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STUDIES ON THE MECHANISM OF PHOSPHOLIPID STORAGE INDUCED BY AMANTADINE AND CHLOROQUINE IN MADIN DARBY CANINE KIDNEY CELLS

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Abstract—Previous studies suggested that hepatic lipidosis caused by cationic amphiphilic drugs in rats is related to the capacity of these drugs to concentrate in liver lysosomes. These drugs inhibit lysosomal phospholipases, causing phospholipid accumulation. Amantadine, an inhibitor of influenza A virus replication, is a cationic amphiphilic drug which concentrates in the lysosomes of the Madin Darby canine kidney (MDCK) cell. In the present study, amantadine and chloroquine were shown to inhibit soluble lysosomal phospholipases isolated from MDCK cell *in vitro*. Both amantadine and chloroquine concentrated in MDCK cell lysosomes. These drugs caused phospholipid storage in cultured MDCK cells, and the amounts of the respective agents required to cause phospholipid storage correlated with the capacities of the agents to inhibit lysosomal phospholipases. The mechanisms involved in this phenomenon are discussed, and a three-step hypothesis is presented predicting which agents will cause phospholipidosis.

A human foam cell lipidosis, caused by treatment with diethylaminoethoxyhexestrol (DH), was first reported in Japan more than ten years ago [1, 2]. It was characterized by enlargement of liver and spleen and by the appearance of foam cells in the bone marrow and vacuolated lymphocytes in the peripheral blood. This disorder could be produced by administration of DH to rats or monkeys, causing an increase in liver phospholipid content [3]. Treatment of experimental animals with chloroquine, another cationic amphiphilic drug, caused a similar increase in tissue phospholipids [4]. This type of iatrogenic lipidosis has now been reported for more than thirty cationic amphiphilic agents in various experimental or clinical situations [5].

In examining the molecular basis for this type of drug-induced lipidosis, we found that choloroquine and DH were localized almost exclusively in the lysosomal compartment in the liver of treated rats [6]. Essentially all of the excess phospholipids in liver could be accounted for by the increased lysosomal phospholipid pool suggesting that a block in intralysosomal phospholipid catabolism might explain the disorder [6, 7]; increased phospholipid synthesis was not apparent [7]. DH and chloroquine were subsequently found to be potent inhibitors of liver lysosomal phospholipases A and C [8]. Further studies showed that a number of other cationic amphiphilic agents, some of which are known to produce lipidosis, inhibit liver lysosomal phospholipases A and C [9].

Amantadine, an inhibitor of influenza A virus replication, also inhibits liver lysosomal phospholipases [9]. In previous studies of the mechanism of amantadine inhibition of influenza A virus replication, we noted that [^{3}H]amantadine enters cells freely and concentrates in the lysosomes of cultured Madin Darby canine kidney (MDCK) cells [10]. These findings suggested that this agent could be used to prospectively test the hypothetical requirements for production of cellular phospholipid storage. We hypothesize that three factors are necessary for production of cellular phospholipidosis by a pharmacologic agent: (1) the drug must enter the cell freely, (2) it must concentrate to a substantial degree in cell lysosomes, and (3) the drug must inhibit the action of lysosomal phospholipases. This paper presents results of studies with amantadine and chloroquine which strongly support this hypothesis.

MATERIALS AND METHODS

Cells. MDCK cells were maintained between passages 60 and 80 in 150 cm^2 plastic cell culture bottles (Costar) at 37° in an atmosphere of 5% CO_2 in air in Dulbecco's modified Eagle's medium (pH 7.4) containing 100 units/ml penicillin and 100 μg/ml streptomycin and 5% fetal bovine serum (DMEM5). The MDCK cells have been free of mycoplasma as noted previously [10].

Preparation of subcellular fractions of MDCK cells. MDCK cells were washed with cold phosphate-buffered saline (PBS) (pH 7.4), harvested by scraping the cell monolayer with a rubber policeman, and pelleted by centrifugation (490 *g* for 10 min). All operations were done at 4°. A cell lysate was prepared in buffer containing 0.25 M sucrose, 10 mM triethanolamine (pH 7.4) and 1 mM EDTA

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(STE) by a modification of the method of Harms *et al.* [11] as previously described [10]. Briefly, the cell lysate was centrifuged at 600 *g* for 10 min, and the resulting nuclear pellet (N) was reserved. The postnuclear supernatant fraction was centrifuged at 10,000 *g* for 10 min. The mitochondrial pellet (M) was obtained and the supernatant fraction was centrifuged at 20,000 *g* for 10 min to obtain the light mitochondrial pellet (L). The resulting supernatant fraction was centrifuged at 120,000 *g* for 60 min to obtain the microsomal pellet (P) and the supernatant fraction (S). The respective pellets were resuspended in buffer containing 0.25 M sucrose, 10 mM triethanolamine (pH 7.4). The total volume of each fraction was measured, and aliquots were stored at −60° until use.

In the experiments with [^{14}C]chloroquine (New England Nuclear Corp., Boston, MA), the cells were incubated in DMEM5 with 61 μM [^{14}C]chloroquine (sp. act. 0.4 mCi/mmole) at 37° for 60 min. The cells were chilled, and the cell monolayer was washed twice with cold PBS. The cells were harvested and subcellular fractions were prepared as noted above. [^{14}C]Chloroquine was measured in the respective fractions by liquid scintillation spectrometry.

Marker enzyme assays. Protein was measured by the method of Lowry *et al.* [12]. Succinate *p*-iodonitrotetrazolium (INT) reductase, a mitochondrial marker, was measured by the method of Pennington [13]. *N*-Acetyl-β-D-glucosaminidase, a lysosomal component, was measured as described by Koldovsky and Palmieri [14]; NADPH cytochrome *c* reductase (microsomes) was assayed according to Sottacasa *et al.* [15].

Solubilization of MDCK lysosomal phospholipases. Acid phospholipases A and C were solubilized from the M + L pellet by ten cycles of freezing and thawing in half-strength STE buffer; membranous protein was removed by centrifugation at 120,000 *g* for 1 hr.

Measurement of phospholipases A and C. Phospholipases A and C were measured by determining the rate of conversion of [9,10-^{3}H]dioleoylphosphatidylcholine to [^{3}H]lysophosphatidylcholine and [^{3}H]diacylglycerol and [^{3}H]monoacylglycerol, respectively, at pH 4.4 as described previously [8, 9]. Detailed assay conditions are given in the legends to the tables. The reaction products were separated by thin-layer chromatography, identified by staining with iodine vapors and counted by liquid scintillation spectrometry as noted previously [8, 9]. Amantadine and chloroquine were dissolved in distilled water and added to the incubation in the final concentrations noted in Table 2.

Chemicals. Sucrose (Ultrapure) was obtained from Schwarz-Mann, Orangeburg, NY. Cytochrome *c* and *p*-iodonitrotetrazolium violet (grade 1) were obtained from the Sigma Chemical Co., St. Louis, MO. Amantadine was the gift of Drs. E. C. Hermann and C. E. Hoffman of E. I. duPont de Nemours & Company, Newark, DE. Chloroquine was supplied by the Winthrop Laboratories, New York, NY. Other chemicals were of analytical reagent grade. [9,10-^{3}H]Oleic acid was purchased from the New England Nuclear Corp. [^{3}H-dioleoyl]-Phosphatidylcholine was synthesized by the method of Warner and Benson [16]. 1,2-Diolein and 2-monoolein were purchased from Serdary Research Laboratories, London, Ontario, Canada.

RESULTS

Subcellular distribution of acid phospholipases and [^{14}C]chloroquine in MDCK cells. The subcellular distribution of [^{14}C]chloroquine, acid phospholipase A and marker enzymes for mitochondria and lysosomes in MDCK cells is shown in Fig. 1. Acid phospholipase A and [^{14}C]chloroquine were not enriched in the microsomal and supernatant fractions of the MDCK cells. Most of the total activity of acid phospholipase A as well as the [^{14}C]chloroquine was recovered in the mitochondrial (M) and light mitochondrial (L) fractions which represented 50–55 and 22–23% of the total activity in both cases respectively. The profile of these two variables was nearly identical to that of *N*-acetylglucosaminidase, a lysosomal enzyme which was also recovered in the M

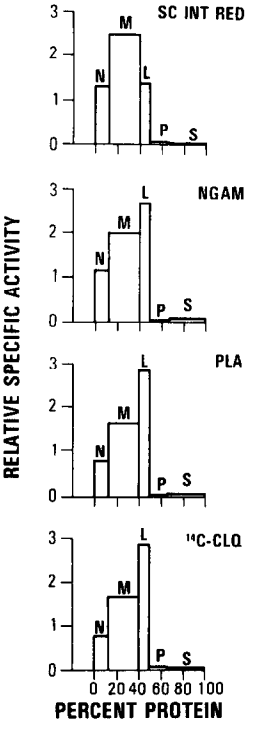

Fig. 1. Subcellular distribution of acid phospholipase A, [^{14}C]chloroquine and marker enzymes in MDCK cells. Each fraction is represented on the ordinate by the relative specific activity (percent total recovered activity/percent total protein) and on the abscissa by its protein content expressed as percent recovered protein. Abbreviations: N, nuclei and debris; M, mitochondria; L, light mitochondria; P, microsomes; and S, soluble. The results in the three upper panels are the average of three separate experiments; [^{14}C]chloroquine (lower panel) represents a single experiment. Recovery of the respective activities from the MDCK cell lysates was as follows: succinate INT reductase (SC INT RED), 84%; *N*-acetyl-β-D-glucosaminidase (NGAM), 83%; acid phospholipase A (PLA) 82%; and [^{14}C]chloroquine ([^{14}C]CLQ), 87%.

Table 1. Activity of soluble lysosomal phospholipases A and C in crude preparations isolated from MDCK cells, rat kidney and rat liver*

Source of lysosomes	Product (nmoles·mg^{-1}·hr^{-1}) Phospholipase A	Phospholipase C	A / C
MDCK cells (10)	58 ± 25	4 ± 1	14.5
Rat kidney (3)	248 ± 42	33 ± 5	13.0
Rat liver (5)	330 ± 45	125 ± 20	2.6

* Results are means ± S.D. The numbers in parentheses represent the numbers of replicates, *N*.

Table 2. Inhibition of MDCK lysosomal phospholipase A by amantadine and chloroquine*

Drug concn (mM)	Phospholipase A activity: Amantadine	Chloroquine
0	100	100
0.5	87 ± 13 (5)	61 ± 12 (4)
1.25	78 ± 7 (6)	45 ± 3 (4)
2.5	85 ± 20 (6)	34 ± 5 (5)
10	13 ± 8 (8)	7 ± 4 (4)
25	0 (3)	0 (4)
50	0 (3)	1 ± 1 (3)

* Results are expressed as percentages of control rate for MDCK lysosomal phospholipase A ± S.D. Numbers in parentheses represent the numbers of replicates. The control rate for phospholipase A was 58 ± 25 nmoles·mg^{-1}·hr^{-1} (N = 10).

and L fractions, representing 57 and 22% of total activity respectively. This indicates that acid phospholipase A and [^{14}C]chloroquine are primarily lysosomal. Acid phospholipase C activity was enriched principally in the M and L fractions of MDCK cells, while the total activity was recovered primarily in the M, L and supernatant fractions (data not shown). Recovery of protein, marker enzymes, acid phospholipase A and [^{14}C]chloroquine from the homogenate ranged from 82 to 87%.

Solubilization and properties of MDCK lysosomal phospholipases. The soluble fraction obtained from the M + L fraction contained 24% of the protein and 99 and 79% of the total acid phospholipase A and C activities respectively. Phospholipase A had a broad pH optimum between pH 3.8 and 4.8, and little activity was evident above pH 5.6. Phospholipase C activity was optimal between pH 3.8 and 4.8; substantial activity of phospholipase C persisted in the pH 5 and 6 range in contrast to phospholipase A. The activity of the crude soluble MDCK lysosome phospholipases was compared with that previously obtained from highly purified kidney [17] and liver lysosomes [9]; the results are shown in Table 1. In the MDCK cell and kidney preparations, the ratio of phospholipase A to phospholipase C activity was 14.5 and 13.0 respectively. However, in the liver the ratio of A to C was only 2.6, due to a relatively greater contribution of phospholipase C. The absolute rates of phospholipase A and C from these three sources cannot be compared directly since the kidney and liver data were obtained from highly purified lysosomal fractions [9, 17]. However, our data suggest that phospholipase A is the principal lysosomal phospholipase activity in the MDCK cell.

Table 3. Inhibition of MDCK cell lysosomal phospholipases A and C by cationic amphiphilic drugs and monovalent cations *in vitro*

Addition	Phospholipase IC_{50}* (mM) A	C
Amantadine	4.0	6.8
Chloroquine	1.0	2.0
Na^+	42	> 50
NH_4^+	43	> 50

* IC_{50} represents the concentration (mM) of agent required to inhibit the control activity by 50% for the respective phospholipases. Monovalent cations were added as the chloride salts.

Table 4. Effects of amantadine, chloroquine and ammonium chloride on the phospholipid content of cultured Madin Darby canine kidney (MDCK) cells*

Additions	Phospholipid (nmoles/mg protein)	P value†
Control, none	225 ± 27 (12)	
Amantadine, 100 μM	238 ± 28 (6)	NS‡
Amantadine, 316 μM	313 ± 40 (6)	< 0.0005
Chloroquine, 10 μM	290 ± 29 (3)	< 0.005
Chloroquine, 31.6 μM	316 ± 6 (3)	< 0.0005
NH_4Cl, 3.16 mM	238 ± 71 (3)	NS
NH_4Cl, 10.0 mM	299 ± 48 (6)	< 0.001

* Means ± S.D.; numbers in parentheses represent the numbers of replicates. Total lipid extracts were prepared by the method of Folch *et al.* [18], and lipid phosphorus was measured by the method of Rouser *et al.* [19].

† Statistical tests of significance were made using Student's *t*-test of the difference of unpaired means.

‡ Not significantly different from the control

Effects of amantadine and chloroquine on lysosomal phospholipases. The soluble lysosomal phospholipase preparation from MDCK cells was incubated at pH 4.0 with increasing concentrations of amantadine and chloroquine. As shown in Table 2, both of these agents inhibited phospholipase A activity. At 25 mM, both agents abolished the activity of lysosomal phospholipase A. Similar results were obtained with phospholipase C (data not shown). Chloroquine was more effective than amantadine in inhibiting MDCK lysosomal phospholipases; 1–2 mM chloroquine or 4–7 mM amantadine reduced enzyme activity by 50% (Table 3). Because chloroquine and amantadine are cations at pH 4.0, the effects of two monovalent cations on the activity of these phospholipases were examined. The monovalent cations Na^+ and NH_4^+ also inhibited MDCK cell lysosomal phospholipase A but much higher concentrations (43–47 mM) were required. This effect was less pronounced with lysosomal phospholipase C.

Effects of amantadine, chloroquine and ammonium chloride on the phospholipid content of cultured MDCK cells. MDCK cells were grown for 72 hr in control medium (DMEM5) or in medium containing amantadine, chloroquine or ammonium chloride. As shown in Table 4, each agent increased the phospholipid content of the cells. Chloroquine was the most effective in producing phospholipid storage in MDCK cells; levels of 10 and 31.6 μM caused the phospholipid content to increase from 225 nmoles/mg protein to 290 and 316 nmoles/mg protein respectively. Ten- to thirty-fold more amantadine (316 μM) was required to produce a statistically significant increase in MDCK cell phospholipid content. Ammonium chloride at a concentration of 10 mM also increased the phospholipid content of MDCK cells.

DISCUSSION

The subcellular distribution of acid phospholipases A and C is virtually idential to that of the lysosomal marker enzyme in the cultured MDCK cell as well as in the rat kidney cortex [17]. In rat kidney cortex [17] and in MDCK cells, phospholipase A activity is much greater than that of phospholipase C. Both of the lysosomal phospholipases of the MDCK cell are substantially inhibited by amantadine and chloroquine, especially at concentrations of 10 mM and above.

[^{14}C]Chloroquine is highly localized to the M + L fraction which contains most of the *N*-acetylglucosaminidase, a lysosomal marker enzyme. [^{3}H]Amantadine is also lysosomotropic in MDCK cells [10]. Seventy-six percent of the labeled chloroquine was present in the M + L fractions versus 55% in the case of radioactive amantadine, as shown previously [10]. Only 8% of the [^{14}C]chloroquine was recovered in the soluble fraction from MDCK cells versus 34% with amantadine [10]. Thus, chloroquine is more lysosomotropic than amantadine in MDCK cells.

Although we have shown that chloroquine and amantadine [10] are highly concentrated in MDCK lysosomes, the exact intralysosomal concentration has not been determined. Recently Hollemans *et al.* [20] made estimates of the intralysosomal concentration of chloroquine in cultured human skin fibroblasts. They estimate the intralysosomal concentration of chloroquine to be 114 mM at a medium concentration of 100 μM. Based on our results (Table 2), this level of chloroquine or amantadine, if achieved in the lysosomes of the MDCK cell, would completely block the intralysosomal degradation of phospholipid.

MDCK cells grown in the presence of chloroquine or amantadine had a significantly increased content of phospholipid. This represents the first demonstration of amantadine-induced phospholipid storage. Amantadine is less active than chloroquine in producing phospholipid storage in MDCK cells. This may be due to its lesser degree of lysosomotropism and its slightly lower capacity to inhibit lysosomal phospholipase A (Table 3). The source of the excess phospholipid is not certain but it may arise from intracellular sources [7] and from lipoproteins taken up from the medium.

Ten millimolar ammonium chloride also produced phospholipid storage in MDCK cells. This was probably due to an increase in the intralysosomal pH rather than a direct inhibitory effect of ammonium chloride [21]. As can be seen in Table 3, inhibition of MDCK lysosomal phospholipase A by ammonium ion was not substantial and was comparable to that obtained by the addition of other monovalent cations such as Na^+.

In conclusion, we believe that a three-step mechanism is involved in the production of phospholipidosis by cationic amphiphilic agents. The proposed requirements are: (1) free entry of agent into the cell, (2) concentration of the agent in lysosomes, and (3) inhibition of lysosomal phospholipases. The inhibition may be direct, indirect (through increases in the intralysosomal pH), or both. In support of this hypothesis, the cationic amphiphilic drugs chloroquine and amantadine [10] were shown to enter MDCK cells and concentrate highly in lysosomes. They both inhibit phospholipase A, the principal MDCK lysosomal phospholipase. Finally, MDCK cells grown in the presence of these agents were shown to have an increased phospholipid content. Taken together, these findings strongly support the three-step hypothesis outlined above.

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