Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5295–5299, December 1977
Biochemistry

Evidence for control of serotonin secretion from human platelets by hydroxyl ion transport and osmotic lysis

(exocytosis/anion transport/chemiosmosis)

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Communicated by DeWitt Stetten, Jr., September 1, 1977

ABSTRACT **Serotonin secretion from human platelets, stimulated either by thrombin or the calcium ionophore A23187, was found to be inhibited by anion transport blocking drugs such as 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), pyridoxal phosphate, probenecid, and suramin. These drugs have previously been shown to inhibit ATP-evoked release of epinephrine from isolated chromaffin granules by blocking chloride uptake and subsequent osmotic lysis. However, in contrast to granule release, platelet secretion was insensitive to chloride and, instead, was dependent on OH^-. Platelet release was suppressed by low pH, and inhibition by the transport blocking drugs was competitive only with respect to OH^-. Serotonin release from platelets was also suppressed by increasing extracellular osmotic strength, and the relationship between suppression and external osmotic strength was quantitatively similar to that observed in the case of chromaffin granules. We conclude that platelet exocytosis could occur when serotonergic granules are closely juxtaposed to the plasma membrane, thus exposing the granule anion transport site to the more alkaline medium. Secretion of serotonin could occur as a consequence of OH^- transport and osmotic lysis of the granule–plasma membrane complex, analogous to the chemiosmotic mechanism of chloride-dependent epinephrine release from isolated chromaffin granules.**

Human platelets secrete serotonin [5-hydroxytryptamine (5-HT)] from storage sites in secretory vesicles by exocytosis (1–4), a well-defined ultrastructural process common to many secretory tissues (5–19). The chemical and energetic bases for regulation of exocytosis in platelets and other cells remain poorly understood although recent studies from our laboratory have implicated permeant anions in the control of this event (20). We found that ATP-evoked epinephrine release from isolated adrenal chromaffin granules (secretory vesicles) depended upon anion permeation and could be inhibited by anion transport blocking drugs such as probenecid, pyridoxal phosphate, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), suramin, and others (21, 22). These drugs have been best studied previously in human erythrocyte membranes where they were found to bind to the anion transport protein (Band III) and block anion exchange (23–29). The drug structures are diverse but are generally anionic aromatic compounds that are impermeant to biological membranes. Suramin (29, 30) attracted our particular attention because of recent reports that it also inhibited phagosome–lysosome fusion in macrophages (31). The granule release event was due to osmotic rupture (21, 32, 33) consequent to chloride uptake, and drug inhibition appeared to be due to competitive inhibition with respect to chloride concentration (21).

It seemed plausible that the release chemistry defined by the chromaffin granule system might be a prototype for exocytotic processes in intact cells (20, 22), and we decided to test the action of anion transport blocking drugs on 5-HT secretion from platelets. Secretion of 5-HT from platelets is evoked by diverse agents (1, 2, 34–43). In the present work, utilizing thrombin and A23187 as releasing agents, we found that anion transport blocking drugs inhibited 5-[^{3}H]HT release from platelets and that the inhibition was competitive with OH^-. In addition, we found that increased extracellular osmotic strength suppressed platelet secretion in a manner similar to that previously shown for chromaffin granule release.

MATERIALS AND METHODS

Preparation of 5-[^{3}H]HT-Labeled Platelets. Fresh human blood was mixed with EDTA (0.15 ml of 10% EDTA, pH 7.0 per 10 ml blood) at room temperature and centrifuged at 1000 × *g* for 3.5 min to obtain platelet-rich plasma (PRP). PRP was then mixed with 0.2 μM 5-[^{3}H]HT and incubated for 45 min at 18°. The labeled platelets were then centrifuged at 4° and resuspended by aspiration in the same volume of cold Rossi's medium (44) with a siliconized pipet. Platelets were again centrifuged and resuspended in 3 ml of ice-cold 0.3 M sucrose containing human serum albumin 1 mg/ml. The platelet count was determined by the method of Brecher and Cronkite (45) and adjusted to contain 10^6 platelets per mm^3 by addition of sucrose/albumin solution.

Assay of Release. The final incubation medium contained 130 mM NaCl, 25 mM 4-morpholinoethanesulfonic acid (MES)/NaOH buffer, pH 7.42, and drugs and activators as described in a total volume of 500 μl. The reaction was initiated by addition of 50 μl of platelet suspension and terminated after 1 min with 10 μl of 10% glutaraldehyde in 10 mM MES/NaOH buffer, pH 7.42. Tubes containing fixed platelets were then centrifuged at 1030 × *g* for 10 min at 4°, and 300 μl of supernatant was withdrawn to determine radioactivity released. Percentage release (%R) was determined from: %R = 100 (released cpm − blank)/(maximum released cpm − blank).

Drugs and Reagents. Human thrombin (EC 3.4.21.5) was a highly purified lyophilized preparation donated by David Aronson of the Bureau of Biologics. A23187, a calcium ionophore, was obtained from R. L. Hammill, Lilly Research Laboratories, Indianapolis, IN, and was stored frozen at 1 mg/ml in dimethyl sulfoxide and diluted for use in 95% ETOH. SITS was obtained from British Drug House. Probenecid and

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Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; PRP, platelet-rich plasma; MES, 4-morpholinoethanesulfonic acid; %R, percentage released.

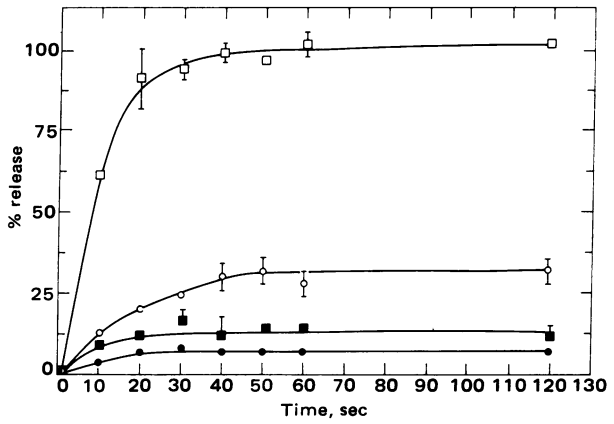

FIG. 1. Time course of A23187-induced 5-[^{3}H]HT release from human platelets. Maximal release was 92,500 cpm above a blank of 6500 cpm. Open symbols: □, 0.76 μM A23187; ○, 0.35 μM A23187. Solid symbols: same as open symbols but plus 1 mM SITS. Error bars, difference between duplicates.

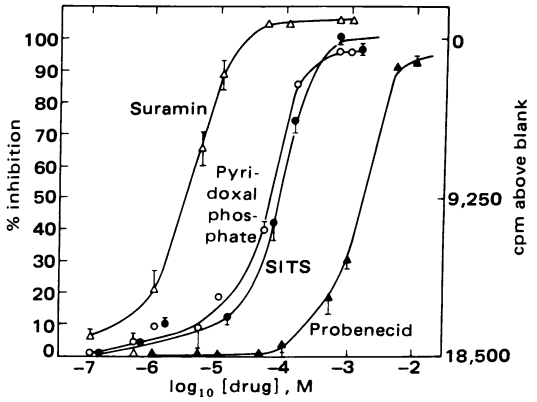

FIG. 2. Influence of anion channel blocking agents on thrombin-stimulated release of 5-[^{3}H]HT from human platelets. The added dose of thrombin was 0.16 unit/ml, corresponding to 38% of the release obtained from a supermaximal thrombin dose of 31 units/ml. Total 5-[^{3}H]HT release, in the absence of inhibitors, was 30,900 cpm over blank; the blank was only 7350 cpm. Suramin (△) and SITS (●) were readily soluble in distilled water and gave neutral solutions. However, pyridoxal phosphate (○) solutions had to be adjusted to pH 7.4 with Tris base. Probenecid (▲) was dissolved in a small amount of 0.1 M NaOH and titrated to pH 7.4 with acetic acid. Similar curves were obtained with platelets from six other donors on 12 separate occasions. Error bars, difference between duplicates.

pyridoxal phosphate were obtained from Sigma. Suramin [bis(*m*-amino-*m*-aminobenzoyl-*p*-methylbenzyl-1-naphthylamine-4,6,8-trisulfonate)carbamide] was obtained from Imperial Chemical Industries.

RESULTS

Properties of Releasing Platelets. Mixing platelets with either thrombin or A23187 resulted in prompt release of up to 60–80% of total bound 5-[^{3}H]HT at pH 7.4. Platelets incubated at 37° without release agents usually released 5–10% of their total 5-[^{3}H]HT, and incubation at 4° resulted in release of only 2–3% of total radioactivity regardless of the presence or absence of release agents. Release in all cases reached an asymptotic value within 1 min, as previously reported for release of nucleotide (2, 43) and calcium (42, 43). In the time course shown in Fig. 1, A23187-induced release of 5-[^{3}H]HT was proportional to dose and ended in less than 1 min. The asymptotic yields with both thrombin and A23187 were found to be directly proportional to the apparent initial rates at 10 sec, up to a total of 50% release. We concluded that the 1-min data could be used as initial rates for conventional kinetic analysis in this release range.

Anion Transport Blocking Agents and Release. The anion transport blocking agents all were capable of completely inhibiting thrombin-induced release of serotonin from platelets (Fig. 2). A23187 alone was found to induce substantial platelet release but addition of 4 mM calcium to the medium raised release levels to those found with thrombin. As indicated in Fig. 1, A23187-induced release was completely suppressed (<5% maximal release) in the presence of 1 mM SITS. Complete suppression was also observed in 1 mM suramin and pyridoxal phosphate and 10 mM probenecid when platelets were exposed to an A23187 dose that induced 50% of maximal 5-[^{3}H]HT release. SITS (1 mM) also suppressed ADP-induced platelet aggregation.

Identity of the Permeant Ion. In contrast to the findings with isolated chromaffin granules, the platelet release reaction was found to be fully active in chloride-deficient media in which NaCl was replaced by either sucrose or Na isethionate ($HO\text{-}C_2H\text{-}SO_3^-$). However, because platelet release by agents such as ADP is known to be decreased at lower pH (46, 47), the possibility of OH^- being the permeant anion was also considered (48). As shown in Fig. 3, thrombin-induced release was optimal between pH 7.5 and 8.0 and decreased to zero at pH 6.0 ($[OH^-] = 10^{-8}$ M). A23187-induced release also had a similar pH dependence. We therefore concluded that the pH dependence of platelet release was a platelet property and not unique to specific release agents.

We further tested the hypothesis that OH^- was the critical permeant anion by analyzing anion transport blocker inhibition as a function of OH^- concentration. Competitive inhibition of anion transport blockers is often analyzed by using the Dixon plot (21, 49) and, as shown in Fig. 4, we found that all four drugs tested were classical competitive inhibitors with respect to OH^- concentration. In the Dixon analysis, competitive inhibition is demonstrated when all plots at different substrate concentration (OH^- in this case) mutually intersect with each other in the upper left hand quadrant. This point of intersection should also coincide with the perpendicular projection of $1/\%R_{max}$ (50).

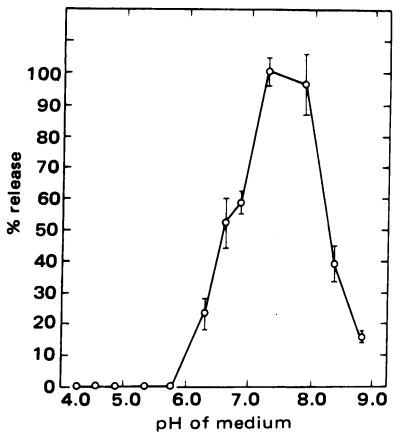

FIG. 3. Influence of pH on thrombin-induced release of 5-[^{3}H]HT from human platelets in 1-min incubations. The added dose of thrombin, 0.16 unit/ml, induced 24% of the release obtained from a supermaximal thrombin dose, 31 units/ml, at pH 7.32. The total 5-[^{3}H]HT release at pH 7.32 was 75,000 cpm over blank. The buffers between pH 4.32 and 7.90 were 10 mM MES and variable amounts of Tris base. The more alkaline buffers were 10 mM Tris and variable amounts of MES. Error bars, difference between duplicates.

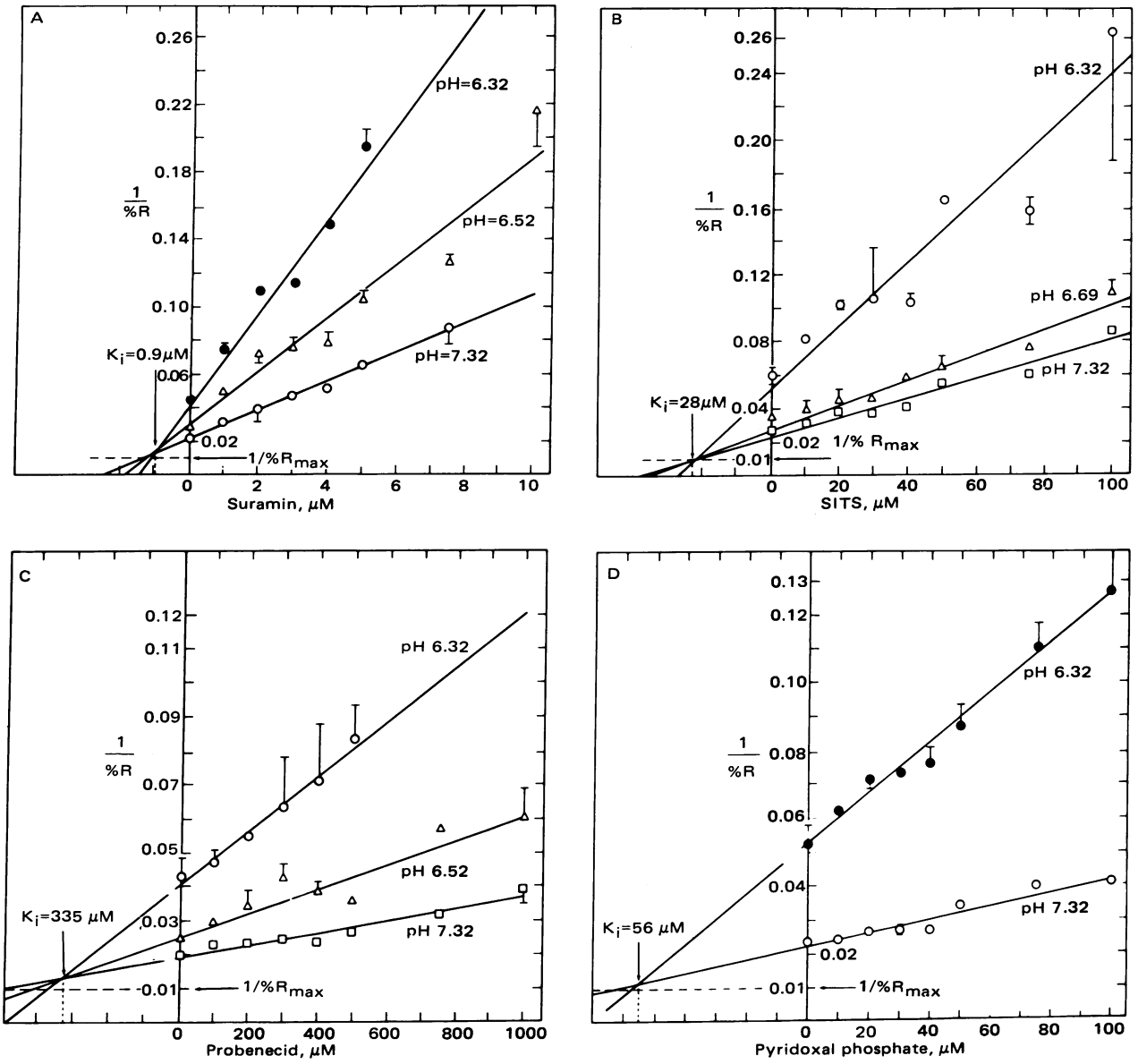

FIG. 4. Dixon plots of anion transport blocker inhibition of platelet release as a function of OH^- concentration. Incubations were for 1 min. All buffers contained 10 mM MES and variable concentrations of Tris. The term $1/\%R_{max}$ is analogous to Dixon's term $1/V_{max}$ and is simply 1/100% release, or 0.01. (*A*) Suramin inhibition. Added dose of thrombin, 0.16 unit/ml, corresponded to 47% of the release obtained from a supermaximal thrombin dose, 31 units/ml. Total 5-[^{3}H]HT release in the absence of suramin at pH 7.32 was 83,000 cpm over blank. Curves were drawn by linear regression analysis of all points and correlation coefficients (*r* values) were 0.99 (pH 7.32), 0.91 (pH 6.52), and 0.94 (pH 6.32). (*B*) SITS inhibition. Added dose of thrombin, 0.16 unit/ml, corresponded to 45% of the release obtained from a supermaximal thrombin dose, 31 units/ml. Total 5-[^{3}H]HT release in the absence of SITS was 80,000 cpm over blank. Curves were drawn by linear regression analysis of all points, and correlation coefficients were 0.94 (pH 7.32), 0.95 (pH 6.69), and 0.88 (pH 6.32). (*C*) Probenecid inhibition. Added dose of thrombin, 0.31 unit/ml, corresponded to 48% of release obtained from a supermaximal thrombin dose, 31 units/ml. Total 5-[^{3}H]HT release in the absence of probenecid at pH 7.32 was 92,000 cpm over blank. Curves were drawn by linear regression analysis of all points and correlation coefficients were 0.93 (pH 7.32), 0.92 (pH 6.67), and 0.97 (pH 6.32). (*D*) Pyridoxal phosphate inhibition. Added dose of thrombin was 0.08 unit/ml, corresponding to 42% of the release obtained from a supermaximal thrombin dose, 31 units/ml. Total 5-[^{3}H]HT released in the absence of pyridoxal phosphate at pH 7.32 was 118,000 cpm over blank. Curves were drawn by linear regression analysis of all points, and correlation coefficients were 0.92 (pH 7.32) and 0.98 (pH 6.32).

The values of K_i (μM), measured for each drug with platelets from different donors' were: suramin, 0.9, 2.9; SITS, 28, 22; pyridoxal phosphate,75, 56, 22; probenecid, 335, 350.

Influence of Osmotic Pressure on Platelet Secretion. If the analogy between releasing granules and releasing platelets were exact, we might also expect the platelet release system to be suppressed by increased external osmotic strength. The osmotic strength of the platelet incubation medium was increased by adding either NaCl or sucrose, and its influence on the platelet release reaction was measured. Base-line release was transiently increased slightly by increased osmotic strength, with a maximum at 600 mOsM but declined to the original base line by 1000 mOsM. Activated release showed a similar transient change with maximum at 600 mOsM and declined to zero by 1000 mOsM. The difference between activated and base-line releases was then plotted as a function of osmotic strength of the medium (Fig. 5*A*) to show the true suppressive influence of external osmotic strength on stimulus-secretion. An alternative way of plotting these data is shown in Fig. 5*B* where log %R is plotted against osmotic strength yielding a straight line and an estimate of the osmotic strength required to suppress platelet release by 99.9%: about 1030 mOsM.

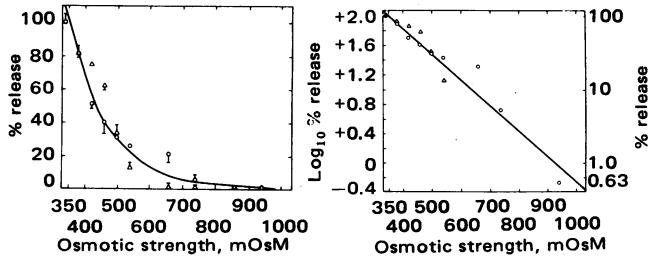

FIG. 5. Influence of osmotic strength on platelet release at pH 7.32. (*A*) Arithmetic plot. The experiment was performed exactly as described in *Materials and Methods* except that water was replaced with different amounts of either 1 M NaCl (○) or 2 M sucrose (△) to change the osmotic strength. The thrombin dose was 0.16 unit/ml, corresponding to 42% of release obtained from a supermaximal thrombin dose, 31 units/ml. A separate blank was measured for each osmotic strength point. The total 5-[^{3}H]HT released in the standard medium (340 mOsM) was 94,350 ± 2500 cpm over blank. The theoretical curve was drawn from the data in (*B*). (*B*) Log plot of the data in (*A*). The curve shown is for the NaCl data and was calculated by linear regression analysis (r = 0.95); 99% suppression of release was obtained in an external osmotic strength of 1030 mOsM.

DISCUSSION

Exocytosis of 5-HT from human platelets was found to be blocked by anion transport blocking agents and suppressed by increased osmotic strength. Similar results were found with ATP-evoked epinephrine release from isolated adrenal chromaffin granules. The main differences between platelets and granules were in the identity of the permeant ion (OH^- *vs.* Cl^-) and in order of activity of the drugs. The drug activity sequence for granules was SITS > probenecid > suramin > pyridoxal phosphate ≫ isethionate. However, for platelets, the sequence was suramin ≫ SITS, pyridoxal phosphate ≫ probenecid; isethionate was inactive. These drug sequences may be related to anion specificity, but further studies on other exocytotic systems will be required to illuminate this point. We conclude from these data that both granules and platelets probably released by analogous mechanisms involving stimulation of anion transport.

The dependency of release on external osmotic pressure in platelets was almost identical to that previously reported (33) for release of epinephrine from chromaffin granules, in which extinction of release occurred at 960 mOsM (660 mOsM above isotonic). It was previously suggested (22) that the increased osmotic content in releasing chromaffin granules might be derived from solubilized core material, because 650 mOsM could be obtained from the total of epinephrine (0.5 M) and ATP (0.12 M) in the core (51). Platelet secretory granules are reported to contain as much or more of serotonin, ATP, and Ca^{2+} (52), and it is possible that the osmotic bases of platelet release and granule release are similar in origin.

Our data indicate that isolated secretory granules and intact platelets release by similar mechanisms, yet this must be reconciled with the fact that isolated granules are simple unitary structures whereas platelets consist of serotonergic granules enclosed by membranes. It is possible that, during exocytosis in platelets, the secretory granule membrane somehow becomes physically integrated with the platelet plasma membrane, thus exposing an anion transport site to the extracellular space. Some insights into how this could occur come from electron microscopic studies of exocytosis in both exocrine pancreas (12, 17) and rat mast cells (18, 19). In these studies, at least two sequential steps of exocytosis have been discerned: juxtaposition or "fusion" of vesicle and plasma membranes, and subsequent "fission" or breakage of the barrier between granular contents and extracellular space (the latter step is the actual release event). According to this analysis, the juxtaposition or fusion state could be equivalent to the time during which functional vesicle membrane properties would appear to be conferred upon the whole cell. The fission step in exocytosis could correspond functionally to the release reaction in isolated vesicles and occur by local osmotic rupture. The origin of the increase in osmotic content of serotonergic granules might be due to either increased ion content or recruitment of the inactive granule core material into osmotic activity by the permeant ions. These concepts are summarized in Fig. 6.

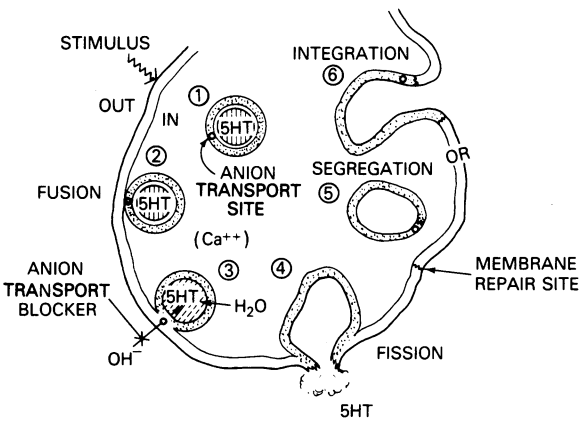

FIG. 6. Anion transport dependent, osmotic lysis model for the fission step in platelet exocytosis. The diagram represents possible events in the platelet after stimulation by thrombin or other agonist. (*1*) Resting serotonergic granule. By analogy with chromaffin granules, the serotonergic granule membrane is shown with an intrinsic anion transport site (round structure). (*2*) Initiation of a fusion or juxtaposition complex between the serotonergic granule and the platelet plasma membrane. No specific timing is implied relating the stimulus to step 2. In addition, calcium may be involved in this and other steps, but our present experiment does not bear on this point. (*3*) The granule and plasma membranes become more intimately connected and the granule anion channel becomes exposed to the outside medium. (We do not exclude the possibility that the plasma membrane could also contribute an anion channel, or indeed that the anion channel could exclusively come from the plasma membrane alone in the platelet case.) After activation of anion transport, OH^- enter passively and water then enters the granule. (*4*) The osmotic pressure of the granule in the fusion complex increases and causes rupture at the weak point of contact between granule and plasma membranes. The subsequent repair events (*5* and *6*) are not part of our data. However, it is evident that the hydrophobic areas of the newly torn granule and plasma membranes could reapproximate, leading either to interior segregation of the granule membrane, as has been observed in adrenal medulla (54), or transient integration, as has been proposed in the frog neuromuscular junction (15).

It is necessary to emphasize that the nature of anion transport occurring in isolated chromaffin granules and postulated to occur in releasing platelets is quite distinct from the anion exchange occurring in erythrocytes. Net increases in anion content of the granule probably occur because increased osmotic strength suppresses uptake. It follows that, the counter ion for the permeant anion in platelets remains to be identified. Protons have been proposed as the granule cation, since the proton ionophore FCCP blocks release (32, 53), and we have recently found it to block platelet secretion as well. The requirement for extracellular sodium or potassium is directly excluded by our experiments, but permeation of granules by these ions from an intracellular source remains possible.

These results have led us to conclude that exocytosis from platelets depends upon a chemiosmotic mechanism involving OH^- transport through specific anion transport sites and subsequent osmotic lysis of vesicles at their sites of fusion or juxtaposition with platelet plasma membranes. The internal pH

of serotonergic granules has been estimated to be approximately 6 (55), suggesting that a chemical gradient of OH^- can occur physiologically. The transport site may be a secretory vesicle membrane component inserted into the plasma membrane, although the exact origin remains a problem for future study. We can anticipate that, if anion transport dependent osmotic lysis of secretory vesicles were a general mechanism for secretion, other exocytotic systems besides platelets should prove sensitive to the anion transport blocking drugs.

The authors are intellectually indebted to Dr. W. W. Douglas by virtue of his unpublished public lecture at the Annual Meeting of the Society of Neuroscience, San Diego, CA, 1974, in which he showed pictures of mast cell granules swelling before release and speculated about an osmotic basis for swelling that could contribute to membrane thinning and rupture.

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