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ELECTRON PROBE MICROANALYSIS OF CHANGES IN DENSE-BODY PHOSPHORUS AND CALCIUM CONTENT FOLLOWING ALTERATIONS IN PLATELET SEROTONIN LEVELS

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Abstract—Using intact, washed human platelets, we have evaluated dense-body phosphorus and calcium content following procedures which alter total or vesicular serotonin (5-HT) content. Platelet vesicular 5-HT stores were labeled by incubation of platelet-rich plasma (PRP) with [^{3}H]-5-HT, and washed cells were treated for varying time periods with thrombin, the ionophore X537A, or the metabolic poisons antimycin A plus 2-deoxyglucose. After each treatment, [^{3}H]-5-HT remaining in the cells was measured by formaldehyde fixation and scintillation counting. Air-dried whole mounts were prepared from the same cell suspensions, and the calcium and phosphorus content of dense bodies was evaluated by electron microprobe X-ray analysis. A large increase or decrease in vesicular [^{3}H]-5-HT can occur without any measurable change in dense-body calcium or phosphorus. In addition, the loss of [^{3}H]-5-HT, calcium, and phosphorus apparently occurs at different times during platelet incubation with metabolic poisons, suggesting that continued maintenance of vesicular stores of each of the three substrates is linked in a distinct manner to cellular energy production.

Recent work from this laboratory has delineated the utility of electron probe X-ray microanalysis for evaluating compositional differences between dense bodies from various populations of platelets [1, 2]. In the present work, we have employed this technique to analyze the phosphorus and calcium content of dense bodies in human-platelet populations subjected to a variety of treatments known to alter platelet serotonin 5-HT storage or to induce 5-HT release. The results suggest that a large increase or decrease in the amount of platelet 5-HT releasable by thrombin (5-HT apparently being stored exclusively in platelet dense bodies [3–6]) can occur with a small or no significant alteration in the phosphorus or calcium content of the dense bodies. Nevertheless, both dense-body phosphorus and calcium are reduced significantly after treatment for several hours with the metabolic poisons antimycin A and deoxyglucose. Dense-body 5-HT content appears to decrease more rapidly and to a much larger extent than either phosphorus or calcium, and calcium, at some point, decreases more rapidly than phosphorus. Thus, it seems likely that in normal cells the storage of the three components is not linked in an obligatory fashion. Furthermore, the continued storage of each component may depend in a unique fashion on the availability of sufficient intracellular energy stores.

MATERIALS AND METHODS

Whole blood, obtained from a donor with no known history of hematologic disorders or drug ingestion for at least 3 weeks prior to donation, was collected in the citrate/EDTA medium of Detwiler and Feinman [7]. In the experiments described here, all platelets studied were obtained from a single blood collection. Platelet-rich plasma (PRP) was prepared by differential centrifugation [8]. Endogenous 5-HT content was measured by a modification of the enzymatic procedure of Saavedra *et al.* [9]. To label vesicular 5-HT stores, PRP was incubated for 30 min at 37° with either 10^{-8} M or 10^{-6} M [^{3}H]-5-HT (Amsheram-Searle Corp., Arlington Hts., IL; sp. act. 14 Ci/ m-mole). Platelets were pelleted and resuspended in a Tris–citrate buffer [10] containing 6 mM dextrose, 0.35% bovine serum albumin (Sigma Chemical Co., St. Louis, MO; crystallized and lyophilized), and 5% Stractan [11]. Aliquots of the resuspended platelets were treated as follows: (1) incubation at 37° for 5 min (control cells), (2) addition of human thrombin (final concentration, 4 units/ml) at 22° or 37°, (3) addition of X537A (Lasolocid, final concentration, 25 μM) at 37°, and (4) addition of antimycin A (final concentration, 7.5 μM) and 2-deoxyglucose (final concentration, 32 mM) at 37°. The amount of [^{3}H]-5-HT remaining inside platelets after each treatment was evaluated by the addition of formaldehyde fixative [3] 60 sec after the addition of thrombin or X537A, and either 30, 180 or 300 min after the addition of antimycin A plus deoxyglucose. After fixation, cells were cooled at once to 0°, pelleted, and solubilized for liquid scintillation counting as described previously [10]. Air-dried platelet whole mounts were prepared at the same time intervals from platelet aliquots as described in more detail elsewhere [1, 2, 6], by placing 10 μl of platelet suspension on copper grids coated with collodion and carbon and rapid blotting of all liquid with Whatman No. 4 filter paper. Evidence that this procedure preserves dense-body membrane integrity and content of calcium (Ca), phosphorus (P), and 5-HT has been discussed in previous publications [1, 2, 5, 6, 12].

Dense bodies in platelet whole mounts were examined in an Hitachi H-500 electron microscope

(Hitachi Division of the Perkin-Elmer Corp., Norwalk, CT), operated in the scanning-transmission (STEM) mode with an acceleration voltage of 75 kV, a beam diameter of approximately 100 nm, and a final magnification of 20,000x. Specimen current density was kept constant at 5×10^{-10} amps/cm^2 (measured with a Faraday cage at the photographic screen). Platelets were selected at random, and in a given platelet every dense body with a diameter $\geqslant 100$ nm was probed with a stationary spot positioned as nearly as possible to its center. Previous studies employing the Hitachi HU-12A and H-500 microscopes and a spot size of 10–20 nm have documented that the beam may be positioned accurately on the specimen and that neither the specimen nor the beam drifts appreciably during a 100-sec counting period [1]. X-ray spectra were recorded for 100 sec, utilizing a KEVEX energy-dispensive Mark V detector and 5100 recording and analysis system (Kevex Corp., Foster City, CA). Total gross counts in windows set for P (1.90–2.10 keV) and for Ca (3.60–3.80 keV) were corrected for the background contribution as described previously [1], utilizing a programmable background subtraction routine.

Table 1. Amount of [^{3}H]-5-HT remaining in cells and changes in human-platelet 5-HT content after various treatments

Experimental protocol	Total amount of [^{3}H]-5-HT present (moles/platelet $\times 10^{20}$, mean ± S. E. M.)	Per cent change in [^{3}H]-5-HT level in cells preincubated with 10^{-8} M [^{3}H]-5-HT
Control	0	
10^{-6} M [^{3}H]-5-HT for 30 min	282 ± 2.3	
10^{-8} M [^{3}H]-5-HT for 30 min	3.34 ± 0.02	
Thrombin (4 units/ml) at 22° for 60 sec	1.63 ± 0.13*	−51.3
Thrombin (4 units/ml) at 37° for 60 sec	0.64 ± 0.07†	−80.8
X537A (25 μM) at 37° for 60 sec	0.35 ± 0.03†	−89.5
Metabolic poisons		
30 min	3.10 ± 0.07‡	−7.2
180 min	0.75 ± 0.07†	−77.5
300 min	0.19 ± 0.02†	−94.3

*$P < 0.01$, when compared to the amount of [^{3}H]-5-HT present prior to treatment (Bonferroni t statistic).

†$P < 0.001$, when compared to the amount of [^{3}H]-5-HT present prior to treatment (Bonferroni t statistic).

‡Not significantly different from the amount of [^{3}H]-5-HT present prior to treatment (Bonferroni t statistic).

RESULTS

The platelets examined in this experiment initially contained $3.07 \pm 0.34 \times 10^{-18}$ moles/platelet of endogenous 5-HT (Table 1). Incubation of the cells with 10^{-6} M [^{3}H]-5-HT for 30 min at 37° added $2.82 \pm 0.02 \times 10^{-18}$ moles of [^{3}H]-5-HT/platelet (an amount equivalent to 92 per cent of the endogenous 5-HT), of which 81 per cent was released by 4 units/ml of thrombin acting at 37° (data not shown). Incubation of the same cells with 10^{-8} M [^{3}H]-5-HT for 30 min at 37° added $3.34 \pm 0.02 \times 10^{-20}$ moles of [^{3}H]-5-HT/platelet (an amount equivalent to 1 per cent of the endogenous 5-HT), of which 81 per cent was released by 4 units/ml of thrombin at 37°. Treatment with 4 units/ml of thrombin (at 22°) or 25 μM X537A (at 37°) for 60 sec prior to fixation also caused significant reductions in the [^{3}H]-5-HT content (51 and 90 per cent respectively). Incubation of aliquots of the same cell suspension with antimycin A plus deoxyglucose led to a progressive loss of [^{3}H]-5-HT from the cell (from 7 per cent after 30 min to 94 per cent after 300 min).

Table 2 compares net X-ray counts per dense body for P and Ca, and the P/Ca ratio, as determined during microprobing of individual dense bodies in cells treated as described above prior to rapid air drying. Neither doubling the platelet 5-HT content (by incubation with 10^{-6} M [^{3}H]-5-HT) nor halving it (by thrombin treatment at 22°) produced changes larger than 10 per cent in net P or Ca counts or larger than 5 per cent in P/Ca ratios, and none of the P or Ca changes observed were statistically significant. In contrast, an approximately 20 per cent decrease in net P and Ca counts followed treatment with X537A for 60 sec ($P < 0.01$). Incubation with metabolic poisons for 30 min produced essentially no change in total [^{3}H]-5-HT or dense-body P and Ca content, while incubation for 180 or 300 min resulted in large decreases in net P and Ca counts (40–55 per cent), the P/Ca ratio (27 per cent), and the [^{3}H]-5-HT content (78–94 per cent) (all statistically significant at the $P < 0.001$ level).

DISCUSSION

This study evaluated changes in dense-body Ca and P content following procedures which simultaneously altered total platelet 5-HT content. Attempts to correlate the two types of measurements are of interest in the light of a number of previous studies suggesting that platelet endogenous 5-HT is stored under normal circumstances exclusively in dense bodies, possibly complexed with ATP and divalent cations. As documented previously and reproduced here, platelets containing 3×10^{-18} moles/platelet of endogenous 5-HT can add both small (1 per cent of endogenous content) and large (92 per cent of endogenous content) amounts of [^{3}H]-5-HT, both of which are released to the same extent by brief thrombin treatment and thus appear to coexist in the same thrombin-releasable (vesicular) compartment [4]. Accordingly, the behavior of [^{3}H]-5-HT can be used as an index of the disposition of the unlabeled endogenous 5-HT. In addition, previous studies and the data presented here indicate that preincubation with 10^{-6} M 5-HT produces a net increase in total 5-HT content without a net change in the number of dense bodies per cell [4].

In the cells studied here, a net increase in thrombin-releasable [^{3}H]-5-HT content results in no statistically significant difference in dense-body P, Ca, or P/Ca ratio. From the experimental data, we cannot rule out some minor changes in one or all of the three parameters, since an actual decrease as large as 20 per cent or an increase of 5 per cent might prove statistically not significant. Nevertheless, it seems clear that the net addition of a large amount of [^{3}H]-5-HT to this compartment has not been accompanied by a change of equivalent magnitude in P or Ca content. Thus, it seems likely that the net movement of 5-HT into the dense body is not coupled in an obligatory fashion to a net influx of either P or Ca.

Table 2. Effect of various treatments on intensities of X-rays derived from the phosphorus and calcium of human-platelet dense bodies.

Experimental protocol (N = number of dense bodies measured)	Number of platelets examined	Net P intensity/dense body (counts/100 sec)			Net Ca intensity/dense body (counts/100 sec)			Ratio P/Ca		
		Mean ± S. E. M.	% Change*	P value	Mean ± S. E. M.	% Change*	P value	Mean ± S. E. M.	% Change*	P value
Control† (N = 102)	11	3957 ± 183			2793 ± 156			1.53 ± 0.03		
10^{-6} M [^{3}H]-5-HT for 30 min (N = 74)‡	11	3588 ± 230	−9.3 (−22.7 to +5.5)	NS§	2516 ± 168	−9.9 (−24.3 to +6.7)	NS	1.46 ± 0.03	−4.7 (−9.9 to +0.1)	NS
Thrombin (4 units/ml) at 22° for 60 sec (N = 56)	27	3486 ± 204	−11.9 (−24.1 to +1.8)	NS	2431 ± 158	−13.0 (−26.6 to +2.8)	NS	1.49 ± 0.05	−2.4 (−9.4 to +5.0)	NS
X537A (25 μM) at 37 for 60 sec (N = 92)‡	15	3186 ± 121	−19.5 (−28.3 to −9.3)	<0.01	2123 ± 85	−24.0 (−33.4 to −12.7)	<0.01	1.55 ± 0.03	+1.0 (−4.8 to +7.2)	NS
Metabolic poisons										
30 min (N = 83)‡	10	3873 ± 170	−2.2 (−13.6 to +11.0)	NS	2692 ± 156	−3.6 (−16.8 to +12.0)	NS	1.50 ± 0.02	−2.4 (−7.3 to +1.0)	NS
180 min (N = 60)‡	10	2317 ± 106	−41.4 (−48.5 to −33.5)	<0.001	1509 ± 80	−45.9 (−53.5 to −37.1)	<0.001	1.58 ± 0.03	+2.8 (−2.6 to +8.6)	NS
300 min (N = 70)‡	11	2381 ± 101	−39.8 (−46.8 to −31.9)	<0.001	1271 ± 64	−54.5 (−60.7 to −47.2)	<0.001	1.95 ± 0.04	+27.0 (+20.1 to +34.4)	<0.001

* Values in parentheses are approximate 95 per cent interval estimates (Bonferroni t statistic) when each treatment group was compared with the control group.

† Cells containing 3.34×10^{-20} moles/platelet of [^{3}H]-5-HT.

‡ Number of bodies per platelet not significantly different from control value.

§ Not significant.

In contrast, treatment with X537A for 60 sec, which removes most (90 per cent) of the [^{3}H]-5-HT from the platelet, leads to no significant change in the total number of dense bodies present and a statistically significant 20 per cent reduction in both P and Ca (with no significant change in the P/Ca ratio). The amount of calcium actually removed from a typical dense body in this case would total approximately 6×10^{-18} moles, using 2.84×10^{-17} moles as an estimate of the total amount of calcium present in a single dense body [12]. The amount of 5-HT removed here was at least 3×10^{-18} moles/platelet, or approximately 5×10^{-19} moles/dense body, assuming an average of six dense bodies per platelet (see also Ref. 6). On a relative scale, then, X537A removes from the dense body a far greater quantity but a significantly lesser percentage of Ca than 5-HT—a phenomenon consistent with the known ability of X537A to transport both divalent cations and biogenic amines across lipid bilayers [13]. The concomitant loss of dense-body P, which is most probably not directly transported by X537A, may result from extrusion of sufficient P to maintain electrical neutrality inside the dense body.

Analysis of platelet 5-HT and dense-body P and Ca after incubation with metabolic poisons for varying times also documents significant changes in the amounts of the three components present. Other studies have suggested that incubation of platelets for 30 min at 37 with the concentrations of metabolic poisons used here leads to movement of essentially all the thrombin-releasable (vesicular) 5-HT to a non-releasable (cytoplasmic) compartment, without a net loss of 5-HT from the cells [14]. If a similar phenomenon has occurred in the cells examined here after a 30 min incubation, vesicles containing essentially no endogenous 5-HT may have a normal P and Ca content. In any case, after both 180 and 300 min of incubation, the percentage of the [^{3}H]-5-HT remaining inside the cells is less than the percentage of either Ca or P. Although both P and Ca are decreased by the same percentage after 180 min, a larger percentage of Ca than P is lost from the dense bodies after 300 min of incubation. In terms of their relative susceptibilities to prolonged incubation with metabolic poisons, 5-HT appears to be lost most readily from dense bodies, followed by Ca and then by P. Since the total dense-body Ca eventually reaches a lower relative level than does total dense-body P (resulting in an increased P/Ca ratio), it seems possible that the maintenance of normal dense-body calcium stores may be somewhat more critically dependent than is phosphorus on the availability of sufficient intracellular stores of metabolic energy.

Treatment with a saturating dose of thrombin (4 units/ml) for 60 sec at 22 releases 52 per cent of total vesicular [^{3}H]-5-HT and a similar percentage of the total number of dense bodies (see also Ref. 12). Since the same cells can release 81 per cent of their total vesicular [^{3}H]-5-HT when incubated with the same amount of thrombin at 37°, this appears to be a partial release reaction. The dense bodies remaining inside the cell after the partial release reaction at 22°

appear to have the same P and Ca content as those in the control group. Thus, the data suggest that the calcium and phosphorus known to appear extracellularly after treatment with thrombin [7, 12, 15] come exclusively from the dense bodies released, rather than from both those released and those not released. These data contribute new evidence that the release of platelet dense bodies is an "all-or-none" process with respect to the contents of a given dense body. In addition, they suggest that non-released "refractory" dense bodies remaining after a given amount of dense-body release are not distinguishable by differences in their P and Ca content from the released "non-refractory" dense bodies.

It is also of interest that the measurements of dense-body phosphorus and calcium presented here display considerable variation. The estimate of variability between dense bodies within individual platelets for both net P and Ca counts averages about 10^6. Similarly, the estimate of variability between platelets is almost the same order of magnitude. The source of this variability is not clear at present. It is probably not related to the number of dense bodies per platelet, however, since the latter parameter is not significantly correlated with the mean number of P or Ca counts obtained from all the dense bodies in a given platelet (correlation coefficients, 0.128 and 0.313 respectively). The average amount of P and Ca present in all the dense bodies in a given platelet thus may not be strongly correlated with the age of that platelet, which is believed to be related to the number of dense bodies per platelet [12]. We can speculate that platelets arrive in the circulation containing dense bodies with widely differing net amounts of phosphorus and calcium, and that this variability is maintained as the platelets age (i.e. lose dense bodies) during their lifetime.

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