Journal of Fish Diseases 1995, **18**, 519–527

Low-pH increases the binding of haemorrhagic septicaemia rhabdovirus to membrane phospholipids

J. M. COLL *INIA, Sanidad Animal, CISA - Valdeolmos, Madrid, Spain*

Abstract. The rhabdovirus causing viral haemorrhagic septicaemia (VHS), a disease of coldwater fish, especially salmonids, binds to sonicated radioactively labelled membrane phospholipids [phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC)]. The extent of binding of VHSV to membrane phospholipids is dependent on pH, occurring at physiological pH, but being maximal at about pH 5·5 and suggesting that a pH-induced conformational change is needed for the VHSV to maximally bind phospholipids. VHSV bound PS more effectively than either PE or PC, as demonstrated by ultracentrifugation and competition experiments.

Introduction

The lack of vaccines against fish rhabdoviruses (viral haemorrhagic septicaemia, VHS; infectious haematopoietic necrosis, IHN; spring viaremia of carp, SVC) make these among the most damaging diseases of international aquaculture. We are trying to understand the determinants of these viral infections to help in the design of recombinant vaccines (Leong & Fryer 1993; Lorenzen, Olesen, Vestergaard-Jorgensen, Etzerodt, Holtet & Thorgersen 1993; Estepa, Thiry & Coll 1994) and/or viral inhibition agents. Since available evidence suggests that the mammalian rhabdovirus-cellular membrane interactions which lead to some of the initial steps in viral infections seem to involve viral-membrane phospholipid(s) interactions, the study of rhabdovirus-fish cellular membrane phospholipid interactions (Bernard, Keroualt & de Kinkelin 1984) could help design new methods for control of these viruses.

Phospholipid-detergent micelles extracted from cellular membranes are capable of inhibiting the attachment and the infection of some mammalian rhabdoviruses, including rabies (Superti, Seganti, Tsiang & Orsi 1984) and vesicular stomatitis virus (VSV) (Schlegel, Willigan & Pastan 1982). Of all the lipids tested, phosphatidylserine (PS) is the strongest inhibitor of VSV attachment to the cells (Schlegel, Sue, Willingham & Pastan 1983) at physiological pH, but 10 times more rabies or VSV attach to the cellular membranes at pH ≤6·5 than at higher pH (Wunner, Reagan & Koprowski 1984). After viral attachment, the internationalization of the virus by a temperature-dependent process occurs immediately. This is followed by the fusion of viral and cellular membranes at pH ≤5·5, as suggested by inhibition of fusion by lysosomotropic amines (Rigaut, Birk & Lenard 1991; Schlegel *et al.* 1982; Superti *et al.* 1984).

Mammalian rhabdoviruses possess a unique glycoprotein G present in the virion as a homotrimeric integral membrane protein forming about 400 protruding 83 Å spikes (Gaudin, Ruigrok, Tuffereau, Knossow & Flamand 1992). The trimers of glycoprotein G are responsible for the virus attachment to the cellular membrane and they also contain the fusion properties of the virus in both mammalian (Gaudin, Ruigrok, Knossow & Flamand 1993) and fish (Lecocq-Xhonneux, Thiry, Dheur, Rossius, Vanderheijden, Martial & DeKinkelin 1994) rhabdoviruses. Exposure of isolated trimers of the rabies glycoprotein G to low pH induces conformational and size changes (Gaudin *et al.* 1993) that expose some of its hydrophobic regions to the surface of the trimer, possibly allowing further interactions with the target cellular membrane (Bourhy, Kissi & Tordo 1993). In influenza, the membrane fusion step also depends on the exposure of a

Correspondence: Dr J. M. Coll, INIA, Sanidad Animal, CISA - Valdeolmos, 28130 - Madrid, Spain.

hydrophobic region (fusion peptide), buried in the native structure, that becomes exposed to the surface when triggered by a conformational low-pH-induced change (Carr & Kim 1993; Bullough, Hughson, Skehel & Wiley 1994). No clear identification of such a fusion peptide has yet been made for rhabdoviruses (Coll 1995a), although the recent mapping of fusion-defective VSV mutants have shown that at least four different regions distributed through the glycoprotein G molecule could be involved (Whitt, Zagouras, Crise & Rose 1990; Li, Drone, Sat & Ghosh 1993). The region from amino acids (aa) 123 to 137 has been recently proposed as a putative fusion domain by site-directed mutagenesis (Zhang & Ghosh 1994), and it has been shown to be localized near regions with a–d hydrophobic aa repeats (Coll 1995b).

We report here that membrane phospholipid binding seems to occur, regardless of the lower temperature requirement for infection, in VHSV, a salmonid fish rhabdovirus with similar properties to mammalian rhabdoviruses.

Materials and methods

Viruses

VHSV 07·71 isolated in France (Le Berre, De Kinkelin & Metzger 1977) from rainbow trout, *Oncorhynchus mykiss* (Walbaum), was grown and assayed for infectivity in epithelial papillosum cyprini (EPC) cells, as described by Basurco, Sanz, Marcotegui & Coll (1991). VHSV was purified (1) by double ultracentrifugation or (2) by polyethyleneglycol concentration and ultracentrifugation (Basurco *et al.* 1991). Briefly, virus concentrated (1) by ultracentrifugation or (2) by polyethyleneglycol was layered on a 15–45% sucrose gradient in TNE (0·15 M Tris, 0·15 M NaCl, 1 mM EDTA, pH 7·6) and spun at 80 000 ***g*** for 270 min in a Beckman ultracentrifuge. The band at 31% sucrose contained most of the viral infectivity, whole virions as seen by electron microscopy and all the viral proteins (purified VHSV); the band at 26% sucrose contained traces of viral infectivity, the N and Nx viral proteins, and showed nucleocapsids by electron microscopy. Each fraction was further purified by ultracentrifugation over a 20% sucrose cushion and kept at 4 °C until used. To radioactively label the viral proteins, a U-^{14}C amino acid (aa) lysate was introduced into the cell cultures at 4 μCi ml^{-1} (Amersham International, Amersham, Buckinghamshire, England). The number of G molecules per VHSV virion was calculated from densitometry of the autoradiography of purified labelled VHSV and estimated to be about 300 pmoles of G per 200 μg of VHSV.

Solid-phase phospholipid binding assays

Immediately prior to the assay, the labelled phospholipids (L-3-phosphatidyl- [L-C3-^{14}C]Serine, 1,2-dioleoyl, PS; L-3-phosphatidylcholine, 1-palmitoyl-2-[1-^{14}C]linoleoyl, PC or L-3-phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]linoleoyl,PE; at 53–55 mCi mmo^{-1}; Amersham International) dissolved in organic solvents were dried under vacuum in glass tubes. PC and PE are major components (41–63% and 16–27%, respectively), whereas PS is a minor component (≤1%) of the membrane lipids of many fish cell lines (Bernard *et al.* 1984). Phosphate-citrate buffer was prepared from 0·1 M citric acid and 0·2 M dibasic sodium phosphate stock solutions (Gaudin *et al.* 1993). After the addition of the phosphate-citrate buffer to the dried phospholipids, the mixture was sonicated for three 1-min periods in the cold and immediately used for the subsequent binding assays.

One hundred microlitres per well volumes of 20–40 μg ml^{-1} of VHSV in distilled water were dried overnight at 37 °C in 96-well plates (Costar, The Netherlands or Nunc, Denmark). Plates were kept at –20 °C in a dried atmosphere until used. Immediately prior to use, the coated plates were washed once with the phosphate-citrate buffer and twice with distilled water. Then several amounts of sonicated

labelled phospholipids in phosphate-citrate buffer were added in 100-μl volume per well. After 4 h incubation at 4 or 20 °C, depending on the experiments, the plates were washed three times with distilled water, and incubated with 100 μl well^{-1} of 2% SDS in 50 mM ethylenediamine, pH 11·5, at 60 °C for 30 min. The supernatants (100 μl well^{-1}) were pipetted into 96-well polyethylene terephthalate plates; Hiload-scintillation liquid (LKB, Loughorough, England) (100 μl well^{-1}) was added, mixed and counted on a 1450-Microbeta scintillation counter (Wallace Oy, Turku, Finland, and Pharmcia Iberica, S.A., Spain).

Sucrose gradient separation of VHSV-phospholipid complexes

^{14}C-labelled sonicated phospholipids were incubated ± purified VHSV at 4 °C overnight in 1 ml of citrate/phosphate buffer at pH 7·6 or 5·6. About 50 000 cpm each of phospholipids, phospholipids with VHSV or ^{14}C-labelled VHSV (U-^{14}C aa, Amersham) were applied to the top of a 3-ml discontinuous sucrose gradient (1 ml of 40% W/V sucrose, 1 ml of 30% sucrose, 1 ml of 20% sucrose). The VHSV was ultracentrifuged at 80 000 *g* for 4 h at 4 °C in a SW55 rotor in a Sorvall ultracentrifuge. Then the gradients were collected from the top in 150-μl fractions and assayed for radioactivity.

Results

Binding of phospholipids to VHSV in solution

Initial experiments were performed by ultracentrifuging pre-incubated mixtures of purified virus and sonicated labelled phospholipids at pH 7·6. Because little or no comigration of viral infectivity and phospholipids could be detected, experiments were performed at pH 5·6 (the pH at which mammalian rhabdoviruses fuse to cellular membranes). At pH 5·6, purified VHSV bound a higher amount of phospholipids, as demonstrated by comigration of VHSV infectivity and labelled PS, PE or PC (Fig. 1). Under the ultracentrifugation conditions used, the isolated purified virus banded between 31 and 35% sucrose as determined by labelled VHSV and by infectivity assays, and the isolated sonicated labelled phospholipids remained in the upper phase even after 20 h of ultracentrifugation (data not shown). Cytopathic effects were detected after 2 days of incubation of ultracentrifugation fractions on EPC monolayers, in fractions at 2·4, 2·55 and 2·7 ml from the top of the gradient of PS + VHSV mixtures at pH 7·6. In the same gradients, cytopathic effects extended from fraction 2·4 to 3·6 ml, 3 days after incubation. Cytopathic effects also resulted after isolation using similar fractions from PS + VHSV mixtures at pH 5·6, but only after 5 days of incubation. Since the infectivity assay was carried out in the continuous presence of the added test volume (without changing the inoculum) there was no possibility that inhibition of viral infectivity could be observed (Schlegel *et al.* 1983).

More than 80% of the total labelled PS recovered in the sucrose gradient after ultracentrifugation comigrated with the VHSV, whereas only 20–30% of the total labelled PE or PC recovered comigrated with the VHSV. The high percentage of PS binding to VHSV obtained suggests that no saturation of the PS binding sites of VHSV was obtained in any of these experiments with the amounts of PS used. In contrast, the majority of the PC or PE remained unassociated to VHSV in parallel experiments.

When the peak of PS radioactivity from PS-VHSV mixtures at pH 5·6 (from fraction 2·5 to 3·5 ml) was pooled and ultracentrifuged through a pH 7·6 sucrose gradient, banding of most of the radioactivity at 31–35% of sucrose was again obtained, confirming the stability of the PS.

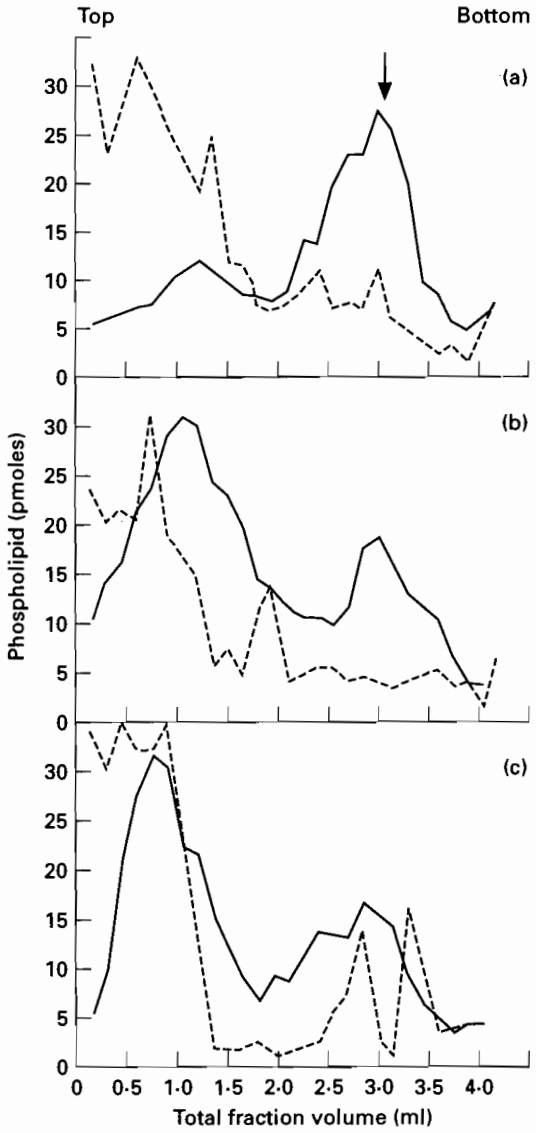

Figure 1. PS-binding to VHSV in solution. Purified VHSV containing about 300 pmoles of glycoprotein G and 500 pmoles of labelled phospholipid were incubated overnight at 0 °C, pH 5·4, after which this was ultracentrifuged in a sucrose discontinuous gradient (1 ml of 40% sucrose +1 ml 30% sucrose +1 ml of 20% sucrose +1 ml of VHSV-phospholipid mixture) at 80 000 ***g*** for 4 h. Fractions of 150 μl were collected from the top of the tubes and counted. Top of the tube to the left, bottom to the right: (—) pH 5·6; (-----) pH 7·6. Labelled, sonicated VHSV-free phospholipids stayed at the top of the tube at any pH and 80 000 ***g*** for 20 h. The vertical arrow indicates both the highest label when isolated labelled VHSV was ultracentrifuged and the fastest VHSV infectivity of ultracentrifuged VHSV + phospholipid fractions at pH 7·6: (a) PS; (b) PE; (c) PC.

Binding of phospholipids to VHSV on a solid-phase

Phospholipid binding to solid-phase VHSV was estimated in order to simplify the assays. Figure 2 shows that PS binding to solid-phase VHSV was two-fold higher at pH 5·6 than at pH 7·6, confirming the ultracentrifugation data and suggesting a higher availability of PS binding sites at the lower pH. The temperature did not influence the binding reaction, since identical curves were obtained at 0 and 20 °C. The binding of PS to solid-phase VHSV increased with time of incubation both at pH 7·6 and 5·6 to reach a near saturation value after 2–4 h. The significant amount of PS binding to solid-phase VHSV at pH 7·6 (Fig. 2), compared with the low PS binding to soluble VHSV (Fig. 1), could be caused by the partial

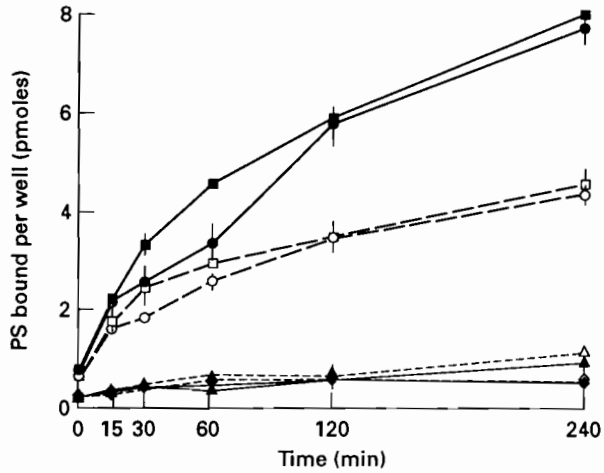

Figure 2. Time course of PS binding to solid-phase VHSV. Binding of PS (47 pmoles well^{-1}) was performed at 4 and 20 °C. Plates were coated with 2 μg of VHSV 07·71: (● — ●) PS binding to VHSV at pH 5·6, 4 °C; (○ --- ○) PS binding to VHSV at pH 7·6, 4 °C; (■ — ■) PS binding to VHSV at pH 5·6, 20 °C; (□ -----□) PS binding to VHSV at pH 7·6, 20 °C; (▲—▲) PS binding to uncoated wells at pH 5·6, 4 °C; (△---△) PS binding to uncoated wells at pH 7·6, 4 °C; (∗—∗) PS binding to uncoated wells at pH 5·6, 20 °C; (∗----∗) PS binding to uncoated wells at pH 7·6, 20 °C. Averages and standard deviations of duplicates are represented.

denaturation of the virion after solid-phase coating and/or because of the relatively high temperature at which coating of the solid-phase was performed (37 °C).

PS showed peak binding to the solid-phase VHSV between pH 5 and 5·5, depending on the virus preparation, whereas binding of PC or PE showed a lower binding level and a continuous decrease of binding with pH (Fig. 3). Maximal phospholipid binding to solid-phase VHSV was obtained with 2–4 μg of total VHSV protein per well, equivalent to about 3–6 pmoles of glycoprotein G per well (Fig. 3). In every case, the relative extent of binding of phospholipids was in the order PS > PC > PE.

No strong evidence for a two-site phospholipid binding (biphasic curves) was obtained when labelled PS, PE or PC binding to VHSV was performed in the presence of increasing amounts of cold phospholipids extracted and purified from animal tissues (Fig. 4). All the curves obtained were monophasic and very similar whether cold PS, PE or PC were used to compete for the binding of any of the apparent three labelled phospholipids.

All of the purified strains of VHSV tested in solid-phase binding assays, including an IHNV strain, showed binding of PS compared to controls (Table 1). In contrast, purified glycoprotein G-free nucleocapsids showed only slightly higher than background levels of binding of PS.

Discussion

Using ultracentrifugation and solid-phase phospholipid binding assays, VHSV has been shown to bind PC, PE or PC, the main phospholipid components of fish cellular membranes (Bernard *et al.* 1984). This finding extends the observation of membrane phospholipid interaction from mammalian rhabdoviruses (VSV and rabies) to fish rhabdoviruses.

PS binding to VHSV was three-fold higher than for PC or PE, as shown by ultracentrifugation, pH dependence and solid-phase VHSV binding assays. Similarly, inhibition of VSV binding to cells by PS

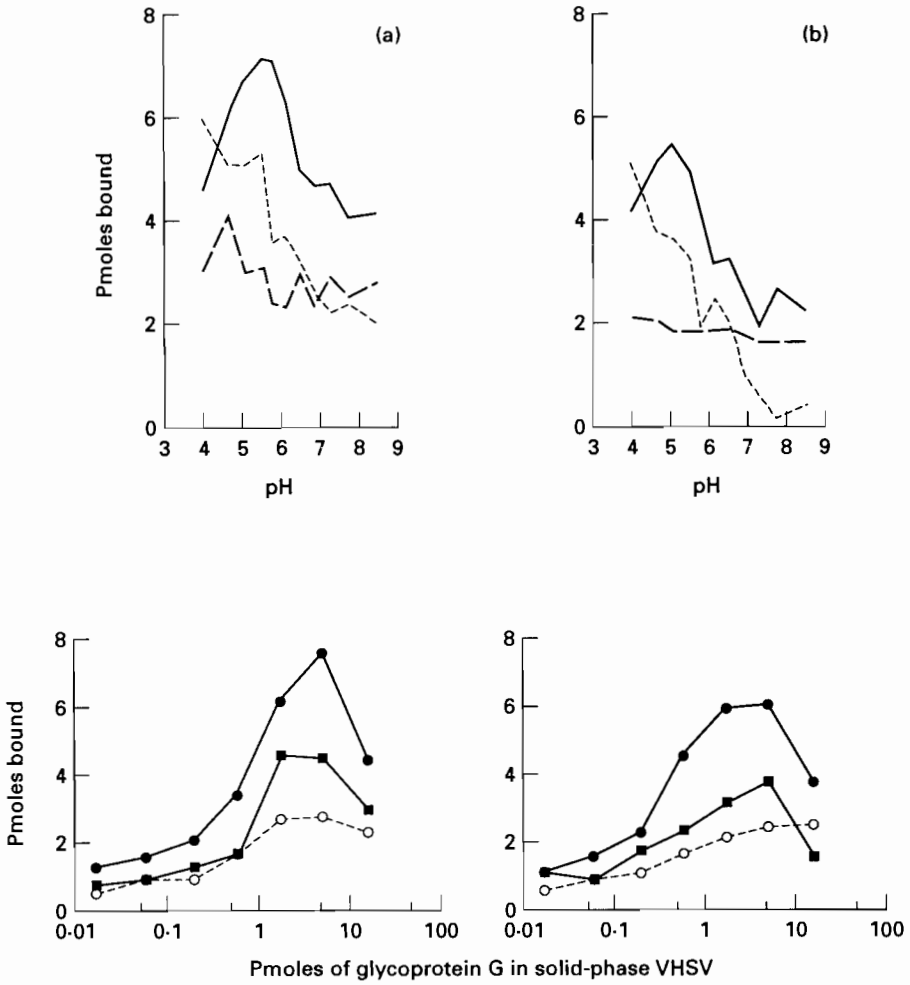

Figure 3. Variables influencing phospholipid binding to solid-phase VHSV. Binding of 30 pmoles of phospholipids at 0 °C for 4 h: (a) VHSV purified by two ultracentrifugations; (b) VHSV concentrated and ultracentrifuged. To estimate the pH dependence on the binding, 2 μg of VHSV protein per well was used to coat the plates. To estimate VHSV concentration dependence of the binding of phospholipids, binding was performed at pH 5·6. The VHSV concentration used to coat the wells is given in equivalent pmoles of glycoprotein G (3 pmoles 2 μg VHSV/well^{-1}): (—) PS; (------) PE; (·····) PC.

dissolved in octylglucoside was ≥80%, whereas PE and PC were only ≈30% and ≤10% inhibitory, respectively (Schlegel *et al.* 1983). No phospholipid specificity for binding has been yet reported for rabies (Wunner *et al.* 1984). PS, PE and PC show similar competition curves with any of the labelled phospholipids bound to VHSV (Fig. 4), suggesting that VHSV possesses either a unique site to bind all three phospholipids, with a preference for the acidic PS, or several sites with similar properties. If several phospholipid binding sites with different binding affinities for PS, PE or PC were present, it might be expected that the competition curves would show more than one S profile but not the monophasic curves in fact obtained. On the other hand, this site could be formed by more than one stretch of glycoprotein G sequence. A 35–60-fold molar excess of cold phospholipid reduced the labelled phospholipid to 50% of the initial label. Although

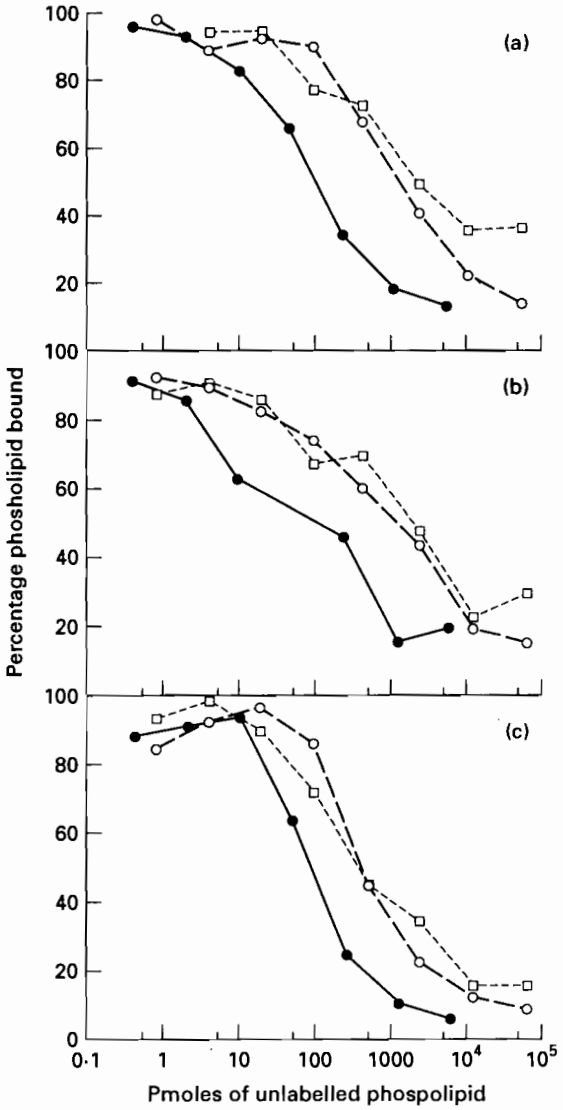

Figure 4. VHSV binding of labelled phospholipids in the presence of increasing amounts of cold phospholipids. Cold phospholipids were obtained from tissue extracts, dissolved in organic solvents, serially diluted five-fold and dried under vacuum. Phosphate citrate buffer was then added and each solution sonicated just before use. Purity as estimated by thin-layer chromatography was 80% for PC, 80% for PC + PE and 10% for PS (contamination with brain glycolipids). No attempt was made to further purify the phospholipids. The amounts of cold phospholipids were calculated according to the estimated purity. Purified VHSV was bound to solid-phase plates at 2 μg $well^{-1}$ and then incubated for 4 h at 4 °C in phosphate-citrate buffer, pH 5·4, in the presence of labelled (20 pmoles) and cold phospholipids: (a) PS, (b) PE and (c) PC indicate the ^{14}C labelled phospholipids: (○ --- ○) cold egg yolk PC (Sigma, MI, USA); (■—■) cold sheep brain PC + PE (Sigma); (●—●) cold bovine brain PS (Sigma).

this larger amount could be a result of the different composition in molecular species between the labelled and the cold phospholipids obtained from mammalian tissues, and/or the micellar physical form of phospholipids in water, it probably also reflects the high binding capacity of the phospholipid binding site(s). On the other hand, it is not likely that the source of phospholipids would influence the results because of the similar compositions between mammalian and fish tissues (Bernard *et al.* 1984).

The binding of PS to VHSV in solution is much higher at low pH (Fig. 1). It seems that conformational changes induced by pH 5·6 are needed for VHSV to bind phospholipids more strongly and are favoured by VHSV coating to hydrophobic solid-phases (a significant level of binding of PS to VHSV on a solid-phase at pH 7·6 was detected compared to its low binding to VHSV in solution at the same pH).

Table 1. PS binding to solid-phase VHSV isolate*

Rhabdoviruses	Optional concentration (μg well^{-1})	PS bound (pmoles)
VHSV-F1	2	3·13 ± 0·32
VHSV-F2	2	3·91 ± 0·56
VHSV-23·75	2	3·24 ± 0·22
VHSV-798	2	3·98 ± 0·26
VHSV-144	2	4·07 ± 0·06
VHSV-471	2	4·23 ± 0·14
VHSV-472	2	4·30 ± 0·18
VHSV-689	2	4·14 ± 0·19
VHSV-07·71	2	3·40 ± 0·21
IHNV-Cedar	2	3·41 ± 0·11
EPC protein	2	0·03 ± 0·20

* Viruses were concentrated and purified by ultracentrifugation (Basurco *et al.* 1991) and used to coat the wells of 96-well plates (solid-phase). PS binding was 20 pmoles of PS at 4 °C, pH 5·6, for 4 h. Optimal rhabdovirus concentrations were determined by binding purified VHSV-07·71 to the wells at between 0·1 and 50 μg well^{-1}. Means ± standard deviations from four-fold replicates are represented. EPC protein was a cell extract from the cell line in which the rhabdoviruses were grown. Backgrounds (0·05 pmoles per well) estimated by PS binding to uncoated wells were subtracted from all the data. IHNV = infectious haematopoietic necrosis virus.

That VHSV-phospholipid binding is higher at pH 5·6 than pH 7·6 might be considered surprising since VHSV infects cells at pH > 7; however, this also occurs with rabies and VSV (Wunner *et al.* 1984). All these results are consistent with a first low-affinity binding (adsorption) of the VHSV to the phospholipids of the cellular membrane of the host at physiological pH, most probably caused by electrostatic interactions. After the VHSV is internalized to the cellular endosomes, the pH is lowered inducing an increase in the VHSV-phospholipid binding, probably because of additional hydrophobic interactions, as seen to occur with other rhabdoviruses (Schlegel *et al.* 1982; Schlegel *et al.* 1983; Superti *et al.* 1984; Gaudin *et al.* 1993).

By analogy with other mammalian rhabdoviruses and as suggested by the lower phospholipid binding obtained with glycoprotein G-free nucleocapsids, the phospholipid binding of VHSV seems to be mediated by glycoprotein G. The phospholipid binding obtained with VHSV could be caused by the necessity of (1) several stretches of glycoprotein G for maximal binding; (2) a trimer structure; (3) other viral proteins; and/or (4) viral membrane anchorage of the glycoprotein G. That other viral proteins are needed for phospholipid interactions with phospholipids is not likely because no evidence of viral surface exposition of any viral protein other than glycoprotein G exists in any rhabdovirus. However, the protein M2 (pI ≈ 9) can interact with the negatively charged phospholipid residues of PS (Zakowsky, Petri & Wagner 1981; Li *et al.* 1993); and it seems responsible for the binding of liposomes to rhabdoviral skeletons (devoid of glycoprotein G and lipids) (Barge, Gaudin, Coulon & Ruigrok 1993).

The study of VHSV-phospholipid interactions could further clarify mechanisms of viral entry into fish cells. At the same time, mapping of the VHSV protein sequences implicated in the binding might show some general rhabdoviral features and/or suggest experimental approaches to study the earliest stage of rhabdoviral infections. A synthetic phospholipid (perhaps an analogue of PS) capable of binding to and inhibiting the attachment of VHSV to fish cells at pH 7–8 (the pH at which most salmonid aquaculture is carried out) might be a possible inhibitor of VHSV infection.

Acknowledgments

We appreciated the help of J. P. Coll in typing the manuscript. This work was supported by Research Grant CT94-1334 from the AIR 2 Programme of the European Economic Community and by INIA project SC94-102.

References

Barge A., Gaudin Y., Coulon P. & Ruigrok R. W. H. (1993) Vesicular stomatitis virus M2 protein may be inside the ribonucleocapsid coil. *Journal of Virology* **67**, 7246–7253.

Basurco B., Sanz F., Marcotegui M. A. & Coll J. M. (1991) The free nucleocapsids of the viral haemorrhagic septicaemia virus contain two antigenically related nucleoproteins. *Archives of Virology* **119**, 153–163.

Bernard J., Keroualt B. & de Kinkelin P. (1984) High-performance thin-layer chromatography of lipids from different fish cell lines and from viral haemorrhagic septicaemia virus. *Annales de Virologie (Institut Pasteur)* **135 E**, 177–182.

Bourhy H., Kissi B. & Tordo N. (1993) Molecular diversity of the lyssavirus genus. *Virology* **194**, 70–81.

Bullough P. A., Hughson F. M., Skehel J. J. & Wiley D. C. (1994) Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature (London)* **371**, 37–43.

Carr C. M. & Kim P. S. (1993) A spring-loaded mechanism for the conformational change of influenza hemagglutinin. *Cell* **73**, 823–832.

Coll J. M. (1995a) The glycoprotein of rhabdoviruses. *Archives of Virology*, **140**, 827–851.

Coll J. M. (1995b) Heptad-repeat sequences in the glycoprotein of rhabdoviruses. *Virus Genes*, in press.

Estepa A., Thiry M. & Coll J. M. (1994) Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout leucocyte anamnestic *in vitro* responses. *Journal of General Virology* **75**, 1329–1338.

Gaudin Y., Ruigrok R. W. H., Knossow M. & Flamand A. (1993) Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *Journal of Virology* **67**, 1365–1372.

Gaudin Y., Ruigrok R. W. H., Tuffereau C., Knossow M. & Flamand A. (1992) Rabies virus glycoprotein is a trimer. *Virology* **187**, 627–632.

Le Berre M., De Kinkelin P. & Metzger A. (1977) Identification sérologique des rhabdovirus des salmonidés. *Bulletin de l'Office International des Epizooties* **87**, 391–393.

Lecocq-Xhonneux F., Thiry M., Dheur I., Rossius M., Vanderheijden N., Martial J. & DeKinkelin P. (1994) A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. *Journal of General Virology* **75**, 1579–1587.

Leong J. C. & Fryer J. L. (1993) Viral vaccines for aquaculture. *Annual Review of Fish Diseases* **3**, 225–240.

Li Y., Drone C., Sat E. & Ghosh H. P. (1993) Mutational analysis of the vesicular stomatitis virus glycoprotein G for membrane fusion domains. *Journal of Virology* **67**, 4070–4077.

Lorenzen N., Olesen N. J., Vestergaard-Jorgensen P. E., Etzerodt M., Holtet T. L. & Thorgersen M. C. (1993) Molecular cloning and expression in *Escherichia coli* of the glycoprotein gene of VHS virus and immunization of rainbow trout with the recombinant protein. *Journal of General Virology* **74**, 623–630.

Rigaut K. D., Birk D. E. & Lenard J. (1991) Intracellular distribution of input vesicular stomatitis virus proteins after uncoating. *Journal of Virology* **65**, 2622–2628.

Schlegel R., Sue T. T., Willingham M. C. & Pastan I. (1983) Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site? *Cell* **32**, 639–646.

Schlegel R., Willigan M. C. & H. Pastan I. H. (1982) Saturable binding sites for vesicular stomatitis virus on the surface of Vero cells. *Journal of Virology* **43**, 871–875.

Superti J., Seganti L., Tsiang H. & Orsi N. (1984) Role of phospholipids in rhabdovirus attachment to Cer cells: brief report. *Archives of Virology* **81**, 321–328.

Whitt M. A., Zagouras P., Crise B. & Rose J. K. (1990) A fusion-defective mutant of the vesicular stomatitis virus glycoprotein. *Journal of Virology* **64**, 4907–4913.

Wunner W. H., Reagan K. J. & Koprowski H. (1984) Characterization of saturable binding sites for rabies virus. *Journal of Virology* **50**, 691–697.

Zakowsky J. J., Petri W. A. & Wagner R. R. (1981) Role of matrix protein in assembling the membrane of vesicular stomatitis virus: reconstitution of matrix protein with negatively charged phospholipid vesicles. *Biochemistry* **20**, 3902–3906.

Zhang L. & Ghosh H. P. (1994) Characterization of the putative fusogenic domain in vesicular stomatitis virus glycoprotein G. *Journal of Virology* **68**, 2186–2193.