A Fluorescent Hydrophobic Probe Used for Monitoring the Kinetics of Exocytosis Phenomena†

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ABSTRACT: A fluorescence method is presented for quantitatively analyzing exocytosis phenomena and monitoring their kinetics. The method is based on the particular properties of a hydrophobic fluorescent probe, 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) [Prendergast, F. G., Haugland, R. P., & Callahan, P. J. (1981) *Biochemistry 20*, 7333–7338; Kuhry, J. G., Fonteneau, P., Duportail, G., Maechling, C., & Laustriat, G. (1983) *Cell Biophys. 5*, 129–140; Kuhry, J. G., Duportail, G., Bronner, C., & Laustriat, G. (1985) *Biochim. Biophys. Acta 845*, 60–67]. When this probe is interacted with intact resting cells in aqueous suspensions, it labels solely the membranes that are in contact with the external medium and is incorporated into them according to a partition equilibrium; i.e., the amount of the probe incorporated is proportional to the available membrane surface. TMA-DPH is highly fluorescent in membranes and not at all in water. Thus, a measurement of the TMA-DPH fluorescence intensity provides a signal proportional to the membrane surface. In secretory cells, the membrane surface available for the probe is increased upon fusion of the membrane of the secretory granules with the cell plasma membranes, directly or via intergranule fusion. Thus, when these cells are stimulated, more TMA-DPH is incorporated than in resting cells since the probe is allowed to also interact with the granule membranes now connected with the external medium by pores. This process results in a proportional increase in the TMA-DPH fluorescence intensity. The response was found to be very rapid and able to follow accurately the exocytosis kinetics. The method was tested on the histamine release process induced from peritoneal rat mast cells by compound 48/80 and ionomycin and from 2H3 rat basophilic leukemia cells (RBL) by ionomycin. The TMA-DPH fluorescence signal was compared with the histamine level, and the validity of the method was controlled by kinetic analyzes, dose–response studies, and response to metabolic inhibitors and to temperature changes. The conclusions were corroborated by a comparative study with the permeant probe 1,6-diphenylhexa-1,3,5-triene (DPH), and its principle was supported by fluorescence microphotography results. Under Discussion, arguments are developed to establish the specificity of the method for exocytosis and to discard the contribution of other processes such as the increase in the membrane permeability, labeling or leaking of secreted products, or changes in membrane potential. Finally, the method appeared to be of value to study thoroughly secretory systems, allowing differentiation of exocytosis from other secretory mechanisms.

Exocytosis is the process by which activated secretory cells release intracellular products stored in granules, which, after fusion with the plasma membrane, open to the external space. A typical and well-documented model of this process is histamine secretion by mast cells (Uvnäs, 1978; Lagunoff & Chi, 1980; Pearce, 1982; Siraganian, 1983). Exocytosis phenomena are generally analyzed by titration of the secreted products, which provides information on the final step of the process. Other approaches such as electron microscopy (Lawson et al., 1975), scanning electron microscopy (Tizard & Holmes, 1974), and high-speed microcinematography (Holstein & Tardent, 1984) are intended to describe the morphological sequence of the mechanism, whereas electrophysiological membranous modifications may be followed with patch-clamp micropipet techniques (Fernandez et al., 1984). These methods require sophisticated equipment with complex experimental procedures. In this paper, we present a simple and sensitive fluorescence method for quantifying exocytosis and monitoring its kinetics. The principle of the method is based on the particular properties of a cationic lipophilic fluorescent probe, 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH)[1] (Prendergast et al., 1981). This probe, when allowed to interact with intact cells in aqueous suspension, has been shown to label solely the plasma membranes (Kuhry et al., 1983). In addition, it exhibits a partition equilibrium (Kuhry et al., 1985) between the aqueous buffer, where it is not fluorescent, and the membranes, where it has a high quantum yield: 0.8 (Cranney et al., 1983). The fraction of TMA-DPH incorporated in membranes at equilibrium is proportional to the cell concentration. Thus, any change in the amount of the membranes in contact with the external medium should be accompanied by a parallel change in fluorescence intensity. Since TMA-DPH is very rapidly incorporated into membranes, this fluorescence effect should take place without delay. These features strongly suggested application to exocytosis. In this case, secretory granules come into fusion with the plasma membrane. The process involves

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[1] Abbreviations: TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene; RBL, rat basophilic leukemia cells; DPH, 1,6-diphenylhexa-1,3,5-triene; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, Eagle's minimum vital medium; Me_2SO, dimethyl sulfoxide.

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not only direct fusion of single granules but also multiple intergranule fusion, which leads to an extended opening to the external medium through pores and, thus, to a marked increase of the membrane surface in contact with the external space (Tasaka & Yamasaki, 1973; Tizard & Holmes, 1974; Chandler & Heuser, 1980; Dvorak et al., 1983; Fernandez et al., 1984). In the presence of TMA-DPH, the probe incorporation is expected to be enhanced, giving rise to a fluorescence signal. The reliability and sensitivity of the corresponding fluorescence signal have been tested on the exocytosis of purified peritoneal rat mast cells and of 2H3 rat basophilic leukemia cells (RBL) (Fewtrell et al., 1981; Beaven et al., 1984), in parallel with the usual histamine release determinations.

Experimental Procedures

Chemicals. The fluorescent probes TMA-DPH (*p*-toluenesulfonic salt, Molecular Probes) and 1,6-diphenylhexa-1,3,5-triene (DPH, KOCHLIGHT), their solvents dimethylformamide and tetrahydrofuran, respectively, the histamine release inducers compound 48/80 (Sigma) and calcium ionophores A23187 (Boehringer-hMannheim) and ionomycin (Calbiochem), the metabolic inhibitors antimycin A (Boehringer-Mannheim) and dinitrophenol (Aldrich), dimethyl sulfoxide as solvent, the buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and the saline components of the buffer solution (see further) were all of first grade purity and were used without further purification. All the chemicals were tested for possible contribution to the spectroscopic properties of the fluorescent labels.

Cell Systems. Peritoneal rat mast cells were prepared fresh as described earlier (Amellal et al., 1984) and purified on a bovine serum albumin gradient (Kuhry et al., 1985). They were resuspended in a saline buffer containing the following (mM): NaCl, 137; KCl, 2.7; $MgCl_2$, 1; $CaCl_2$, 1; NaH_2PO_4, 0.4; glucose, 5.6, HEPES, 10 (pH 7.4). they were examined under the microscope for purity (more than 97%) and for viability by exclusion of Trypan blue (higher than 95%). A high viability level is important because damaged cells become permeable for TMA-DPH (Kuhry et al., 1985).

Rat basophilic leukemia cells (RBL) of the secreting subline 2H3 (Barsumian, 1981) were maintained as monolayer cultures in Eagle's minimum vital medium (MEM) (Gibco) with Earl salts, supplemented with 15%, heat-inactivated (56 °C), fetal calf serum, and buffered with 7 mM $NaHCO_3$. The cells were incubated at 37.4 °C in a 5% CO_2 atmosphere in 80-cm^2 Nunclon sterile culture flasks. They were harvested at confluency, after extensive washing of the layers with saline buffer, by gentle scraping and then resuspended in saline buffer. The viability was over 98%.

Cell Labeling with TMA-DPH: Fluorescence Intensity Measurements. The cell suspensions were adjusted to a concentration of (1.6–1.8) × 10^5 cells/mL and introduced into 1-cm quartz fluorescence cuvettes. The cell concentration was determined accurately by turbidity measurements: 0.10 ± 0.01 absorbance unit at 350 nm corresponded to (1.60 ± 0.05) × 10^5 mast cells/mL and to (1.80 ± 0.05) × 10^5 2H3 RBL cells/mL. The cuvette was preincubated for 5 min at 37 °C in the thermostated cuvette holder of a JY 3D Jobin Yvon spectrofluorometer under gentle stirring to first ensure thermal equilibrium and then to perform the preliminary measurement of background scattered light. The apparatus was then set to zero, in order to substract the contribution of this background light from the subsequent fluorescence intensity measurements. TMA-DPH from a 5 × 10^{-4} M stock solution in dimethylformamide was added to the cell suspension to give a final concentration of 2.0 × 10^{-7} M. The sample was allowed to stand for 2 min, although the incorporation was almost immediate, as shown by the stabilization of the TMA-DPH fluorescence intensity. Fluorescence intensity was then continuously monitored both before and after induction of histamine release. The excitation and observation wavelengths were 350 and 430 nm (maximal emission).

Histamine Release Induction. The mast cell secretion was challenged by compound 48/80 and by the calcium ionophore ionomycin. For RBL cells, compound 48/80 was found to be inactive, and only ionomycin was used. Calcium ionophore A23187 was discarded because it seriously interfered with TMA-DPH fluorescence.

The inducers were introduced into the samples after the basal fluorescence level was recorded for 2 min. Compound 48/80 (20 μL) dissolved in saline buffer was added to give final concentrations from 0.01 to 1 μg/mL (plateau response). The increase in fluorescence intensity was followed until completion of the secretion from mast cells (90 s). Ionomycin from a 10^{-3} M stock solution in dimethyl sulfoxide (Me_2SO) was intermediately diluted in saline buffer and then added to the sample to give concentrations between 10^{-8} and 10^{-6} M. The incubation was continued for 5 min for mast cells and 20 min for RBL cells, depending upon the respective kinetic features of the secretion processes.

Histamine Titrations. The secreted histamine levels were determined with the fluorometric test of Shore et al. (1959), omitting the extraction procedure. The assays were performed parallel to the fluorescence intensity measurements, under the same conditions (simultaneous induction). Aliquots from the cell suspensions at a concentration of 1.6–1.8 cells/mL (100 μL for mast cells and 500 μL for RBL cells, which are poorer in histamine) were taken at regular time intervals after induction (every 7 s for mast cells with compound 48/80 and every 30 s for RBL cells with ionomycin) and immediately pipetted into 1 mL of ice-cold saline buffer to stop the reaction. The histamine content was determined in the supernatant from a low-speed centrifugation (220*g*, 2 min). The spontaneous histamine release level was measured in the same way, before induction, and the total histamine content was obtained after treatment of the sample with 10% trichloroacetic acid.

Experiments with 1,6-Diphenylhexa-1,3,5-triene (DPH). To confirm the validity of the method, negative control experiments were done with DPH, the neutral parent molecule of TMA-DPH, which has similar spectroscopic properties (Cranney et al., 1983) and has formerly been widely used in membrane fluidity studies (Shintzky & Barenholz, 1978) but behaves as a totally permeant probe and labels all the hydrophobic regions of the cells uniformly (Berlin & Fera, 1977; Johnson & Nicolau, 1977; Kuhry et al., 1983). In particular, in resting secretory cells, the granule membranes are labeled too, so that, when exocytosis takes place, there should be no change in the fluorescence intensity. The experiments were done with mast cells. The cell suspension (2 × 10^5 cells/mL) was incubated with DPH (10^{-6} M) from a 10^{-3} M stock solution in tetrahydrofuran at 37 °C for 30 min, the required time for complete incorporation of the probe at 37 °C (Kuhry et al., 1983). Fluorescence intensity before and after induction with compound 48/80 was measured as already described for TMA-DPH.

TMA-DPH Fluorescence Microphotography. The specificity of TMA-DPH as a plasma membrane label in intact cells was demonstrated earlier by UV fluorescence microphotography on cultured L 929 mouse fibroblasts (Kuhry et al., 1983). In the present study, the same technique was applied to mast cells and RBL cells to investigate the labeling evolution

upon exocytosis. Mast cells were observed as suspensions and RBL cells as monolayer cultures on cover slips, 24 h after seeding. The cells were incubated with 5×10^{-6} M TMA-DPH at 37 °C. The high probe concentration was necessary to overcome the rapid bleaching of TMA-DPH due to photoisomerization of the DPH moiety of the molecule under strong UV illumination (Duportail & Weinreb, 1983). The samples were observed through a Leitz Ortholux II photomicroscope equipped for epiillumination (Ploemopack system), with filters at 340–380 nm for excitation and at 430 nm for observation. Control phase-contrast pictures were obtained with the same equipment under visible light. The original magnification was 600×; 400 ASA ILFORD HP 5 films were used for recordings. Unstimulated and stimulated cells (mast cells, compound 48/80, 0.2 μg/mL; RBL cells, ionomycin, 3×10^{-7} M) were examined on separate preparations.

Results

Preliminary Controls. TMA-DPH has been shown not to alter the viability or the functionality of the cells studied on the basis of the following observations: after incubation of the cell suspensions (mast and RBL cells) in saline buffer with TMA-DPH (5×10^{-7} M) at 37 °C under gentle stirring for various time intervals up to 60 min, cell viability, determined by Trypan blue exclusion, remained higher than 90%, and no difference was observed from unlabeled controls. Histamine secretion was not affected either in the presence of TMA-DPH: the inducers–dose response curves of labeled and control cells could be superimposed. The histamine release inducers compound 48/80 and ionomycin were checked for noninterference with the excitation and emission spectra of TMA-DPH. The incorporation features of TMA-DPH in mast and RBL cells were similar to that described earlier for L 929 mouse fibroblasts (Kuhry et al., 1983, 1985); i.e., the kinetics of the process were very rapid (less than 3 s at 37 °C to reach the equilibrium incorporation), and quantitatively, the rate of incorporated probe was proportional to the cell concentration in the range examined [$(0.6–3) \times 10^5$ cells/mL]; in other words, when the concentration of membranes available for the probe was increased, proportionally more TMA-DPH was taken up.

Fluorescence Micrographs. Representative micrographs of TMA-DPH staining in mast cells and RBL cells, before and after histamine release was induced, are shown in Figure 1. Because of the already mentioned strong photobleaching of TMA-DPH, the photographs were not as clear as direct observation, but the following features stand out: in both types of cell, the probe appears to be confined to the peripheral region before induction. After induction, the evolution of the staining is particularly clear in RBL cells. The labeled border line is markedly entlarged, and in some cases, the staining is extended to almost the entire cell. However, the staining is discontinuous and should be attributed to internal granule labeling after extensive intergranule fusion rather than to uniform labeling of the inside of the cell resulting from an increased membrane permeability for the probe. Further arguments to corroborate this interpretation are developed in the following sections. It should be noted that the process does not necessarily result in an increase of the overall cell size, since the membrane surface available for TMA-DPH is mainly increased by the formation of intracellular granule strings communicating with the external space through pores after fusion with the plasma membrane (Chandler & Heuser, 1980). The characteristics of the staining of mast cell are basically similar to those of RBL cells described above, albeit with more intense staining of the cells after induction. This can be attributed to a higher concentration of granules, which is typically 1200 per mast cell (Helander & Bloom, 1974) and about 4–5 times less in basophilic cells, as estimated by comparing electron micrographs (Dvorak et al., 1983). Probable granule ejection into the extracellular space is shown in the picture selected for RBL cells, but this process was seldom observed, as confirmed by visible light phase-contrast photographs obtained in the same conditions (not represented).

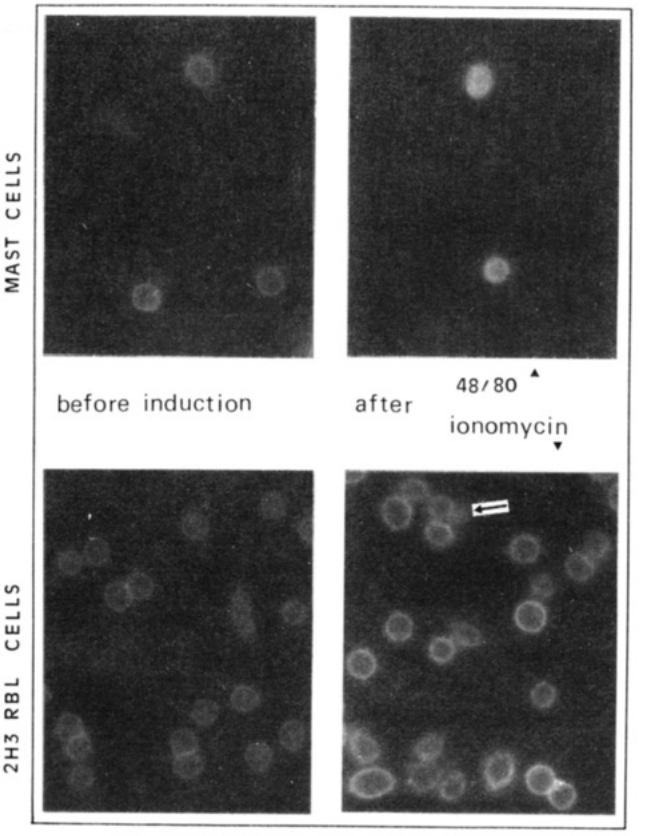

FIGURE 1: Fluorescence microphotographies of TMA-DPH-labeled rat peritoneal mast cells and 2H3 RBL cultured cells before and after histamine release induction. The cells were incubated with 5×10^{-6} M TMA-DPH at 37 °C and observed on cover slips through a Leitz Ortholux II photomicroscope with Ploemopak system. The original magnification was 600×. Excitation wavelength was 340–380 nm and observation wavelength 430 nm. The mast cell density was lower than that of RBL cells because of the rather low yield of the preparation and purification process. The induction was challenged by compound 48/80 (0.2 μg/mL) for mast cells and by ionomycin (3×10^{-7} M) for RBL cells. Stimulated and unstimulated cells were observed on separate preparations. No better contrast could be obtained because of the considerable photobleaching of TMA-DPH under strong UV illumination. The pictures show that after induction the surface of the peripheral staining by TMA-DPH is markedly enlarged. In mast cells, the staining seems to cover the cell surface. This is interpreted (see text) by intergranule fusion. Possible ejection of granules (arrow) could be observed but was a rather seldom phenomenon.

Kinetic Studies. Figure 2 shows typical curves for the evolution with time of TMA-DPH fluorescence intensity, parallel with those for histamine release, for representative inducer doses selected from assays at 10 different concentrations. Histamine levels were corrected for spontaneous release and expressed as absolute amounts per 10^6 mast cells and per 5×10^7 RBL cells, i.e., 25 ± 1 μg. The TMA-DPH fluorescence intensity results are given in relation to the basal fluorescence intensity, before induction, set equal to unity. Because of the proportionality of the fluorescence intensity to the cell (i.e., to the membrane) concentration, the increase in fluorescence intensity is directly interpretable as an increase in membrane concentration, attributed to the granule mem-

Table I: Compared Dose-Response Results for Histamine Release and TMA-DPH Fluorescence Intensity Increase in Rat Peritoneal Mass Cells and RBL 2H3 Cells Stimulated by Ionomycin[a]

	ionomycin (M)			
	3×10^{-8}	10^{-7}	2×10^{-7}	3×10^{-7}
mast cells				
increase in TMA-DPH fluorescence intensity (% of basal fluorescence)	23.5 ± 1.6	34.2 ± 2.2	71.5 ± 0.6	85.1 ± 1.1
histamine secretion				
$\mu g/10^6$ cells	2.2 ± 0.3	4.5 ± 0.4	16.5 ± 0.6	19.5 ± 1.9
% of total	8.9 ± 1.3	18.0 ± 1.6	66.0 ± 2.6	77.8 ± 0.8
RBL 2H3				
increase in TMA-DPH fluorescence intensity (% of basal fluorescence)	0.9 ± 0.5	7.9 ± 2.0	17.3 ± 0.7	19.1 ± 1.5
histamine secretion				
$ng/10^6$ cells	45.6 ± 2.6	123.0 ± 20.4	234.0 ± 17.8	296.4 ± 8.2
% of total	9.1 ± 5.2	24.6 ± 4.1	46.0 ± 3.6	58.5 ± 1.5

[a] The results were obtained from the plateau values of kinetic curves, i.e., 5 min after induction for mast cells and 20 min for RBL 2H3 cells. Values are the means ± SEM of four determinations.

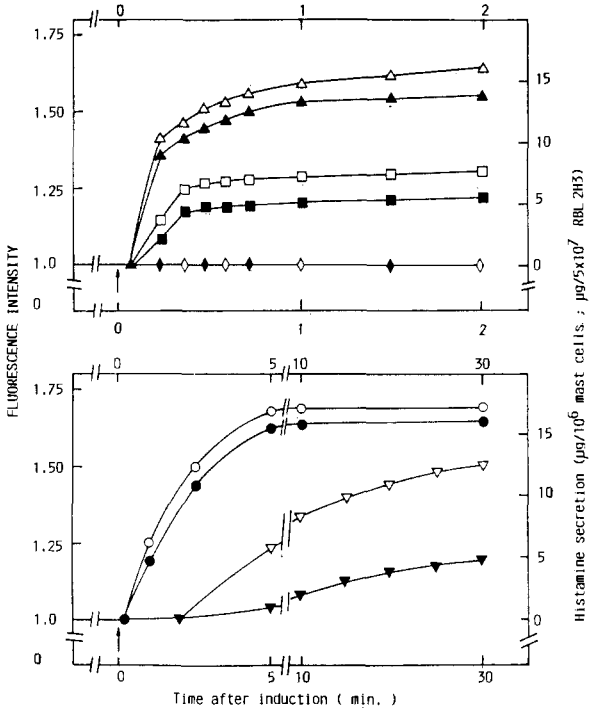

FIGURE 2: Comparative kinetics of histamine release and increase in TMA-DPH fluorescence intensity in rat peritoneal mast cells and 2H3 RBL cells. The upper part of the figure represents the kinetics for mast cells stimulated by compound 48/80, for three selected doses: 0.01 (◇, ◆), 0.05 (□, ■), and 0.2 µg/mL (△, ▲). The results for mast cells (○, ●), and 2H3 RBL (▽, ▼) cells stimulated by ionomycin (2×10^{-7} M) are presented in the lower part of the figure. Open symbols are used for the TMA-DPH fluorescence intensity and full symbols for released histamine levels. The fluorescence results are referenced to the basal intensity before induction, and histamine levels are expressed as absolute yields for typical cell densities. Temperature was 37 °C and TMA-DPH concentration 2×10^{-7} M.

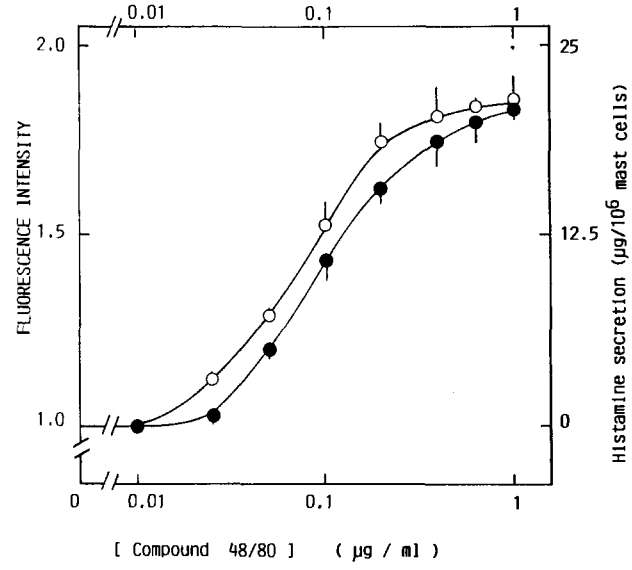

FIGURE 3: Compared responses of mast cells to increasing doses of compound 48/80 for histamine release and for increase in TMA-DPH fluorescence intensity. Open symbols represent the fluorescence results and full symbols histamine levels. Units are the same as in Figure 2. The results were obtained from the plateau values of kinetic curves (90 s after induction). Temperature was 37 °C and TMA-DPH concentration 2×10^{-7} M. Vertical bars denote SEM from four determinations.

branes concerned in the exocytosis, assuming that no other process is involved in the fluorescence effect; this condition will be discussed further. In the systems examined, there was a remarkable correspondence between the kinetics of the histamine release and the fluorescence signal. The two processes matched each other within better than 2 s. Moreover, the results were in satisfactory agreement with earlier reported kinetic studies (Sullivan et al., 1975; Beaven et al., 1984). It is of particular interest that both types of curves have simultaneous plateaus, which rules out the possibility that the fluorescence effect could be due to increased membrane permeability of the probe: in that case, fluorescence would have increased continuously for more than 1 h because of the progressive labeling of the inner hydrophobic regions or because of interaction of TMA-DPH with biological material leaking from the cells into the saline buffer. As a matter of fact, such behavior was commonly observed in damaged cells (Kuhry et al., 1985). A complementary experiment was performed to confirm this point. TMA-DPH labeled and unlabeled mast cells suspensions, at the same concentration (2×10^5 cells/mL), were subjected to low-speed centrifugation, once the histamine secretion induced by compound 48/80 (0.4 µg/mL) was over. The supernatants were examined respectively for fluorescence intensity and scattered light intensity levels. No significant difference in these two parameters was found between the treated cells and unstimulated controls handled in the same way. This indicates that no leakage in the mast cell membrane resulted from the exocytosis process and that the expulsion of secretory granules into the external medium was a minor phenomenon, as already suggested.

Dose–Response Results. The fluorescence increase of TMA-DPH was, such as histamine secretion, correlated to the dose of the chemical inducer used to trigger the secretory process. The results are reported in Figure 3 for mast cells with compound 48/80 and in Table I for both cell systems with ionomycin. These two representations were assumed to be complementary, the first giving more emphasis to the general aspect of the evolution and the second a better overall view

of the sensitivity and prevision of the method. Satisfactory correspondence between the data from the two methods was observed in both type of cells, except at the very low inducer concentrations (<0.02 μg/mL for compound 48/80 and 3×10^{-8} M for ionomycin). Below these values, the fluorescence response was more sensitive in mast cells, and in contrast, histamine release was relatively higher in RBL cells. For clear interpretation of this threshold behavior, further experiments will be necessary. Nevertheless, these observations emphasize that secretion and exocytosis cannot necessarily be superimposed, and one advantage of the method described is precisely that it reveals discrepancies and, thus, should be of value in their study. From a quantitative point of view, the fluorescence signal after induction by ionomycin is about 4 times greater in mast cells than in RBL cells, which matches the relative difference in granule concentration in the two cell types (see Fluorescence Micrographs).

Use of Metabolic Inhibitors: Role of Temperature. Respiratory chain inhibitors (antimycin and cyanide) and an uncoupling agent (dinitrophenol, DNP) were assayed in mast cells to block exocytosis, in order to see whether they also abolished the increase in TMA-DPH fluorescence intensity. Unfortunately, DNP and cyanide caused a dramatic TMA-DPH fluorescence quenching and could not be used. Antimycin interfered with the fluorescence of TMA-DPH; still, it was possible to show that this product, at a final concentration of 10^{-6} M, totally suppressed the fluorescence response corresponding to the histamine release. The effect of decreasing the temperature was examined for the same purpose. The results for purified mast cells (2×10^5 cells/mL in saline buffer; compound 48/80, 0.4 μg/mL; TMA-DPH, 2×10^{-7} M) exhibited remarkable parallels between the decrease in the TMA-DPH intensity and the decrease in released histamine. Both parameters appeared to undergo a sharp simultaneous drop when the temperature decreased below 20 °C.

Compared Labelings with TMA-DPH and DPH. The variations in fluorescence intensity of the plasma membrane probe TMA-DPH and of the permeant probe DPH, in mast cells, before and after induction with compound 48/80 (0.4 μg/mL), are compared in Figure 4. Unlike TMA-DPH, DPH is incorporated into all regions of the cell and labels the granule membranes before induction. This explains why no increase in DPH fluorescence intensity was observed, when the cells were stimulated, as was the case for TMA-DPH, although a high histamine release (>50%) was noticed. The inocuous nature of DPH at the concentration used (10^{-6} M) was controlled by viability test (Trypan blue exclusion). The striking difference between the behavior of the two probes is thought to provide strong support for the validity of the method.

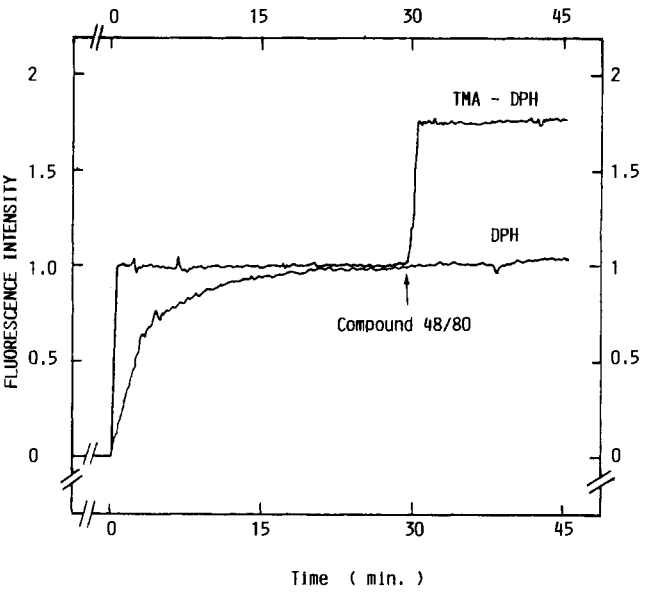

FIGURE 4: Compared evolution with time of fluorescence intensity of TMA-DPH and DPH, in rat peritoneal mast cells before and after histamine release induction by compound 48/80. The left part of the figure shows the incorporation kinetics of the two probes, which are very rapid for TMA-DPH and take as long as 30 min until equilibrium for DPH, at 37 °C (TMA-DPH, 5×10^{-7} M; DPH, 10^{-6} M). After induction (compound 48/80, 0.4 μg/mL), a typical increase in the TMA-DPH fluorescence intensity is observed, whereas the fluorescence intensity of DPH remains constant. This is due to the fact that DPH, contrary to TMA-DPH, labels the inside of the cell, before induction, and among others the granule membranes. No increase in fluorescence intensity is therefore to be expected for DPH, upon exocytosis, since the granule labeling was already completed before induction.

DISCUSSION

We have presented a fluorescence assay permitting continuous, sensitive, and quantitative monitoring of exocytosis phenomena. The method has been tested on two types of cells, peritoneal rat mast cells and 2H3 RBL cells, challenged by histamine releasing agents, but it is quite general in its principle and is thought to be extendable, with no a priori restrictions, to any secretory system. It only requires simple fluorescence intensity measurements and applies to pure intact cells. Moreover, the cells remain preserved during the assay. It is based on the property of the hydrophobic fluorescent probe TMA-DPH, which is usually incorporated restrictively into the plasma membranes of the cells, to label in the case of secretory cells the granule membranes when the exocytosis process is triggered, resulting in a corresponding increase in the fluorescence intensity. To our knowledge, no similar method has been reported before. A fluorescence method has also been proposed for measuring fusion kinetics between biological membranes (Hoechstra et al., 1984), which relied on the reduction in fluorescence self-quenching of octadecylrhodamine B chloride, when labeled membranes fuse with unlabeled membranes. However, the application of this approach to exocytosis was not envisaged, and the behavior and localization of the probe in whole cells was not investigated. The value of the method presented here lies in the analysis of the exocytosis phenomenon itself. The results of this study show that the incorporation of TMA-DPH is proportional to the available membrane concentration and that the fluorescence signal triggered by the induction of exocytosis followed the appropriate kinetics without delay and displayed a sensitive response to dose. The effect could be abolished, correlatively with secretion, by inhibiting the cell metabolism. Moreover, the signal intensity was found to be related to the number of granules in each type of cell examined. Contribution from other types of mechanism in generating the signal was ruled out: the products released did not interact with TMA-DPH, and the increase of their concentration in the external medium did not enhance fluorescence. The effect could not be attributed either to any increase in the plasma membrane permeability for TMA-DPH; otherwise, no plateau would have been observed in the kinetic curves. The shape of the kinetic curves also ruled out any detection of membrane potential changes. Such changes have been reported in mast cells (Sugiyama & Utsumi, 1974) and in RBL cells (Kanner & Metzger, 1983), but their kinetics are markedly different. Besides, TMA-DPH behaves quite different from membrane potential fluorescent dyes, which are almost insensitive to membrane concentration (Waggoner, 1979). In experiments in the laboratory with phosphatidylcholine vesicles, loaded inside with potassium ions (150 mM) and suspended in a

buffer containing 0.25 M sucrose and 10 mM HEPES, pH 7.0, it was shown that no change in TMA-DPH (10^{-6} M) fluorescence intensity was denoted after addition of valinomycin (10^{-6} M) to equilibrate the internal and external K^+ concentration (G. Duportail and J. G. Kuhry, unpublished data). Thus, TMA-DPH was insensitive to the membrane potential modification induced in this way. Our aim in this study was mainly to establish the relevance of this fluorescence method for monitoring exocytosis. Nevertheless, some light was also shed on various structural and kinetic aspects of the histamine release from mast and RBL cells. The ejection of secretory granules into the external space was shown to be of very minor importance, by the absence of TMA-DPH fluorescence intensity in the supernatants of low-speed centrifugation of stimulated mast cell suspensions and by the fluorescence micrographs. Another observation was the different threshold behavior of the dose–response of the fluorescence signal in mast cells and RBL cells. In mast cells, the fluorescence approach was more sensitive than histamine release, but in RBL cells it was the contrary: the histamine release started at lower doses than the fluorescence increase, and this was assumed to indicate a contribution of a secretion not mediated by exocytosis, as has been described in some neurotransmitter systems (Uvnäs & Åborg, 1984; Vizi, 1984) in which exocytosis and cation exchange were competitive mechanisms. In such situations, the method described, coupled with classical titrations of the mediator released, should be able to resolve part of each mechanism and to analyze the corresponding kinetics.

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