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DEMONSTRATION AND EVALUATION OF APPARENT CYTOPLASMIC AND VESICULAR SEROTONIN COMPARTMENTS IN HUMAN PLATELETS

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Abstract—Thrombin has been used in conjunction with formaldehyde treatment to investigate the distribution of labeled serotonin between releasable and non-releasable compartments during the serotonin uptake process. The data indicate that serotonin accumulates in a predictable fashion in both compartments, which may represent, respectively, vesicular and cytoplasmic amine pools. The results also suggest that the cytoplasmic pool may reach sizable proportions under certain incubation conditions.

Previous work has indicated that human platelets store both endogenous and newly added serotonin (5-HT) primarily in membrane-bound vessicles [1, 2]. The actual uptake of 5-HT is, however, thought to occur in two steps: (a) transport through the plasma membrane into the platelet cytoplasm, and (b) transport through the vesicular membrane into the storage vesicle [1, 3–5]. Whether 5-HT transported through the plasma membrane accumulates in appreciable quantities in a cytoplasmic pool prior to its translocation into vesicles has not been evaluated to date.

This paper describes the use of thrombin treatment in conjunction with formaldehyde fixation to examine the early phases of 5-HT uptake. The addition of formaldehyde to platelets appears to stop 5-HT uptake very rapidly, permitting more accurate evaluation of the time course of uptake than has previously been possible [6]. Thrombin releases endogenous platelet 5-HT into the extracellular medium, presumably from vesicular storage sites [1, 2]. This work suggests that the effect of thrombin on platelet serotonin stores over relatively short periods is limited to the exocytotic release of storage vesicles.

MATERIALS AND METHODS

Blood was collected from volunteers without known hematologic problems or a history of drug ingestion for 10 days prior to collection, utilizing a collection medium in which the final concentration of citrate in whole blood was 18 mM and that of EDTA was 1 mM [7]. Platelet-rich plasma (PRP) was prepared by differential centrifugation at 4° [8]. PRP was incubated with 1×10^{-7} M [^{14}C]5-HT (55 mCi/m-mole; Amersham/Searle, Elmhurst, Il.) at 37° for 30 min. The PRP was then cooled to 4°, and the platelets were spun into a pellet and resuspended to a final count of 1 to 2×10^{8}/ml in buffer of the following composition: 116 mM NaCl, 4 mM KCl, 1.8 mM KH_2PO_4, 1.1 mM $MgSO_4$, 25 mM Tris, 10.9 mM citrate, 5.9 mM dextrose, and 0.35% bovine serum albumin (crystallized and essentially fatty-acid free, obtained from Sigma Chemical Co., St. Louis, MO), at a pH of 7.35.

Duplicate 500-μl aliquots of platelet resuspension were brought to 37°, and [^{3}H]5-HT (500 mCi/m-mole; Amersham/Searle, Elmhurst, Il.) was added to varying initial concentration levels. This and all subsequent additions were performed by placing an appropriate volume (5–25 μl) of the solution to be added to the platelets on a plastic plumper (CalBiochem, La Jolla, CA) and dropping the plumper into the aliquot of resuspended platelets. At appropriate times after the addition of [^{3}H]5-HT, aliquots were either fixed with formaldehyde [6] or treated with 2 units/ml of bovine or human thrombin.

Formaldehyde was prepared daily from trioxymethylene, as described by Karnovsky [9], and added so that the final concentration was 1.5%. Thrombin, obtained through the courtesy of Dr. John Fenton, had been purified according to the method of Glover and Shaw [10]. Formaldehyde was also added to thrombin-treated aliquots 1 min after the addition of thrombin. All samples were kept in an ice bath after formaldehyde addition.

After fixation, platelets were pelleted by centrifugation at 4°. Aliquots of the supernatant were taken for scintillation counting, the remainder of the supernatant was aspirated, and 550 μl of 0.4 N perchloric acid was added to the platelet pellet. A 500-μl aliquot of the perchloric acid was added to the scintillation fluid. Samples were scintillation counted using separate channels for tritium and carbon-14 evaluation. The spillover of carbon-14 into the tritium channel was corrected for by utilizing ^{14}C-labeled samples to which no tritium had been added.

Dense-body content of platelet populations was evaluated by counting the dense bodies in a random selection of 100 platelets seen in whole mounts, prepared by the air-drying procedure utilized previously [11].

RESULTS

Examination of the amount of prelabeled [^{14}C]5-HT released from resuspended platelets by thrombin shows that the per cent [^{14}C]5-HT released remains essentially constant over a 60-min period

Table 1. Thrombin-induced release of prelabeled [^{14}C]5-HT and dense bodies from platelets as a function of incubation time in 5-HT-free media*

	Time of incubation at 37° (min)					
	2	5	10	20	30	60
Donor No. 1						
Per cent release of [^{14}C]5-HT (mean ± S.E.M., $N = 6$)	78.8 ± 0.8	78.7 ± 0.6	78.9 ± 2.0	80.0 ± 0.9	82.1 ± 0.8	81.9 ± 0.9
Per cent release of dense bodies from 100 platelets	76.1					82.0
Donor No. 2						
Per cent release of [^{14}C]5-HT (mean ± S.E.M., $N = 6$)	84.1 ± 1.0	83.3 ± 0.4	82.6 ± 1.6	83.9 ± 0.8	84.0 ± 0.9	81.0 ± 1.5
Per cent release of dense bodies from 100 platelets	86.1					81.9

* PRP from each donor was incubated for 30 min at 37° with [^{14}C]5-HT (initial concentration 1×10^{-7} M). Platelets were cooled to 4°, spun into a pellet, and resuspended in 5-HT-free buffer (platelet concentration 1 to 2×10^8/ml of solution). Duplicate 500 μl-aliquots, incubated at 37°, were either fixed with formaldehyde at the times indicated or treated with thrombin (final concentration 2 units/ml) and then fixed with formaldehyde 60 sec after thrombin addition. Labeled material in platelet pellets was determined as described in Materials and Methods. The per cent release of [^{14}C]5-HT was calculated from the amount of ^{14}C-labeled material remaining in the pellet after thrombin treatment. The per cent release of dense bodies was calculated from the number of dense bodies remaining in 100 platelets after thrombin treatment (see Materials and Methods).

(Table 1). Similarly, the per cent of the total dense-body complement of 100 platelets released by thrombin remains unchanged over this time period, and is similar in amount to the total per cent release of [^{14}C]5-HT.

To evaluate the rate of entry of [^{3}H]5-HT into platelet storage vesicles, [^{3}H]5-HT was added at varying initial concentrations, up to 10^{-6} M, to resuspended platelets (prelabeled in PRP with 1×10^{-7} M [^{14}C]5-HT as described above)(Table 2). Comparison of total platelet [^{3}H]5-HT with the amount released by thrombin over time intervals from 10 to 300 sec after the addition of [^{3}H]5-HT suggests that the amount of [^{3}H]5-HT releasable by thrombin as a proportion of total [^{3}H]5-HT accumulated in the platelet (i.e. that in the apparent vesicular storage pool) is at first relatively small. After longer time periods, progressively more of the total platelet [^{3}H]5-HT becomes thrombin-releasable. At longer time intervals and with the lower [^{3}H]5-HT concentrations used here, the per cent [^{3}H]5-HT released approaches (but never exceeds) the per cent [^{14}C]5-HT released. Both total platelet [^{14}C]5-HT and thrombin-releasable [^{14}C]5-HT remain unchanged over the time periods studied here (Table 2).

Table 3 summarizes changes in the levels of extracellular [^{3}H]5-HT in the same series of experiments described in Table 2. As indicated, the extracellular 5-HT concentration decreases steadily over time at all concentrations studied.

Table 4 presents a comparison of changes in total and releasable [^{14}C]5-HT and [^{3}H]5-HT after the addition of [^{3}H]5-HT at initial concentrations of 10^{-6} and 10^{-5} M. For Table 4, we have used the per cent release of prelabeled [^{14}C]5-HT prior to [^{3}H]5-HT addition as an index of the per cent release of the total vesicular pool. From this figure, we have calculated the amount of material in vesicular and cytoplasmic compartments. The non-releasable cytoplasmic pool of [^{3}H]5-HT declines to zero after a 30-min incubation with 10^{-6} M [^{3}H]5-HT, but increases after a similar incubation with 10^{-5} M [^{3}H]5-HT. Total [^{14}C]5-HT, although apparently unchanged after a 2-min incubation period with 10^{-6} or 10^{-5} M [^{3}H]5-HT, declines by 30 min after the addition of the [^{3}H]5-HT. The per cent of [^{14}C]5-HT released by thrombin 30 min after the addition of 10^{-5} M [^{3}H]5-HT indicates that some vesicular [^{14}C]5-HT has moved from the vesicular compartment into the cytoplasm during this time period.

Table 5 indicates that after a 10-min incubation with 10^{-5} M [^{3}H]5-HT, the amount of labeled material released by thrombin increases when platelets are resuspended in a 5-HT-free medium and incubated briefly at 37°.

DISCUSSION

The studies presented here describe the use of thrombin and formaldehyde fixative to study the uptake and vesicular storage of radioactively labeled 5-HT. Some points not previously appreciated emerge from the data.

First, the concentration of extracellular 5-HT can change significantly within tens of seconds after 5-HT addition at initial concentrations of 10^{-6} M or lower, when measured in platelet suspensions containing 1 to 2×10^8 platelets/ml. This seems reasonable in view of the fact that, at an initial concentration of 10^{-8} M 5-HT, there are 3×10^{12} molecules of [^{3}H]5-HT/500-μl aliquot. With 10^{-8} M [^{3}H]5-HT, each platelet need only accumulate 10^4 molecules of [^{3}H]5-HT in order to remove essentially all the labeled material from the medium.

Table 2. Evaluation of the thrombin-induced release of prelabeled [^{14}C]5-HT and newly added [^{3}H]5-HT as a function of time from 10 to 300 sec after [^{3}H]5-HT addition*

	Time after addition of [^{3}H]5-HT before fixation or addition of thrombin (sec)						
	0	10	20	30	60	120	300
Prelabeled [^{14}C]5-HT							
Total (moles/platelet × 10^{19})	1.833	1.857	1.828	1.842	1.829	1.837	1.862
Per cent released by thrombin	91.2	91.1	91.0	90.8	90.9	91.5	92.0
Newly added [^{3}H]5-HT							
5×10^{-8} M							
Total (moles/platelet × 10^{20})	0.220†	0.695	1.354	1.701	2.787	5.894	10.648
Per cent released by thrombin	0	32.1	50.7	72.4	79.0	87.9	90.9
1×10^{-7} M							
Total (moles/platelet × 10^{20})	1.220†	0.845	1.801	2.766	4.588	10.394	17.314
Per cent released by thrombin	0	35.3	62.3	62.0	80.4	85.9	87.4
5×10^{-7} M							
Total (moles/platelet × 10^{20})	1.988†	2.541	5.403	9.422	16.425	31.343	74.055
Per cent released by thrombin	0	12.2	34.7	52.1	64.2	76.6	84.9
1×10^{-6} M							
Total (moles/platelet × 10^{20})	3.663†	3.557	7.807	12.806	24.268	43.239	104.60
Per cent released by thrombin	0	6.6	36.0	52.4	62.5	72.4	80.3

* Platelets were prelabeled with [^{14}C]5-HT and resuspended in 5-HT-free buffer as described for Table 1. For the 0 sec time point, 500-μl aliquots of platelet suspension were fixed prior to the addition of [^{3}H]5-HT. The other time points were obtained as follows: 25 μl of [^{3}H]5-HT solution was placed on a plastic plumper, which was then dropped into a 500-μl aliquot of platelet resuspension (final concentration as specified in this table). At the specified time after the addition of [^{3}H]5-HT, either 25 μl of formaldehyde fixative (final concentration 1.5%) or 10 μl thrombin (final concentration 2 units/ml) was added to platelets using a second plumper. Formaldehyde fixative (25 μl, final concentration 1.5%) was added to thrombin-treated aliquots 60 sec after the addition of thrombin. Labeled material in platelet pellets was determined as described in Materials and Methods, and the per cent release calculated as outlined for Table 1.

† Labeled material associated with the platelet pellet when platelets were fixed with formaldehyde prior to the addition of [^{3}H]5-HT. This value was subtracted from the amounts of ^{3}H-labeled material associated with pellets at each of the subsequent time points, since it is believed to represent material trapped in the pellet extracellular space (see Ref. 6).

Second, measurement of the thrombin-induced release of prelabeled [^{14}C]5-HT in platelets resuspended in a 5-HT-free medium may serve as an index of the per cent release of the total vesicular pool. Previous studies have shown that incubating human PRP with 10^{-7} M [^{14}C]5-HT at 37° for 30 min adds to platelet endogenous 5-HT a relatively small amount of labeled material (on the average about 1 per cent of the endogenous stores) [12]. These data and those of others [1, 2] suggest that the behavior and per cent release by thrombin of the ^{14}C-labeled material may be used as an index of the disposition of the endogenous 5-HT. Of more importance for interpreting some of the data presented here, the association between release of dense bodies and release of [^{14}C]5-HT in the absence of any exogenous 5-HT, and the stability of the two figures over a 60-min period at 37°, suggest that the per cent release of prelabeled [^{14}C]5-HT provides an index of measurement of the extent to which the entire population of platelet

Table 3. Changes in extracellular [^{3}H]5-HT concentration during uptake of [^{3}H]5-HT into platelets*

Initial concn of [^{3}H]5-HT (M)	Control (no uptake)	Activity (cpm) of [^{3}H]5-HT/platelet aliquot in supernatant after uptake for varying times (mean ± S. D.)					
		10 sec	20 sec	30 sec	60 sec	120 sec	300 sec
5×10^{-8}	9404	8880	8595	8093	7453	6043	3769
	± 265	± 282	± 122	± 213	± 714	± 615	± 636
		(−5.6%)	(−8.6%)	(−13.9%)	(−20.7%)	(−35.7%)	(−59.9%)
1×10^{-7}	18,421	17,795	17,541	16,395	14,705	12,747	8815
	± 2959	± 478	± 637	± 544	± 732	± 1692	± 1426
		(3.4%)	(−4.8%)	(−11.0%)	(−20.2%)	(−30.8%)	(−52.1%)
5×10^{-7}	88,720	87,123	85,615	80,070	77,320	66,673	50,504
	± 2579	± 2345	± 2574	± 5210	± 5604	± 8013	± 8104
		(−1.8%)	(−3.5%)	(−9.7%)	(−12.8%)	(−24.9%)	(−43.1%)
1×10^{-6}	184,832	184,139	184,047	174,711	165,656	157,200	125,131
	± 10,643	± 1384	± 1571	± 4165	± 14,625	± 12,417	± 17,467
		(−0.4%)	(−0.4%)	(−5.5%)	(−10.4%)	(−14.9%)	(−32.3%)

* Experimental procedures were as described for Table 2. Aliquots of the supernatant remaining after fixed platelets had been spun into a pellet were taken for liquid scintillation counting.

Table 4. Comparison of the thrombin-induced release of prelabeled [^{14}C]5-HT and newly added [^{3}H]5-HT at 2 and 30 min after [^{3}H]5-HT addition*

Initial concn of [^{3}H]5-HT (M)	Time after addition of [^{3}H]5-HT before fixation or addition of thrombin					
	Zero (no [^{3}H]5-HT uptake)		Two min		Thirty min	
	[^{14}C]5-HT	[^{3}H]5-HT	[^{14}C]5-HT	[^{3}H]5-HT	[^{14}C]5-HT	[^{3}H]5-HT
1×10^{-6}						
Total (moles/platelet $\times 10^{19}$)	1.759	3.351†	1.766	4.754	1.711	28.214
Per cent of label released by thrombin	81.4	0	81.9	63.4	81.5	72.5
Calculated amount in vesicles (moles/platelet $\times 10^{19}$)	1.759	0	1.766	3.680	1.711	28.214
Calculated amount in cytoplasm (moles/platelet $\times 10^{19}$)	0	0	0	1.074	0	0
1×10^{-5}						
Total (moles/platelet $\times 10^{19}$)	1.832	3.457†	1.839	11.777	1.428	66.004
Per cent of label released by thrombin	85.8	0	85.9	62.7	82.7	75.1
Calculated amount in vesicles (moles/platelet $\times 10^{19}$)	1.832	0	1.839	8.597	1.375	57.423
Calculated amount in cytoplasm (moles/platelet $\times 10^{19}$)	0	0	0	3.180	0.053	8.581

* Experimental procedures were as described for Table 2. Since the per cent release of platelet-dense bodies induced by thrombin is similar to that of the prelabeled [^{14}C]5-HT (see Table 1), it was assumed that the thrombin-releasability of the prelabeled [^{14}C]5-HT could be used as an index of the total per cent release of the vesicular pool. The per cent of the ^{3}H-labeled material released by thrombin was thus corrected for the per cent of the total vesicular pool released by thrombin to derive the actual amount of ^{3}H-labeled substance in the vesicles. For example, when 85 per cent of both [^{14}C]5-HT and dense bodies was released prior to the addition of [^{3}H]5-HT, the actual amount of [^{3}H]5-HT in vesicles was calculated by multiplying the per cent actually released by 100/85. Thus 62.7 per cent release of [^{3}H]5-HT indicates that 73.8 per cent of the [^{3}H]5-HT present is contained in vesicles.

† Labeled material associated with the platelet pellet when platelets were fixed with formaldehyde prior to the addition of [^{3}H]5-HT. See legend to Table 2.

vesicles is being released by thrombin. Thus, measurement of the thrombin-induced release of prelabeled [^{14}C]5-HT in 5-HT-free media, as described here, can serve as an index to which release of either prelabeled [^{14}C]5-HT or newly added [^{3}H]5-HT can be compared. Comparison of the different sets of values can be used to detect and delineate the magnitude of apparent platelet cytoplasmic and vesicular pools of both ^{14}C- and ^{3}H-labeled material.

The amount of 5-HT in the cytoplasmic (non-releasable) compartment may be quite large under certain incubation conditions, such as exposure over

Table 5. Changes in thrombin-induced release of [^{3}H]5-HT after resuspension in 5-HT-free media*

	Before resuspension	After resuspension
Total (moles/platelet $\times 10^{19}$)	29.469	30.944
Per cent of label released by thrombin	75.5	87.7
Calculated amount in vesicles† (moles/platelet $\times 10^{19}$)	25.373	30.944
Calculated amount in cytoplasm† (moles/platelet $\times 10^{19}$)	4.096	0

* Platelets in PRP were spun into a pellet and resuspended in 5-HT-free buffer (platelet concentration 2.672×10^{8}/ml of solution). Aliquots were incubated with [^{3}H]5-HT (initial concentration 10^{-5} M) for 10 min, and the total amount of label accumulated plus the amount releasable with thrombin was determined as described previously. Some 5-HT-incubated aliquots were cooled to 0° and spun into a pellet. Platelets were resuspended in 5-HT-free buffer and the total amount of label plus that releasable by thrombin was again evaluated in platelet aliquots warmed to 37° prior to the addition of fixative or thrombin. The amount of ^{3}H-labeled material in vesicles and cytoplasm was calculated according to the assumption that the thrombin released 87.6 per cent of the total vesicular population—a figure derived from the per cent release of prelabeled [^{14}C]5-HT added to a separate PRP aliquot from the same donor (see legends to Tables 1 and 4).

† Platelets from the same donor incubated in PRP with 1×10^{-7} M [^{14}C]5-HT (30 min at 37°) and resuspended in 5-HT-free media released 87.6 per cent of their [^{14}C]5-HT after the addition of thrombin.

a 30-min period to an initial extracellular 5-HT concentration of 10^{-5} M. Its retention in the platelet after a 1-min thrombin treatment suggests that thrombin acting over this time period does not act simply to permit the leakage of 5-HT through the platelet plasma membrane. Rather, these data are more compatible with the hypothesis that, at these concentrations and times, thrombin acts as a specific releasing agent for 5-HT contained in platelet vesicles.

The lack of change in the release of prelabeled [^{14}C]5-HT after prolonged incubation at 37° suggests that no net movement of [^{14}C]5-HT from vesicles to cytoplasm occurs during incubation in the absence of extracellular 5-HT. When extracellular [^{3}H]5-HT is added, platelets appear to move the ^{3}H-labeled material in sequence from a cytoplasmic to a vesicular compartment. Furthermore, when additional net cytoplasmic accumulation is stopped by removing the platelets from a 5-HT-containing medium, material originally sequestered in the cytoplasm is apparently capable of moving into the vesicles.

Since the newly added vesicular [^{3}H]5-HT from an extracellular pool of 10^{-6} M or less does not alter the amount of [^{14}C]5-HT in the vesicles during a relatively short incubation period, the [^{3}H]5-HT must initially be entering the vesicles by a process of net uptake rather than exchange. The data render it unlikely that vesicular 5-HT circulates continually between vesicle and cytoplasm in the "pump-leak" fashion proposed by others [13, 14]. It this were the case, [^{14}C]5-HT from the vesicles would mix with, and be diluted by, cytoplasmic [^{3}H]5-HT. Both components of the cytoplasmic mixture would then compete for uptake into the vesicles, decreasing the apparent amount of [^{14}C]5-HT present in this compartment.

Incubation of ^{14}C-prelabeled platelets at higher extracellular [^{3}H]5-HT concentrations (10^{-6} to 10^{-5} M) or for longer times (30 min) indicates that, at some point during the addition of [^{3}H]5-HT to the vesicular compartment, the amount of [^{14}C]5-HT in this pool begins to decrease. These data suggest that each vesicle has a certain maximal amount of 5-HT it can contain, a value which defines its "maximal packet size." Addition of cytoplasmic [^{3}H]5-HT to "full" vesicles apparently continues to occur, but produces loss of some of the prelabeled [^{14}C]5-HT (and presumably loss of endogenous 5-HT as well). The amount of [^{3}H]5-HT which can be added to a given vesicle before prelabeled [^{14}C]5-HT begins to be lost may depend on the amount of endogenous 5-HT present, a figure which can vary considerably among individuals [12].

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