Vol. 119, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1984 Pages 116-123

COMPARISON OF SEROTONIN UPTAKE BY DENSE BODIES INSIDE AND OUTSIDE HUMAN PLATELETS

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Received January 9, 1984

A technique has been developed for quantitating the absolute number of dense bodies present in solution following isolation from human platelets. The amount of [^{3}H]-5HT accumulated per dense body was measured following either sonication alone or sonication plus isolation utilizing a Metrizamide density gradient; the dense bodies in each case were washed and resuspended in sodium or potassium-rich buffer. Uptake per dense body following removal from the cell was less than 10% of the amount of uptake per dense body by intact platelets. It thus seems possible that residence of dense bodies inside intact platelets is required for 5HT transport into dense bodies to proceed at a maximal rate.

Examination of the subcellular processes associated with amine uptake into chromaffin granules has been greatly facilitated by the availability of methods for the isolation of these organelles (Winkler and Westhead, 1980). The development of techniques for isolating the dense bodies (amine storage vesicles) from human platelets (Fukami et al., 1978) has permitted similar types of studies, and the work to date suggests that amine uptake into the of two types vesicles is mediated by similar mechanisms. In both cases, the vesicular membrane is believed to play a pivotal role by virtue of its content of amine transport proteins and its ability to generate pH and ionic potential gradients (Johnson et al., 1979; Salama et al., 1980; Carty et al., 1981; Scherman et al. 1981; Wilkins et al., 1981; Fishkes et al., 1982).

The nature of amine transport into vesicles inside intact cells has proved more difficult to define. Although the plasma membrane or components in the cell cytoplasm may be important, their role has not been possible to delineate because of the lack of methods for direct comparison of uptake kinetics in isolated vesicles and intact cells. We report here the

0006-291X/84 $1.50

the development of a technique for quantitating the amount of serotonin (5HT) taken up per dense body by dense bodies isolated from human platelets. When coupled to accurate measurement of 5HT uptake per dense body by the dense bodies in intact platelets, this method can be used to estimate the relative of contribution structures outside the vesicle membrane to 5HT uptake by intact platelets.

MATERIALS AND METHODS

For a typical dense body preparation, 200 ml of whole blood was collected from volunteers with no history of hematologic disorders or drug ingestion for 10 days prior to donation. Blood was collected into the citrate-EDTA medium of Detwiler and Feinman (1973), and platelet-rich plasma (PRP) was prepared by differential centrifugation (Murphy et al., 1969).

For measurement of platelet dense bodies, air-dried whole mounts were prepared by rapid blotting of 10 microliter aliquots of PRP placed on carbon-coated grids (E.F. Fullam Co., Schenectady, NY) (Costa et al., 1974; Costa et al., 1977). Platelet uptake of 5HT was evaluated by centrifuging the PRP at 1000 g for 20 minutes and resuspending the platelets in a buffer ("Tris-citrate-albumin buffer") at pH 7.35 with the following composition: 116 mM NaCl, 4.2 mM KCl, 1.8 mM KH_2PO_4, 1.1 mM $MgSO_4$, 10.9 mM trisodium citrate, 25 mM Tris, 5.9 mM dextrose, and 0.35% bovine serum albumin (crystallized, lyophilized, and essentially fatty-acid free; Sigma Chemical Co. St. Louis, MO) (Costa et al., 1977). Aliquots of platelets containing 10^7 platelets per ml were warmed to 37°C and incubated with 10^{-6}M [^{3}H]-5HT (30.1 Ci/mmole; New England Nuclear Corp., Boston, MA) and 10^{-5}M [^{14}C]-inulin (1.9 Ci/mg; Amersham Corp., Arlington Hts., IL) for 10 or 30 minutes. Platelets were fixed with formaldehyde (Costa and Murphy, 1975) and pelleted; platelet radioactivity was mobilized with 0.4 N perchloric acid and counted in a liquid scintillation counter (Tracor Mark III; Tracor Analytic, Des Plaines, IL).

Dense bodies were separated from other cellular components utilizing the procedure of Fukami et al. (1978), which was modified to give a high yield of intact dense bodies, to eliminate intact platelets from the dense-body preparation, and to avoid the use of metabolic poisons. PRP was centrifuged (1000 g x 20 minutes) and the platelets were resuspended in 14 ml of 0.25 M sucrose containing 10 mM Tris and 2 mM ATP ("sucrose buffer;" pH 7.35), and maintained at 0°C while being sonicated at the lowest power setting for 25 seconds (Ultrasonics Sonifier W-350 with a 1/2-inch tip). The sonicate was spun at 11,500 g for 20 minutes, and one portion of the pellet was washed twice and resuspended in Tris-citrate-albumin buffer containing 2 mM ATP (for uptake studies, see below). A second portion was resuspended in 1 ml of 0.25 M sucrose buffer and layered over a continuous density gradient of Metrizamide (Accurate Chemical Co., Hicksville, NY). Gradients were formed in cellulose nitrate tubes from isosmotic solutions of 16 gms of Metrizamide and 37 ml of Tris-citrate albumin buffer at pH 7.4 per 100 ml final volume, and 35 gms of Metrizamide and 37 ml of 10 mM TES buffer at pH 7.4 (N-tris [hydroxymethyl] methyl-2-aminoethane sulfonic acid; Sigma Chemical Co.) per 100 ml final volume. Gradients were centrifuged for 60 minutes at 100,000 g in Beckman L2-65B ultracentrifuge with a swinging-bucket rotor (SW-40, Beckman Instruments, Palo Alto, CA), and the appropriate bands were aspirated with a Pasteur pipette bent to a 90° angle. We chose to study uptake utilizing the bottom band (band 3), which contained the highest number of dense bodies per unit area. Essentially no intact platelets were present when this material was spun onto grids in a quantitative fashion (see below).

For measurement of [^{3}H]-5HT uptake, dense bodies were washed two times by centrifugation (11,500 g for 20 minutes), resuspended in 10 ml of sucrose-Tris buffer, and centrifuged again. Dense bodies were then resuspended in Tris-citrate-albumin buffer containing 2 mM ATP and either held at 0°C or warmed to 37°C. A final concentration of 10^{-6}M [^{3}H]-5HT (30.1 Ci/mmole) and 10^{-5}M [^{14}C]-inulin (1.9 mCi/mg; Amersham Corp., Arlington Hts, IL) was added. After incubation for 10 or 30 minutes, dense bodies were cooled to 0°C and centrifuged for 1 minute at top speed (30 psi; approximately 95,000 g) in the angle head rotor of a Beckman Airfuge (Beckman Instruments). Radioactivity in the pellet was mobilized with 0.4 N $HClO_4$ and counted as described above. The amount of [^{3}H]-5HT accumulated intracellularly was calculated by subtracting from the total amount of tritium present that which was trapped in the pellet (as estimated from the amount of [^{14}C]-inulin present in the pellet of both the intact platelets and the isolated dense bodies).

Dense bodies were quantitated in intact platelets by electron-microscopic examination of air dried whole mounts (Costa et al., 1974, 1977). Isolated dense bodies were quantitated using the Beckman Airfuge and EM-90 rotor, in a modification of published procedures for counting virus particles in suspension (Hammond et al., 1981). Carbon-coated electron-microscope grids (E.F. Fullam Co.) were held in place at the bottom of the rotor by placing them on top of 5 mm x 5 mm pieces of Mylar film lying against the bottom wall. The Mylar was coated prior to use with 3140 RTV silicone curing rubber (Dow Corning, Midland, MI). Aliquots of the dense-body suspension were centrifuged at approximately 95,000 g (top speed, or 30 psi) for 1 minute, after which the grid was removed, blotted dry, and examined in the electron microscope. Counting was done utilizing pictures (5000 x or 10,000 x) taken in the scanning-transmission mode of an Hitachi H-700H microscope equipped with a scanning attachment. Identification of dense bodies was confirmed by electron microprobe analysis (Costa et al., 1981).

RESULTS

In our hands, the cell disruption procedure which provided the best total yield of morphologically-identifiable dense bodies, and the lowest number of intact platelets, was a relatively short sonication at a low power setting (average yield, 45.3%) (Table 1). Centrifuging the particulate

Table 1. Recovery of human platelet dense bodies following sonication and isolation on a Metrizamide gradient

Experiment Number	Total Number of Dense Bodies in Platelets	Percent Platelet Dense Bodies Remaining After Sonication	Percent Platelet Dense Bodies in Band 3 of Metrizamide
1	6.3×10^{10}	23.2%	5.9%
2	1.2×10^{11}	78.7%	6.0%
3	1.1×10^{11}	44.0%	7.7%
4	7.8×10^{11}	52.5%	17.2%
5	6.0×10^{10}	28.2%	6.5%

portion of this sonicate over sucrose gradients according to the method of Fukami et al. (1978) produced a pellet containing less than 5% of the total number of dense bodies present in the sonicate. With a Metrizamide gradient, no pellet was present and three distinct bands were visible within the body of the gradient. Both dense bodies and alpha granules were present in all three bands (Figure 1), but the lowermost band (band 3) contained no observable intact platelets and the largest number of dense bodies per unit area. On the average from five experiments, 21.3% of the dense bodies present in the sonicate were recovered in band 3; the average overall yield was 8.7% of the dense bodies available prior to sonication.

Previous studies have shown that, under normal circumstances, more than 90% of an initial concentration of 10^{-6}M [^{3}H]-5HT added to platelets

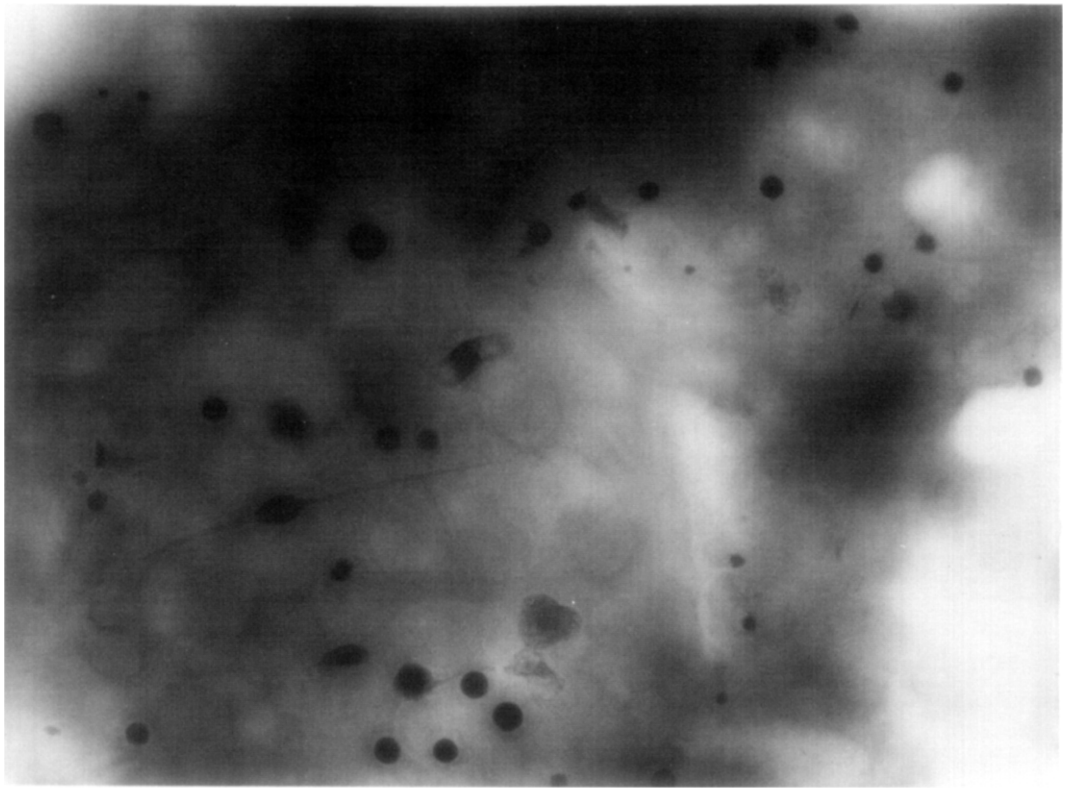

Figure 1. Typical field on a Airfuge-prepared grid containing the subcellular components present in band 3 of the Metrizamide gradient. Dense bodies are recognizable because of their relative electron opacity as compared with other structures. Dense bodies with "tails" and "handles" resemble those seen in whole amounts of intact platelets (Sweetman et al., 1980). x 17,000.

at 37°C enters the dense bodies during a 10-30 minute incubation period (Costa et al., 1977). Total platelet uptake of 10^{-6}M [^{3}H]-5HT can therefore be used as an index of the amount of [^{3}H]-5HT accumulated by the dense bodies in intact platelets. Intact platelets were first diluted to a final cell concentration of 10^7 platelets/ml in order to produce a concentration of dense bodies per ml similar to that existing in the solutions containing isolated dense bodies. The uptake of dense bodies inside platelets averaged 4.1 x 10^{-20} moles/dense body after 10 minutes at 37°C and 7.4 x 10^{-20} moles/dense body after 30 minutes at 37°C (Table 2). Uptake at 0°C was negligible.

In contrast, a single dense body after sonication, washing, and incubation at 37°C took up 0.25 x 10^{-20} moles of [^{3}H]-5HT after 10 minutes and 0.40 x 10^{-20} moles after 30 minutes--6% and 5% of the amounts accumulated, respectively, by dense bodies in intact platelets. Even at 0°C, isolated dense bodies took up 0.10 x 10^{-20} moles of

Table 2. Comparison of the uptake of [^{3}H]-5HT by dense bodies in intact human platelets and after sonication alone, or after sonication plus isolation on a Metrizamide gradient

Incubation Conditions	Amount of [^{3}H]-5HT Accumulated Per Dense Body in Intact Platelets (moles x 10^{20})	Amount of [^{3}H]-5HT Accumulated Per Dense Body After Isolation (moles x 10^{20})	
		Sonication Only	Sonication + Metrizamide Isolation
0°C, 10 minutes	0.049[1] ±0.001	0.101 ±0.007	0.139 ±0.009
37°C, 10 minutes	4.07 ±0.05	0.251[2] ±0.003	0.228 ±0.002
37°C, 30 minutes	7.41 ±0.05	0.404[2] ±0.012	0.269 ±0.014

[1]Represents [^{3}H]-5HT trapped in pellet; equivalent to percentage of [^{14}C]-inulin associated with pellet.

[2]In Tris-citrate-albumin buffer in which the sodium was 4 mM and the potassium 120 mM, the uptake of [^{3}H]-5HT was 0.23 x 10^{-20} moles/dense body after 10 minutes at 37°C, and 0.50 x 10^{-20} moles/dense body after 30 minutes at 37°C.

[^{3}H]-5HT in 10 minutes (40% of the amount accumulated during a similar time period at 37°C). Metrizamide-isolated dense bodies at 0°C or 37°C took up amounts of [^{3}H]-5HT comparable to those accumulated by sonicated-washed dense bodies. In order to evaluate the possibility that dense bodies after removal from the cell might lyse following incubation in chloride-rich buffer, we compared the numbers of dense bodies present after sonication in sucrose buffer and after washing and incubation in Tris-citrate-albumin buffer at either 0°C or 37°C for 10-30 minutes. The sonicate in sucrose contained a total of 2.55×10^{10} dense bodies; after washing and incubation for 30 minutes at 37°C, 1.95×10^{10} dense bodies were present. Isolation utilizing a Metrizamide gradient produced 5.50×10^9 dense bodies in band 3; 5.51×10^9 were counted after incubation at 0°C for 30 minutes and 7.33×10^9 after incubation for 30 minutes at 37°C.

DISCUSSION

These studies were initiated to develop methods for the comparison of uptake by dense bodies in intact human platelets and following isolation. We were reluctant to employ the rotenone/deoxyglucose procedure of Fukami et al. (1978) for isolation, since exposure of dense bodies to an inhibitor of mitochondrial oxidative phosphorylation might also be expected to alter the uptake mechanism (Bashford et al., 1976; Costa et al., 1978). As reported by Fukami et al. (1978), we also obtained very low yields of dense bodies when we omitted this preincubation with metabolic inhibitors prior to sonication and centrifugation over a sucrose gradient. However, a combination of relatively gentle sonication alone, or gentle sonication followed by centrifugation over a Metrizamide gradient, produced dense body yields comparable to those reported by Fukami et al. (1978).

Once isolation and quantitation techniques were developed, we were able to determine that dense bodies do not lyse following incubation in chloride-rich buffer, and that the amount of [^{3}H]-5HT taken up per dense body is less than 10% of the amount accumulated by dense bodies in intact platelets. The presence of appreciable numbers of alpha-granules and lyso-

somes in the sonicates or Metrizamide band 3 appears not to present a problem, since it does not interfere with measurement of the total number of dense bodies present. Furthermore, accumulation of [^{3}H]-5HT by all three types of organelles would be expected only to increase the apparent amount of uptake per dense body.

Uptake by isolated dense bodies at 0°C was almost half of that seen at 37°C. It therefore seems likely that the observed accumulation of [^{3}H]-5HT by dense bodies removed from human platelets might be low because it represents, in part, a diffusion-mediated process. It is possible that some type of damage to dense bodies during the removal procedure might have diminished greatly their active uptake capacity. Nevertheless, previous studies have suggested that the calcium and phosphorous in the dense body core remain intact even during isolation on a Metrizamide gradient (Costa et al., 1981). Our data is thus also compatible with the previous hypothesis that an important determinant of the rate and extent of 5HT uptake into dense bodies is their relationship to other cellular components such as the plasma membrane (Costa et al., 1981). Further insights into this question might be gained by comparison of amine uptake per vesicle in intact cells with that in vesicles isolated from platelets of other species (DaPrada and Pletscher, 1968; Salganicoff et al., 1975) or from chromaffin cells (Sudhof, 1982).

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