Lipolytic activity of ricin from *Ricinus sanguineus* and *Ricinus communis* on neutral lipids

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The present study was carried out with a view of determining ricin lipolytic activity on neutral lipids in emulsion and in a membrane-like model. Using 2,3-dimercapto-1-propanol tributyrat (BAL-TC₄) as substrate, the lipolytic activity of ricin was found to be proportional to ricin and substrate concentrations, with an apparent K_m ($K_{m,app}$) of 2.4 mM, a k_{cat} of 200 min⁻¹ and a specific activity of 1.0 unit/mg of protein. This work was extended to *p*-nitrophenyl (*p*NP) fatty acid esters containing two to twelve carbon atoms. Maximum lipolytic activity was registered on *p*NP decanoate (*p*NPC₁₀), with a $K_{m,app}$ of 3.5 mM, a k_{cat} of 173 min⁻¹ and a specific activity of 3.5 units/mg of protein. Ricin lipolytic activity is pH and galactose dependent, with a maximum at pH 7.0 in the presence of 0.2 M galactose. Using the monolayer technique with dicaprin as substrate, ricin showed a lipolytic activity proportional to the ricin concentration at 20 mN/m, which is dependent on the surface pressure of the lipid monolayer and is detectable up to 30 mN/m, a surface pressure that is of the same order of magnitude as that of natural cell

membranes. The methods based on *p*NPC₁₀ and BAL-TC₄ hydrolysis are simple and reproducible; thus they can be used for routine studies of ricin lipolytic activity. Ricin from *Ricinus communis* and *R. sanguineus* were treated with diethyl *p*-nitrophenylphosphate, an irreversible serine esterase inhibitor, and their lipolytic activities on BAL-TC₄ and *p*NPC₁₀, and cytotoxic activity, were concurrently recorded. A reduction in lipolytic activity was accompanied by a decrease in cytotoxicity on Caco2 cells. These data support the idea that the lipolytic activity associated with ricin is relevant to a lipase whose activity is pH and galactose dependent, sensitive to diethyl *p*-nitrophenylphosphate, and that a lipolytic step may be involved in the process of cell poisoning by ricin. Both colorimetric tests used in this study are sensitive enough to be helpful in the detection of possible lipolytic activities associated with other cytotoxins or lectins.

Key words: cytotoxin, *Euphorbiaceae*, lipase, phospholipase.

INTRODUCTION

Ricin, one of the most thoroughly studied plant toxins, is produced by the beans of *Ricinus communis* [1] and *R. sanguineus* (family *Euphorbiaceae*) [2]. Its high toxicity found an application in the production of immunotoxins that are used in various trials for cancer treatment [3]. It is also a powerful tool for studying intracellular trafficking [4].

Ricin is a heterodimeric glycoprotein consisting of an A-chain (RTA, ricin toxin A-chain) disulphide-linked to a B-chain (RTB, ricin toxin B-chain); see Lord et al. [5] for a review. RTA is an N-glycosidase, responsible for ricin cytotoxicity, that depurinates adenine residue 4324 of the 28 S rRNA, thus inactivating protein synthesis [6]. Since its endocytotic properties are very limited, RTA alone shows a very low level of cytotoxicity [7]. RTB is a galactose-specific lectin containing three galactose-binding sites [8–10]. Mutation or deletion of any one of these sites reduces the ability of ricin to penetrate into the cell [11]. It was found that the efficiency of RTA-immunotoxins was enhanced by (i) the presence of RTB [12], (ii) partially inactivated RTB (having lost its ability to recognize sugar units on cell membranes) [13], and (iii) RTB with blocked sugar-binding sites [14]. These findings raised the question of the presence of another mechanism by which ricin could penetrate the cellular membrane and which was not limited to the lectin activity of RTB. More recent studies also showed that RTB [15] and ricin [16] could induce membrane fusion on liposomes. The ability of ricin and RTB to interact with lipids is

believed to play a role in ricin liberation into the cell [16]. All together, this information is consistent with the participation of a lipid–protein interaction step during the mechanism of cellular membrane penetration by ricin. This lipid–protein interaction step could be mainly due to the presence of a non-lectin activity on RTB. In a previous study, we observed that, *in vitro*, RTB showed a high lipolytic activity on tributyrin (54 units/mg) in the presence of an acetone-soluble extract from *Euphorbia characias* (another plant belonging to the *Euphorbiaceae* family) [17]. Unfortunately, the exact role of this extract in the expression of the lipolytic activity of RTB has not yet been elucidated. Since this lipolytic activity might be involved in the non-lectin enhancement of poisoning by ricin [18] and might contribute to the biological activity of ricin, we aimed at identifying, in the absence of any cofactor, the presence of a lipolytic activity in the native ricin in which the A and B chains were associated by a disulphide bond. The ability of ricin to hydrolyse phospholipids, which are the major components of cellular membranes, was observed *in vitro* [19]. These observations concerning the lipolytic activity of ricin on glycerophospholipids were attributed to a lipolytic activity more relevant to a non-specific lipase rather than that of a classical phospholipase of the A₁/A₂ type. Later, the level of cytotoxicity of ricin isoforms was correlated with their lipolytic activity [2]. Hence, if one can hypothesize that the mechanism of cell intoxication by ricin involves the hydrolysis of some of the phospholipids of the cell membranes, one does not know whether glycerophospholipids are the unique substrate for

Abbreviations used: RSA₆₀, 60 kDa *Ricinus sanguineus* ricin; RCA₆₀, 60 kDa *Ricinus communis* ricin; RTA, ricin toxin A-chain; RTB, ricin toxin B-chain; ATCL6B, acid-treated cross-linked sepharose 6B; *p*NPC₂, *p*-nitrophenyl acetate; *p*NPC₄, *p*NP butyrate; *p*NPC₆, *p*NP hexanoate; *p*NPC₈, *p*NP octanoate; *p*NPC₁₀, *p*NP decanoate; *p*NPC₁₂, *p*NP dodecanoate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); BAL-TC₄, 2,3-dimercapto-1-propanol tributyrat; DC₁₀, dicaprin; E₆₀₀, diethyl *p*-nitrophenylphosphate; τ , lag time.

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Table 1 Presence of the lipase's consensus sequence Gly-Xaa-Ser-Xaa-Gly among lectins

Protein	Source	Consensus Sequence	Reference, Accession number
Ricin (RCA ₆₀)	<i>R. communis</i>	Gly-Tyr-Ser ⁷⁰ -Pro-Gly	[43]
Abrin-a (B chain)	<i>Abrus precatorius</i>	Gly-Cys-Ser ²²³ -Asn-Gly	[34], gi 476564
RIPT	<i>Polygonatum multiflorum</i>	Gly-Arg-Ser ⁴⁶² -Val-Gly	[37], gi 7141131
Petroglauclin 1	<i>Petrocoptis glaucifolia</i>	Gly-Pro-Ser ²⁴ -Pro-Gly	[38], gi 927614
Lectin-like protein	<i>Arabidopsis thaliana</i>	Gly-Ala-Ser ¹¹⁹ -Asp-Gly	gi 9279652
Vegetative lectin 58 kDa	<i>Dolichos biflorus</i>	Gly-Leu-Ser ²¹⁵ -Glu-Gly	gi 115133374
Agglutinin I	Jerusalem artichoke	Gly-Gly-Ser ¹⁴ -Gly-Gly	gi 11358955
Lectin LECCLA4	<i>Clivia minia</i>	Gly-Ile-Ser ⁷³ -His-Gly	gi 2144225
Lectin LECCLA1	<i>Cl. minia</i>	Gly-Val-Ser ⁷² -Ser-Gly	gi 632104
Agglutinin II (LECCLAII)	<i>Cl. alu.</i>	Gly-Ser-Ser ¹⁴¹ -Gly-Gly	gi 4033446
Agglutinin II (LECCLAII)	<i>Cl. alu.</i>	Gly-Arg-Ser ²⁵² -Ala-Gly	gi 4033444
Agglutinin	<i>Urtica dioica</i>	Gly-Val-Ser ³⁴⁶ -Tyr-Gly	gi 7435354
Lectin/endochitinase	<i>Urt. dioica</i>	Gly-Val-Ser ³⁴⁶ -Tyr-Gly	gi 113510
Lectin CLAII	<i>Cladastis lutea</i>	Gly-Ser-Ser ¹⁴¹ -Gly-Gly	gi 2147660
Storage protein/lectin	<i>Cl. lutea</i>	Gly-Arg-Ser ²⁵² -Gly-Gly	gi 2147630
Lectin CLA II	<i>Cl. lutea</i>	Gly-Ser-Ser ¹⁴¹ -Gly-Gly	gi 1141759
Lectin CLA	<i>Cl. lutea</i>	Gly-Arg-Ser ¹⁴¹ -Ala-Gly	gi 1141755
Agglutinin I (RPBA I)	<i>Robinia</i>	Gly-Lys-Ser ⁷⁷ -Leu-Gly	gi 4033447
Lectin UEA Ii	<i>Ulex europaeus</i>	Gly-Phe-Ser ²¹⁵ -Gly-Gly	gi 7245915
Lectin UEA II	<i>Ule. europaeus</i>	Gly-Ser-Ser ¹⁰⁴ -Ala-Gly	gi 11511
Lectin (bark)	<i>A. thaliana</i>	Gly-Arg-Ser ¹⁹⁵ -Tyr-Gly	gi 3894158
Ripening-associated protein	<i>Musa acuminata</i>	Gly-Gly-Ser ⁵⁸ -Gly-Gly	gi 6224973
Concanavalin A	<i>Canavalia brasiliensis</i>	Gly-Ser-Ser ¹⁶⁹ -Val-Gly	gi 2554766
Lectin	—	Gly-Gln-Ser ³⁷ -Leu-Gly	gi 592278
Lectin	<i>Griffonia simplifolia</i>	Gly-Phe-Ser ²¹⁸ -Ala-Gly	gi 494245
Lectin	<i>Sambucus nigra</i>	Gly-Leu-Ser ¹²⁴ -Asn-Gly	gi 2138271
Kinase/receptor	<i>A. thaliana</i>	Gly-Trp-Ser ²⁵⁹ -Phe-Gly	gi 1418331
Lectin	<i>A. thaliana</i>	Gly-Ala-Ser ¹⁵⁰ -Gly-Gly	gi 995619
Lectin	—	Gly-Lys-Ser ⁷⁷ -Leu-Gly	gi 606716
Gélonin	<i>Gelonium multiflorum</i>	Gly-Asn-Ser ⁸⁰ -His-Gly	gi 542012
Cinnamomin	<i>Cinnamomum camphorum</i>	Gly-Tyr-Ser ³⁵⁵ -Ala-Gly	gi 10337767
GAP31	<i>G. multiflorum</i>	Gly-Asn-Ser ⁸⁰ -His-Gly	gi 132593
Ribosome-inactivating protein	<i>S. nigra</i>	Gly-Asp-Ser ³⁷⁴ -Ser-Gly	gi 1929497
Endochitinase gosh I	—	Gly-Val-Ser ³⁰² -Tyr-Gly	gi 3334147
Endochitinase (B chain)	tobacco	Gly-Val-Ser ³⁰² -Pro-Gly	gi 116321
Endochitinase (A chain)	tobacco	Gly-Val-Ser ³⁰⁷ -Pro-Gly	gi 116314
Basic endochitinase 2	<i>Oryza sativa</i>	Gly-Val-Ser ³⁰⁶ -Tyr-Gly	gi 116317
Galectin 16 kDa	Nematode	Gly-Pro-Ser ⁸⁵ -Asn-Gly	[44]
Lectin	<i>Bauhinia purpurea</i>	Gly-Xaa-Ser-Xaa-Gly	[45]
β (1–4)-Endoxylanase	<i>Streptomyces lividans</i>	Gly-Xaa-Ser-Xaa-Gly	[46]

ricin lipolytic activity. The present study was carried out with a view to document the ability of ricin to act as a lipase and to catalyse the hydrolysis of neutral lipids, which were suspected to be better substrates, in emulsion and in a membrane-like model. Furthermore, as ricin is a galactose-specific lectin, we tested its lipolytic activity in the presence of galactose and glucose (control), at various pH values. The organophosphorus compound diethyl *p*-nitrophenylphosphate (E₆₀₀) is a specific, irreversible serine esterase and protease inhibitor very active on lipases but not on most phospholipases A₂, whose active centre is devoid of serine [20]. Since E₆₀₀ has been shown to react with RTB, which bears the amino acid sequence, Gly-Xaa-Ser-Xaa-Gly, characteristic of lipases [17], we treated ricin with E₆₀₀, and tested concurrently its lipolytic activity on neutral lipids and its cytotoxicity on Caco-2 cells, in order to correlate lipase activity and biological function. It is expected that the demonstration of higher levels of lipolytic activity of ricin on neutral lipids and the characterization of more sensitive tests for the detection of this lipolytic activity, through chromogenic substrates, would facilitate further investigations aimed at a better understanding of the mechanism of cell penetration. In addition, since the amino-acid sequence Gly-Xaa-Ser-Xaa-Gly is widespread among cytotoxins and lectins, as shown in Table 1, such tests may help to

reveal some hidden lipolytic activities involved in biological processes.

MATERIALS AND METHODS

Materials

Ricin from *R. communis*, *p*NP acetate (*p*NPC₂), *p*NP butyrate (*p*NPC₄), *p*NP hexanoate (*p*NPC₆), *p*NP octanoate (*p*NPC₈), *p*NP decanoate (*p*NPC₁₀), *p*NP dodecanoate (*p*NPC₁₂), E₆₀₀, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), BSA, D-galactose and D-glucose were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Dicaprin (DC₁₀), mixture of 1,2 and 1,3 isomers, was also purchased from Sigma and repurified by flash chromatography [21] in our laboratory. Lipase from *Rhizopus arrhizus* was from Boehringer (Mannheim, Germany). 2,3-dimercapto-1-propanol tributylate (BAL-TC₄) was purchased from Aldrich (L'Isle d'Abeau Chesnes, France). Acetonitrile was of maximum purity grade and was from SDS (Peypin, France).

Caco 2 cells were a gift from Professor A. Zweibaum (INSERM U178, Villejuif, France). All cell culture reagents and materials were from Life Technologies (Cergy Pontoise, France). Cytotoxicity assays were performed with the Cell Titer 96®

Non-Radioactive Cell Proliferation Assay kit (Promega, Charbonnières, France).

Ricin purification

Ricin beans of the variety *R. sanguineus*, which is similar to *R. communis*, were purchased from a local seed shop (Girerd & fils, Le Thor, France). Ricin was purified in the laboratory as described previously [2], using cross-linked Sepharose 6B (Pharmacia, Saclay, France) that was acid treated (ATCL6B) as described by Ersson et al. [22] and adapted for affinity chromatography with a BioCad sprint HPLC apparatus (Perseptive Biosystem, Cambridge, MA, U.S.A.). The three active isoforms obtained at the end of the purification were pooled and reloaded on to ATCL6B. Their molecular masses were controlled by mass spectrometry [2]. The fraction eluted here was used for all experiments. SDS/PAGE, in reducing and non-reducing conditions, and isoelectric-focusing electrophoresis were performed to check ricin purity (results not shown).

Ricin lipolytic activity on BAL-TC₄

The method used was based on that described by Furukawa et al. [23]. Reactions were made in 1 ml of 100 mM Tris/HCl buffer, pH 8.0, in which 0.1 ml of 20 mM DTNB (in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of 20 mM BAL-TC₄ (in ethanol) were added. Reactions were performed at 37 °C following the addition of various quantities of protein. After 30 min incubation, the reaction was stopped by the addition of 2 ml of acetone, and the absorbance was then measured at 412 nm using a Beckman DU 40 spectrophotometer (Beckman, Gagny, France). A molar absorption coefficient of 14200 M⁻¹·cm⁻¹ was used to quantify the concentration of reaction products released. Variations in the reactional volume were taken into consideration to calculate the lipolytic activity. Lipolytic activities are expressed in units; 1 unit represents 1 μ mol of substrate hydrolysed per min under the experimental conditions.

Ricin lipolytic activity on pNPC₂ and pNPC₄

Reactions were performed at 25 °C in 1 ml of 50 mM phosphate buffer, at pH 7.4, containing 40 μ l of acetonitrile. Immediately before the experiments, pNPC₂ or pNPC₄ (300 mM in acetonitrile) was added to give a final concentration of 30 mM, and the kinetics were started for 10 min following the addition of 10 to 100 μ l of protein. The absorbance, at 400 nm, was recorded every minute. The activity was calculated from the slope of the curve obtained by plotting, after correction for the reaction volumes, the absorbance versus time. A molar absorption coefficient of 18400 M⁻¹·cm⁻¹ was used to quantify the *p*-nitrophenol released.

Ricin lipolytic activity on pNPC₆, pNPC₈, pNPC₁₀ and pNPC₁₂

Immediately before the experiments, pNPC₆, pNPC₈, pNPC₁₀ or pNPC₁₂ (300 mM in acetonitrile) was added to 1 ml of 50 mM phosphate buffer, pH 7.4, containing 100 μ l of 2% (v/v) Triton X-100 in water, to give a final concentration of 30 mM. The reaction was started by the addition of various quantities of ricin. After 10 min incubation at 25 °C, the reaction was stopped by the addition of 2 ml of acetone, briefly centrifuged, and the absorbance was measured at 400 nm. The amount of *p*-nitrophenol released was determined using a molar absorption coefficient of 18400 M⁻¹·cm⁻¹ after correction for the reaction volume.

Dependence of ricin lipolytic activity on pH and galactose

Experiments were done using BAL-TC₄ or pNPC₁₀ as substrates, including either 0.2 M galactose or 0.2 M glucose in the reactional medium, with a fixed quantity of ricin. pH was adjusted to the desired values with concentrated HCl. Variations of *p*-nitrophenol for molecular absorption with pH were taken into account to calculate the concentration of product of hydrolysis. Controls were done in the same conditions of pH, presence or absence of sugar, using either BSA or a lipase from *R. arrhizus*. Results are the means of three measurements and the corresponding standard error. ANOVA correlation test was used to compare statistically the values obtained with and without sugar at each pH value.

Ricin lipolytic activity on DC₁₀ monolayers

The activity of a lipase, on DC₁₀ monolayers, results in the release of products (decanoic acid and monodecanoylglycerol) which are freely soluble in the aqueous phase. The lipolytic activity can be recorded by monitoring either the decrease of the surface pressure while using a constant surface area, or the reduction of the surface occupied by the DC₁₀ monolayer while a constant surface pressure value is kept throughout the experiment by means of a mobile barrier. The experiments presented here were conducted on DC₁₀ monolayers under controlled surface pressure. A KSV minitrough system (KSV, Helsinki, Finland), including a Wilhelmy plate for recording the surface pressure, was utilized with all the recommended precautions [24] to obtain reproducible results. Only one of the two substrate reservoirs of the enzymic trough was used for the kinetic measurement in order to increase the sensitivity. The monolayer experiments were performed at 25 °C using a 10 mM Tris/HCl, pH 8.0, buffer containing 100 mM NaCl, 21 mM CaCl₂ and 1 mM EDTA.

Ricin treatment with E₆₀₀

E₆₀₀ was dissolved in acetonitrile (200 mM) to reduce spontaneous hydrolysis. The 60 kDa *R. sanguineus* ricin (RSA₆₀) and 60 kDa *R. communis* ricin (RCA₆₀) samples (1.5 μ M) were incubated with E₆₀₀ at a final concentration of 10 mM (corresponding to a molar excess of 6600) for 48 h at 4 °C, in the cell culture medium. The various ricin concentrations tested for their cytotoxicity were obtained by dilution of this stock solution, whose lipolytic activity was checked. Controls were done under the same conditions using ricin alone and E₆₀₀ in acetonitrile or acetonitrile alone, to check the cytotoxicity of these reagents.

Cell culture and cell viability assays

Caco₂ cells were grown in Dulbecco's modified Eagle's medium supplemented with 1% (v/v) non-essential amino acid solution (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) and 20% (v/v) filtered and inactivated foetal bovine serum, under 10% CO₂. Cells were trypsinized and seeded at 2 × 10⁴ cells per well in 100 μ l of media in 96-well flat-bottomed plates (Corning-Costar, Cambridge, MA, U.S.A.). The cells were then cultured under the same conditions as for the monolayer preparation for 24–48 h before use.

At the next step, the medium was removed from the wells, 100 μ l of native or E₆₀₀-treated ricin solution (10⁻⁷ M) in the same medium was added and the cells were incubated for 24 h at 37 °C under 10% CO₂. After incubation, ricin was removed and the cells were washed with PBS for 3 min at room temperature (23 °C) before evaluation of the number of living cells with the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation

Assay kit [25]. The attenuation was measured using a multiwell spectrophotometer at 492 nm wavelength (Labsystems, iEMS Reader MF, Helsinki, Finland).

Incubations without ricin or E_{600} were referred to as 100% living cells, and the cell survival was expressed as a percentage. This percentage was calculated as the ratio of attenuation values corresponding to surviving sensitive cells in the assay (E_{600} -treated ricin) to that of non-treated sensitive cells. The attenuation corresponding to surviving sensitive cells in the assay is the difference in the attenuation measured for cells under assay and that measured for cells having been incubated with 10^{-7} M ricin, a dose that was assumed to kill all the sensitive cells. The attenuation corresponding to non-treated sensitive cells is the difference in the attenuation measured for non-treated cells and that measured for cells having been incubated with 10^{-7} M ricin. Each determination was carried out on five samples; results are expressed as the mean of the five measurements and the corresponding standard error.

RESULTS

The lipolytic activity is associated with RSA_{60}

To be sure that the activity recorded was associated with RSA_{60} and not with any other non-specific contaminant, the three RSA_{60} isoforms, once purified, were pooled and reloaded onto an ATCL6B column, washed, and then eluted by a galactose gradient (0–0.2 M) in the same buffer (phosphate buffer 5 mM, pH 7.5, containing 0.2 M NaCl). The enzymic activity was measured before, during, and after the elution of the absorbance peak, on the same volume of aqueous phase, using different techniques. As shown in Figure 1, almost all the lipolytic activity was found to be associated with the RSA_{60} peak. It was observed

Figure 1 Lipolytic measurements of ricin fractions during its elution from an ATCL6B column

Lipolytic activity measurements, on equal volumes (100 μ l) of each fraction obtained from the ATCL6B column, along the elution step with a galactose gradient (0–0.2 M) before (fraction 1), during (fraction 2) and after (fraction 3) the absorbance peak. Lipolytic activity was tested by the monolayer technique and the BAL-TC₄ and pNPC₁₀ assays. The activity is expressed as number of molecules of DC₁₀ hydrolysed/min per cm² of the reaction compartment of the trough at constant surface pressure (20 mN/m).

Figure 2 RSA_{60} lipolytic activity on BAL-TC₄

Relationship between RSA_{60} lipolytic activity (expressed in units) and BAL-TC₄ concentration (expressed in mM) in the presence of 1 mg of RSA_{60} (Inset) and different amounts of RSA_{60} (expressed in mg). Experiments were performed in 1 ml of Tris/HCl buffer (100 mM, pH 8.0) containing 0.1 ml of 20 mM DTNB, when using 20 mM of BAL-TC₄. See the Materials and methods section for details.

that no activity occurred after heat treatment of RSA_{60} (30 min, 100 °C), nor was any activity recorded when the same amount of 120 kDa *R. sanguineus* agglutinin protein was used.

Lipolytic activity of RSA_{60} on BAL-TC₄

Figure 2 shows the lipolytic activity of RSA_{60} on BAL-TC₄ as a function of RSA_{60} quantities. This lipolytic activity is linear up to 30 min (results not shown) and was found to be proportional to substrate concentration up to 3 mM (Figure 2, inset), then plateaued from 30 to 100 mM. Using the method of Hanes [26] we calculated for RSA_{60} an apparent K_m ($K_{m,app}$) of 2.41 mM and a k_{cat} of 200 min⁻¹. Activity is proportional to RSA_{60} concentration and from this slope a specific activity of 1.0 unit/mg of protein was calculated (Figure 2).

Lipolytic activity of RSA_{60} on pNP esters

The activity of RSA_{60} was tested on pNP fatty acid esters of different chain length. RSA_{60} was active in the lipolysis of pNP esters, the activity increasing with the fatty acid's chain length up to ten carbon atoms (Table 2), then decreasing for twelve carbon atoms on pNPC₁₂. RSA_{60} lipolytic activity on pNP esters was found to be proportional to RSA_{60} concentration (Figure 3), as well as substrate concentration (Figure 4). The maximal specific activity was measured on pNPC₁₀, for which a value of 3.5 units/mg of protein was calculated. On this substrate RSA_{60} showed a $K_{m,app}$ of 3.5 mM and a k_{cat} of 173 min⁻¹. Taking into consideration the ratio of the specific activity to the blank value (Table 2), BAL-TC₄ and pNPC₁₀ show the highest signal-to-noise ratios. This makes these substrates good candidates for routine measurement of ricin lipolytic activity.

Dependence of ricin lipolytic activity on pH and galactose

Due to the spontaneous hydrolysis of pNPC₁₀ at high pH and low molar absorption coefficient of p-nitrophenol at low pH, the

Table 2 Specific activity of RSA₆₀ on various substrates in comparison with the signal-to-noise (blank) ratio

Activity was calculated on the basis of the quantities of palmitic acid* or oleic acid† released, and adapted from Helmy et al. [19]. POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; C₁₆, palmitic acid; C_{18:1}, oleic acid; NS, not significant; ND: not detectable. Data are the mean ± S.E.M. for 3 to 12 determinations from 3 to 12 experiments (*n*). ‡ Calculated from the slope of the RSA₆₀ amount (mg) versus activity (units) curve.

Substrate	Specific activity‡ (units/mg of protein)	Blank (units)	Specific activity/blank (mg ⁻¹)
BAL-TC ₄	1.01 ± 0.093 (5)	0.31 ± 0.005 (12)	3.3
<i>p</i> NPC ₂	NS	0.51 ± 0.031 (12)	0
<i>p</i> NPC ₄	1.16 ± 0.191 (5)	0.83 ± 0.015 (12)	1.4
<i>p</i> NPC ₆	0.37 ± 0.039 (3)	1.32 ± 0.045 (12)	0.3
<i>p</i> NPC ₈	2.52 ± 0.445 (5)	0.49 ± 0.015 (12)	5.1
<i>p</i> NPC ₁₀	3.51 ± 0.366 (5)	0.26 ± 0.016 (12)	13.3
<i>p</i> NPC ₁₂	0.98 ± 0.151 (3)	0.25 ± 0.013 (12)	3.9
POPC*(C ₁₆)	1.2 × 10 ⁻³	ND	—
POPC†(C _{18:1})	1.6 × 10 ⁻³	ND	—

Figure 3 RSA₆₀ lipolytic activity using *p*-NP esters

Relationship between RSA₆₀ lipolytic activity (expressed in units, $\mu\text{mol}/\text{min}$) and RSA₆₀ amount (expressed in mg) using *p*-NP esters as substrates. $\triangle$, *p*NPC₆; $\square$, *p*NPC₈; $\blacktriangle$, *p*NPC₁₀; $\blacksquare$, *p*NPC₁₂. Experiments were performed in 1 ml of phosphate buffer (50 mM, pH 7.4) containing 100 μl of 2% Triton X-100 in water and 30 mM substrate (see the Materials and methods section).

lipolytic activity of ricin was solely tested between pH values of 5.5 and 7.5, while BAL-TC₄ allowed the use of a wider pH range between 5 and 8. All assays were performed in the absence and presence of 0.2 M galactose or 0.2 M glucose. As a control, the same assays were run using BSA and a lipase from *R. arrhizus*. No hydrolysis occurred with *p*NPC₁₀ and BAL-TC₄ when BSA was tested. With the *R. arrhizus* lipase, it was observed that on *p*NPC₁₀ hydrolysis no change in activity occurred between pH 5.5 and 7.5. Conversely, on BAL-TC₄ the presence of 0.2 M glucose decreased the lipolytic activity by an average of 55%, as shown in Figure 5(C).

Figure 5(A) shows on a comparative basis the data obtained for ricin with *p*NPC₁₀ as substrate. The activities measured in the presence of galactose, with the exception of that measured at pH 6.5, are significantly higher, over 2-fold, than those in its absence. In the presence of glucose, significantly lower activities were recorded compared with the control. On *p*NPC₁₀ the

Figure 4 RSA₆₀ lipolytic activity using *p*-NP decanoate

Relationship between RSA₆₀ lipolytic activity (expressed in units, $\mu\text{mol}/\text{min}$) and *p*NPC₁₀ concentration (expressed in mM) in the presence of 0.1 mg of RSA₆₀. Experiments were performed in 1 ml of phosphate buffer (50 mM, pH 7.4) containing 100 μl of 2% Triton X-100. *p*NPC₁₀ was added at a final concentration of 30 mM.

lipolytic activity of ricin is maximal between pH 6.5 and 7.0 in absence of galactose, while in its presence the maximum is shifted to pH 7.0.

The use of BAL-TC₄ as substrate allowed the investigation of a larger range of pH values (Figure 5B). On this substrate, in the absence of sugar in the media, the maximum lipolytic activity is measured between pH 6.5 and 7.0, the same observed with *p*NPC₁₀ as substrate. Comparatively, in the presence of 0.2 M galactose, a shift in the optimum pH of activity to 7.0 was observed while the activity was increased by a factor of 4.5.

On the other hand, the presence of 0.2 M glucose was associated with decreased activities compared with that of the control without sugar. Hence, data from Figures 5(A) and 5(B) support the conclusion that galactose stimulates the lipolytic activity of ricin on *p*NPC₁₀ and BAL-TC₄, while glucose shows the opposite effect. Furthermore, in the presence of galactose the dependence of the lipolytic activity associated with ricin on pH is strongly reduced at pH 6.0 and below by a factor of 3.8–13.

Dependence of RSA₆₀ lipolytic activity on the surface pressure of DC₁₀ monolayers

The monolayer technique was used to measure the relationship between the surface pressure of the lipid monolayer and the

Figure 5 Effect of glucose, galactose and pH on RSA₆₀ lipolytic activity

Relative lipolytic activities of ricin on pNPC₁₀ (A) and BAL-TC₄ (B), and *R. arrhizus* lipase (C), in the absence (○) or presence of 0.2 M galactose (●) or 0.2 M glucose (■). 100% activity corresponds to the maximal activity obtained in the presence of 0.2 M galactose for ricin and in the absence of any added sugar for *R. arrhizus* lipase. Values are the mean of three measurements and the corresponding standard error. These values were statistically compared at the same pH, for the presence or absence of sugar; a, significant difference at 99.9%; b, at 99%; c, at 95%.

lipolytic activity of RSA₆₀. DC₁₀ monolayers were spread at 20 mN/m all over the surface of the trough. As shown in Figure 6, when RSA₆₀ is injected (50 µg) into the reaction compartment of the trough (12.5 ml) underneath the monolayer, a transient increase in the surface pressure of approx. 0.1–0.5 mN/m is observed. The increase in surface pressure is attributable to the tensioactivity of RSA₆₀ that adsorbs to and penetrates into the monolayer. This step was followed by a decrease in the surface pressure which is due to the hydrolysis of the DC₁₀ monolayer. As the experiments were designed at a constant surface pressure (20 mN/m) with a regulation set in order to compensate for the decrease in surface pressure, the barrier began to move only when the surface pressure decreased below the pre-set value. The

Figure 6 Screen print-out of a monolayer experiment

The arrow indicates the moment of RSA₆₀ injection (0.1 mg) into the reaction compartment (12.5 ml) under a monolayer of DC₁₀. The experiment was performed at a constant surface pressure of 20 mN/m.

Figure 7 RSA₆₀ lipolytic activity on a DC₁₀ monolayer

Relationship between surface pressure and lipolytic activity (□) and τ (■) after the injection of 0.1 mg of RSA₆₀ into the reaction compartment (12.5 ml).

lipolytic activity of RSA₆₀ (measured by the reduction of the area covered by the barrier, cm²/min, and expressed as the number of molecules hydrolysed per unit of time, calculated from the area occupied by a molecule of DC₁₀ at 20 mN/m, i.e. 55 Å²/molecule) was found to be proportional to the RSA₆₀ concentration. The time elapsed between the injection of the protein and the barrier movement (Figure 6) is the lag time (τ) and is the sum of two types of equilibrium: one corresponding to the equilibrium of RSA₆₀ penetration into the monolayer and the other corresponding to the equilibrium of DC₁₀ monolayer hydrolysis by RSA₆₀ [27]. The rate of hydrolysis and τ are both known to be dependent on the surface pressure, as shown in Figure 7.

Association of the lipolytic and cytotoxic activities of ricin

When ricins from *R. communis* and *R. sanguineus* were treated with E₆₀₀, an irreversible serine hydrolase inhibitor, a decrease in the lipolytic and cytotoxic activities was observed (Table 3). Treatment was made with 10 mM E₆₀₀ to ensure that, when diluted in cell culture medium together with ricin, no toxic effect of this product could be seen on cells (results not shown). When incubated with 10⁻⁷ M ricin, all the sensitive cells are killed in the assay. When 10⁻⁷ M E₆₀₀-treated ricin was tested, 70 ± 2.4 % of the sensitive cells remained alive, using commercial ricin from *R. communis*, and 60 ± 4.1 % in the case of home-purified ricin from *R. sanguineus*. These values correspond to a reduction of over 2 to 3 log units of ricin cytotoxicity. The same preparations of

Table 3 Effect of E_{600} on cytotoxic and lipolytic activities of ricin from *R. communis* and *R. sanguineus* seeds

Caco 2 cells received 10^{-7} M control or E_{600} -treated ricin solution diluted in culture medium for 24 h. Incubations without ricin or E_{600} were referred to as 100% living cells, and the cytotoxicity was expressed as the percentage of sensitive cell death; the results are expressed as means $\pm$ S.E.M. for five samples. Lipolytic activity was measured on BAL-TC₄ and *p*NPC₁₀, as described in the Materials and methods section, using 50 μ g of control or E_{600} -treated ricin; the results are means $\pm$ S.E.M. for three determinations.

	Remaining activity (% of control)		
	Cytotoxic	Lipolytic	
		BAL-TC ₄	<i>p</i> NPC ₁₀
RCA ₆₀	30 $\pm$ 2.4	11 $\pm$ 5.0	2 $\pm$ 2.1
RSA ₆₀	40 $\pm$ 4.1	52 $\pm$ 2.4	32 $\pm$ 8.4

non-treated and E_{600} -treated ricin were tested for their lipolytic activity. The latter decreased by $89 \pm 5\%$ (*R. communis*) and $48 \pm 2.4\%$ (*R. sanguineus*) on BAL-TC₄, and by $98 \pm 2.1\%$ (*R. communis*) and $68 \pm 8.4\%$ (*R. sanguineus*) on *p*NPC₁₀, compared with control protein.

DISCUSSION

When using the pH-stat technique (titration carried out at constant pH) and tributyrin, triolein or castor oil as substrates, we were unable to measure any lipolytic activity associated with ricin as we did before for RTB. This is likely to be due to the lack of sensitivity of this assay. The measure of the lipolytic activity of ricin was extended to various substrates: (i) one analogue of triacylglycerol, BAL-TC₄; (ii) various chromogenic substrates such as *p*-NP esters of aliphatic short to medium chain acids, and (iii) monomolecular films of a pure natural diacylglycerol, DC₁₀.

Data from Figure 2 show that RSA₆₀ is active in hydrolysing BAL-TC₄ and that the rate of hydrolysis is proportional to the concentration of the protein. A $K_{m,app}$ of 2.41 mM and a k_{cat} of 200 min⁻¹ were calculated. A specific activity of 1.0 unit/mg of protein was calculated for RSA₆₀. The use of *p*-NP esters allowed the evaluation of the dependence of this lipolytic activity on fatty acid chain length, as shown in Figure 3. The decanoate appeared to be the best substrate, and data derived from Figure 4 allowed us to calculate a $K_{m,app}$ of 3.5 mM and a k_{cat} of 173 min⁻¹. A specific activity of 3.5 units/mg of protein was calculated for RSA₆₀. Data in Table 2 allowed a comparison of the various specific activities calculated for all these substrates and the ratio of the specific activity measured on each substrate to the corresponding blank value. *p*NPC₁₀ showed the highest signal-to-noise ratio. We made use of this apparent specificity for decanoic acid in a subsequent assay of this lipolytic activity in a monolayer system using DC₁₀ monomolecular films spread at the air–water interface. It was observed that RSA₆₀ hydrolysed the DC₁₀ monolayer at a rate proportional to its concentration (results not shown). As shown in Figure 7, at 10 mN/m and like any other protein, RSA₆₀ penetrates into the monolayer, and due to its relatively high concentration the increase in the surface pressure overwhelms the hydrolysis for a long period of time, resulting in a very long lag period (τ values of more than 180 min). At higher surface pressures, the τ values measured were much lower, but they were still relatively high in comparison with those usually recorded with, for example, pancreatic lipases or phospholipases A₂ [28]. This difference was attributed to the

high concentration of RSA₆₀ used, 100 nM, compared with the range of concentrations, 0.1 nM to a few nM, generally used in the case of pancreatic lipases or phospholipases A₂. As the lipolytic activities recorded were in the same range as those of the pancreatic enzymes, this means that the specific lipolytic activity of RSA₆₀ is probably 100–1000-fold lower than that of the pancreatic enzymes [29]. This finding is not surprising, considering the fact that with RSA₆₀ we are dealing with an activity whose biological role may serve simply to destabilize the membrane lipids by hydrolysing just a few molecules, contrary to what occurs with pancreatic lipase and phospholipase A₂, whose role is to provide the organism with a mass of lipolytic products which can be absorbed by the intestine. It is worth noting that the lipolytic activity of RSA₆₀ was observed up to 30 mN/m, a surface pressure that is equivalent to that of natural cell membranes [30].

Demonstration of ricin lipolytic activity implied that it was essential to be sure that the activity recorded was associated with the RSA₆₀ and not with any other non-specific contaminant. Data from Figure 1 show that all the lipolytic activity was associated with the absorbance peak corresponding to the RSA₆₀ pool of three pure isoforms, previously separated and characterized by mass spectrometry as described in [2]. The levels of activity measured before and after this peak range within the reproducibility of the blank values, if one refers to the data from Table 2, and hence can be considered as nil. Another difficulty lay in the fact that the weak levels of activity we measured declined with time. This study was conducted on various samples of ricin, some of which were commercial (Sigma), some kindly provided by Professor F. Stirpe (University of Bologna, Bologna, Italy) and some purified in our laboratory. From our experiments, we noted that the level of activity measured with the various samples was not completely reproducible except for our fresh home-purified ricin. This may be partly attributable to the weak lipolytic activity of ricin, which even under the most suitable conditions is detectable under our conditions for only a few weeks. This is likely to be due to the already observed self-aggregation of ricin [7] and also probably to the way in which the samples have been processed and preserved (freeze-drying, presence of reducing agent, sodium azide, glycerol, etc.).

Data from Figures 5(A) and 5(B) illustrate the effect of galactose and pH on the lipolytic activity of ricin measured on two different substrates, *p*NPC₁₀ (Figure 5A) and BAL-TC₄ (Figure 5B). As a constant feature the presence of 0.2 M glucose induced a decrease in activity, while that of galactose increased the lipolytic activity by a factor of 2.5 on *p*NPC₁₀ and of 4.5 on BAL-TC₄ at pH 7.0. Such effects are not observed with the lipase of *R. arrhizus* (Figure 5C). It is tempting to correlate the effect of galactose on lipolytic activity with the well-known specificity of the B-chain for this sugar [9] and hence to associate lipolytic activity and B-chain, since RTB shows a strong sequence similarity (43.5%) in its first 30 N-terminal amino acids to a lipase purified from *Euphorbia characias* latex. Furthermore, its protein backbone that cross-reacts with the lipase's antibodies, bears the consensus sequence for a lipase, Gly-Xaa-Ser-Xaa-Gly [31–33], and hydrolyses tributyrin with an activity of 54 units/mg of protein in the presence of an acetone extract of *E. characias* latex [17]. Hence, the lipolytic activity we measured for ricin is attributable to the ricin molecule and not to an impurity, and furthermore RTB is very likely to be responsible for this lipolytic activity. It is worth noting that the pentapeptide Gly-Xaa-Ser-Xaa-Gly is present in the primary sequence of many other ribosome-inactivating proteins like Abrin-a [34–36], *P. multiflorum* ribosome-inactivating protein [37], petroglaucin [38] and many other lectins as shown in Table 1. Hence the primary

structure of ricin and other cytotoxins is consistent with the possible presence of a lipase catalytic centre.

In order to address the question of whether this lipolytic activity was related to ricin cytotoxicity, ricin from *R. communis* and *R. sanguineus* were treated with E_{600} , an organophosphorus compound which reacts with the activated serines of the catalytic centre of esterases and lipases [20], and at the same time the lipolytic and cytotoxic activities were monitored. The results of these experiments are presented in Table 3. One can see that for both species of ricin a parallel exists between the loss of lipolytic activity and the loss of cytotoxic activity. One can note here that the decreases observed in lipolytic and cytotoxic activities with E_{600} -treated ricin are in a different range, but this reflects two distinct biological roles for the toxin, and supports the idea that a small fraction of the initial lipolytic activity may allow the full cytotoxic activity to be expressed. Furthermore, one must keep in mind that the cytotoxic effect is measured here through an indirect assay based on the oxido-reduction status in the cell, rather than on the direct assessment of the protein synthesis capacity of the cells. E_{600} strongly inhibits the lipolytic activity, supporting the conclusion that an activated serine residue is critical for this enzymic activity.

The decrease in cytotoxicity leads to the conclusion that a correlation exists between lipolytic activity and the cell poisoning mechanism. Such a conclusion is supported by the pH dependence of the lipolytic activity which, in presence of galactose, is reduced at pH 6.0 and below by a factor of 3.8–13 as shown in Figures 5(A) and 5(B). This decrease in activity correlates with the decrease in ricin toxicity observed on Vero cells at pH 6.0 and below, although the binding of ricin to cells was unaffected by pH variations [39]. These results are to be considered in the view of the co-existence of different mechanisms for ricin entry into the cell. The toxin enters by either clathrin-dependent or -independent endocytosis, reaches the cytosol via retrograde transport through the Golgi apparatus, reaches the endoplasmic reticulum via the endoplasmic-reticulum-associated protein pathway ('ERAD'), where translocation occurs via the Sec61 translocon [40]. The lipolytic activity of a lipase-type documented here, and observed on different lipidic substrates, could permit the destabilization of some membrane phospholipids, revealing another pathway by which the toxin enters the cell and reaches the cytosol.

In conclusion, we can say that ricin from *R. sanguineus* or *R. communis* is capable of hydrolysing different lipid classes (triacylglycerols, partial acylglycerols and esters) with activities comparable with that documented for other plant lipases [41]. Ricin equally hydrolyses phospholipids that are the major components of cellular membranes [19]. The activities of RSA₆₀ on BAL-TC₄ and pNPC₁₀ were found to be approx. 600 and 3000 times higher respectively than that measured on phospholipids (Table 2), and are maximal at pH 7.0 in the presence of 0.2 M galactose, corresponding to specific activities of 4.5–16 units/mg of protein. Furthermore, the data from Table 2 show that these two assays are reproducible and sensitive enough to allow an easy characterization and quantification of the lipolytic activity of ricin. These assays may be useful to document possible lipolytic activities associated with other cytotoxins, in order to gain a better understanding of the various processes of toxin entry into cells. In addition, these findings, added to the action of E_{600} on ricin lipolytic activity, support the idea that the lipolytic activity of ricin is more relevant to that of a lipase than that of a phospholipase. Hence the action of ricin on membrane phospholipids could occur through a phospholipase A₁ activity which is very often a minor activity of lipases [42]. The observation according to which different isoforms of ricin show a lipolytic

activity that correlates with their cytotoxicity [2], added to the observation that E_{600} switches off both lipolytic and cytotoxic activities at the same time, supports equally the involvement of a lipolytic step, of a lipase type, in the mechanism of cell intoxication. The response of the lipolytic activity to pH and galactose bring additional evidence for this conclusion.

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