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AY9944 inhibits early activation of phosphatidylinositol metabolism in concanavalin A-stimulated lymphocytes

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AY9944, an agent which blocks cholesterol synthesis [1], can inhibit blastic transformation of lymphocytes in a cholesterol-free medium [2]. The inhibition does not occur if plasma low density lipoproteins (LDL) are added to the medium. Since LDL provide exogenous cholesterol to cells, it was thought that AY9944 inhibits blastic transformation by means of a blockade of cholesterol synthesis.

Nevertheless, in a plasma-containing medium, despite the presence of exogenous cholesterol, an excess of AY9944 can inhibit blastic transformation [3, 4]. Thus, another mechanism, irrespective of cholesterol synthesis, could be involved. We have hypothesized that AY9944, which is an amphiphilic substance with strong affinity for the phospholipid moiety of biological membranes [5], inhibits blastic transformation of lymphocytes by means of an effect on the plasma membrane of cells, during the first stages of the mitogen's action [4]. In order to check this point, we studied the effect of AY9944 on the early activation of phosphatidylinositol (PI) metabolism by lectins ("PI response") [6]. We observed that AY9944 inhibits the activation of PI metabolism during the first hours of the action of concanavalin A on lymphocytes, and that it blocks cell entry into G1.

Materials and Methods

Preparation of lymphocytes. Human peripheral lymphocytes were prepared by Böyum's method [7], under precise conditions previously described [8].

Cell cultivation. Cells were incubated in RPMI medium, supplemented with 20 mM HEPES buffer, 0.3 g of L-glutamine, 300,000 I.U. of penicillin and 0.3 g of streptomycin per liter, and with 10% autologous plasma. The incubation was performed at 37°, in a 5% CO_2 atmosphere. When present, AY9944 (Ayerst Lab., U.S.A.) was used at a concentration of 10, 20 or 40 μM.

[^{32}P]*Phosphate incorporation into phospholipids.* Four million cells were incubated in 1.5 mL of medium (final volume), in glass tubes (in which subsequent direct extraction of phospholipids was possible). Cells were first preincubated for 48 hr, with or without AY9944. Then, they received 300 μCi (15 μg) of [^{32}P]phosphate (CEA, France) per tube, and simultaneously, when stimulated, 100 μg of concanavalin A (the Sigma Chemical Co., Poole, U.K.). We verified that this dose of concanavalin A (66 μg/mL) corresponds, in our experimental conditions (4 million cells per tube), to the optimal rate of [^{3}H]thymidine incorporation into DNA (unpublished results).

For pulse experiments, 5 hr after the onset of incorporation, the incubation was stopped by the addition of 10 mL of methanol–chloroform (1/1, v/v). Phospholipids were extracted, separated and counted for radioactivity as previously described [8].

For pulse-chase experiments, 5 hr after the onset of incorporation, cells were centrifuged, washed with RPMI and re-suspended in 1.5 mL of the supernatant from ^{32}P-free cultures, which were performed simultaneously. The incubation was then carried on for a further 19 hr-period and stopped as described above.

Cell viability. The percentage of dead cells was evaluated, after 48 hr-preincubation with or without AY9944, by the method of trypan blue exclusion.

[^{3}H]*Thymidine incorporation into DNA.* Four hundred thousand cells were incubated per tube, in 150 μL of medium, with 10 μg of concanavalin A. Forty-eight hours after the onset of incubation, each tube received 2 μCi of [^{3}H-methyl]thymidine (CEA). Two hours later, the incorporation was stopped by the addition of 1 mL of a 25% solution of trichloracetic acid (TCA). After 15 min, the TCA-insoluble material was centrifuged, washed four times with TCA and directly transferred into scintillation phials, where it was dried and its radioactivity counted.

Results and Discussion

Table 1 indicates that, as previously described [2–4], AY9944 inhibits [^{3}H]thymidine incorporation into cell

Table 1. Effect of AY9944 on [^{3}H]thymidine incorporation into DNA and on cell viability

AY9944 (μM)	0	10	20	40
[^{3}H]Thymidine incorporation*	100†	37 (±7)	8 (±2)	—
Cell mortality‡	3 (±1)	2 (±1)	7 (±1)	16 (±3)

* Rate of [^{3}H]thymidine incorporation (2 hr-pulses), evaluated after 48 hr-incubation with concanavalin A, and with or without AY9944. Percentage of the value obtained in the absence of AY9944.

† This corresponds to 8480 cpm/10^5 cells (±1270).

‡ Percentage of dead cells evaluated after 48 hr-preincubation of unstimulated lymphocytes, with or without AY9944.

Average of six experiments (±SD).

DNA, in a dose-dependent manner. In our experiments, the inhibition is incomplete at a concentration of AY9944 of 10 μM and quite complete at a concentration of 20 μM.

Table 1 also shows the viability of cells after 48 hr-preincubation with AY9944, that is to say just before that [^{32}P]phosphate pulses were performed. At concentrations of AY9944 of 10 and 20 μM, the percentage of dead cells is negligible. It is somewhat higher at a concentration of 40 μM, which was thus discarded.

The effects of AY9944 on [^{32}P]phosphate incorporation into PI are described in Table 2. Cells were first pre-incubated for 48 hr with or without AY9944. Then, they received [^{32}P]phosphate for 5 hr pulses, and they were either simultaneously stimulated by concanavalin A or kept unstimulated.

In unstimulated cells, AY9944 slightly enhances the rate of [^{32}P]phosphate incorporation into PI. It has previously been observed that other cationic amphiphilic substances, such as chlorpromazine or local anesthetics, have a similar effect, by means of an inhibition of phosphatidic acid hydrolysis (so that phosphatidic acid is redirected into the synthesis of phospholipids which follow the CDPdiacylglycerol pathway) [9].

In concanavalin A-stimulated cells, early activation of [^{32}P]phosphate incorporation into PI [6] is strongly lowered by AY9944. We thus conclude that AY9944 inhibits the first stages of cell stimulation by concanavalin A, which are the locus of activation of phosphoinositide breakdown and resynthesis [9]. Accordingly, as indicated in Table 3, the disappearance of ^{32}P from labelled PI is also inhibited by AY9944.

Table 2. [^{32}P]Phosphate incorporation into PI

AY9944 (μM)	0	10	20
Unstimulated cells	1*	1.67 (±0.26)	1.70 (±0.29)
Stimulated cells	3.89 (±0.58)	3.72 (±0.72)	2.48 (±0.37)
Ratio	3.89	2.23	1.46

Lymphocytes were first preincubated for 48 hr with or without AY9944. Then, they received [^{32}P]phosphate and simultaneously, when stimulated, concanavalin A, for further 5 hr-incubation. [^{32}P]Phosphate incorporation into PI was referenced to the standard value obtained in unstimulated cells in the absence of AY9944. Average of seven experiments (±SD).

* This corresponds to 3840 cpm/million cells (±580) (i.e. 47% of the radioactivity of total phospholipids).

Table 3. Disappearance of ^{32}P from labelled PI

AY9944 (μM)	0	20
Unstimulated cells	106 (±15)	86 (±5)
Stimulated cells	55 (±6)	86 (±9)

[^{32}P]Phosphate pulses were performed as described in the legend of Table 2. Cells were then centrifuged and re-suspended in the supernatant from unlabelled cultures. The disappearance of ^{32}P from labelled PI was evaluated after a subsequent 19 hr-incubation, and expressed in percentage of the initial radioactivity. Average of five experiments.

Table 4. [^{32}P]Phosphate incorporation into PC

AY9944 (μM)	0	10	20
Unstimulated cells	1*	1.45 (±0.36)	0.99 (±0.16)
Stimulated cells	2.42 (±0.50)	2.29 (±0.51)	1.51 (±0.31)
Ratio	2.42	1.58	1.53

[^{32}P]Phosphate incorporation into PC was evaluated as described for PI in the legend of Table 2.

* This corresponds to 4650 cpm/million cells (±930) (i.e. 49% of the radioactivity of the total phospholipids).

Since AY9944 blocks the early stages of the mitogen's action, it may inhibit cell entry into the G1 phase. The observation that phosphatidylcholine (PC) synthesis is lowered by AY9944 (Table 4) is in agreement with this point of view. Thus, it is clear that, contrary to previous conclusions [2], the blockade of cholesterol synthesis, which would occur later in G1, is not a possible mechanism for the inhibitory effect of AY9944 on blastic transformation. It is more likely that, in concanavalin A-stimulated lymphocytes, AY9944 inhibits earlier events which normally lead to cell activation.

It has been previously established that, to be efficient, AY9944 must be added within the first 24 hr after the cell stimulation by concanavalin A [2]. This agrees with an early blockade of the mitogen's action, since the stimulation of lymphocytes by concanavalin A is mitogen-dependent for these first 24 hr [10].

In this study, we demonstrated that AY9944 inhibits the early stages of lymphocyte stimulation. Thus, it may be thought that AY9944 acts similarly to other cationic amphiphilic substances, such as phenothiazines or local anesthetics, which also inhibit the first steps of blastic transformation [11]. These substances involve a blockade of calmodulin [11], and AY9944 is itself a calmodulin antagonist [12]. Moreover, as for these other amphiphilic substances possessing an ionizable amine function [9], it may be hypothesized that AY9944 inhibits enzymes which have an acidic phospholipid as substrate, such as phosphatidic acid phosphohydrolase (see above), and also PI specific phospholipase C. Further experiments are needed to test these hypothesis.

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Time-dependent effects of chloroquine on pH of hepatocyte lysosomes

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Cationic amphiphilic compounds (CACs) are chemicals composed of a hydrophobic aromatic ring(s) and positively charged, hydrophilic side chains [1, 2]. These compounds have high pK values (e.g. 8–10) due to their positively charged side chains and can act as weak bases [2]. Indeed, chloroquine, a prototypic CAC commonly used in studies in cell biology, can accumulate in acidic intracellular compartments, including lysosomes and endosomes; its sequestration in these organelles results in an increase in their pH due to proton consumption [3, 4]. The resultant elevated pH (i.e. weak base action) can cause major alterations in the intracellular trafficking and degradation of macromolecules, affecting receptor-mediated endocytosis, intralysosomal digestion, exocytosis, and the biosynthesis of secretory proteins [5]. Moreover, generalized phospholipidosis (in which the liver is often the major site of phospholipid accumulation) has emerged as a serious adverse effect of the clinical use of some CACs (e.g. amiodarone) [2, 6]. While the exact pathogenesis of the phospholipidosis is obscure, increased intralysosomal pH may play a role.

Since there have been no reports systematically evaluating the time dependency of the weak base actions of chloroquine, we studied the serial *in vivo* effects of chloroquine on the pH of hepatocyte lysosomes.

Methods and Results

Male Sprague–Dawley rats (Harlen Sprague–Dawley, Indianapolis, IN) were fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) and allowed access to water *ad lib*. Chloroquine hydrochloride (Aralen hydrochloride, Winthrop-Breon Laboratories Division of Sterling Drug Inc., New York, NY) was administered intraperitoneally at a dose of 40 mg/kg body weight as chloroquine base. To examine the acute effects of chloroquine, rats were killed at 30 min and at 1, 2, 2.5, 3, 12, 24 and 48 hr after a single injection. To examine the chronic effects of chloroquine, rats were dosed every other day for a total of 4, 6, 8, 10 and 12 days. Control animals received saline injections intraperitoneally using identical dose schedules. Body weights at the start of the experiments were not different between rats in control and treatment groups and ranged from 200 to 245 g. After their respective days of treatment, changes in body and liver weights were not different between control and treatment groups (data not shown).

Intralysosomal pH was measured by flow cytometry in isolated hepatocytes using a modification of a technique described by Murphy [7] for analysis of endocytic vesicles. Fluorescein isothiocyanate dextran (FITC-Dex, mol wt. 70,000; Sigma Chemical Co., St. Louis, MO), a pH-sensitive lysosomotropic fluorescent probe [8], was loaded into hepatocyte lysosomes by intraperitoneal injection 16 hr prior to sacrifice as described [9]. Isolated hepatocytes were prepared from rat liver as previously described [10] yielding approximately 500×10^6 hepatocytes per liver with a viability of greater than 90% by Trypan blue exclusion and a purity of 99% by electron microscopy. Selective sequestration of FITC-Dex in lysosomes was confirmed by subcellular fractionation [9]. Isolated hepatocytes were suspended in Krebs–Ringers–Hepes* solution, and lysosomal pH was measured with a Becton Dickinson FCSD IV flow cytometer (Mountain View, CA) with an excitation wavelength of 488 nm. Emission fluorescence was measured using 530 and 585 nm filters with a 570 nm beam splitter. The 530/585 nm ratio was calculated after counting 20,000 cells. A standard curve for lysosomal pH was prepared by placing the hepatocytes in phosphate-citrate buffers ranging in pH from 4.5 to 6.5. Intralysosomal pH was equilibrated with the buffer pH through the addition of ionophores (10 μM monensin and 10 μM nigericin). Metabolic inhibitors (50 mM 2-deoxyglucose and 50 mM sodium azide) were also added to inhibit proton pump activity. For each point on the standard curve, 20,000 hepatocytes were counted and the 530/585 nm ratio was plotted against buffer pH.

The effect of chloroquine on the pH of lysosomes in isolated hepatocytes is shown in Fig. 1. An immediate chloroquine effect was apparent with pH rising from a

*Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.