# Fluorescent Detection of $Zn^{2+}$-Rich Vesicles with Zinquin: Mechanism of Action in Lipid Environments

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ABSTRACT High concentrations of free $Zn^{2+}$ ions are found in certain glutamatergic synaptic vesicles in the mammalian brain. These terminals can be visualized histochemically with quinoline sulfonamide compounds that form fluorescent complexes with $Zn^{2+}$. The present study was undertaken to examine the interaction of the water-soluble quinoline sulfonamide probe, Zinquin (2-methyl-8-(toluene-*p*-sulfonamido)-6-quinolyloxyacetic acid) with the complex heterogeneous cellular environment. Experiments on rat hippocampal and neocortical slices gave indications that Zinquin in its free acid form was able to diffuse across the plasma and synaptic vesicle membranes. Further experiments were undertaken on unilamellar liposomes to study the interaction of Zinquin and its metal complexes in membranes. These experiments confirmed that Zinquin is able to diffuse across lipid bilayers. Steady-state and time-resolved fluorimetric studies showed that Zinquin in aqueous solution mainly forms a 1:2 (metal:ligand) complex with small amounts of a 1:1 complex. Formation of the 1:1 complex was favored by the presence of lipid, suggesting that it partitions into membranes. Evidence is presented that Zinquin can act as a $Zn^{2+}$-ionophore, exchanging $Zn^{2+}$ for two protons. The presence of a pH gradient across vesicles traps the $Zn^{2+}$-probe complex within the vesicles. Zinquin is useful as a qualitative probe for detecting the presence of vesicular $Zn^{2+}$; however, its tendency to partition into membranes and to serve as an ionophore should be borne in mind.

## INTRODUCTION

Zinc has a multiplicity of roles in the cellular economy, acting prominently as a structural part of proteins or at the heart of the catalytic site of enzymes (Berg and Shi, 1996). There is, however, a fraction of zinc (termed "vesicular" or "chelatable") that is found in synaptic vesicles and appears not to be in strong association with organic molecules (Frederickson, 1989). The precise role of this pool of zinc, although known for a number of years, remains enigmatic. Recently it was shown that a single zinc transporter (ZnT3) (Wenzel et al., 1997) is responsible for loading a subset of glutamatergic terminals with zinc, as knocking out ZnT3 eliminates the histologically manifest $Zn^{2+}$ in these terminals (Cole et al., 1999).

Until recently the only methods for detecting this pool of zinc were histological stains of fixed tissue, such as Timm's stain (Danscher, 1981), which relies on the silver intensification of metal precipitated by disulfide. Fluorimetric probes such as Fura-2 (Grynkiewicz et al., 1985) have greatly advanced the biological characterization of calcium, and similar advantages could be anticipated for a zinc-sensitive probe. Some progress has been made in this regard by using $Ca^{2+}$ probes (Atar et al., 1995; Cheng and Reynolds, 1998; Sensi et al., 1997) in conjunction with the heavy metal chelator TPEN (*N,N,N′,N′*-tetrakis (2-pyridylmethyl)-ethylenediamine) (Arslan et al., 1985; Snitsarev et al., 1996), which has a low affinity for $Ca^{2+}$ and $Mg^{2+}$. Zinquin (Zalewski et al., 1993, 1994) (we will use the name "Zinquin" for the acid form of the probe) and TFLZn (Budde et al., 1997) (Fig. 1), two probes sensitive to $Zn^{2+}$ and applicable to live tissue, were developed over the last few years.

We will give a synopsis of the development of quinoline sulfonamide probes, as much of the early work was reported in the Russian literature. To the best of our knowledge Bozhevolnov and Serebriakova (1961) were the first to introduce the quinoline sulfonamide moiety (compound II in Fig. 1) as the basis for $Zn^{2+}$- and $Cd^{2+}$-sensitive fluorescent probes. Serebriakova et al. (1964) systematically explored the fluorescence sensitivity of derivatives of compound I to metals (Fig. 1). Of the range of compounds they investigated, compounds I and II fluoresced brightest in the presence of $Zn^{2+}$ and $Cd^{2+}$. They presented evidence in the form of Job's plots for the formation of 1:2 complexes. Subsequently, Krasavin et al. (1969) used compound II to visualize high $Zn^{2+}$ concentrations in pancreatic tissue. On the basis of these studies Frederickson et al. (1987) tested a number of commercially available quinoline derivatives and found the best results with TSQ (Fig. 1), which is now quite widely used as a histochemical stain in frozen sections.

Fluorescence is conferred on quinoline sulfonamides by chelation of $Zn^{2+}$ by the two nitrogens. At neutral pH the free ligands exist in the forms depicted in Fig. 1, and binding of the metal displaces the proton from the amide group. The free ligand exhibits little fluorescence and complexation of $Zn^{2+}$ substantially increases the fluorescence,

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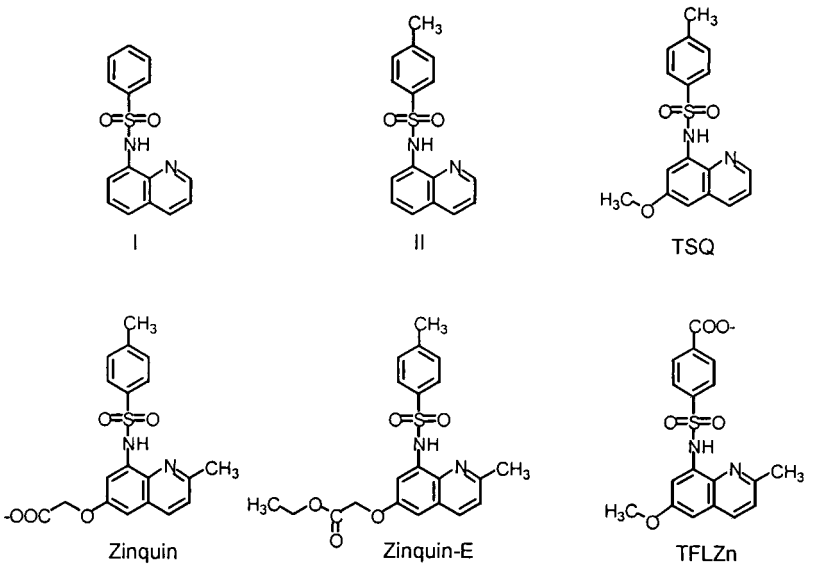


FIGURE 1 Structure of quinoline sulfonamide probes.

while $Ca^{2+}$ and $Mg^{2+}$ have little effect and iron and copper ions quench its fluorescence.

Fluorimetric probes are typically introduced into the cytoplasm of cells by using an AM (acetoxymethyl ester) form of the probe (Tsien, 1981) ("AM loading"). Modifying the carboxyl groups to AM groups renders the probe membrane permeant, and once the AM form has diffused into the cytoplasm endogenous esterases remove the AM group, trapping the free acid form of the probe in the cytoplasm. In the case of synaptic $Zn^{2+}$, the ion is in an internal compartment and AM loading might not be the best route for introducing probes. In this paper we show that both the free acid forms of Zinquin and TFLZn are able to move into synaptic vesicles by diffusion. Although these probes have been used in a number of different biological applications (Berendji et al., 1997; Brand and Kleineke, 1996; Budde et al., 1997; Nasir et al., 1999; Qian et al., 2000), their behavior in complex heterogeneous systems has not been characterized. In order to understand the interactions of $Zn^{2+}$ probes in such systems we have used unilamellar liposomes as a model membrane system. We show that the stoichiometry of the complexes formed is influenced by the presence of a membranous phase, and that the metal-probe complex can partition into the membrane. Moreover, under certain conditions Zinquin can act as an ionophore, counter-transporting one $Zn^{2+}$ for two protons, and that this movement is prevented by the proton gradient that normally exists in synaptic vesicles.

## MATERIALS AND METHODS

### Reagents

Unless otherwise stated, all chemicals were from Fluka (Milwaukee, WI); Zinquin and TFLZn were from TefLabs (Austin, TX), phospholipids from Avanti (Alabaster, AL).

### Preparation of hippocampal and neocortical slices

Long-Evans rat (22–45 days old) hippocampal or neocortical slices were cut on a McIlwain chopper (400 $\mu$m). The slices were transferred to physiological saline (124 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 23 $NaHCO_3$, 10 glucose, bubbled with 95/5% $O_2/CO_2$) in an interface holding chamber at room temperature.

Slices were transferred to a chamber on the microscope and perfused continuously with saline at room temperature. Slices were stained with Zinquin by perfusing them with physiological saline containing the probe. Images were acquired on an Olympus BX50WI upright microscope. Illumination was provided by a monochromator at 360 nm (TILL Photonics, Martinsried, Germany), passed through a dichroic (400DCLP, Chroma, Brattleboro, VT) and then though a filter (D510/80m Chroma) onto the faceplate of a Princeton Instruments cooled CCD camera. Data was acquired by the Metafluor program (Universal Imaging Corp., Downington, PA).

### Fluorimetry

Excitation-emission spectra were determined on a Hitachi F-4500 spectrofluorimeter using methacrylate cuvettes whose temperature was controlled by a circulating water bath. For the neocortical experiments, a slice was attached to a coverslip by superfusing it with a thin layer of low gelling-point agarose (Sigma, St. Louis, MO) dissolved in physiological saline (40°C). The coverslip with the slice was suspended in the cuvette at a 45° angle to the excitation beam. The slice was continuously perfused by recirculating the saline (50 ml) through an oxygenation chamber. For the determination of quantum yields, fluorescein (1 $\mu$M, assuming a yield of 0.9 in 0.1 M NaOH at 25°C) was used as a standard, using the method of Demas and Crosby (1971).

### Frequency domain fluorescence spectroscopy

The modulation values and the phases were measured at multiple modulation frequencies using a multiharmonic Fourier transform phase-modulation spectrofluorimeter SLM 48000MHF (Spectronic Instruments, Inc, Rochester, NY). An HeCd laser was used to provide excitation at 325 nm. Fluorescence emission was collected at right angles and a 345 nm long-

pass filter was used in the emission channel to minimize scattered laser light. A photomultiplier tube (R1477, Hamamatsu) was used to detect the fluorescence.

Data analysis was performed with a commercially available software package (Globals Unlimited, Urbana, IL) for global analysis (Beechem et al., 1991). Various decay models, including both discrete and distributed fluorescence lifetimes, were used to find the best fit to the data. Gaussian distribution was used in all distributed models.

Fluorescence decay models, including the sum of up to four discrete fluorescence decays, the sum of one Gaussian distribution and up to two discrete fluorescence lifetimes, and the sum of two Gaussian distributions, were used to fit the experiment data. The best models were selected to be the ones with the fewest number of parameters, lowest $\chi^2_R$ value, and residues randomly distributed around zero.

### Liposomes

Liposomes were prepared by ultrasonicating lipids (Avanti) at a concentration of 10 mg/ml in liposomal saline (concentrations in mM: 120 KCl, 20 NaCl, 10 Hepes, pH 7.4). Chloroform was evaporated from the lipid by passing a stream of nitrogen over the solution, 2.5 ml of liposomal saline was added to the lipid, and the mixture was vortexed and allowed to hydrate for 1 h. The solution was added to a screw-topped glass vial and then ultrasonicated (Laboratory Systems, Hicksville, NY) until the solution clarified. All experiments were performed at lipid concentrations of 1 mg/ml in liposomal solution at 26°C unless otherwise stated. Liposomal experiments were repeated at least three times, but single exemplars are shown in the figures.

## RESULTS

### Characteristics of Zinquin and TFLZn in aqueous solution

Both TFLZn and Zinquin are soluble in aqueous solution (~1 mg/ml) and fluoresce when complexed with $Zn^{2+}$ (Fig. 2 *A*). Details of the specificity of TFLZn and Zinquin binding and fluorescence have been presented elsewhere (Budde et al., 1997; Fahrni and O'Halloran, 1999; Zalewski et al., 1994) and will not be reviewed here, only to remark that Zinquin fluoresces when associated with $Cd^{2+}$, while TFLZn does not.

In aqueous solution with 1 $\mu$M of the $Zn^{2+}$ probe and 10 $\mu$M zinc the quantum yields for Zinquin and TFLZn were 0.36 and 0.07, respectively (26°C). Because of its higher quantum yield, we have chosen to concentrate our study on Zinquin; however, most of the results also held for TFLZn.

The titration of Zinquin (10 $\mu$M) with $ZnCl_2$ in aqueous solution showed an inflection at the $Zn^{2+}$ concentration (5 $\mu$M) corresponding to a 1:2 (metal:ligand) complex (Fig. 2 *B*). Increasing $Zn^{2+}$ above this level led to a further increase in intensity, probably as a result of the formation of a 1:1 complex. The method of continuous variation (Job's method) (Huang, 1982) was used to determine the stoichiometry of the Zinquin-$Zn^{2+}$ complex. The fluorescence of a set of solutions prepared with varying mole fractions of metal and ligand, with the sum of the metal and Zinquin held constant (10 $\mu$M), was determined. The mole fraction at which the fluorescence peaks indicates the stoichiometry

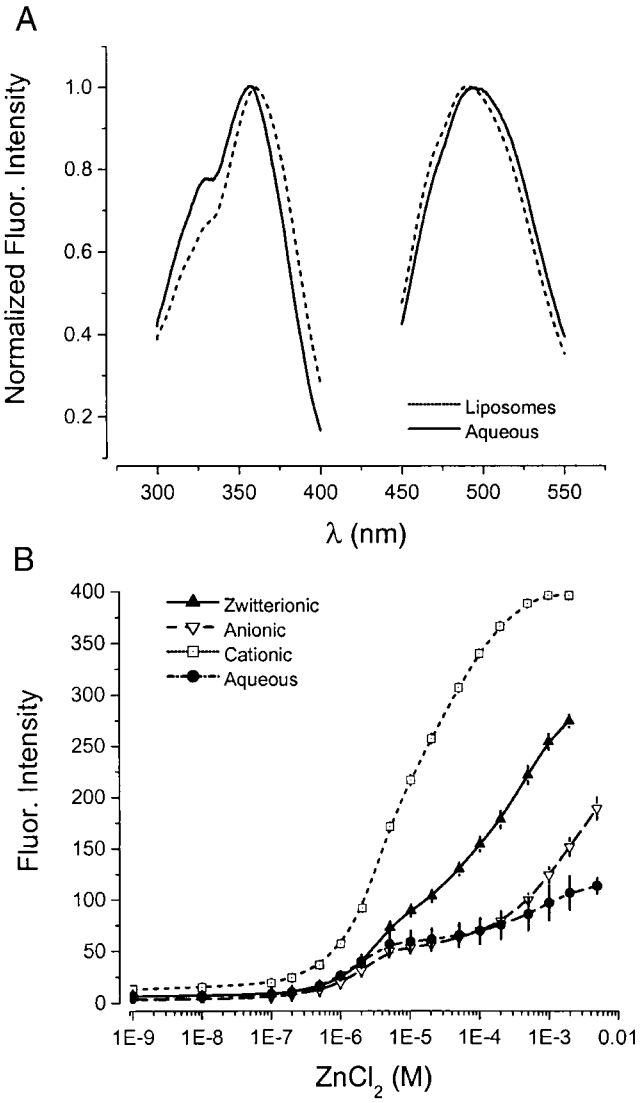


FIGURE 2 (*A*) Normalized excitation and emission spectra of Zinquin-$Zn^{2+}$ complexes in aqueous and liposomal (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) solutions. 10 $\mu$M Zinquin and 100 $\mu$M $ZnCl_2$. (*B*) Fluorescence as a function of $Zn^{2+}$ at a fixed Zinquin concentration (10 $\mu$M) (ex = 358 nm; em = 497 nm). Liposomes were formed in liposomal saline containing 100 $\mu$M $MgCl_2$. Zwitterionic liposomes: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (1.27 mM); cationic liposomes: 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (1.23 mM); anionic liposomes: 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (1.25 mM). Mean ± SEM, $n = 3$.

of the metal-ligand complex; 0.33 in this case, corresponding to the formation of a 1:2 complex (Fig. 3). Our conclusion is similar to that of Hendrickson et al. (1997) who found that Zinquin predominantly forms a 1:2 complex with traces of 1:1 in 50% aqueous ethanol solution. Fahrni and O'Halloran (1999) found no evidence for 1:1 complexes in potentiometric titrations performed in an 80:20 mixture of DMSO and water; however, their experiments were performed at 1:2 ratio of $Zn^{2+}$ and Zinquin, which would tend to minimize the formation of the 1:1 complex.

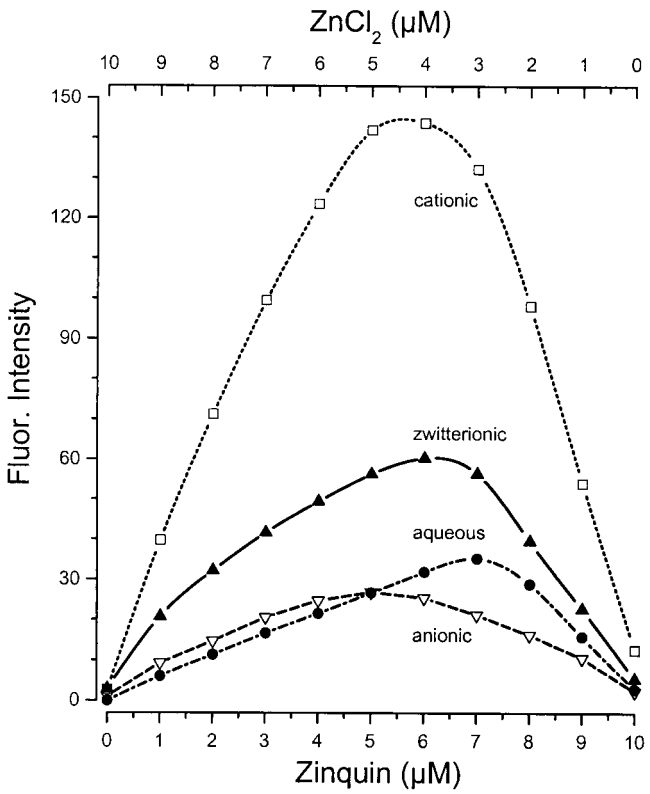


FIGURE 3 Job's plots of Zinquin in aqueous solution and liposomes with different headgroups. Lipids as in Fig. 2.

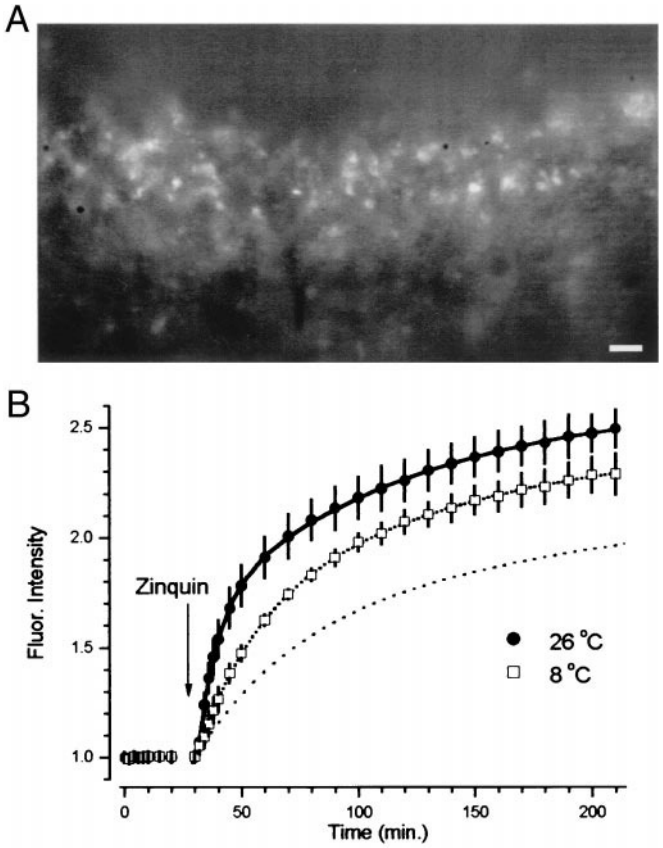


FIGURE 4 Loading of rat brain with Zinquin. (*A*) High-power view of mossy fiber terminals in a hippocampal slice. Cell bodies can be seen in relief toward the bottom of the image. Scale bar 10 $\mu$m, 1-s exposure. (*B*) Loading of rat neocortical slices with Zinquin at two different temperatures. The slices were mounted on a coverslip (see Materials and Methods) held in a cuvette in a spectrofluorimeter, where the fluorescent intensity was measured every second. Zinquin (10 $\mu$M) was added at the time indicated by the arrow. Data have been normalized with respect to the initial fluorescence (mean $\pm$ SEM, $n = 3$). The sum of two exponential processes was fitted to the data; 26°C: $\tau_1 = 8.5 \pm 0.3$ min, $a_1 = 0.68$, $\tau_2 = 91 \pm 5$ min, $a_2 = 0.94$; 8°C: $\tau_1 = 22 \pm 2$ min, $a_1 = 0.54$, $\tau_2 = 99 \pm 17$ min, $a_2 = 0.9$ The dotted line shows the expected time course at 8°C if the process was governed by a $Q_{10}$ of 2.5 ($\tau_1 = 30$ min, $a_1 = 0.68$, $\tau_2 = 473$ min, $a_2 = 0.94$).

## Loading hippocampal slices with Zinquin

Hippocampal slices incubated in 25 $\mu$M Zinquin gave the same characteristic labeling as the Timm's method, that is, fluorescence was observed in the hilus of the dentate gyrus and mossy fiber projections, with little labeling of cell bodies. Under high-power magnification individual mossy fiber boutons could be discerned (Fig. 4 *A*).

We suggested previously that the protonated form of TFLZn might cross the membrane (Budde et al., 1997). However, from the measured $pK_a$ of the carboxyl group of Zinquin (Hendrickson et al., 1997), the concentration of the protonated species would be only 0.02% of the total concentration at pH 7.4. The rapidity with which Zinquin crossed membranes (see below) makes this mechanism unlikely. What seems more plausible is that the deprotonated species of Zinquin and TFLZn are membrane-permeant, which is consistent with the known permeability of various monocarboxylic acid derivatives with hydrophobic tails (Walter and Gutknecht, 1984).

$Zn^{2+}$ probes may simply diffuse across membranes passively or be actively taken up by a transporter or endocytosis. To examine these possibilities the rate of Zinquin uptake was measured as a function of temperature, as temperatures below 15°C are known to block endocytosis and would be expected to retard active transport more severely than passive transport. The uptake of Zinquin was measured in rat neocortical slices held in a cuvette while circulating oxygenated saline at 26 or 8°C. The time course of loading at both temperatures was roughly biexponential (Fig. 4 *B*), with $Q_{10}$ values of 1.6 and 1.0 for the fast and slow processes, respectively, which is consistent with diffusion accounting entirely for the movement of Zinquin (Weiss, 1996). The predicted time course of loading at 8°C if the process were active and had a $Q_{10}$ of 2.5 is shown by the dotted line in Fig. 4 *B*.

## Spectral characteristics of Zinquin in liposomes

In order to characterize the interaction between Zinquin and membranes we have used a unilamellar liposome prepara-

tion. We will show here that the stoichiometry of $Zn^{2+}$-Zinquin complexes and their quantum yields are influenced by the chemical environment, and that the $Zn^{2+}$-probe complexes have a tendency to partition into membranes.

The excitation-emission characteristics of Zinquin-$Zn^{2+}$ complexes in aqueous solution and in liposomes are shown in Fig. 2 *A*. In liposomes the excitation spectrum is shifted to higher wavelengths and the emission spectrum to lower wavelengths, typical of a fluorophore moving into a more hydrophobic environment (Lakowicz, 1983).

When a fixed concentration of the Zinquin was titrated with $Zn^{2+}$ the results shown in Fig. 2 *B* (trace labeled "zwitterionic") were obtained. In liposomes the $Zn^{2+}$-fluorescence intensity curves were biphasic, exhibiting an inflection close to the 1:2 ratio (i.e., at 5 $\mu$M $ZnCl_2$) and then increasing to a peak with a magnitude twofold higher than the first inflection point. Our interpretation is as follows: at low $Zn^{2+}$ concentrations the 1:2 complex predominates and partitions partially into the bilayer, raising the fluorescent intensity. At higher concentrations the 1:1 complex forms and partitions into the membrane. The results are consistent with the 1:1 and the 1:2 having approximately the same quantum yield, so the maximum intensity is almost twofold higher than the intensity at the inflection point.

The 1:2 complex has a charge of $-2$ while the 1:1 complex is uncharged. The equilibrium between these different species might be expected to be influenced by the charge on the liposomes. To test this possibility titrations were performed on cationic and anionic liposomes (Fig. 2 *B*). In cationic liposomes the titration curve is shifted to the left, which is consistent with the negatively charged 1:2 complex associating more strongly with the membrane than in the case of zwitterionic liposomes, and, because of the increased shielding from water, experiences an increase in quantum yield. Cationic liposomes would be expected to decrease the $Zn^{2+}$ concentration in the electric double layer, therefore it is unlikely that the liposomes exert their effect directly on the $Zn^{2+}$. Titrations with anionic liposomes are consistent with the negative charge on the phospholipid headgroups militating against the 1:2 complex partitioning into the bilayer, while allowing passage of the 1:1 complex into the bilayer (Fig. 2 *B*).

To estimate the stoichiometry of the complexes formed, Job's plots (Fig. 3) were performed in liposomal solutions with different headgroups to estimate the influence of membrane charge on speciation. In anionic liposomes the plots were indicative of a 1:1 stoichiometry, while the cationic and zwitterionic liposomes appeared to give mixtures of the 1:1 and 1:2 complexes.

## Fluorescence relaxation of Zinquin

The form of the excitation-emission curves in liposomes at different $Zn^{2+}$ concentrations showed no discernible differences, indicating that steady-state spectral characteristics of the 1:1 and 1:2 complexes were indistinguishable (data not shown). Fluorescence relaxation times provide a more sensitive measure of the chemical environment about the fluorophore than do steady-state spectra. The relaxation spectra of Zinquin and TFLZn were determined in aqueous solution using frequency domain techniques (Lakowicz et al., 1984) (Fig. 5). The frequency domain data were fitted to a variety of models that included both discrete and distributed lifetimes (Gaussian) models (see Methods for details).

Under conditions that favor the formation of a 1:2 complex (10 $\mu$M Zinquin and 1 $\mu$M $Zn^{2+}$) in aqueous solution, a single relaxation time (4.6 ns) was observed. Increasing the $Zn^{2+}$ concentration to 100 $\mu$M led to the development of an additional component (18 ns), probably arising from the 1:1 complex. TFLZn exhibited a single relaxation time (1.8 ns) at mole ratios of $Zn^{2+}$ to TFLZn of 0.1 and 10 (data not shown), implying that only the 1:2 complex was formed in significant amounts in aqueous solution.

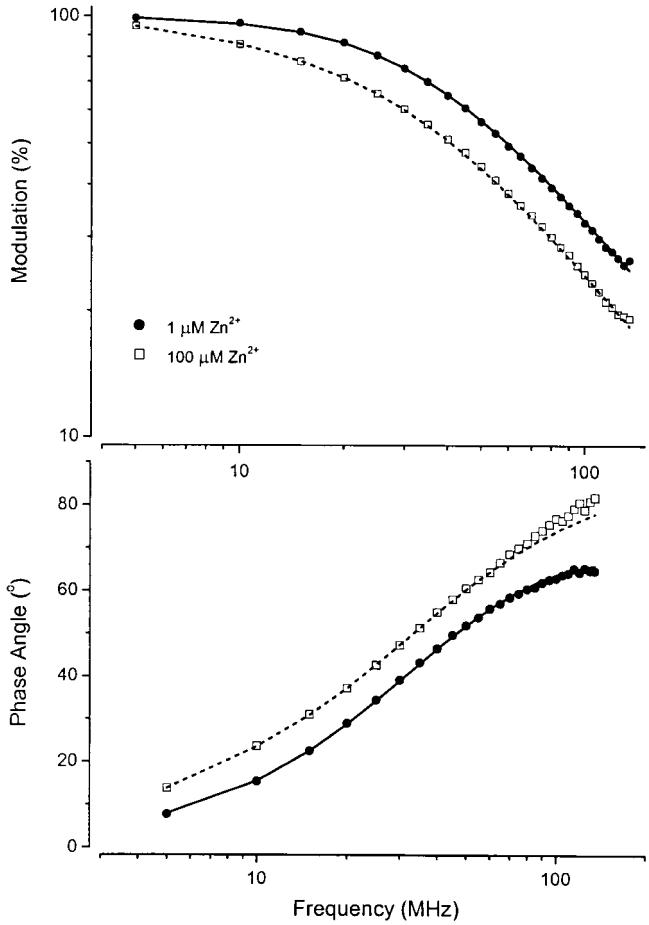


FIGURE 5 Frequency domain relaxation spectra of Zinquin (10 $\mu$M) in aqueous solutions with different mole ratios of $Zn^{2+}$ to Zinquin. For 1 $\mu$M $Zn^{2+}$ $\tau$ = 4.6 ns and amplitude ($\alpha$) = 0.95; a faster relaxation was also observed that could be attributed to scattering. For 100 $\mu$M $Zn^{2+}$ $\tau_1$ = 5.1 ns, $\alpha_1$ = 0.75 and $\tau_2$ = 18.2 ns, $\alpha_2$ = 0.25.

## Zinquin passage across bilayers

We have shown previously that TFLZn (Budde et al., 1997) in the free acid form crosses membranes without the need for an acetoxymethyl ester group to render the molecule membrane-permeant. As shown above, the same held true for Zinquin. Unilamellar liposomal preparations were used to determine the mechanism of Zinquin's passage across membranes. Liposomes were formed in the presence of $Zn^{2+}$ or $Mg^{2+}$ and then diluted in saline to produce a divalent ion concentration gradient. Addition of 1 $\mu$M Zinquin to a liposomal solution containing 100 $\mu$M $ZnCl_2$ inside the liposomes and 1 mM EDTA extracellularly to reduce the extraliposomal $Zn^{2+}$ concentration led to a relatively slowly developing increase in fluorescence, with no decrement of fluorescence observed over a period of 45 min (Fig. 6 *A*). Addition of the membrane-permeant $Zn^{2+}$ chelator TPEN (Arslan et al., 1985) quenched the fluorescence, while no signal was observed when the liposomes were preincubated with TPEN (data not shown), supporting the notion that the dye crosses the membrane to interact with intraliposomal $Zn^{2+}$. These results are consistent with Zinquin diffusing across the membrane and forming a complex with $Zn^{2+}$ that does not cross the membrane and does not serve as a $Zn^{2+}$ ionophore. When the same experiments were performed with higher Zinquin concentrations, as expected the fluorescence rose more rapidly on addition of the probe; however, after reaching a peak the fluorescence declined monotonically at a rate proportional to the amount of Zinquin added. The decline suggests that at higher concentrations of Zinquin the probe can act as an ionophore, shuttling $Zn^{2+}$ out of the liposomes, where it is then chelated by EDTA. The decline does not result from self-quenching, as in the absence of EDTA. Addition of 100 $\mu$M Zinquin led to a sustained increase in fluorescence that did not decline like that in Fig. 6 *A* (data not shown).

To further explore Zinquin's action as an ionophore, the same experiments as described in the preceding paragraph were performed in liposomes bearing different charged headgroups. In anionic liposomes, addition of Zinquin at high and low concentrations led to a slow monotonic increase of fluorescence that was sustained for 45 min (Fig. 6 *B*). The slowness of the increase is expected from the unfavorable interaction between the Zinquin free acid and the negatively charged headgroups. The absence of an indication of $Zn^{2+}$-shuttling is consistent with the anionic headgroups impeding the movement of the negatively charged 1:2 complex across the membrane.

The situation was reversed in the case of cationic liposomes (Fig. 6 *C*). Now at high Zinquin/$Zn^{2+}$ ratios, ionophoresis was enhanced, consistent with the favorable charge interaction between the 1:2 complex and the positively charged headgroups. Note that the high levels of fluorescence evident in the case of 100 $\mu$M Zinquin after the addition of TPEN show that the uncomplexed free Zinquin has a relatively high level of intrinsic fluorescence in cationic liposomes, again consistent with charge driving the probe into the membrane.

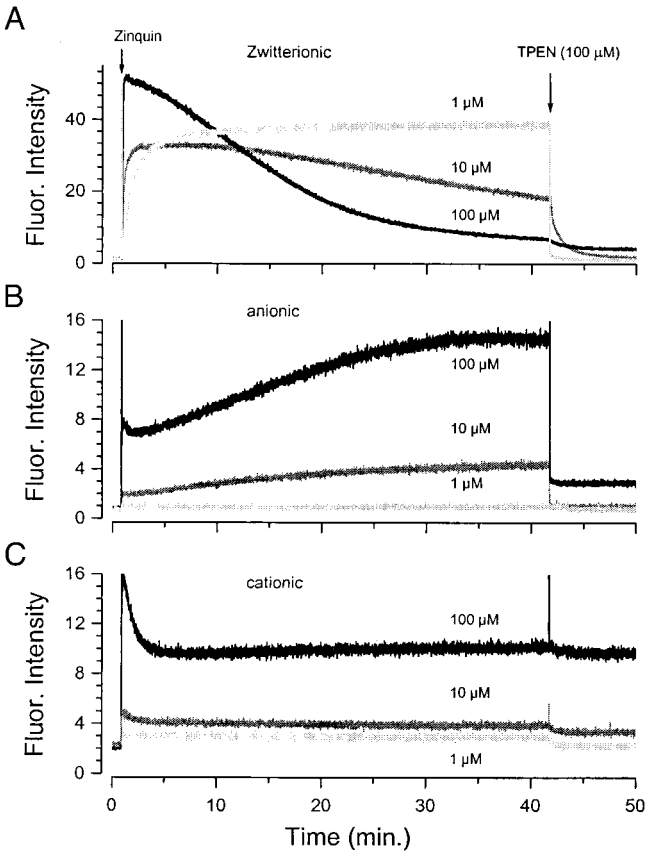


FIGURE 6 Passage of Zinquin across liposomal membranes containing $Zn^{2+}$. Liposomes were used that contained 100 $\mu$M $ZnCl_2$ inside and 10 $\mu$M outside; 1 mM NaEDTA was added at time 0. Zinquin was added at different concentrations (indicated near the traces) at the time indicated by the arrow. Lipids as in Fig. 2.

## Effect of transliposomal pH gradients

If the 1:2 complex acts as a zinc ionophore, it is expected that for each $Zn^{2+}$ ion moved across the membrane two protons will be transported in the opposite direction (see Fig. 8). If a proton gradient is set up in the same direction as the $Zn^{2+}$ gradient, it should retard the movement of $Zn^{2+}$ across the membrane. This was found to be the case. If a proton gradient of 7.4/5.5 (outside/inside) was set up across liposomes, containing 100 $\mu$M $Zn^{2+}$, the ionophoric activity of Zinquin was abolished (Fig. 7 *A*). This does not result from a change of the state of protonation of the lipids, as a pH of 5.5 on both sides of the liposomes did not inhibit $Zn^{2+}$ ionophoresis (data not shown). That the pH gradient is the essential factor was demonstrated by the abolition of $Zn^{2+}$ transport by a pH gradient of 9.6/7.4 (Fig. 7 *B*). The slower entry of Zinquin into the liposomes at pH 9.6 may

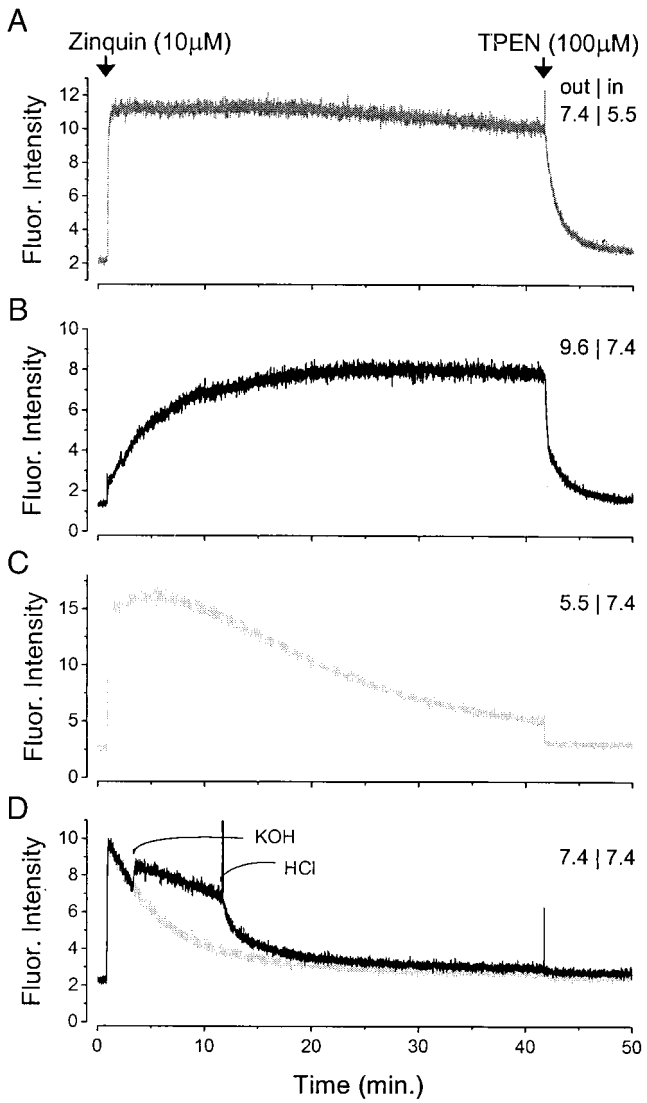


FIGURE 7 Influence of transmembrane pH gradient on $Zn^{2+}$ flux. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine liposomes with 100 $\mu$M $ZnCl_2$ and 10 $\mu$M extraliposomal $ZnCl_2$; 1 mM NaEDTA was added at time 0. Liposomes were formed with different intravesicular pH values as indicated on the right of the panels (inside/outside pH). (*D*) KOH: 21 mM KOH added; HCl: 21 mM HCl added. Gray line, control (7.4/7.4).

result from the deprotonation of the amide nitrogen, resulting in reduced lipid solubility. As expected, a pH gradient (5.5/7.4) counter to the $Zn^{2+}$ gradient did not stop $Zn^{2+}$ transport (Fig. 7 *C*). It is unlikely that the trapping of Zinquin by a pH gradient could result from changes in the state of protonation of the phospholipid phosphate group, as its $pK_a$ is $< 1$. Addition of KOH to liposomes with no pH gradient retarded the rate of $Zn^{2+}$ efflux, while the subsequent addition of HCl accelerated it (Fig. 7 *D*).

The 7.4/5.5 pH gradient is close to that encountered in synaptic vesicles, and this could account for the absence of any sign of $Zn^{2+}$ transport in synaptic terminals (Fig. 4 *B*).

## DISCUSSION

Provision of the acid form of Zinquin in the solution bathing hippocampal slices leads to the clear delineation of synaptic terminals replete with free zinc. That this pattern develops without the need for an acetoxymethyl or ethyl ester derivative suggests that the probe is able to passively cross the membrane. Our experiments with unilamellar liposomes confirmed that Zinquin is indeed able to cross lipid bilayers by simple diffusion, and that the provision of an acetoxymethyl or ethyl ester is unnecessary.

Fig. 8 shows our proposed mechanism for the transport of $Zn^{2+}$ across membranes by Zinquin. $Zn^{2+}$ binds to Zinquin, displacing a proton and the complex that has no net charge, then partitions into the membrane. This 1:1 complex then interacts with a free Zinquin molecule, forming a complex with a charge of $-2$, releasing one more proton. The 1:2 complex moves to the other side of the membrane, releases the $Zn^{2+}$ ion, and then each Zinquin binds two protons. The net effect of this mechanism is to move one $Zn^{2+}$ ion across the membrane and two protons in the opposite direction. The mechanism also accounts for why a pH gradient might be expected to limit $Zn^{2+}$ passage and for the influence of the charge on the headgroup on $Zn^{2+}$ ionophoresis.

We hypothesize that the charged 1:2 complex is located within the electrical double layer, perhaps with hydrophobic portions dipping into the bilayer. The complex is likely to be partially shielded from interactions with water molecules, minimizing quenching and boosting its quantum yield. It is likely that the 1:1 complex inserts into the membrane having no charge.

It is difficult to access intracellular membrane compartments, such as synaptic vesicles, with fluorimetric probes.

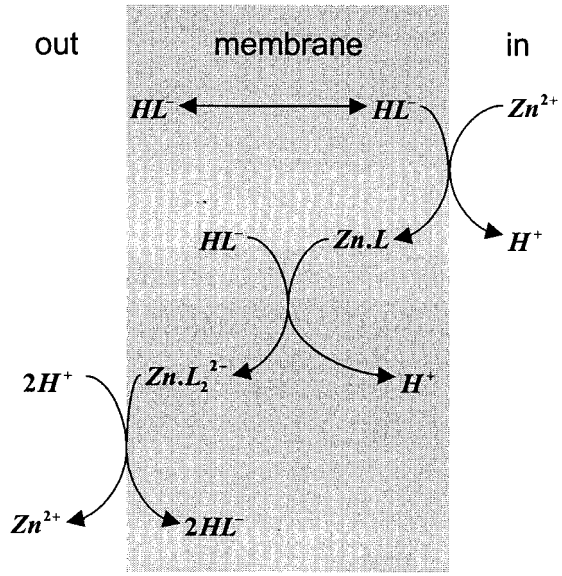


FIGURE 8 A possible mechanism for Zinquin-induced $Zn^{2+}$ passage across a bilayer. $HL^-$ is Zinquin in the state depicted in Fig. 1.

AM probe derivatives are of little value for such compartments because the probe becomes trapped in the cytoplasm. The carboxylated quinoline sulfonamide probes, Zinquin and TFLZn, permeate freely across membranes and render the high $Zn^{2+}$ concentration in synaptic vesicles visible.

It might be thought that Zinquin could remove $Zn^{2+}$ from zinc-binding proteins and shuttle $Zn^{2+}$ into vesicles. However, Zinquin only stains terminals that are known to be marked by Timm's stain, which only highlights a specific subpopulation of glutamatergic terminals. If Zinquin were stripping $Zn^{2+}$ from proteins in an indiscriminant fashion, then all somata and nuclei, which have a higher total $Zn^{2+}$ concentration than terminals (Frederickson, 1989), should be stained. The fact that this does not occur suggests that Zinquin specifically highlights terminals with high free-zinc concentrations, and only minimally, if at all, perturbs the status of $Zn^{2+}$ bound to proteins.

The sensitivity of Zinquin is an issue of some interest, as it essentially determines the minimum $Zn^{2+}$ concentration that can be detected. For synaptic vesicles with a diameter of 50 nm (Peters and Palay, 1996), a single $Zn^{2+}$ ion per vesicle translates into a concentration of 25 $\mu$M, setting a lower limit to the vesicular $Zn^{2+}$ concentration. Fahrni and O'Halloran (1999), on the basis of their measurements, have suggested that Zinquin could detect $Zn^{2+}$ at a concentration of 4 pM. This assumes that $Zn^{2+}$ is buffered, i.e., that there is sufficient chelated $Zn^{2+}$ available, to saturate Zinquin without perturbing the free $Zn^{2+}$ concentration. That this should obtain is by no means self-evident; it could indeed be the case that the cellular $Zn^{2+}$ chelators are so avid as to prevent abstraction by Zinquin. The case is most acute in the diminutive synaptic vesicles, where Zinquin might rapidly deplete the free $Zn^{2+}$.

Zinquin is a useful probe for making qualitative observations on free zinc in live preparations, with the proviso that changes in intensity my result from changes in intravesicular pH. The quinoline sulfonamide moiety provides admirable selectivity for $Zn^{2+}$ against calcium and magnesium, while being quenched by copper and iron, and may thus serve as a useful starting point for refining $Zn^{2+}$ probes without the drawbacks of the current generation of probes.

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