Concentration-Dependent Staining of Lactotroph Vesicles by FM 4-64

Matjaž Stenovec,* Igor Poberaj,† Marko Kreft,*‡ and Robert Zorec*‡
*Celica Biomedical Sciences Center, 1000 Ljubljana, Slovenia; ‡Laboratory of Neuroendocrinology-Molecular Cell Physiology, Medical School, University of Ljubljana, 1000 Ljubljana, Slovenia; and †Department of Physics, Faculty of Mathematics and Physics, University of Ljubljana, 1001 Ljubljana, Slovenia

ABSTRACT Hormones are released from neuroendocrine cells by passing through an exocytotic pore that forms after vesicle and plasma membrane fusion. An elegant way to study this process at the single-vesicle level is to use styryl dyes, which stain not only the membrane, but also the matrix of individual vesicles in some neuroendocrine cells. However, the mechanism by which the vesicle matrix is stained is not completely clear. One possibility is that molecules of the styryl dye in the bath solution dissolve first in the plasma membrane and are then transported into the vesicle by lateral diffusion in the plane of the membrane, and finally the vesicle matrix is stained from the vesicle membrane. On the other hand, these molecules may enter the vesicle lumen and reach the vesicle matrix by permeation through an open aqueous fusion pore. To address these questions, we exposed pituitary lactotrophs to different concentrations of FM 4-64 to monitor the fluorescence increase of single vesicles by confocal microscopy after the stimulation of cells by high K^+. The results show that the membrane and the vesicle matrix exhibit different concentration-dependent properties: the plasma membrane staining by FM 4-64 has a higher affinity in comparison to the vesicle matrix. Moreover, the kinetics of vesicle loading by FM 4-64 exhibited a concentration-dependent process, which indicates that FM 4-64 molecules stain the vesicle matrix by aqueous permeation through an open fusion pore.

INTRODUCTION

Exocytosis represents the fusion of the vesicle and the plasma membrane, an essential step in the process of neuronal and neuroendocrine release of neurotransmitters and hormones. A great deal about this process at the single-vesicle level was acquired by using electrophysiological, amperometric (Neher and Marty, 1982; Neher, 1998), and optical methods, in particular those employing the styryl dyes, such as FM 1-43, to track vesicle recycling (Cochilla et al., 1999). These dyes are amphiphilic and fluoresce when inserted in a hydrophobic environment, such as the plasma membrane, but cannot cross the membrane bilayer. Therefore, an increase in the cell membrane area can readily be reported by these dyes (Neves and Lagnado, 1999). In addition to labeling the membrane, in some cells these dyes also stain the matrix of individual vesicles (Angleson et al., 1999). However, the mechanism by which the vesicle matrix is stained, when a fusion pore forms between the docked vesicle and the plasma membrane, is not entirely clear.

At least two mechanisms have been considered in the past. One possibility is that styryl dye molecules may be transported into vesicles by lateral diffusion in the plane of the membrane (Takahashi et al., 2002), although the dye transport within the membrane into the vesicle is restricted due to high local curvature (Tse et al., 1993). Once the dye molecules are in the vesicle membrane, they can cross to the vesicle matrix. On the other hand, it is also possible that the dissolved styryl dye molecules in the extracellular solution may stain the vesicle matrix directly by aqueous permeation through an open fusion pore. To distinguish between the two mechanisms, Takahashi et al. (2002) studied vesicles in β-cells at different concentrations of the styryl dye in the bath. They found that the rate of FM 1-43 loading into individual vesicles was independent of FM 1-43 concentration, contrary to what one would expect if aqueous permeation of FM 1-43 molecules into vesicles is the mechanism of vesicle staining. Hence they concluded that β-cell vesicles are loaded with FM 1-43 by lateral diffusion in the plane of the membrane.

By using confocal microscopy, we here used a similar approach to test the mechanism of styryl dye loading into the vesicle matrix of pituitary lactotrophs. These cells secrete prolactin and appear to have ~100 vesicles morphologically docked to the plasma membrane (Angleson et al., 1999; Cochilla et al., 2000; Bauer et al., 2004; Stenovec et al., 2004). It was shown previously that staining cells with styryl dyes allows the monitoring of prolactin secretion from a single vesicle, since the release from a single vesicle is associated with a rapid increase in vesicle fluorescence due to staining of the vesicle membrane and the vesicle matrix (Angleson et al., 1999). The increase in styryl dye fluorescence is also associated with a loss of fluorescently labeled peptide hormone preloaded into prolactin-containing vesicles (Stenovec et al., 2004). Here we show that the FM 4-64 staining of the plasma membrane and the vesicle matrix exhibit distinct properties. Moreover, the results show that the kinetics of vesicle loading by FM 4-64 is concentration-dependent. These results indicate that in stimulated cells, the vesicle matrix is stained mainly by aqueous diffusion of FM 4-64 molecules through an open fusion pore.

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Address reprint requests to Robert Zorec, E-mail: robert.zorec@mf.uni-lj.si.

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METHODS

Cell cultures and confocal microscopy

Pituitaries were obtained from male Wistar rats after decapitation that followed CO_2 anesthesia. The animals were sacrificed in accordance to the following ethical codes and directives: International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences, and Directive on Conditions for Issue of License for Animal Experiments for Scientific Research Purposes (Official Gazette of the Republic of Slovenia, Nos. 40/85 and 22/87). Isolated pituitaries were enriched for lactotrophs as described (Ben-Tabou et al., 1994). Cell-loaded coverslips were placed into a recording chamber on an inverted confocal microscope (Zeiss LSM 510, Jena, Germany) and supplied with 400 μl of extracellular bathing solution containing appropriate concentration of FM 4-64 (Molecular Probes, Leiden, The Netherlands).

The apparent FM 4-64 binding properties of the cell surface membrane and the vesicles were examined on stimulated cells that were kept in K^+-enriched solution initially containing 0.1 μM FM 4-64. Subsequently, FM 4-64 dye concentration was increased in a stepwise manner by a series of bolus applications of the dye (stocks of FM 4-64 of 10–250 μM prepared in the extracellular solution). Increments in FM 4-64 concentration were made after 5 min of the equilibration of the bolus in the bathing solution. The recordings were made during the last minute of the cell exposure to a particular FM 4-64 concentration. Fluorescent images were acquired by a plan-apochromatic oil immersion objective (63×, 1.4 numeric aperture) by using 488 nm Ar-Ion laser excitation and filtered at long pass 580 nm. In kinetic experiments, the rate of image acquisition was increased to 300 ms per frame. Images were quantitatively analyzed by using LSM 510 software (Zeiss). A circular field (diameter 14 pixels) was positioned over different regions of the stained plasma membrane alone and over the plasma membrane with a vesicle. The average fluorescence intensity of such an image was measured as a function of time at a particular FM 4-64 dye concentration.

Solutions

Extracellular solution contained (in mM): NaCl 130, KCl 5, $CaCl_2$ 8, $MgCl_2$ 1, D-glucose 10, HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] 10, and pH 7.2/NaOH. K^+-enriched solution contained (in mM) NaCl 35, KCl 100, $CaCl_2$ 8, $MgCl_2$ 1, D-Glucose 10, HEPES 10, and pH 7.2/NaOH. Chemicals were obtained from Sigma (Darmstadt, Germany) and were of highest purity grade.

Modeling

To model the fluorescence intensity increase in stimulated fusion events, we considered the geometry of the fusion pore, the vesicle, and the matrix inside the vesicle. When the fusion pore opens, dye molecules start to move through the pore into the vesicle. They become experimentally clearly visible after they enter the matrix. To explain the observed dependence of the rate of fluorescence intensity increase as a function of external dye concentration C_0, we considered the following two-step model of dye transport from external solution into the vesicle matrix. In the first step, dye molecules diffuse through a fusion pore, increasing their concentration around the vesicle matrix. While in the proximity of vesicle matrix, the next step follows: dye molecules penetrate into the vesicle matrix and their number within the matrix increases. The intensity of fluorescence signal grows correspondingly until saturation is eventually reached.

In this model, there are two rate-limiting mechanisms. The first one is the Fickian dye diffusion through the fusion pore, which is concentration-dependent. The second one is the dye transfer into the vesicle matrix, which is distinct from the dye diffusion in the solution. The vesicle matrix in lactotrophs consists of densely aggregated molecules of prolactin, which can be isolated by centrifugation as insoluble particles (Giannattasio et al., 1975). The structure of these aggregates appears not to be amorphous and seems to be formed by oligomerization of prolactin molecules involving specific sites (Keeler et al., 2003).

Therefore, it is reasonable to suppose that the migration of dye molecules into the matrix is a much more complicated process than the simple Fickian diffusion. Dye molecules can enter the matrix structure through a limited number of surface entry sites. Once a dye molecule is docked on the matrix surface, it can migrate into the matrix through a mesh of tiny pores/pathways. Such a transport can be described in terms of a single-file-diffusion, studied previously from experimental (Lutz et al., 2004) and theoretical (Schutz, 2003, and references therein) points of view. An additional factor that may influence the transport of the dye molecules within the matrix is the dye molecule interaction with the matrix itself. In both cases, the rate-limiting dye influx into the vesicle matrix is governed mainly by the properties of the dye transport mechanism(s) within the matrix and not by the external dye concentration. The dye influx is proportional to the number of occupied entry sites on the matrix surface, and its maximum value is limited by the total number of entry sites on the matrix surface.

Fluorescence intensity $I(t)$ is proportional to the total number of dye molecules within the matrix. In the first approximation, it grows linearly with time

$$I(t) = \eta\alpha N t, \tag{1}$$

where η is fluorescence quantum efficiency, α represents the transport coefficient through the vesicle matrix, and N is the number of occupied entry sites at the surface of the matrix. The maximum initial fluorescence intensity increase rate is given by a derivative of Eq. 1 and is limited by the number of available surface binding sites as has already been shown above. To obtain a relation between fluorescence intensity and dye concentration, we assume that the constants η and α do not depend on the dye concentration. The number of occupied surface states N is determined by chemical equilibrium between unbound dye molecules in solution and bound molecules at the surface of the matrix. It is given by

$$\frac{(N_0 - N)C}{N} = \beta, \tag{2}$$

where β is a dye binding constant, N_0 the total number of available binding sites on the matrix surface, and C the dye concentration in a solution at the matrix surface. Dye concentration C can be estimated from a rate equation

$$\frac{dC}{dt} = \mu(C_0 - C) - \alpha N, \tag{3}$$

where C and C_0 are dye concentrations within the vesicle (more precisely at the vesicle matrix surface) and external concentration, respectively; μ is the transport coefficient through a fusion-pore, α is the transport coefficient through the vesicle matrix, and N is the number of occupied states. The first term on the right side of Eq. 3 represents the diffusive current of dye molecules through the fusion pore into the vesicles. Diffusion constant, pore dimensions, and any necessary correction for pore geometry are lumped together into transport coefficient μ. The second term in Eq. 3 represents the transport of molecules into the matrix. The magnitude of this transport of molecules is taken to be proportional to the number of occupied dye entry sites at the surface of the matrix N with a proportionality transport coefficient α.

The volume of the liquid between the matrix and the vesicle wall is tiny compared to the total volume of the vesicle (Angleson et al., 1999). After fusion-pore opening, equilibrium concentration determined by Eq. 3 is quickly established. In equilibrium, the time derivative in Eq. 3 is zero. The stationary number of occupied binding sites N_{st} is given by

$$N_{st} = \left(\frac{1}{2N_0} \left(1 + \frac{\beta + \frac{\alpha N_0}{\mu}}{C_0} + \sqrt{\frac{4\beta}{C_0} + \left(\frac{\beta + \frac{\alpha N_0}{\mu}}{C_0} - 1 \right)^2} \right) \right)^{-1}. \tag{4}$$

From Eqs. 1 and 4, one can calculate fluorescence rise time ($t_{20–80\%}$) and its maximum time derivative. Both quantities are experimentally measurable, and can be used as a direct verification of agreement between experimental results and theoretical predictions.

The fitting functions

Experimentally measured dependencies of rise times $t_{20–80}$ on extracellular dye concentrations C_0 were fitted with the following function obtained from Eqs. 1 and 4:

$$t_{20-80} = P_1\left(1+\frac{P_2}{C_0}+\sqrt{\frac{P_3}{C_0}+\left(\frac{P_2}{C_0}-1\right)^2}\right). \quad (5)$$

The fitting function contains three independent fitting parameters: P_1, P_2, and P_3. P_1 is the scaling parameter. Parameters P_2 and P_3 are combinations of the above-described constants and are given by $P_2 = \beta+(\alpha N_0/\mu)$and $P_3 = 4\beta$, respectively. A similar procedure was used for fitting maximum fluorescence intensity increase rates. We used the fitting function given by

$$\frac{dI}{dt} = P_1'\left(1+\frac{P_2}{C_0}+\sqrt{\frac{P_3}{C_0}+\left(\frac{P_2}{C_0}-1\right)^2}\right)^{-1} \quad (6)$$

with the same fitting parameters P_2 and P_3 as above and a scaling parameter P_1'.

RESULTS

To study the mechanisms of styryl dye loading into single vesicles, lactotrophs were stimulated by exposing them to extracellular solution containing 100 mM K^+. As reported previously, this resulted in the appearance of bright spots on the plasma membrane perimeter (Fig. 1, *right*), which are due to FM 4-64 wash-resistant, apparently homogeneous staining of individual vesicles docked to the plasma membrane (Angleson et al., 1999; Stenovec et al., 2004). After high K^+ bath application, the FM 4-64 stained only vesicle like puncta close to the plasma membrane, which contain peptide hormones (Fig. 1; see Stenovec et al., 2004). Lower panels (Fig. 1) show single-vesicle fluorescence intensity, recorded with 10 μM FM 4-64, at the time of applying bathing solution containing high K^+ (*left*) and 70 s later (*right*). To determine the concentration-dependence of single-vesicle fluorescence intensity, we exposed cells to different FM 4-64 concentrations. The fluorescence intensity of single-vesicle images was compared to the fluorescence intensity of the same size images of the membrane only. Panels *A* and *B* in Fig. 2 show lines at which the intensity profiles at different concentrations of FM 4-64 were obtained. The line intensity profiles revealed a fluorescence intensity increase as a function of concentration increase (Fig. 2, *C* and *D*). The intensity increase was larger in vesicle profiles in comparison to those drawn through the membrane only. This may be due to a larger membrane area acquired due to the vesicle membrane fused to the plasma membrane, and/or due to the staining of the vesicle content, such as the vesicle matrix, as well.

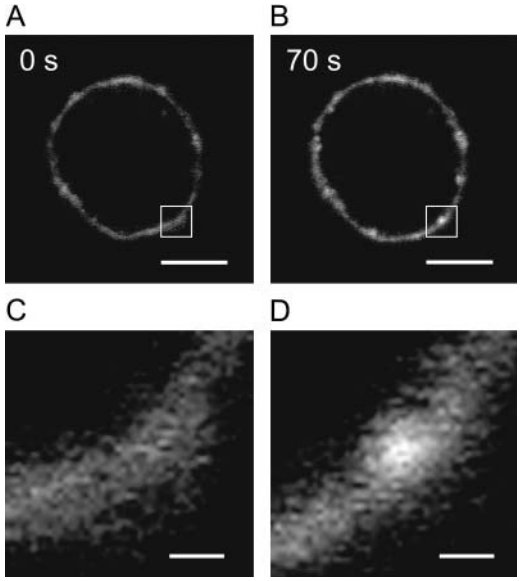

FIGURE 1 Staining of lactotrophs with styryl dye FM 4-64 before and after depolarization. Confocal images of isolated lactotroph stained with FM 4-64 (10 μM). Top images show FM 4-64 stained before (0 s, *A*) and 70 s after (*B*) bath application of 100 mM K^+. Note that the cell membrane has only a few bright fluorescent spontaneously present spots. However, after K^+ stimulation, the number of these spots, representing individual vesicles, is increased. Scale bar = 5 μm. Lower panels are enlarged portions of panels *A* and *B*, showing details of the stained cell membrane and the vesicle before (*C*) and after K^+ stimulation (*D*). Scale bar = 0.5 μm.

The membrane area monitored in the images equals 4.00 μm^2. By assuming a lactotroph vesicle diameter of 200 nm (Angleson et al., 1999), with an area of 0.12 μm^2, one can expect an increase in fluorescence intensity of ~3% upon the fusion of the vesicle with the plasma membrane. Since the recorded fluorescence intensity after vesicle fusion is from ~2- to 3-fold higher than this (Figs. 2 and 3 *A*), it is likely that in addition to the vesicle membrane, FM 4-64 also stains something else in the vesicle lumen, probably the vesicle matrix, as described earlier (Angleson et al., 1999).

Fig. 3 plots the fluorescence intensity in images of membrane only (Fig. 3 *A*), and in images with a vesicle and the plasma membrane (Fig. 3 *B*) exposed to increasing concentrations of FM 4-64. The solid lines represent best fits to the first-order kinetics equation: $I = I_{max} \times C/(K_D + C)$, where I and I_{max} denote the intensity in arbitrary units (AU), C the FM 4-64 micromolar concentration, and K_D the FM 4-64 micromolar concentration at which the fluorescence intensity is half-maximal. The fitting of the data revealed that the apparent binding of FM 4-64 to membrane alone exhibits a higher apparent affinity ($K_D = 0.5 \pm 0.1$ μM) in comparison to the vesicle and the plasma membrane together ($K_D = 2.6 \pm 0.8$ μM). Moreover, assuming that the FM 4-64 quantum yield in the membrane and vesicle environment is similar, the significantly higher I_{max} of 1194 ± 158 AU indicates that there is a two- to threefold higher binding capacity for FM 4-64 molecules in the vesicle/membrane

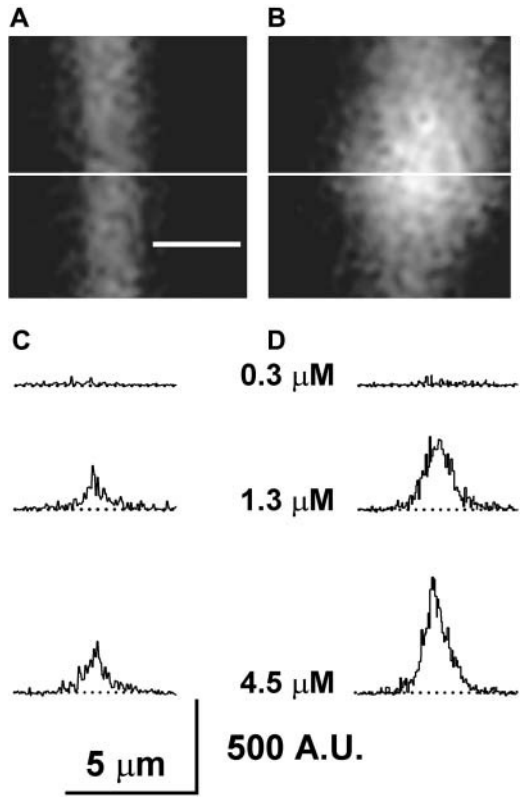

FIGURE 2 Properties of stained cell plasma membrane by FM 4-64 with and without a stained vesicle. Confocal images showing detailed FM 4-64 staining of the cell plasma membrane (*A*) and the vesicle with the plasma membrane (*B*). Thin lines, perpendicular to the plasma membrane, indicate the position at which the fluorescence intensity profiles (see panels *C* and *D*) have been obtained. Scale bar = 1 μm. The fluorescence intensity profiles of the plasma membrane only (*C*) and of the plasma membrane with the vesicle (*D*) obtained at different FM dye concentrations are indicated in the middle of the panels. Note that the fluorescence intensity profile of the association of the membrane with the vesicle is two- to threefold higher in comparison to the intensity of the plasma membrane alone. Moreover, it appears to have a considerably broader space distribution in comparison to the profile of the cell surface membrane alone. Dotted lines represent 0 AU.

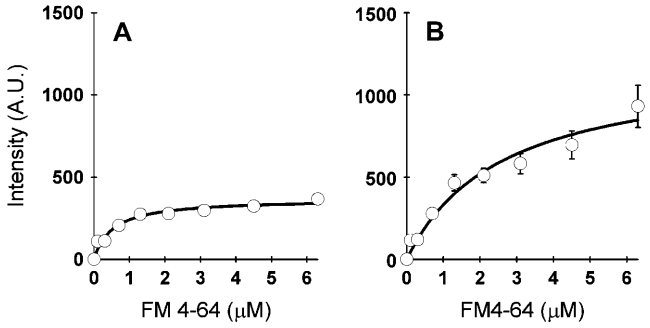

FIGURE 3 Concentration-dependent staining by FM 4-64 of the plasma membrane alone and of the vesicle and the plasma membrane together. The average FM 4-64 fluorescence intensity (mean ± SE, *open symbols*) of the plasma membrane alone (*A*, $n = 8$) and of the vesicle with the plasma membrane (*B*, $n = 4$) at different FM 4-64 concentrations. Fluorescence data were fitted to the first-order kinetics equation $I = I_{max} \times C/(K_D + C)$ (I, intensity in AU; I_{max}, maximal intensity in AU; C, FM 4-64 micromolar concentration; K_D, FM 4-64 dye micromolar concentration at which the fluorescence intensity is half maximal) to obtain an apparent $K_D = 0.5 \pm 0.1$ μM and $I_{max} = 366 \pm 22$ AU for the plasma membrane and an apparent of $K_D = 2.6 \pm 0.8$ μM and $I_{max} = 1194 \pm 158$ AU for the vesicle and the plasma membrane together, respectively.

association in comparison to the binding capacity in the plasma membrane alone (I_{max} of 366 ± 22 AU).

Next we examined the time-course of vesicle staining to test whether the delivery of FM 4-64 molecules to vesicles is indirect by lateral diffusion in the plane of the membrane (Takahashi et al., 2002). Once the dye molecules are in the vesicle membrane, they can cross to the vesicle matrix. On the other hand it is also possible that the styryl dye molecules dissolved in the extracellular solution may enter the vesicle matrix directly by aqueous permeation through an open fusion pore. To distinguish between the two mechanisms, Takahashi et al. (2002) studied β-cells, and found that the rate of FM 1-43 loading into individual vesicles was independent of dye concentration. If FM 1-43 molecules enter into vesicles by aqueous permeation through an open fusion pore, a concentration-dependent kinetics of vesicle staining is expected (Takahashi et al., 2002). Hence they concluded that β-cell vesicles are loaded with FM 1-43 by lateral diffusion in the plane of the membrane.

Representative time-dependent changes of vesicle staining by FM 4-64 are shown in (Fig. 4 *top panels*). Maximal staining is attained much faster in stimulated lactotrophs at 4 μM in comparison to that recorded at 2 μM FM 4-64. Measurements of the time required for a 20–80% change of the fluorescent signal increase revealed that the dye loading was 2.9 s at 4 μM and 14.1 s at 2 μM FM 4-64, respectively. In agreement with this, the maximal time derivative of fluorescence intensity increase was also strongly dependent on FM 4-64 concentration (Fig. 4, *bottom panels*). To further verify that the rate of vesicle staining is a function of FM 4-64 concentration, the time course of vesicle staining was studied in a range of FM 4-64 concentrations. The average results are plotted in Fig. 5, clearly showing that the time required for a 20–80% change and the rate of fluorescence intensity are more rapid at higher FM 4-64 concentrations. Lines in Fig. 5 (*top* and *bottom*) represent best fits to Eqs. 5 and 6. The derivative was fitted with P_1 fixed. Its value was determined from a derivative magnitude at high concentrations (it was taken to be two times the derivative value at 30 μM). Other two parameters were fitted. The rise time was also fitted with P_1 fixed. However, the fitting procedure did not converge if both P_2 and P_3 were fitting parameters. We have made P_3 constant and equal to the one obtained from the derivative fit. Then, P_2 was fitted and approximately the same result was obtained as in the previous case. The results indicate that two processes determine the loading of vesicles by FM 4-64: the concentration-dependent component reflects the concentration-dependent diffusion of FM 4-64 molecules through an open fusion-pore, whereas the

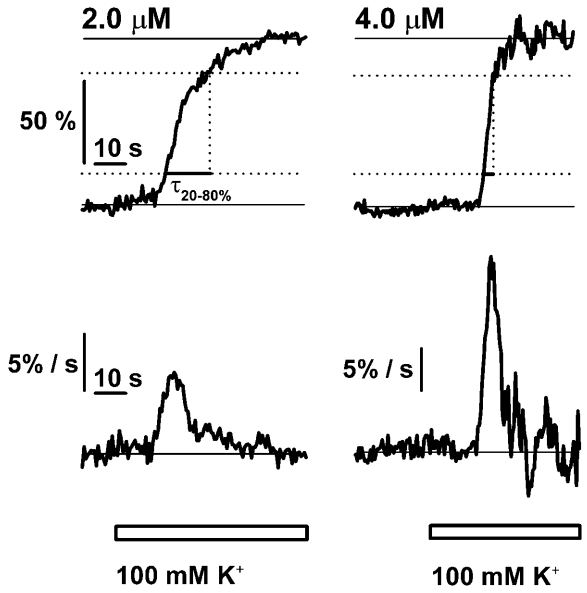

FIGURE 4 Kinetics of single-vesicle loading with FM 4-64. Normalized time-dependent FM 4-64 fluorescence intensity increase in a single vesicle at 2 μM (*top left*) and at 4 μM (*top right*). Fluorescence intensity was measured relative to the same sized image of the plasma membrane only. Note the slower FM 4-64 fluorescence intensity increase when the cell is stimulated by K^+-enriched solution containing 2 μM FM 4-64 dye (*left*) in comparison to the more rapid FM 4-64 fluorescence intensity increase when the cell is stimulated by a solution containing 4 μM FM 4-64 (*right*). The traces represent two typical responses recorded in two different cells. The thin solid lines represent the basal level of fluorescence intensity. Bottom panels show time derivatives of the signals shown on top panels. Note that the maximal derivative of the signal obtained at 2 μM FM 4-64 (*bottom left*) is at least twofold smaller than the one obtained at 4 μM FM 4-64 (*bottom right*).

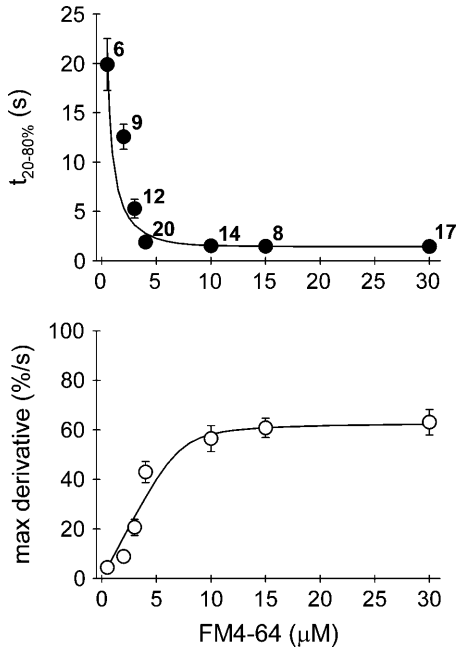

FIGURE 5 Concentration dependence of the average rise time ($t_{20-80\%}$ (s)) and the maximal derivative (%/s) of single-vesicle loading by FM 4-64. Solid circles represent the average rise time of FM 4-64 fluorescence intensity (mean ± SE) determined as the time of amplitude increase from 20 to 80% of its maximal level ($t_{20-80\%}$) at different FM 4-64 concentrations. Open circles represent the maximal derivative of FM 4-64 fluorescence intensity increase (mean ± SE) at different FM 4-64 concentrations. Note the reduction of the FM 4-64 fluorescence intensity rise time as the concentration of FM 4-64 is increased. The solid lines represent the best fits to the rise-time data ($t_{20-80} = P_1(1+(P_2/C_0)+\sqrt{(P_3/C_0)+((P_2/C_0)-1)^2})$) and the maximal derivative data ($((dI/dt) = P_1'(1+(P_2/C_0)+\sqrt{(P_3/C_0)+((P_2/C_0)-1)^2})^{-1}$), where P_1 and P_1' are respective scaling parameters, $P_2 = \beta + \alpha N_0/\mu$, $P_3 = 4\beta$ (see Materials and Methods). The numbers adjacent to the solid circles represent the number of vesicles studied.

saturation is likely due to the dye transfer within the vesicle matrix.

DISCUSSION

The aim of this work was to investigate the mechanism of single-vesicle staining by the FM 4-64 dye. Styryl dyes have been demonstrated to be a useful tool to study vesicle recycling in nerve terminals (Betz and Bewick, 1992; Cochilla et al., 1999; Arvanis et al., 2003) and in neuroendocrine cells (Stafford et al., 1993; Smith and Betz, 1996). These amphiphilic dyes fluoresce when inserted in a hydrophobic environment, such as the plasma membrane, but cannot cross the membrane bilayer and are therefore used as a marker of the cell membrane area, which is fluctuating due to exo- and endocytosis (Henkel et al., 1996; Neves and Lagnado, 1999). However, it was shown that in addition to staining the plasma membrane in neuroendocrine lactotrophs, these dyes also stain the vesicle matrix (Angleson et al., 1999; Cochilla et al., 2000).

The mechanism by which vesicles are stained with styryl dyes is still under debate. In neuronal cells, vesicle staining is thought to be mediated via aqueous diffusion of dye molecules through an open fusion pore (Henkel and Betz, 1995). On the other hand, lateral diffusion along the inner wall of the open fusion pore is also a mechanism of staining in pancreatic β-cells (Takahashi et al., 2002). The latter conclusion is based on measurements of the dynamics of vesicle staining at different FM 1-43 styryl dye concentrations. If aqueous permeation is the predominant mechanism of such staining, the vesicle staining should depend on the concentration of styryl dye, given that aqueous inflow is proportional to dye concentration. Contrary to this expectation, Takahashi et al. (2002) found that the time course of vesicle staining was independent of the concentration of FM 1-43 between 5 and 50 μM (see their Fig. 3 *D*). We performed similar experiments on lactotrophs and found that the time course of vesicle staining was strongly dependent on the concentration of FM 4-64 between 0.5 and 4 μM, indicating that in lactotroph vesicles, aqueous permeation does play an important role in vesicle staining. The discrepancy between the results obtained on the two cell types is unlikely due to the

different molecules used, since both have similar molecular weights, and similar results were obtained with FM 1-43 in lactotrophs (data not shown). It is more likely that the discrepancy between the two results can be accounted for by the different concentration ranges used in the two sets of experiments. It is also possible that the structure of the open fusion pore in our experiments differs from that in β-cells. It was reported previously that the transport of dye molecules into vesicles by lateral diffusion in the plane of the membrane is restricted by high local curvature (Tse et al., 1993). Our results may therefore indicate a strongly curved membrane in the open fusion pore. A different open fusion pore geometry in comparison to the one described in β-cells is also indicated by the comparison of the fitted parameter α of Eq. 3 (Fig. 5), which is ~5-fold smaller in our experiments as compared to the estimate from Takahashi et al. (2002). This difference may in part be due to the different saturating concentration of FM dye in the membrane found in β-cells (apparent $K_d = 7\ \mu M$) and in lactotrophs (apparent $K_d = 0.5\ \mu M$, but similar to that reported by Smith and Betz, 1996) and/or due to the fusion pore geometry. A wider open fusion-pore diameter with a shorter neck would reduce the parameter α (see Eq. 3, Material and Methods). The structure of the fusion-pore in the two cells may further be different since the shortest rise time of lactotroph vesicle staining is ~5-fold slower in comparison to the fastest staining in β-cell vesicles. This may be due to the presence of the vesicle matrix in lactotrophs, which is stained by FM 4-64 and appears to have a lower apparent binding affinity for FM 4-64 in comparison to the membrane alone (Fig. 3). In addition to the aqueous diffusion of FM 4-64 molecules through the fusion pore, transfer of dye molecules into the vesicle matrix is possibly also a rate-limiting process for vesicle staining at higher concentrations of FM 4-64. Vesicle matrix in lactotrophs consists of densely aggregated molecules of prolactin, which can be isolated by centrifugation as insoluble particles (Giannattasio et al., 1975). The structure of these aggregates appears not to be amorphous but is generated through polymerization of prolactin molecules involving specific sites (Keeler et al., 2003). The interaction and migration of FM 4-64 dye molecules within vesicle matrix may involve special binding at interaction sites. Therefore, to explain the observed dependence of the rate of fluorescence intensity increase as a function of external dye concentration, we considered a two-step model of dye transport from external solution into the vesicle matrix. In the first step, dye molecules diffuse through a fusion pore, increasing their concentration around the vesicle matrix. In the next step, dye molecules penetrate into the vesicle matrix. This latter process is rate-limiting at higher dye concentrations, which is consistent with experimental results.

CONCLUSIONS

In conclusion, the results indicate that in rat lactotrophs, the loading of vesicles with FM 4-64 dye is strongly concentration-dependent, which is consistent with the aqueous transport of dye molecules through an open fusion pore. We propose that at higher dye concentrations, the dye transfer/interaction with vesicle matrix is the rate-limiting step of vesicle staining.

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