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EVALUATION OF THE UPTAKE OF VARIOUS AMINES INTO STORAGE VESICLES OF INTACT HUMAN PLATELETS

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1 The uptake of various ^{3}H-labelled amines into a thrombin-releasable compartment of human platelets, thought to represent the platelet vesicular storage pool, has been evaluated. Measurable amounts of 5-hydroxytryptamine (5-HT), dopamine, tyramine, octopamine, and tryptamine accumulate in this pool, and also in a non-thrombin-releasable (cytoplasmic) pool during a 30 min incubation period with 10^{-5} M extracellular amine concentrations.

2 No differences in the accumulation of vesicular or cytoplasmic 5-HT, dopamine, and tyramine are found in platelets treated with deprenyl to inhibit platelet monoamine oxidase as compared to controls.

3 Extracellular tyramine or dopamine in concentrations as high as 10^{-5} M does not alter the initial rate of 5-HT uptake across the platelet plasma membrane. Similarly, sizable cytoplasmic pools of either amine do not alter the initial rate at which small amounts of 5-HT enter platelet cytoplasm or storage vesicles. 5-HT thus appears to be the preferred substrate for uptake into platelets and for movement from cytoplasm to vesicles.

Introduction

Human platelets appear to accumulate a variety of biogenic amines against a concentration gradient (for reviews see Murphy & Kopin, 1972; Sneddon, 1973). The reviews indicate the extent to which debate continues over whether amines other than 5-hydroxytryptamine (5-HT) can enter human platelet storage vesicles, although dopamine appears to enter vesicles in cat platelets (Boullin, McMahon & O'Brien, 1970), and isolated platelet vesicles of rabbit have been reported to accumulate dopamine and several other amines (Da Prada & Pletscher, 1969).

Recently-developed techniques, using formaldehyde fixation (Costa & Murphy, 1975) in conjunction with thrombin (Costa, Murphy & Kafka, 1977a), permit evaluation of the accumulation of radioactively-labelled substances into a platelet thrombin-releasable compartment, believed to represent the vesicular storage site. Using these methods, we have compared the distribution in intact platelets of 5-HT and other amines of biological interest in platelet cytoplasm and storage vesicles. The results of these studies suggested other experiments, also presented here, which examine the interaction between 5-HT and other amines at the platelet plasma and vesicular membranes.

Methods

Human platelets from normal volunteers were collected in citrate/disodium edetate (EDTA), and platelet-rich-plasma (PRP) was prepared by differential centrifugation at 4°C as described previously (Costa *et al.*, 1977a). All platelet counts were performed with an Electrozone Celloscope (Particle Data, Inc., Elmhurst, Ill.).

Calculation of the amounts of radioactively-labelled material in the vesicular compartment was made as described previously, using formaldehyde fixation and thrombin (Costa *et al.*, 1977a). Briefly, PRP was incubated with 2×10^{-7} M [^{14}C]-5-HT for 30 min at 37°C. Platelets were cooled to 0°C, spun into a pellet, and resuspended to a final concentration of $1-2 \times 10^8$/ml in a Tris-citrate buffer (Costa *et al.*, 1977a). Under these conditions, the [^{14}C]-5-HT can be used to follow the behaviour of the endogenous 5-HT

stores, since in control preparations, the percentage release of label induced by thrombin serves as an index of the percentage release of the total number of vesicular storage sites (Costa & Murphy, 1976; Costa *et al.*, 1977a). The actual amount of vesicular amine was calculated as described previously (Costa *et al.*, 1977a).

Evaluation of amine uptake and 5-hydroxytryptamine loss

Platelets pre-labelled in PRP with 2×10^{-7} M [^{14}C]-5-HT as above were resuspended in buffer. ^{3}H-labelled amines (5-HT, 0.50 Ci/mmol; dopamine, 0.50 Ci/mmol; tyramine, 1.1 Ci/mmol; octopamine, 3.7 Ci/mmol; and tryptamine, 0.87 Ci/mmol) were added with the use of plastic plumpers (Costa *et al.*, 1977a) to 500 µl aliquots of resuspended platelets (held at 37°C) to a final concentration of 10^{-5} M for each of the amines tested. Thirty minutes after the addition of amine, aliquots were either fixed with formaldehyde, or treated with human thrombin (2 units/ml for 60 s) and subsequently fixed. ^{14}C- and ^{3}H-labelled material in the pellets was measured by conventional methods.

Evaluation of amine uptake in control and deprenyl-treated platelets

Platelets in PRP were prelabelled with 10^{-5} M cold 5-HT (30 min at 37°C). During the final 10 min of incubation, the irreversible monoamine oxidase (MAO) inhibitor, deprenyl (final concentration 10^{-4}M), was included in the incubation medium in some aliquots. Unpublished experiments in this laboratory have shown that this procedure results in essentially complete inhibition of the production of neutral and negatively-charged metabolites from tyramine and tryptamine by either intact, resuspended platelets or by platelet sonicates. Control and deprenyl-treated platelets were pelleted and resuspended in buffer. ^{3}H-amines (5-HT, dopamine, or tyramine; final concentrations 10^{-5} M) were added to 500 µl aliquots of resuspended platelets held at 37°C. Ten minutes after the addition of amine, platelets were either fixed or treated with thrombin and then fixed. The amount of ^{3}H-amine in platelets and platelet vesicles was evaluated as above.

Effect of extracellular dopamine or tyramine on uptake of extracellular 5-hydroxytryptamine into platelets

Uptake of 10^{-7} M [^{3}H]-5-HT into unlabelled platelets, resuspended in buffer, was measured over a 10 s period at 37°C (Costa & Murphy, 1975). Acid (0.01N HCl) was added to control aliquots immediately before [^{3}H]-5-HT addition; to other aliquots, unlabelled dopamine or tyramine in 0.01 N HCl (final concentrations either 10^{-6} M or 10^{-5} M) was added immediately before the addition of [^{3}H]-5-HT.

Effect of intra-platelet dopamine or tyramine on the uptake of exogenous 5-hydroxytryptamine

Platelets from PRP were resuspended in buffer without prelabelling with [^{14}C]-5-HT. In order to obtain appreciable cytoplasmic concentrations of [^{14}C]-dopamine or [^{14}C]-tyramine, aliquots were incubated for either 10 min or 30 min at 37°C with either [^{14}C]-dopamine (10^{-5} M) or [^{14}C]-tyramine (10^{-5}M) and cooled to 0°C. Pelleted platelets were resuspended to a final concentration of $1-2 \times 10^{8}$/ml in fresh, amine-free buffer. The uptake of 10^{-8} M exogenous [^{3}H]-5-HT was examined over a 1 min period in 500 µl aliquots at 37°C, using formaldehyde and thrombin as described above. Control platelets were similarly incubated for 30 min with diluent (0.01 N HCl), and resuspended in fresh buffer before the measurement of [^{3}H]-5-HT uptake. The percentage release of vesicular [^{14}C]-5-HT was evaluated in separate, PRP-prelabelled platelets from the same donor.

Effect of cytoplasmic 5-hydroxytryptamine on the uptake of exogenous 5-hydroxytryptamine

For this experiment, we wanted to compare the effect of cytoplasmic 5-HT with that of dopamine or tyramine on the movement of new 5-HT into vesicles. However, we could not employ the same protocol as used above, since relatively large pools of cytoplasmic 5-HT will migrate completely into vesicles following resuspension in an amine-free medium (Costa *et al.*, 1977a; Costa, Silber & Murphy, 1977b). In order to obtain widely differing cytoplasmic 5-HT concentrations, two different incubation protocols were employed. For incubation A, platelets in PRP were kept at 37°C for 20 min, pelleted, and resuspended. Resuspended platelets were incubated with 10^{-5} M [^{14}C]-5-HT for 30 min (37°C), and resuspended again in fresh buffer. For Incubation B, platelets in PRP were incubated with 10^{-5} M cold 5-HT for 20 min at 37°C and resuspended in fresh buffer. Resuspended platelets were subsequently incubated with 10^{-5} M [^{14}C]-5-HT (37°C for 30 min), and again resuspended in amine-free buffer. Control platelets were incubated following similar protocols, but diluent was used instead of 5-HT. The 1 min uptake of 10^{-8} M [^{3}H]-5-HT in the resuspended platelets from each incubation was measured at 37°C, as described above, and the amounts of both ^{3}H- and ^{14}C-labelled material in vesicular and cytoplasmic compartment were calculated.

Results

Table 1 delineates the cytoplasmic and vesicular concentrations of various ^{3}H-amines, and summarizes the loss of vesicular [^{14}C]-5-HT during the amine uptake process. In terms of the amounts of labelled material accumulated, 5-HT appears to be the preferred substrate, with dopamine second. All amines examined produced sizable non-thrombin-releasable (cytoplasmic) pools, although this fraction (expressed as a percentage of the total labelled material inside the platelet) was the smallest with octopamine.

The amount of total [^{14}C]-5-HT lost from *platelets* during the accumulation of the different amines varied from <2% to 5% of the pretreatment [^{14}C]-5-HT content for all amines except for [^{3}H]-5-HT, which produced greater (up to 22%) losses of [^{14}C]-5-HT. The amount of [^{14}C]-5-HT lost from *vesicles*, in contrast, ranged from <2% to almost 30% of the pretreatment content for all amines including 5-HT. Since the amount of [^{14}C]-5-HT in the vesicles prior to ^{3}H-amine addition represents only 1–5% of the total endogenous 5-HT content, and is released by thrombin to the same extent as endogenous 5-HT and the total dense-body pool, it can serve as a marker for the behaviour of the endogenous (unlabelled) 5-HT (Costa & Murphy, 1976; Costa *et al.*, 1977a). Thus, the loss of relatively small amounts of [^{14}C]-5-HT from a given compartment indicates that from 20–100 times as much total 5-HT is actually being displaced. The relatively greater amount of [^{14}C]-5-HT lost from vesicles than from the platelet would appear to be explained by material displaced from vesicles which accumulates in platelet cytoplasm.

The data presented in Table 1 raised two questions which we attempted to address by another series of experiments. Data from these experiments are presented in Tables 2–5. (1) What is the nature of the ^{3}H-labelled material in the cytoplasmic pool? Could, for example, this represent an aldehyde or acid amine metabolite, perhaps bound in some fashion to cytoplasmic protein? (2) What is the significance of the loss of [^{14}C]-5-HT from vesicles? Can, for example, 5-HT lost from vesicles during the addition of dopamine or tyramine re-enter the vesicular compartment?

Table 2 indicates that similar cytoplasmic and vesicular pools of 5-HT, dopamine, and tyramine were formed after 10 min of incubation with these ^{3}H-amines, regardless of whether platelet MAO was inhibited or not. Deprenyl appeared to inhibit slightly the uptake of [^{3}H]-5-HT, although the uptake of dopamine and tyramine appeared unchanged. It thus seems likely that cytoplasmic radioactivity does not represent oxidatively deamineated metabolites from these amines.

Tables 3 and 4 show the effects of extracellular and intracellular dopamine and tyramine on the uptake of [^{3}H]-5-HT. Over a 10 s period, neither amine at concentrations 10-fold and 100-fold higher than [^{3}H]-

Table 1 Measurement of total and vesicular ^{3}H-amine concentrations and the loss of vesicular [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT) following a 30 min incubation with 10^{-6} M extracellular amine

	[^{3}H]-5-HT		*[^{3}H]-dopamine*		*[^{3}H]-tyramine*		*[^{3}H]-octopamine*		*[^{3}H]-tryptamine*	
	Total	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*
^{3}H-amine accumulated (mol/platelet × 10^{19})	110.5 ±3.71	88.47 ±1.00	21.14 ±1.09	13.38 ±0.29	7.04 ±0.35	2.55 ±0.16	3.48 ±0.16	3.10 ±0.15	5.17 ±0.63	2.11 ±0.21
[^{14}C]-5-HT lost* (mol/platelet × 10^{19})	0.98 ±0.09	1.27 ±0.05	<0.10 to 0.37†	<0.10 to 1.81†	<0.10†	0.52 ±0.15	<0.10 to 0.15†	0.65 ±0.18	0.28 ±0.02	0.99 ±0.26

Values are mean ± s.e. mean.
* Platelet storage vesicles contained 5.39×10^{-19} mol/platelet of [^{14}C]-5-HT before incubation.
† Amounts of [^{14}C]-5-HT less than 0.10×10^{-19} mol/platelet could not be measured.

Table 2 Measurement of total and vesicular amine concentrations in control and deprenyl-treated platelets following a 10 min incubation with 10^{-5} M extracellular amine

	Amount of ^{3}H-amine present (mol/platelet × 10^{19})					
	[^{3}H]-5-HT		*[^{3}H]-dopamine*		*[^{3}H]-tyramine*	
Pre-treatment	*Total*	*Vesicular*	*Total*	*Vesicular*	*Total*	*Vesicular*
None (control)	32.69 ± 1.15	23.30 ± 2.07	5.448 ± 0.188	1.765 ± 0.686	1.549 ± 0.112	0.117 ± 0.054
Deprenyl (10^{-4} M)	29.36 ± 0.32*	20.58 ± 2.61	5.362 ± 0.297	1.915 ± 0.373	1.462 ± 0.259	0.318 ± 0.175

Values are mean ± s.d.
* Significantly less than control value, $P < 0.005$, Student's t test.

Table 3 Lack of effect of various extracellular concentrations of tyramine and dopamine on the initial rate of uptake of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) into human platelets

Amine added with [^{3}H]-5-HT	*Amount of [^{3}H]-5-HT in platelets (mol/platelet per s × 10^{21})*
None	1.84 ± 0.34
Tyramine	
10^{-6} M	2.08 ± 0.35
10^{-5} M	1.91 ± 0.07
Dopamine	
10^{-6} M	1.99 ± 0.05
10^{-5} M	2.11 ± 0.20

Values are mean ± s.d.

Table 4 Effect of increasing concentrations of cytoplasmic and vesicular [^{14}C]-dopamine and [^{14}C]-tyramine on the uptake of exogenous [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT)

	Amount of [^{3}H]-5-HT accumulated in control, [^{14}C]-dopamine or [^{14}C]-tyramine prelabelled preparations after incubation for 1 min (mol/platelet × 10^{20})		*Amount of [^{14}C]-dopamine or [^{14}C]-tyramine accumulated after incubation for 10 min or 30 min (mol/platelet × 10^{19})*	
	Total	*Vesicular*	*Total*	*Vesicular*
Control	1.493 ± 0.013	1.081 ± 0.281	0	0
[^{14}C]-dopamine (10^{-5} M)				
10 min	1.507 ± 0.054	1.097 ± 0.186	11.735 ± 0.238	7.299 ± 0.390
30 min	1.633 ± 0.143	1.132 ± 0.202	33.590 ± 0.848	20.037 ± 1.702
[^{14}C]-tyramine (10^{-5} M)				
10 min	1.558 ± 0.111	1.396 ± 0.490	3.807 ± 0.132	0.987 ± 0.111
30 min	1.622 ± 0.095	1.217 ± 0.207	7.336 ± 0.134	1.673 ± 0.112

Values are mean ± s.d.

5-HT, appeared to have a significant effect on the movement of 10^{-7} M [^{3}H]-5-HT across the plasma membrane. Similarly, over a 1 min period, the amount of ^{3}H-labelled material (initial concentration 10^{-8} M) accumulating in platelet cytoplasm and vesicles was not significantly affected by either dopamine or tyramine in both vesicles and cytoplasm. It is of interest that [^{3}H]-5-HT uptake was unchanged in the face of cytoplasmic and vesicular ^{14}C-amine concentrations varying over a 2- to 3-fold range. Under these experimental conditions, the amount of [^{3}H]-5-HT moving into the vesicles during a 1 min period was approximately 100-fold less than the total amount of [^{14}C]-dopamine or tyramine present in the cytoplasm.

Table 5 indicates that the presence of cytoplasmic 5-HT significantly lowered the total amount of new 5-HT entering platelets and vesicles over a 1 min period. This experiment, it should be noted, is not strictly comparable to that presented in Table 4, since Incubation A and B platelets contained large cytoplasmic pools of both labelled and unlabelled (endogenous) 5-HT, the latter displaced from vesicles during the addition of labelled material to this compartment.

Discussion

The experiments described here focus on the uptake and intra-platelet distribution of 5-HT and several other biogenic amines. Initially, we measured net accumulation of amines and concomitant changes in the content of a tracer amount of vesicular [^{14}C]-5-HT. The initial data suggested some hypotheses which we then investigated with subsequent experiments.

(1) Under the experimental conditions employed here, 5-HT appears to be the preferred substrate for uptake into both platelets and vesicles, although dopamine is a surprisingly good second-place contender. Octopamine is also of interest in that a larger percentage of the total accumulated by the platelet is sequestered in vesicles (90%), as compared to the range found for other amines (from 36% to 87%). This finding is consistent with a previous report that some of the octopamine present in human platelets appears to be located in platelet vesicles (Murphy, Cahan & Molinoff, 1975).

(2) The cytoplasmic pools of all amines examined are relatively large. The labelled material in this compartment does not appear to represent acid or aldehyde oxidative metabolites, since a pool of similar size forms even after treatment of intact platelets with the irreversible MAO-inhibitor deprenyl (Donnelly & Murphy, 1977). For these particular experiments, we chose to examine 5-HT, dopamine, and tyramine, since dopamine and tyramine are rapidly-deaminated substrates for the deprenyl-sensitive MAO-B found in human platelets (Donnelly & Murphy, 1977).

(3) Some of the data presented here suggest that the rate of entry of amines into vesicles may not be limited by the rate of entry of new amine into the cytoplasm. As shown in Table 4, a considerable increase in the cytoplasmic pools of dopamine and tyramine can occur between 10 and 30 min of incubation (from 5 to 13×10^{-19} mol/platelet for dopamine, and from 3 to 5×10^{-19} mol/platelet for tyramine). Despite this increase, the entry of dopamine into vesicles appears linear, while the vesicular pool of tyramine does not increase in size in parallel with the cytoplasmic pool. We can speculate that the rate of entry of a given amine into vesicles with 10^{-5} M extracellular amine concentrations may be determined by the rate of transport across the vesicular membrane, or by the availability of intra-vesicular binding sites for that amine, rather than by the rate at which material is transported across the plasma membrane.

(4) Taken together, our data are compatible with the hypothesis that once displaced from vesicles into platelet cytoplasm, 5-HT is not subsequently re-accumulated by the vesicles. Newly-added cytoplasmic 5-HT, in contrast, is a substrate for vesicular accumulation even in the absence of extracellular 5-HT, a property incidentally not shared by newly-added cytoplasmic dopamine or tyramine. Vesicular [^{14}C]-5-HT, and presumably endogenous 5-HT, is lost from the platelet during the net addition of new [^{3}H]-5-HT. If the incoming and outgoing 5-HT pools were identical, mixing within the cytoplasm would prevent any net loss of [^{14}C]-5-HT (see also Costa *et al.*, 1977a; Costa, Fauci & Wolff, 1976). In addition, neither dopamine nor tyramine appears to inhibit the entry of new [^{3}H]-5-HT into platelet cytoplasm or vesicles. Thus re-uptake of vesicular [^{14}C]-5-HT should occur if the [^{14}C]-5-HT behaved in a fashion similar to newly-added [^{3}H]-5-HT.

These experiments suggest that the dynamics of the platelet amine uptake and storage system are quite complex, despite negligible contributions from *de novo*

Table 5 Effect of cytoplasmic 5-hydroxytryptamine on the uptake of exogenous [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT)

	Amount of [^{3}H]-5-HT accumulated during a 1 min incubation (mol/platelet × 10^{21})	
	Total	*Vesicular*
Control	9.936 ± 0.185	5.405 ± 0.524
Incubation A*	6.427 ± 0.461	1.330 ± 0.440
Incubation B†	5.796 ± 0.398	0.721 ± 0.131

Values of mean ± s.d.

* Platelets contained 1.163×10^{-18} mol/platelet of labelled 5-HT in a cytoplasmic (non-vesicular) compartment.

† Platelets contained 2.176×10^{-18} mol/platelet of labelled 5-HT in a cytoplasmic compartment.

amine synthesis (Murphy & Kopin, 1972). The data are difficult to interpret in terms of any simple model, despite the fact that these experiments provided a means to explore the platelet under conditions where metabolism appeared unimportant, and where accurate assessment of cytoplasmic and vesicular pools of both newly-added and vesicular 5-HT was possible. In the light of our experience, obtaining and interpreting amine-flux data with other aminergic systems, such as synaptosomes, may present more problems than previously anticipated.

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