[b] $P < 0.05$.

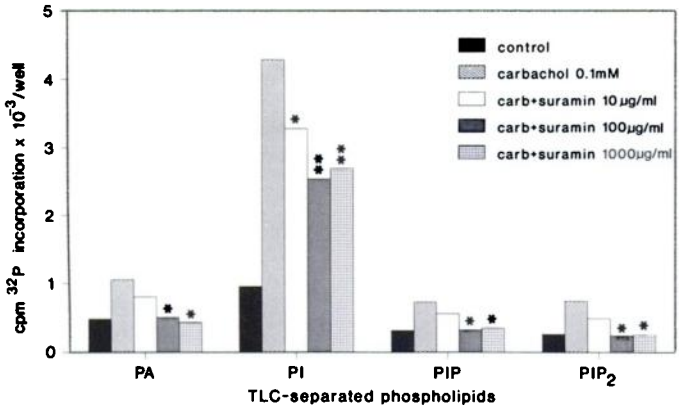
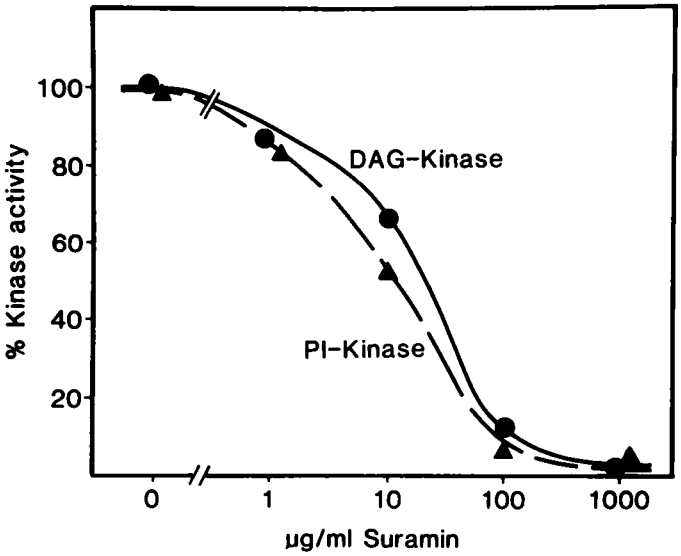


Fig. 5. Effect of suramin on carbachol-stimulated [^{32}P]phospholipid labeling. ^{32}P incorporation under nonequilibrium conditions was performed in unstimulated cells (control) and during incubation (30 min) of HT-29 cells with carbachol (carb) (0.1 mM), in the presence of indicated concentrations of suramin (0.1–1000 μg/ml). Data show results of three independent experiments, each performed in triplicate. *, $P < 0.05$; **, $P < 0.01$, as compared to carbachol-stimulated ^{32}P content of indicated phospholipids. TLC, thin-layer chromatography.



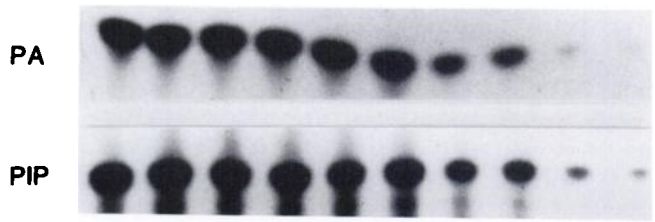


Fig. 6. Determination of PI and DAG kinase activity. PI and DAG kinase from whole cell homogenates were eluted from DEAE cellulose as described in "Materials and Methods." Phosphatidylinositol (200 μg/ml) and diacylglycerol (100 μg/ml) were used as substrates and ^{32}P incorporation in PIP and PA was measured. Phospholipids were extracted, separated by thin-layer chromatography, and exposed to autoradiography film. [^{32}P]phospholipids comigrating with commercial standards of PIP and PA were scraped into vials and radioactivity was determined by Cerenkov counting. Data show results (means ± SEM) of PI and DAG kinase activity in the presence of suramin from three independent experiments, each performed in duplicate. Autoradiographs show representative experiments demonstrating ^{32}P content in PIP and PA, representing PI and DAG kinase activity, respectively. Specific kinase activity of PIP and DAG kinase corresponds to 163 and 107 cpm/min/tube, respectively.

The reduction of ^{32}P incorporation by suramin into phosphatidylinositol, polyphosphoinositides, and phosphatidic acid was observed only in the presence of carbachol, indicating a reduced phospholipid synthesis under stimulated conditions. Inositol

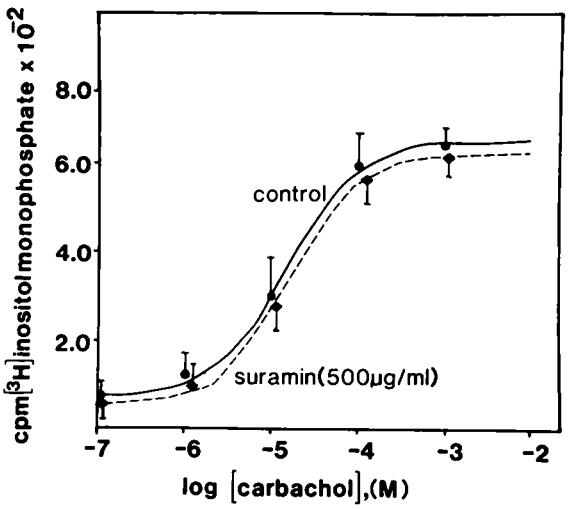


Fig. 7. Effect of suramin on inositol phosphate accumulation. Cells were stimulated with indicated concentrations of carbachol for 30 min, either alone or after pretreatment with suramin for 60 min (500 μg/ml). Results represent means ± SEM (*bars*) of three independent experiments performed in triplicate. A 24-h pretreatment with suramin gave similar results (data not shown).

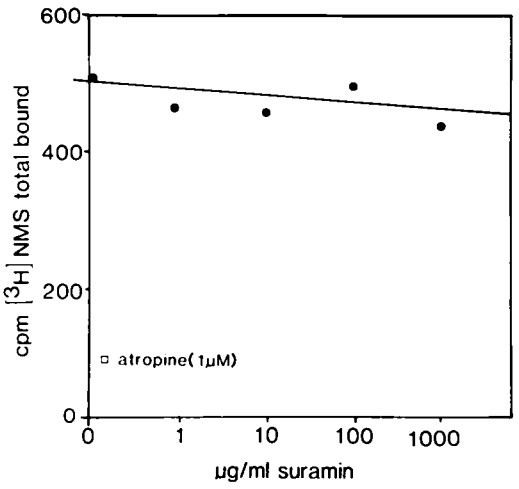


Fig. 8. Effect of suramin on muscarinic receptor binding. Data show muscarinic receptor binding on HT-29 cells by determination with [^{3}H]*N*-methylscopolamine (0.25 nM) in the presence of the indicated concentrations of suramin. Cells were pretreated with suramin 60 min prior to the addition of tritiated NMS. Results represent means of one typical experiment, out of three, each performed in triplicate.

phosphate release representing phospholipase C activity was not altered by suramin during a 30-min stimulation with carbachol. The fact that phospholipase C did not appear to be inhibited by suramin despite inhibition of resynthesis of phosphoinositides suggests that cellular pools of phosphoinositides were sufficient to serve as a substrate for phospholipase C-induced hydrolysis. However, under conditions of long-term stimulation, such as growth factor-induced cell proliferation, a permanently activated phosphoinositide metabolism should be dependent on active phospholipid kinase-mediated phosphoinositide resynthesis. Therefore, growth inhibitory effects of suramin may involve inhibition of phosphoinositide synthesis. In addition, previous reports have indicated a proliferative potential of phosphatidic acid, depending on the content of unsaturated fatty acid residues (32). Since our results show a potent inhibition of phosphatidic acid synthesis by an inhibition of diacylglycerol kinase, this effect may contribute to the antiproliferative effects of suramin.

Remarkably, suramin showed divergent effects on basal and stimulated phosphoinositide metabolism. These findings would support the hypothesis that cells use different phospholipid pools or kinases for basal and receptor-activated phospholipid metabolism. This suggestion would also explain our results, which indicate that incubation with suramin (0.1–1 mg/ml) for up to 24 h did not change phosphoinositides quantitatively and did not interfere with basal phosphoinositide turnover.

Suramin did not alter basal ^{32}P incorporation into phospholipids at lower concentrations, although carbachol-stimulated ^{32}P labeling was potently inhibited (Figs. 3 and 5). However, direct measurements of PI kinase and DAG kinase in cell homogenates showed a potent reduction of PI kinase and DAG kinase (Fig. 6). These results may lead to speculations that kinases involved in basal phospholipid metabolism might be localized in compartments not easily accessible for suramin by exogenous addition to the culture medium.

The ^{32}P labeling of unstimulated HT-29 cells in the presence of suramin resulted in an increase of [^{32}P]phosphatidylinositol at concentrations >200 μg/ml. However, the increase of basal phosphatidylinositol metabolism did not alter carbachol-activated phospholipase C activity. This may be expected due to a possible increase in substrate. The inhibition of carbachol-stimulated PI turnover, which was present already at 10 times lower concentrations of suramin (Fig. 6), was also observed at these high concentrations of 1 mg/ml suramin. This again may indicate a dissociation of basal and stimulated phosphoinositide synthesis. Furthermore, suramin inhibited carbachol-stimulated resynthesis of PI, while basal PI turnover was increased at higher concentrations of suramin. This might suggest additional effects of suramin on pathways related to phosphatidylinositol synthesis, which may involve PI synthase activity or the metabolism of CDP-diacylglycerol.

Several reports of the pathways involved in the resynthesis of phosphatidylinositol and phosphatidylglycerol in type II pneumocytes described a common CDP-diacylglycerol pool for the *de novo* synthesis of phosphatidylinositol in microsomal preparations (33, 34). In our experiments with HT-29 cells suramin potently reduced the ^{32}P labeling of a phospholipid comigrating with phosphatidylglycerol (concentration producing 50% inhibition, <50 μg/ml).[4] We speculate from these data that suramin may additionally inhibit the synthesis of phosphatidylglycerol, probably due to a decrease of CDP-diacylglycerol glycerol phosphate phosphatidyltransferase activity. This would explain the reported increase of basal phosphatidylinositol resynthesis, due to a predominant use of CDP-diacylglycerol pools for phosphatidylinositol formation. Furthermore, this may suggest a connective pathway between these two phospholipids during basal and stimulated phosphoinositide turnover and possibly indicates an auxiliary pathway for the resynthesis of phosphatidylinositol in intestinal cells. However, further experiments will be necessary to clearly demonstrate the effect of suramin on additional pathways involved in phosphatidylinositol synthesis.

Misset and Opperdoes (35) reported inhibition of various phosphoglycerol kinases in *Trypanosoma brucei* by suramin, suggesting that the inhibitory activity of the negatively charged compound suramin on phosphokinases may be related to its affinity to kinases with high isoelectric point values. Butler *et al.* (29) reported a noncompetitive interaction of suramin with a pertussis toxin-sensitive GTP-binding protein in neuronal cell membranes involved in opioid receptor function. Our findings do not indicate that the reduction of stimulated phosphoinositide turnover by suramin is due to reduced GTPase activity in HT-29 cells, since carbachol-activated inositol phosphate release was not affected. Changes in the labeling of cellular ATP pools after suramin treatment are unlikely to account for the changes in [^{32}P]P_i incorporation, since suramin caused divergent effects on various phospholipids.

In A 431 cells, a cell line overexpressing EGF receptors, EGF was found to increase the phosphorylation of phosphoinositides, although results concerning the correlation between the activation of the phosphoinositide system and Ca^{2+} or Na^+/H^+ fluxes are controversial at present (36, 37). Since HT-29 cells were reported to produce EGF-like growth factors (11) suramin could reduce ^{32}P contents of phosphoinositides as a result of decreased autocrine stimulation of phosphoinositide metabolism. In this case suramin should show measurable effects on basal phosphoinositide metabolism. In contrast, our results indicate that suramin-induced changes of phosphoinositide metabolism were not due to a reduction of EGF receptor-mediated autocrine stimulation of phosphoinositide metabolism. Incubation of HT-29 cells with EGF at concentrations varying from 0.1 to 1000 ng/ml did not stimulate the phosphoinositide system as measured by inositol phosphate release or determination of ^{32}P incorporation into membrane phospholipids.[5]

Recent reports have shown inhibitory effects of suramin on protein kinase C activity (38). Since PI kinase is positively regulated by protein kinase C (39) and DAG kinase has been shown to be a protein kinase C substrate (40), suramin may interfere with mechanisms of phosphoinositide resynthesis mediated by protein kinase C.

In summary, we report remarkable effects of suramin on stimulated phosphoinositide metabolism, due to inhibition of PI kinase and DAG kinase activities. This indicates that effects of suramin on second messenger-related phospholipid metabolism need to be considered when suramin is used during investigation of growth factor-related metabolic pathways. Since development and proliferation of tumor cells may be related to increased metabolic activities of growth factor-dependent pathways, persistent retroviral replication, or altered phosphoinositide metabolism, the effects of suramin on phosphoinositide synthesis reported in this study may support the probable value of suramin as a potential anticancer drug.

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[4] Unpublished results.

[5] Unpublished data.

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