Enhancement of phospholipase activity during poliovirus infection

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Infection of human cells with poliovirus leads to modification of phospholipase activity. Phospholipase C, which generates inositol triphosphate, is stimulated, whereas the activation of phospholipase A2 by the calcium ionophore A23187 is inhibited. Analysis of phospholipid moieties in media of HeLa cells infected with poliovirus indicates that the release of fatty acids is not enhanced during infection, suggesting that phospholipase A1 and A2 activities are not stimulated. The release of choline into the medium is significantly higher 3 h after infection, indicating that a phospholipase that has phosphatidylcholine as its substrate becomes activated. This activation requires viral gene expression because inhibitors of poliovirus gene expression added at the beginning of infection block choline release, but continuous viral protein synthesis is not required.

Choline and phosphorylcholine are released into the medium, but the pools of both are gradually depleted in poliovirus-infected cells, perhaps as a consequence of their release into the medium and the increased synthesis of phospholipids that takes place in poliovirus-infected cells. Inhibitors of phospholipase activity such as mepacrine, zinc or cadmium ions significantly reduce this increased release of choline from poliovirus-infected cells. Labelling of cells with \([\text{H}]\)phosphatidylcholine suggests that the choline released from infected cells comes, at least in part, from the hydrolysis of this compound. These results indicate that, in addition to the activation of the phospholipase C which hydrolyses phosphatidylinositol in poliovirus-infected cells, a phospholipase C that acts on a phosphatidylcholine is also activated.

Introduction

Animal viruses profoundly modify the permeability of cellular membranes after infection of the host (Carrasco et al., 1989). These modifications in membrane permeability lead to an imbalance of cell membrane-maintained ionic gradients (Carrasco & Smith, 1976; Egberts et al., 1977; Lacal & Carrasco, 1982; López-Rivas et al., 1987; Nair et al., 1979). In addition, molecules that are usually excluded by cells readily pass into the cell at the time when maximal synthesis of viral proteins is taking place (Carrasco, 1987; Contreras & Carrasco, 1979). This phenomenon has been analysed using hydrophilic translation inhibitors that selectively enter animal virus-infected cells (Carrasco & Vázquez, 1983), and although the changes in membrane permeability have been studied in detail (Carrasco et al., 1989) there are still two fundamental aspects of virus-induced membrane leakiness that remain unexplained. One is the viral component responsible for modifying the cellular membranes and the other is the molecular mechanism underlying the virus-induced membrane leakiness.

Steps towards the elucidation of the mechanism responsible for the increased membrane permeability led us to investigate the activity of cellular lipases during poliovirus infection (Guinea et al., 1989). A striking increase in phospholipase C activity was observed, as measured by the level of inositol triphosphate (IP3) present in infected cells (Guinea et al., 1989). At 3 h post-infection (p.i.) a sustained augmentation of IP3 took place and reached a five-fold increase by 5 h p.i. Phospholipase A2, however, was not enhanced by poliovirus. In fact, the stimulation of this enzyme by the ionophore A23187 was hampered soon after infection with poliovirus, suggesting that not all phospholipase activities were stimulated even at late stages of infection (Guinea et al., 1989).

Potent cellular mediators are generated by the action of both phospholipase C and phospholipase A2 (Bailey, 1985; Berridge, 1987; Hokin, 1985; Needleman et al., 1986; Waite, 1987). Thus, the activity of phospholipase C generates diacylglycerol and IP3, whereas phospholipase A2 leads to the formation of molecules with potent biological activities, such as prostaglandins and prostacyclins (Bailey, 1985; Berridge, 1987; Hokin, 1985; Needleman et al., 1986; Waite, 1987). The activation of phospholipase C is involved in signal transduction of the cellular responses induced by a number of agonists, such as peptide hormones and other biological activators.
Apart from the phospholipase C that hydrolyses phosphatidylinositol, evidence increasingly suggests that hydrolysis of phosphatidylcholine is also enhanced by agonists (Billah & Anthers, 1990; Pelech & Vance, 1989). Therefore, it was of interest to measure this phospholipase C activity during poliovirus infection of human cells in order to correlate these modifications with the concurrent increased membrane permeability.

**Methods**

**Cell cultures and viruses.** HeLa cells were grown in Petri dish (Nunc) culture with 10 ml DMEM (E4D) supplemented with 10% calf serum (Gibco) and were incubated in a 5% CO₂ atmosphere at 37 °C in a Forma incubator.

Poliovirus type 1 (Mahoney strain) was grown on HeLa cells at an m.o.i. of 0.1 p.f.u./cell in DMEM supplemented with 2% calf serum (E4D2). One day later intracellular and extracellular virus was harvested and titrated in HeLa cell monolayers by plaque assay.

**Release of radioactive precursors.** HeLa cells were prelabelled in E4D2 medium for 18 h before infection with the following radioactive precursors: [5,6,8,9,11,12,14,15-αH]arachidonic acid (150 to 230 Ci/mmol, Amersham), [2-αH(N)]myo-inositol (12.8 Ci/mmol, Du Pont), [1(3)-αH]glycerol (1 to 3 Ci/mmol, Amersham), [methyl-3H]choline chloride (75 to 85 Ci/mmol, Amersham), [9,10(n)-αH]palmitic acid (40 to 60 Ci/mmol, Amersham), [9,10(n)-αH]oleic acid (2 to 10 Ci/mmol), t-3-phosphatidyl[N-methyl-3H]choline-1,2-dipalmitoyl (40 Ci/mmol, Amersham). Labelled medium was removed and cells were washed three times with E4D prior to infection at an m.o.i. of 50 in E4D2. After 1 h of viral adsorption at 37 °C, the medium was removed and replaced by a fresh medium. This time was taken to be 0 h.p.i. In each experiment the medium was collected and centrifuged to pellet detached cells at set time intervals p.i. Three-quarters of the total volume was added to 2 ml of Bray’s solution in order to determine the radioactivity released. In order to discover how much [αH]choline was released within each hour of infection, medium was replaced at the appropriate time by an unlabelled medium so that radioactivity released during set time intervals could be measured. In some experiments zinc chloride (Merck), cadmium chloride (Fluka), mepacrine dihydrochloride (Sigma), neomycin sulphate (Sigma), dibucaine hydrochloride (Sigma), diltiazem hydrochloride (Sigma) and xanthate D-609 (kindly provided by G. Sauer, German Center for Cancer Research, Heidelberg, Germany) were added to the monolayers in E4D2 and assayed for 10 min. Afterwards media were treated as described above.

**Estimation of [αH]phosphatidylcholine incorporation into cells.** Monolayers of HeLa cells were labelled with t-3-phosphatidyl[N-methyl-3H]choline-1,2-dipalmitoyl. At appropriate times p.i., labelled medium was discarded and the cell monolayers were scraped from dishes into distilled water and extracted with methanol–chloroform (2:1, v/v), 2.4 M HCl and chloroform. After centrifugation the apolar phase was washed with methanol–1 M HCl (1:1, v/v). The polar fractions were pooled and lyophilized to be counted with 2 ml of Bray’s solution. Apolar phases were desiccated under vacuum and were also counted.

**Analysis of proteins by polyacrylamide gel electrophoresis.** HeLa cells infected with poliovirus at an m.o.i. of 50 were washed twice with methionine-free medium and incubated for 1 h with this medium.
supplemented with 20 μCi/ml [3H]methionine (1-45 Ci/mmol, Amer-
sham) at the times indicated. The radiolabelled cell monolayers were
dissolved in sample buffer (62.5 mM-Tris-HCl pH 6.8, 2% SDS, 0.1 M-
DTT and 17% glycerol, with 0.024% bromophenol blue as indicator).
Samples were sonicated at 12 Hz, heated to 100 °C for 5 min, applied to
a 15% polyacrylamide gel and electrophoresed overnight at 80 V.
Fluorography was carried out in 20% (w/v) 2,5-diphenyloxazole in
dimethylsulfoxide. Finally, the gels were dried and exposed to Kodak
X-ray films.

**Results**

**[3H]Choline release during poliovirus infection**

HeLa cells infected with poliovirus undergo modifica-
tions in membrane permeability during infection (Car-
rasco et al., 1989). These changes correlate in time with
activation of the phospholipase C that hydrolyses
phosphatidylinositol (Guinea et al., 1989). Thus, a
significant and sustained increase in IP3 occurs in HeLa
cells infected with poliovirus from 3 h.p.i. (Guinea et al.,
1989). To determine whether poliovirus also triggered
the activation of other phospholipases, the release into
the medium of several phospholipid moieties was measured
at different times after infection. Fig. 1(a) shows that fatty acids are not released into the culture
medium, suggesting that neither phospholipase A1 nor
phospholipase A2 are activated by poliovirus. This result
agrees with our previous findings that the activation of
phospholipase A2 by the calcium ionophore A23187 is
blocked from early in the poliovirus life cycle (Guinea et al.,
1989). A significant release of choline occurs after
3 h.p.i., suggesting that the phospholipase C that hydrolyses phosphatidylcholine is activated (Irving & Exton, 1987; Muir & Murray, 1987). The labelled choline that appears in the culture medium may also come from the intracellular choline pool. A parallel experiment (Fig. 1b) indicated that the synthesis of poliovirus proteins preceded the release of choline and this latter phenomenon was subsequent to the inhibition of host protein synthesis. A more detailed study of the kinetics of choline release to the medium is shown in Fig. 1(c). No differences were observed between mock-infected HeLa cells and poliovirus-infected cells during the first 3 h.p.i. At the times when choline appeared in the medium, however, (Fig. 1a) the rate of choline liberation from cells was significantly enhanced in poliovirus-infected cells.

To test whether viral gene expression was required for the increased choline release observed during late stages of poliovirus infection, the experiment shown in Fig. 2 was performed. Two inhibitors of poliovirus genome replication, guanidine and Ro 09-0179 (Castrillo & Carrasco, 1987; González et al., 1990) or the translation inhibitor cycloheximide, were added at the beginning or later stages of poliovirus infection. In all cases, the choline liberated in the medium was assayed at 5 to 6 h.p.i. Addition of any of the above mentioned inhibitors at the beginning of infection, but not later, blocked subsequent enhancement of choline levels, suggesting that viral gene expression is required for this phenomenon to take place. Once the viral genomes have been replicated and some poliovirus proteins have been made, however, addition of these inhibitors had no effect on choline release. Continuous poliovirus gene expression is therefore not necessary for the augmented release of choline. The synthesis of proteins was monitored in parallel and the labelled proteins were analysed by SDS-PAGE (results not shown) to ensure that the inhibitors were effective.
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Analysis of products released into the medium

After uptake, choline is phosphorylated before conversion to cytidine diphosphate choline and incorporation into phospholipids as phosphatidylcholine (Pelech & Vance, 1989; Sleight & Kent, 1983). The choline moiety present in phosphatidylcholine can be released as such by the action of phospholipase D (Kiss et al., 1991; Thompson et al., 1991), or as phosphorylcholine by phospholipase C (Waite, 1987). Activation of phospholipase C by agonist action also leads to an increase in both choline and phosphorylcholine, suggesting that both compounds are interconverted (Irving & Exton, 1987; Pelech & Vance, 1989). We therefore analysed the levels of choline and phosphorylcholine present in intracellular pools and in the culture medium of both control and poliovirus-infected cells at different times p.i.

HeLa cells were prelabelled with choline overnight and the label was removed upon infection with poliovirus. At different times p.i. the choline and phosphorylcholine released to the medium and present in the cellular pool were analysed. Fig. 3(a) shows that the labelled products released both in mock- and in poliovirus-infected cells are a mixture of choline and phosphorylcholine, in an approximate ratio of 3:1. In contrast, the amounts of both compounds liberated from infected HeLa cells are significantly higher than from mock-infected cells. Even at 3 h p.i. there is about 300 to 400 % more phosphorylcholine in the medium than in mock-infected cells cultures. At later stages levels are even higher (1200 % of control at 5 h p.i.) (Fig. 3a). Analysis of these two metabolites in the pool of mock-infected cells indicates that choline is gradually phosphorylated (Fig. 3b). In addition, both metabolites clearly disappear from the

Fig. 4. Incorporation of $[^{3}H]$choline in mock-infected and poliovirus-infected cells. (a) Sixty mm Petri dishes of HeLa cells were labelled with $[^{3}H]$choline (5 μCi/ml) from 0 h p.i. At different times, monolayers were harvested and phospholipids were extracted and applied to TLC plates. PC, Phosphatidylcholine; S, sphingomyelin; LPC, lysophosphatidylcholine; OR, origin. (b) Densitometry scan of phosphatidylcholine from TLC plate. (c) As (a), but polar $[^{3}H]$choline metabolites were extracted and applied to TLC plates. C, choline; P, phosphorylcholine. (d, e) Densitometry scans of choline (d) and phosphorylcholine (e) from (c). U or ■, uninfected cells; I or □, infected cells. S.E.M. is given.
present in the pool and phospholipids synthesized. In agreement with phosphorylcholine. These results suggest that the decrease of choline and phosphorylcholine in the infected cells, or that the phosphorylcholine synthesized is used more efficiently.

In order to discover whether the increased release of choline also takes place in cell-free systems, a membrane fraction was prepared (Takegami et al., 1983) from mock-infected or poliovirus-infected cells at 5 h p.i. The release of choline to the soluble fraction was analysed (Fig. 5a). Increased release of label was observed from membranes obtained from the infected cells compared with the membranes from control cells.

Since the activation of phospholipase C or D leads to choline release (Thompson et al., 1991; Waite, 1987) we measured the activity of phospholipase D by assaying the formation of phosphatidylethanol after incubation of the cells with ethanol (Kiss et al., 1991). No evidence of increased activity of phospholipase D in poliovirus-infected cells was observed (results not shown). Finally, the amount of diacylglycerol (DAG) present in poliovirus-infected cells was quantified. Fig. 5 shows that the radioactive DAG was the same in control and poliovirus-infected cells. The quantity of DAG at a given time depends on the balance between its generation and its uptake for synthesis of new phospholipids. Since it is known that phospholipid biosynthesis is increased after poliovirus infection we used the inhibitor of DAG phosphorylation, R 59022 (De Chaffoy et al., 1989; Gómez-Cambronero et al., 1987; Musch & Goldstein, 1990). Fig. 5 demonstrates that more DAG is made in poliovirus-infected cells in the presence of this inhibitor, suggesting that the phospholipase C activity is increased.

**Effect of phospholipase inhibitors on choline release**

To determine whether the choline released comes from hydrolysis of phosphatidylcholine, or from the cytoplasmic pool of choline and phosphorylcholine, the release of choline was measured in the presence of a number of previously described phospholipase inhibitors, such as zinc, cadmium ions (Wolf & Gross, 1985), mepacrine (Grunicke, 1985), neomycin (Langeland, 1986) and D-609 (Müller-Decker, 1989). The effect of all these agents was assayed at 5 h p.i. (Fig. 6a) and 6 h p.i. (Fig. 6b). Fig. 6 shows that these agents have almost no effect on choline release in control cells. All of them except D-609, however, powerfully blocked the increase of choline present in the medium of poliovirus-infected cells, suggesting that the inhibition of phospholipase activity leads to a drop in the amount of choline liberated into the medium of poliovirus-infected cells.

To further corroborate the conclusion that the choline released to the medium comes at least in part from hydrolysis of phosphatidylcholine upon phospholipase activation, cells were prelabelled with [3H]choline metabolites in a cell-free system and 1,2-diacylglycerol generation in poliovirus-infected cells. (a) Membranes from mock-infected or poliovirus-infected HeLa cells were isolated at 5 h p.i. (Takegami et al., 1983). The assay of phospholipase C activity was carried out as previously described (Soo Nam et al., 1990) using [3H]phosphatidylcholine labelled membranes as substrate. The final volumes were 400 µl, incubation was for 45 min and the reaction was stopped with cold methanol. Polar choline metabolites were extracted as indicated in Methods, lyophilized and the radioactivity was estimated. (b) HeLa cells were prelabelled with 4 µCi/ml [3H]arachidonic acid for 18 h. Then the cells were mock-infected or infected with poliovirus at an m.o.i. of 50. At 6 h p.i., 10 µM R 59022 or 442 (control) were added to cells. After 1 h, monolayers were harvested, extracted and neutral lipids separated by TLC. The diacylglycerol spot was densitometrically quantified. ■ Mock-infected cells or membranes; □ cells or membranes infected with poliovirus. S.E.M. is given.

Fig. 5. Release of [3H]choline metabolites in a cell-free system and 1,2-diacylglycerol generation in poliovirus-infected cells. (a) Membranes from mock-infected or poliovirus-infected HeLa cells were isolated at 5 h p.i. (Takegami et al., 1983). The assay of phospholipase C activity was carried out as previously described (Soo Nam et al., 1990) using [3H]phosphatidylcholine labelled membranes as substrate. The final volumes were 400 µl, incubation was for 45 min and the reaction was stopped with cold methanol. Polar choline metabolites were extracted as indicated in Methods, lyophilized and the radioactivity was estimated. (b) HeLa cells were prelabelled with 4 µCi/ml [3H]arachidonic acid for 18 h. Then the cells were mock-infected or infected with poliovirus at an m.o.i. of 50. At 6 h p.i., 10 µM R 59022 or E4D2 (control) were added to cells. After 1 h, monolayers were harvested, extracted and neutral lipids separated by TLC. The diacylglycerol spot was densitometrically quantified. ■ Mock-infected cells or membranes; □ cells or membranes infected with poliovirus. S.E.M. is given.

cytoplasm of poliovirus-infected cells as infection proceeds (Fig. 3b). These results suggest that the choline and phosphorylcholine present in the pool of poliovirus-infected cells are released to the medium because of membrane leakiness and, in part, can be more efficiently incorporated into phosphatidylcholine by the more active synthesis of phospholipids in poliovirus-infected cells (Carrasco et al., 1989; Guinea & Carrasco, 1990).

To measure the extent to which the synthesis of phosphatidylcholine was contributing to the decrease of choline and phosphorylcholine the experiment shown in Fig. 4 was performed. Cells were labelled with choline from the beginning of infection until the cells were processed in order to measure the amounts of choline present in the pool and phospholipids synthesized. In agreement with previous findings, there was more synthesis of phospholipids in poliovirus-infected cells (Carrasco et al., 1989; Guinea & Carrasco, 1990), particularly at late stages of infection, even though at those times the transport of choline clearly drops in the infected cells. Choline levels decrease in infected cells compared with mock-infected cells, however, but this decrease is much lower than the differences observed with phosphorylcholine. These results suggest that the release of choline and phosphorylcholine to the medium is a result of the hydrolysis of phosphatidylcholine and not only release from the cellular pool because of increased membrane permeability. It is also possible that
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Fig. 6. [3H]Choline release in the presence of phospholipase inhibitors. HeLa cells were prelabelled with [3H]choline as described in Methods. After 1 h of poliovirus adsorption, fresh E4D2 was added to both control and infected cells. Cells were incubated at 5 h p.i. (a) or 6 h p.i. (b) during 10 min with ■, new E4D2 medium, or media containing: □, 0.4 mM-ZnCl₂; □, 0.4 mM-CdCl₂; □, 0.1 mM-mepacrine; □, 1 mM-neomycin; □, 10 μg/ml xanthate D-609. After that time media were collected and radioactivity was counted as described in Methods. S.E.M. is given.

Fig. 7. Uptake of [3H]phosphatidylcholine and release into the medium during poliovirus infection. (a) Two μCi/ml of [3H]phosphatidylcholine was added to HeLa cells growing in E4D2. At intervals preinfection the medium was removed and the cells were washed to eliminate extracellular radioactivity and extracted as described. Polar and apolar fractions containing soluble [3H]choline metabolites from cellular pool (○) and cellular phosphatidylcholine (△), respectively, were counted in a liquid scintillation spectrometer. (b) Release of [3H]choline metabolites from (■), uninfected cells or (●) poliovirus-infected HeLa cells prelabelled with 2 μCi/ml of [3H]phosphatidylcholine.

Phosphatidylcholine (Fig. 7a) and the choline liberated was quantified late during infection (Fig. 7b). The data shown in Fig. 7(a) indicate that after overnight labelling with [3H]phosphatidylcholine most of the label is incorporated into phospholipids and only about 10% of the label is already in the pool in the form of choline and phosphorylcholine.

Poliovirus infection induces the appearance of labelled...
choline in the medium, mainly from phosphatidylcholine. In this instance, it seems clear that the choline present in the medium of infected cells must come from hydrolysis of phosphatidylcholine. Moreover, phospholipase inhibitors and compounds such as dibucaine and diltiazem (Hostetler, 1985; Soo Nam et al., 1990; Waite, 1987; Waite & Sisson, 1972) significantly block this hydrolysis and the subsequent increase in levels of choline in the medium of poliovirus-infected cells (Fig. 8).

**Discussion**

Although the poliovirus proteins involved in the modification of membrane permeability have not yet been identified, the mechanisms underlying these changes have been partly elucidated (Carrasco, 1987; Carrasco et al., 1989). The modification of the membrane by an unknown viral protein involves the selective activation of phospholipases of type C and perhaps D, whereas the action of other phospholipases such as A1 or A2 seems not to be enhanced after infection (Guinea et al., 1989).

Since the final outcome of virus infection is cell lysis, it seemed logical that the virus would have developed mechanisms to activate all kinds of phospholipases. However, this seems not to be the case since poliovirus is able to induce cytotoxic effects and cell rounding by activating only phospholipase C, with no activation of phospholipase A2 (Guinea et al., 1989). Of course, apart from the activation of phospholipase C, poliovirus might disturb the integrity of the membrane by a mechanism similar to that of other lytic proteins such as melittin (Batenburg, 1988; Carrasco, 1987; Gelehter & Rozengurt, 1980; Katsu et al., 1989; Pellkofer et al., 1982; Walenga, 1980). Such peptides possess a hydrophobic region that is inserted into the membrane, displacing an interior part of the phospholipid bilayer leaflet and thereby altering the permeability of the membrane. In doing so, melittin is also able to increase phospholipase activity (Batenburg, 1988; Carrasco, 1987; Gelehter & Rozengurt, 1980; Katsu et al., 1989; Pellkofer et al., 1982; Walenga, 1980).

The release of choline or phosphorylcholine could therefore result from the modifications made to the membrane by a viral protein that leads to disruption of the phospholipid bilayer, allowing the release from the cellular pool of molecules such as small metabolites and potassium ions (Carrasco et al., 1989; Carrasco & Smith, 1976). In addition, these membrane alterations can lead to an increased activity of phospholipase C or D. The action of phospholipase C hydrolyses phosphatidylinositol to produce IP3, which releases calcium ions from the endoplasmic reticulum which, in turn, activate phospholipases C and D (Billah & Anthers, 1990). Moreover, the generation of DAG stimulates protein kinase C which causes further activation of phospholipase C (Billah & Anthers, 1990; Exton, 1990; Slivka et al., 1988). In conclusion, it seems plausible that membrane alteration by a still unidentified poliovirus protein and phospholipase C activation can lead to the alterations in membrane permeability which we have described previously (Carrasco, 1978; Carrasco et al., 1989), and ultimately to the development of cytopathic effects (Carrasco, 1978; Castrillo et al., 1987). The identification of poliovirus proteins involved in this phenomenon and the study of their mode of action will further clarify the molecular mechanisms involved in cell lysis by poliovirus. It must be stressed that as yet very little is known about the molecular events involved in cell lysis by any animal virus nor about the viral components involved (Carrasco, 1978; Carrasco et al., 1989).

Another area of interest is the consequence of the activation of phospholipase C, apart from its effects on cell lysis at late stages of infection. Poliovirus stimulates the synthesis of phospholipids from 3 h.p.i. This activation is related to the synthesis of new membrane structures that are connected with the replication of poliovirus genomes (Guinea & Carrasco, 1990). The inhibition of phospholipid synthesis immediately halts this replication (Guinea & Carrasco, 1990). Nothing is known about the mechanisms that initiate the stimulation of phospholipid synthesis and the modifications in membrane traffic. It is tempting to speculate, however, that the activation of phospholipase C is connected with the stimulation of phospholipid synthesis. In fact, addition of phospholipase C to cells in culture increases phospholipid synthesis (Sleight & Kent, 1983). The activation of phospholipase C or the phospholipids liberated from the inner leaflet of the bilayer may therefore trigger the augmented phospholipid synthesis in poliovirus-infected cells (Cornell & Vance, 1987; Pelech & Vance, 1989).

The expert technical assistance of Ms M. J. Almela, Ms M. Chorro and Mr M. A. Sanz is acknowledged. A. Irurzun and L. Pérez are holders of Fundación R. Rich and Comunidad Autónoma de Madrid fellowships respectively. Plan Nacional project number (BIO 92-0715), DGICYT (PB90-0177) and Fundación Ramón Areces are acknowledged for financial support.

**References**


(Received 21 September 1992; Accepted 17 February 1993)