Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2998–3002, May 1986
Neurobiology

Restricted diffusion of tyrosine hydroxylase and phenylethanolamine *N*-methyltransferase from digitonin-permeabilized adrenal chromaffin cells

(catecholamines/lactate dehydrogenase)

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Communicated by Bernhard Witkop, November 8, 1985

ABSTRACT **Tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase; L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] and phenylethanolamine *N*-methyltransferase (PMTase; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.28) are involved in catecholamine biosynthesis and are considered soluble proteins. However, they may actually be localized on the surface of the chromaffin granule. We have used the detergent digitonin to permeabilize the plasma membrane of cultured adrenal chromaffin cells to investigate the subcellular localization of TyrOHase and PMTase. A digitonin titration of the release of proteins and catecholamines revealed the existence of at least three subcellular compartments that are distinguished by their digitonin sensitivity: (*i*) soluble proteins, which were released upon treatment of the cells with low digitonin concentrations (5 μM), (*ii*) a "digitonin-sensitive" cytoplasmic protein pool, which required higher concentrations of digitonin for release (10 μM) and included TyrOHase and PMTase, and (*iii*) the chromaffin granule, which was insensitive to digitonin. Analysis of the rates of release of all of these proteins revealed that the rate of TyrOHase and PMTase release was slower at 10 μM than at 40 μM digitonin, while the rates of release of the other proteins were similar at both concentrations and varied in proportion to their respective sizes. Treatment with cytoskeletal disrupting agents had no effect on TyrOHase or PMTase efflux. These data suggest that TyrOHase and PMTase are in a detergent-labile association in the cell. This is consistent with the concept that TyrOHase and PMTase may be localized on the surface of the chromaffin granule.**

In epinephrine-forming cells of the adrenal medulla, the catecholamine biosynthetic pathway is composed of four enzymes, tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase; L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2], aromatic amino acid decarboxylase (aromatic L-amino acid carboxy-lyase, EC 4.1.1.28), dopamine β-hydroxylase [dopamine β-monooxygenase; 3,4-dihydroxyphenethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] and phenylethanolamine *N*-methyltransferase (PMTase; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.28). Dopamine β-hydroxylase is localized in the interior of the chromaffin granule and on the internal face of the granule membrane (1), while TyrOHase, aromatic amino acid decarboxylase, and PMTase are generally considered to be "soluble" cytoplasmic enzymes and are synthesized on free ribosomes (2–4). However, morphological and subcellular fractionation studies suggest the possible particulate localization of PMTase and TyrOHase, perhaps on the surface of the chromaffin granule. By immunocytochemistry, both PMTase and TyrOHase have been seen to be associated with chromaffin granules (5–9), and one report shows TyrOHase to be localized on microtubules (8). In purification procedures, 5–90% of the TyrOHase activity and 15–20% of the PMTase activity in the adrenal medulla have been reported to be found in the particulate fraction (10–13). TyrOHase is also found in chromaffin granule membranes, where it is the major substrate for protein kinase (14).

It is difficult to study such loose molecular associations between enzymes and cytoplasmic organelles. Both fractionation and fixation techniques can cause redistribution of proteins during tissue handling. However, a recent report suggests that information about the *in situ* state of intracellular proteins can be gained by measuring the efflux rate of proteins from "stripped" cells. Morris and Lasek (15) have investigated the polymerization state of tubulin and actin in the squid giant axon by examining their rates of efflux from extruded axoplasm. The unpolymerized portion of the protein pool diffused out of the axoplasm at the rate predicted by the molecular weight of the protein, while the fraction that was polymerized diffused out more slowly, retarded by the rate-limiting depolymerization step. Thus, the proportion of the intracellular protein bound *in situ* could be calculated.

We have modified this approach for application to chromaffin cells by chemically "stripping" the plasma membrane with the detergent digitonin, thereby simulating the isolated axoplasm preparation (15). We then analyzed the rates of efflux of the catecholamine biosynthetic enzymes, as well as those of enzymes known to be soluble.

We took advantage of the differential digitonin sensitivity of various cellular compartments. Digitonin binds specifically to 3-11a-hydroxysterols and selectively permeabilizes cellular membranes on the basis of their cholesterol content. The plasma membrane of isolated chromaffin cells becomes leaky to proteins and small molecules, while the chromaffin granule membrane is unaffected and the calcium-sensitive secretory machinery remains relatively intact (16, 17). Digitonin has been used to investigate the subcellular distribution of many small molecules and proteins (18–23).

We have characterized the time and concentration dependence of the detergent-evoked release of proteins from cultured chromaffin cells and have defined a unique detergent-labile compartment that contains both TyrOHase and PMTase. This may represent the association of these proteins with particulate components of the cytosol, possibly the chromaffin granule.

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Abbreviations: PMTase, phenylethanolamine *N*-methyltransferase; TyrOHase, tyrosine hydroxylase.

MATERIALS AND METHODS

Cell Culture and Permeabilization. Chromaffin cells were isolated from fresh bovine adrenal glands by collagenase digestion and maintained in primary culture as described (23). On day 3 after initial plating, each well was washed with isotonic salt solution (118 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/2.2 mM $CaCl_2$/10 mM Hepes, pH 7.35). Digitonin medium (0.25 ml) was then added to each well. This medium contained 140 mM Na-glutamate, 20 mM Pipes (pH 6.8), 5 mM glucose, 5 mM $MgSO_4$, 5 mM ATP, 0.5 mM ascorbic acid, 5 mM EGTA, and 10 or 40 μM digitonin. Cells were kept at 37°C. At the appropriate time points, the permeabilizing medium was removed, centrifuged to remove any cells, and frozen. The cells remaining in the well were lysed in 0.5 ml of water, subjected to a freeze/thaw cycle and centrifuged (10,000 × *g* for 5 min), and the supernate was frozen. Aliquots of the medium and the cell lysate were taken for enzyme assays. The percentage of a particular enzyme or protein in the medium was calculated by adding the amount of substance in the medium and cell lysate at each time point to determine the total amount in each well and calculating the percentage of this total that was in the medium.

Assays. Dopamine β-hydroxylase was measured by a modified version of the method of Nagatsu and Udenfriend (24). The assay mixture contained 200 μM sodium acetate, pH 5.5/10 mM disodium fumarate/100 μg of catalase/20 mM *N*-ethylmaleimide/20 μM $CuSO_4$/10 mM ascorbic acid/10 mM tyramine/200 μl of sample (final vol, 500 μl). After incubation at 37°C for 20 min, the reaction was stopped with 1 M perchloric acid. After removal of the catecholamines by aluminum hydroxide-gel adsorption, the samples were transferred to ion-exchange columns (AG 50W×8, 200–400 mesh; Bio-Rad). The columns were washed with water and the adsorbed amines were eluted with 4 M ammonium hydroxide. Octopamine was converted to *p*-hydroxybenzaldehyde by periodate oxidation and quantitated at 330 nm.

Lactate dehydrogenase was measured by monitoring the formation of NADH from NAD. Aliquots (100 μl of cell lysate or digitonin medium) were incubated with 60 mM lactate/100 mM Tris, pH 9.0/1 mM NAD in a final vol of 1.1 ml at room temperature for 30 min.

PMTase was measured in cell lysates by the modification of the method of Axelrod (3) reported by Pollard *et al.* (25). Each sample (50 μl) was incubated with 1 μCi of *S*-[^{3}H]-adenosylmethionine (1 Ci = 37 GBq; New England Nuclear)/13.3 μM *S*-adenosylmethionine/200 mM Tris·HCl, pH 8.6/2.4 mM phenylethanolamine at 37°C (final vol, 200 μl). After 30 min, 0.5 M potassium borate (pH 10) was added and the ^{3}H-labeled product extracted into 4 ml of Betafluor (National Diagnostics, Somerville, NJ) containing 3% (vol/vol) isoamyl alcohol and analyzed by scintillation spectroscopy. Since digitonin in the medium interfered with the assay, PMTase levels were measured only in the cell lysates.

TyrOHase was measured by a modified version of the method of Nagatsu *et al.* (10, 26). Aliquots (200 μl) of the cell lysate or media were incubated with 200 mM Tris acetate, pH 6.0/1 mM $FeSO_4$/100 μM tyrosine (New England Nuclear)/1 mM 6,7-dimethyl-5,6,7,8-tetrahydropterin/100 mM 2-mercaptoethanol at 37°C for 30 min (final vol, 500 μl). The reaction was stopped with 1 M perchloric acid and the ^{14}C-labeled product was isolated by the aluminum hydroxide gel adsorption procedure (27).

Solubilized protein was defined as that protein found in the digitonin permeabilizing medium after various times of incubation. Aliquots of the medium were precipitated for 1 hr at 4°C with 10% trichloroacetic acid. Total protein was measured by subjecting chromaffin cells to water lysis, a freeze/thaw cycle, and centrifugation at 14,000 × *g* for 5 min. The amount of protein in the supernate was taken to be 100%. Protein was measured by the method of Lowry *et al.* (28).

Epinephrine and norepinephrine were separated and measured by reverse-phase HPLC (29). Catecholamines were eluted from a 3 μm (100 × 4.6 mm) ODS column (Chromanetics) with 10 mM phosphate/10 mM trichloroacetic acid/0.02% $NaDodSO_4$, pH 2.8. An ESA electrochemical detector (Model 5100A Dual Electrode) and Gilson Datamaster/Apple IIE were used for detection and peak area analysis.

Polyacrylamide Gel Electrophoresis. Trichloroacetic acid-precipitated material was washed once with water-saturated ether and resuspended in sample buffer. Aliquots were applied to a 10–20% $NaDodSO_4$/polyacrylamide gel (30). Proteins were stained with Coomassie blue, and the bands quantitated with a Beckman Spectrophotometer. A mixture of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme was used to standardize the molecular weight.

RESULTS

Chromaffin cells were exposed to various concentrations of digitonin for 20 min and the amounts of TyrOHase, dopamine β-hydroxylase, PMTase, and epinephrine released were measured. For comparison, the cytoplasmic protein, lactate dehydrogenase, and solubilized protein (as defined in *Materials and Methods*) were also quantitated. Fig. 1 shows that at concentrations up to 40 μM digitonin there was a dose-dependent efflux of each substance. A substantial fraction of each protein was released, while <10% of the intragranular compounds, epinephrine and norepinephrine (data not shown), came out of the cells. In general, increasing the digitonin concentration from 40 to 100 μM had no further effect on the percentage of each protein found in the medium. However, over this concentration range, there was a gradual increase in the percentage of epinephrine released (Fig. 1). Norepinephrine behaved similarly (data not shown).

TyrOHase and PMTase required higher digitonin concentrations for initial release than did lactate dehydrogenase and solubilized protein. An enlargement of the left portion of Fig. 1 (Fig. 2) shows that a significant amount of solubilized protein and lactate dehydrogenase was released into the medium at 5 μM digitonin, while it required 10 μM digitonin to liberate a similar fraction of PMTase and TyrOHase.

The time dependence of the release of the catecholamine synthetic enzymes, as well as lactate dehydrogenase and solubilized protein, was monitored at 10 μM and 40 μM

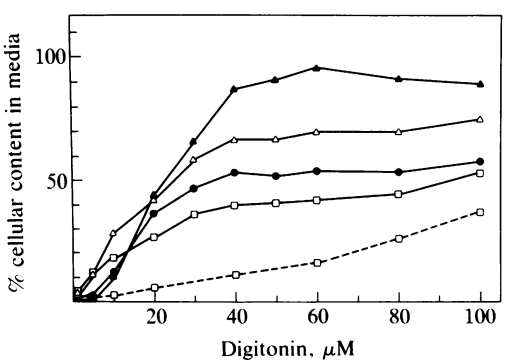

FIG. 1. Digitonin titration of release of cellular proteins and catecholamines. Cells were incubated with various concentrations of digitonin for 20 min. Aliquots of the medium and cell lysate were assayed for the various substances. Each point is the mean of two independent experiments with cells from different preparations (two wells per point per experiment). △, Lactate dehydrogenase; □—□, soluble protein; ●, PMTase; ▲, TyrOHase; □—□, epinephrine.

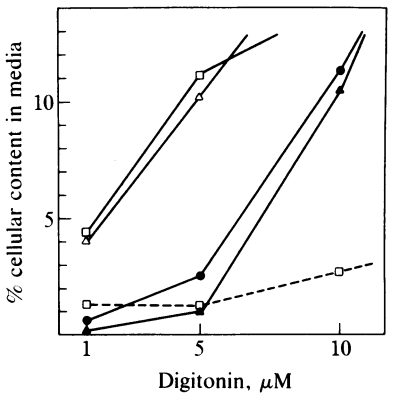

FIG. 2. Digitonin titration of release of cellular proteins and catecholamines. An enlargement of the left-hand portion of the titration curve in Fig. 1.

digitonin over a 30-min period. Ten micromolar digitonin (Fig. 3*A*) was representative of a concentration where release was strongly dependent on the detergent concentration (see Fig. 1), and 40 μM (Fig. 3*B*) was a point on the plateau of the titration curve (see Fig. 1). In 10 μM digitonin, lactate dehydrogenase diffused out of the chromaffin cell promptly, as did solubilized protein (Fig. 3*A*). In contrast, the release of both TyrOHase and PMTase exhibited a distinct lag, with TyrOHase showing a more pronounced delay than PMTase. However, >80% of the intragranular components, dopamine β-hydroxylase and the catecholamines, remained within chromaffin granules, even after 30 min of treatment, reflecting the insensitivity of the granule to digitonin.

The same kinetic experiment performed in 40 μM digitonin showed somewhat different results (Fig. 3*B*). Chromaffin granule contents, dopamine β-hydroxylase and the catecholamines, still remained within the cell at this concentration.

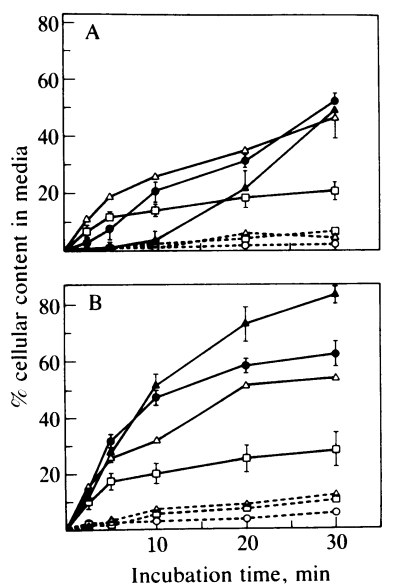

FIG. 3. Time courses of release of cellular proteins and catecholamines in 10 μM (*A*) and 40 μM (*B*) digitonin. Cells were incubated with 10 μM and 40 μM digitonin for various times. Aliquots of the medium and cell lysate were assayed for the various substances. Each point is the mean of several independent experiments with cells from different preparations. △—△, Lactate dehydrogenase (n = 2); □—□, solubilized protein (n = 5); ●, PMTase (n = 4); ▲, TyrOHase (n = 3); △---△, norepinephrine (n = 2); □---□, epinephrine (n = 2); ○, dopamine β-hydroxylase (n = 2). Error bars are the standard of the mean, calculated when $n > 2$.

However, PMTase and TyrOHase showed no transient lag and were promptly released, as were lactate dehydrogenase and solubilized protein, indicating that the slower rate of release seen at 10 μM was not evident at 40 μM digitonin.

To compare the rates of release of these substances quantitatively, we analyzed the data in a manner analogous to that used by Morris and Lasek (15). Assuming that the release of protein occurred by diffusion according to Fick's Law, the logarithm of release as a function of time should be a straight line that has a slope proportional to the diffusion coefficient. The transformed data are linear (Fig. 4), suggesting that the proteins are released from the cells by a simple diffusion process. The rates of release of solubilized protein (Fig. 4*A*), lactate dehydrogenase (Fig. 4*C*), dopamine β-hydroxylase, and catecholamines (Fig. 4*D*) were the same in 10 μM digitonin as they were in 40 μM digitonin. In contrast, TyrOHase and PMTase (Fig. 4*B*) show a slower rate of release in 10 μM digitonin than in 40 μM digitonin, reflecting the lags in TyrOHase and PMTase release (Fig. 3*A*) and implying that the apparent diffusion rate of these proteins was increased at 40 μM digitonin.

The calculated rates of release in 10 and 40 μM digitonin are shown in Table 1. An index of the digitonin dependence of the release rate (40:10 micromolar ratio) indicates that, for TyrOHase and PMTase, release was affected by the concentration of digitonin (40:10 micromolar ratio = 3.04 and 3.16, respectively), while release rates for the other cytosolic and intragranular substances tested were not (mean 40:10 micromolar ratio = 0.78; see Table 1). The high 40:10 micromolar ratio seen for TyrOHase and PMTase is due to a rate of leakage in 10 μM digitonin that is somewhat slower than that for the other cytosolic substances, lactate dehydrogenase and solubilized protein. At 40 μM digitonin these differences among the proteins are not evident.

To evaluate the behavior of other proteins under these conditions, proteins released from chromaffin cells by 10 and 40 μM digitonin were analyzed by $NaDodSO_4$/polyacrylam-

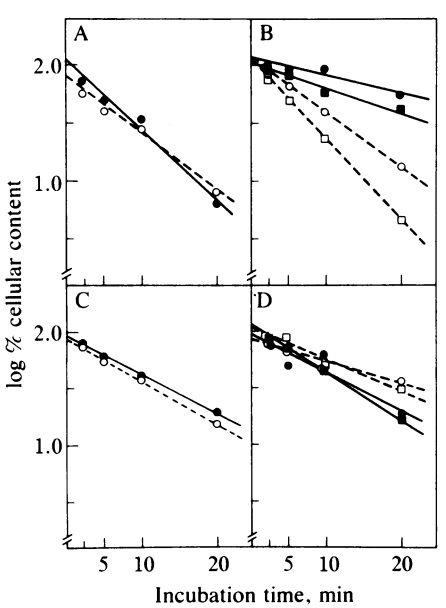

FIG. 4. Release rates of cellular proteins and catecholamines in 10 μM and 40 μM digitonin. Data from Fig. 3 were expressed as percentage maximum release (the value at 30 min was taken as the maximum) and then the logarithm of percentage maximum release of each value was calculated. Closed symbols are data from the time courses in 10 μM digitonin; open symbols are data from the time courses in 40 μM digitonin. Solubilized protein (*A*), TyrOHase (circles) and PMTase (squares) (*B*), lactate dehydrogenase (*C*), dopamine β-hydroxylase (circles) and epinephrine (squares) (*D*).

Table 1. Rates of release of proteins and catecholamines from adrenal chromaffin cells in 10 μM and 40 μM digitonin

	Rate of release, log %/min × 10^{-3}		Digitonin dependence (40:10 micromolar ratio)
	10 μM digitonin	40 μM digitonin	
Solubilized protein	59.1	48.8	0.83
Lactate dehydrogenase	34.6	37.5	1.08
TyrOHase	15.2	46.2	3.04
PMTase	21.4	67.6	3.16
Dopamine β-hydroxylase	32.0	18.4	0.58
Epinephrine	42.0	27.0	0.64

The rates of release of each protein or catecholamine were calculated by least-squares linear regression from the values plotted in Fig. 4. A ratio of these rates at 40 μM and 10 μM was calculated as an index of digitonin dependence of the release rate. The number of experiments contributing to each point is indicated in Fig. 3.

ide gel electrophoresis. Most of the visible intracellular proteins are extracted to some extent by 40 μM digitonin (Fig. 5), suggesting that permeabilization of the cells results in nonselective leakage of cytoplasmic substances. However, a few bands are selectively retained (dots) or selectively extracted (circles).

Quantitative scanning of these proteins allowed analysis of the rates of release in 10 μM and 40 μM digitonin for four of them (Fig. 5, asterisks). Their release rates were similar to each other and to those of some of the previously measured proteins. Like lactate dehydrogenase and solubilized protein, but in contrast to TyrOHase and PMTase, the rate of release, and thus the apparent diffusion coefficient, was not affected by the concentration of digitonin. The mean rate of release in 10 μM digitonin was 0.056 log percent/min and 0.061 log percent/min in 40 μM digitonin.

As predicted by diffusion theory, in 40 μM digitonin the rates of release of TyrOHase, PMTase, lactate dehydrogenase, and the proteins quantitated by densitometry were inversely proportional to their size. The M_r of each protein was estimated from its behavior on NaDodSO$_4$/PAGE, with the exception of the tetramer, lactate dehydrogenase, which has a M_r of 134,000. Each protein was assumed to be spherical and the radius of a sphere of equal volume was calculated by the formula

$$\left(\frac{3M_r}{4\pi\,\rho N}\right)^{1/3} = r,$$

where M_r = molecular mass of the proteins, ρ = the average anhydrous density of protein (estimated, 1.33 g/cm^3), N = Avogadro's number, and r = radius of the molecule. The inverse of the radius is directly proportional to the diffusion coefficient of the protein. Although it was not possible to calculate the diffusion coefficient directly, the experimentally determined release rates were directly proportional to this value. A plot showing the relationship between release rate and $1/r$ is shown in Fig. 6. This relationship defined a line with a correlation coefficient of 0.883. The deviation of several of the proteins from this line may be due to the necessity of making oversimplifying assumptions regarding the shapes and monomeric nature of the molecules. Since at 10 μM digitonin most of the proteins showed the same release rate as at 40 μM, the plot is nearly unchanged if the data from 10 μM digitonin are used. The exceptions to this are the release rates for TyrOHase and PMTase, which are significantly lower at 10 μM digitonin and yield points in Fig. 6 (open symbols) that deviate significantly from the line ($P < 0.01$).

To test the possible involvement of cytoskeletal elements in the slow efflux of TyrOHase and PMTase in 10 μM digitonin,

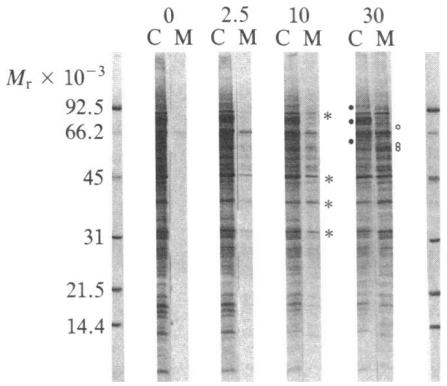

FIG. 5. Time course of cellular and medium proteins in 40 μM digitonin analyzed by Coomassie blue staining after NaDodSO$_4$/PAGE. After incubation of cells in 40 μM digitonin for 0, 2.5, 10, or 30 min, 0.25-well equivalents of cellular lysate (lanes C) and 1.0-well equivalents of released medium (lanes M) were applied to a 10–20% NaDodSO$_4$/polyacrylamide gel and stained with Coomassie blue. Time points (in min) are above the lanes. Molecular weight standards are in the extreme left and right lanes and their sizes are at left.

we pretreated the cells with drugs that alter the configuration of the cytoskeleton. Pretreatment of the cells for 30 min with 50 μM phalloidin (to stabilize F actin), 50 μM colchicine (to depolymerize microtubules), or 10–100 μM cytocholasin D (to depolymerize F actin) had no effect on the efflux rate of PMTase or TyrOHase in 10 or 40 μM digitonin. In contrast, treatment of permeabilized cells with 50 μM colchicine or 100 μM cytocholasin D substantially potentiated catecholamine release induced by increasing the free Ca^{2+} concentration to 20 μM (unpublished observations). This indicated that these drug doses were effective on some processes in the cells and led us to conclude that points of attachment of TyrOHase or PMTase were not likely to be cytoskeletal elements such as microtubules or F actin.

DISCUSSION

Exposure of chromaffin cells to a range of digitonin concentrations indicated the presence of three cellular compartments. We used the extracellular appearance of proteins and catecholamines to define these compartments, characterized by their digitonin dependencies. (*i*) The first was the cyto-

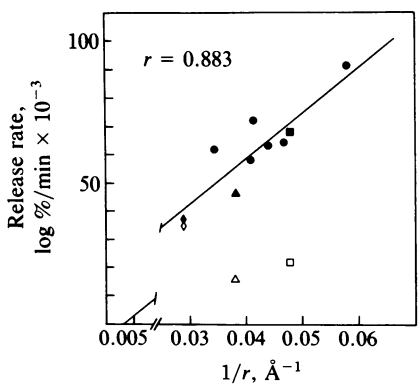

FIG. 6. Correlation between protein release rate and molecular size. The size of each protein was calculated as described in *Results* and $1/r$ was plotted against the release rate in 40 μM digitonin: proteins quantitated by NaDodSO$_4$/PAGE (●); lactate dehydrogenase (◆); TyrOHase (▲); PMTase (■). $1/r$ was plotted against release rate in 10 μM digitonin for lactate dehydrogenase (◇); TyrOHase (△); PMTase (□). The line defined by the solid symbols was calculated by least-squares linear regression and had a correlation coefficient of 0.883. The points for TyrOHase and PMTase release in 10 μM digitonin fall outside the 95% confidence limits of this line ($P < 0.01$).

plasmic protein pool, which was released when the plasma membrane was permeabilized. At low digitonin concentrations (<5 μM), a substantial fraction of solubilized protein (an index of how an average of all proteins behave) and lactate dehydrogenase were released. Digitonin is known to cause the plasma membrane of adrenal chromaffin cells to become leaky, resulting in the release of intracellular proteins that are freely soluble in the cytoplasm of the cell (22, 23). (*ii*) The second compartment was a "digitonin-resistant" cytoplasmic protein pool, which was released only after disruption of both the plasma membrane and a detergent-labile "sequestration" of these proteins, the nature of which is unknown. At higher digitonin concentrations (≈10 μM), the proteins of this second group, TyrOHase and PMTase, began to appear in the extracellular medium. Thus, these proteins exist within the cell in a state that is more resistant to digitonin solubilization than is the plasma membrane, perhaps in association with an intracellular binding site. An analogous situation exists for the enzyme hexokinase, which binds to a proteinaceous binding site in the membrane of mitochondria. This enzyme is more resistant to digitonin solubilization than adenylate kinase, an enzyme whose liberation depends only on the permeabilization of the mitochondrial membrane (20, 21). (*iii*) The third compartment is the chromaffin granule, which is relatively insensitive to digitonin permeabilization (16, 17) and therefore retains intragranular components such as dopamine β-hydroxylase and catecholamines.

We were able to confirm that TyrOHase and PMTase existed in a state that restricts their diffusion by examining the rates at which these substances were released from the cell at 10 μM and 40 μM digitonin and comparing these rates to those of other proteins under these same circumstances. With the exception of TyrOHase and PMTase, all the indices (lactate dehydrogenase, solubilized protein, dopamine β-hydroxylase, and catecholamines, as well as four proteins quantitated by $NaDodSO_4$/PAGE and densitometry) were released from the cell at the same rate at both concentrations of digitonin. This suggested that the disruption of the plasma membrane was complete at 10 μM digitonin and therefore allowed the efflux of most proteins at their diffusion-limited rate at both 10 μM and 40 μM digitonin. This is reinforced by the fact that at both 10 μM and 40 μM digitonin, these proteins diffuse at a rate that is proportional to their size, behavior consistent with a freely diffusable state.

In contrast to the behavior of most of the proteins, at 10 μM digitonin TyrOHase and PMTase were markedly retarded in their rate of release and diffused more slowly than expected for their sizes. When the concentration of digitonin was increased to 40 μM, the rates of TyrOHase and PMTase efflux were increased 3-fold and these higher release rates were consistent with their molecular weights. We conclude that TyrOHase and PMTase exist in a state within the chromaffin cell that restricts their diffusion in 10 μM digitonin. This could be an association with other proteins or organelles, although the lack of effect of cytoskeletal disrupting drugs on TyrOHase and PMTase efflux argues against their binding to cytoskeletal elements. This putative association is disrupted by 40 μM digitonin, allowing free diffusion of these two proteins. These findings confirm that TyrOHase and PMTase exist in an undefined, but clearly detergent-labile and therefore noncovalent, association within the cell. In contrast, lactate dehydrogenase, other soluble proteins, and the intragranular components, catecholamines and dopamine β-hydroxylase, are truly soluble and depend only on the permeabilization of their respective boundary membranes for diffusion-limited release.

The impaired diffusion of the two catecholamine biosynthetic enzymes, TyrOHase and PMTase, from chromaffin cells and the apparent lack of involvement of cytoskeletal structures is consistent with their suggested localization in association with the surface of the chromaffin granule, perhaps bound to a specific binding site (5–13). It would seem to be in the interest of cellular economy to place these two enzymes in close proximity to the third catecholamine biosynthetic enzyme, dopamine β-hydroxylase, which is found in the membrane of and within the chromaffin granule. This association might ensure ready availability of substrates and products for catecholamine biosynthesis and reduce the time necessary for diffusion out of and uptake into the granule during this process. This type of multienzyme association has been described for the glycolytic enzyme pathway (31) and may be a common cellular organizational scheme. In fact, it has recently become clear that most proteins within the cell are not completely free to diffuse throughout the cytosol (32) and that they behave as if the viscosity they experience is many times higher than that expected in an aqueous compartment (33). This implies that "cytosolic" proteins are commonly subject to noncovalent associations or barriers within the cell that impair their diffusion and are probably important for their optimal function.

This work was performed while K.L.K. was supported by a Pharmacology Research Associate Training Fellowship from the National Institute of General Medical Sciences, National Institutes of Health.

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