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The Relationship between Extracellular pH and Control of Blood Platelet Serotonin Secretion

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SUMMARY

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Hydroxyl ions (OH^-) have been proposed as the permeant anions responsible for triggering serotonin release from blood platelets. This hypothesis is based on the observations that (i) platelets exhibit a much higher efficiency of secretion at pH 8.2 than at pH 6.8 and (ii) agents which block anion channels are inhibitors of release. We have investigated this mechanism by testing two predictions of the hypothesis. First, the model implies that the pH of the medium at the time of exocytosis is the determining factor in release. We have observed that platelets *do* secrete serotonin at pH 6.8 if they are pretreated with a secretagogue at pH 7.6. Second, the hypothesis predicts that the actions of OH^- should be confined to the interior of the secretory granule. We have found this not to be the case, since the cellular processes of shape change and protein phosphorylation, neither of which is confined to granules, are markedly dependent on extracellular pH. These results demonstrate that hydroxyl ions affect platelets at a more general and probably earlier stage of activation than would be required by the "hydroxyl as permeant ion" hypothesis.

INTRODUCTION

As a part of their role in hemostasis, blood platelets carry out high-affinity uptake, granular storage, and stimulus-coupled secretion of 5-hydroxytryptamine (5-HT, serotonin; see Ref. 1 for a review). The mechanism by which serotonergic granules are transported to and fused with the topological equivalent of a plasma membrane is unknown, although several interesting hypotheses have been advanced (2–4). Recent studies by Pollard *et al.* (4) implicate anions, in particular hydroxyl (OH^-), as important permeant ions in platelet secretion. The authors suggest that extracellular OH^- enters a serotonergic granule upon the granule's apposition to the plasma membrane, thereupon inducing osmotic swelling of the granule, fission of the plasma membrane, and release of granular contents. From this intriguing hypothesis emerge two experimentally testable predictions: (i) that hydroxyl ion acts at a rather late stage in the series of events culminating in release (i.e., extracellular pH is the final determinant of release); and (ii) that hydroxyl ion enters only the granule, and thus would not be expected to influence extragranular metabolic processes, especially the earlier stages of secretion. Several such intracellular processes are known to occur in platelets between the events of stimulation and secretion, two of these being the "shape change" from a discoid to a more spherical shape (5) and the specific phosphorylation of two soluble proteins, MW 20,000 and 40,000 (6–8). The experiments reported here were intended to identify whether pH effects are in fact exerted at the level of release as suggested by the model, or whether some other cellular process which might be required for secretion is the pH-sensitive component. We have used concanavalin A (Con A)[1] as a secretagogue since recent work has shown that in the absence of extracellular calcium this ligand will cause shape change and specific protein phosphorylation but only minimal release (8, 10); the addition of calcium with Con A to the incubation medium then produces a robust release (8–10). This system can be used to measure the effects of pH on shape change and protein phosphorylation in the absence of secretion (by using Con A in the absence of Ca^{2+}) and, more importantly, to test for the possibility that release could occur under relatively acidic conditions, provided the initial interactions with the lectin have transpired in an alkaline milieu.

METHODS

Measurement of pH. A Transidyne 814-20 pH micro-

[1] Abbreviations used: BSA, bovine serum albumin; CP/CPK, phosphocreatine, creatine phosphokinase; Con A, concanavalin A; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate (sodium laural sulfate).

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electrode and Transidyne 334 reference electrode were used to assess the pH of supernatant fluids following various treatments. These electrodes were calibrated for each experiment against a Radiometer GK-2401C combination electrode using a Radiometer PHM-64 pH meter. The microelectrode system gives a linear response of approximately 50 mV/pH unit in the pH range 5–10 and can be used reliably on volumes as small as 10 μl.

Solutions for platelet suspension. Buffer A: 0.12 M NaCl, 4.3×10^{-3} M KCl, 8.5×10^{-4} M $MgCl_2$, 3×10^{-3} M glucose, 1×10^{-2} M Hepes, pH 7.4.

Buffer B: Same as Buffer A with the additions of 2×10^{-2} M phosphocreatine, 40 μg/ml creatine phosphokinase, and 1×10^{-2} M Tris, pH 7.6, unless stated otherwise.

Buffer C: 0.12 M NaCl, 4.3×10^{-3} M KCl, 8.5×10^{-4} M $MgCl_2$, 3×10^{-3} M glucose, 1×10^{-2} M phosphocreatine, 40 μg/ml CPK, 1×10^{-2} M Mes, 5×10^{-3} M Tris, pH 7.6.

Preparation of platelets. Whole blood was collected from the jugular veins of lightly ether-anesthetized rats into a plastic syringe containing acid–citrate–dextrose. To each volume of whole blood, $\frac{1}{8}$ vol of Buffer A was added to facilitate cell separation. The blood was transferred to 1.5-ml conical plastic centrifuge tubes and spun at $200g_{max}$ for 15 min in a Beckman microfuge equipped with a stroboscopic tachometer and variable speed control. Platelet-rich plasma was removed with a siliconized pasteur pipet and layered over 0.20 ml of an aqueous 30% solution of bovine serum albumin, then spun for 5 min at $200g_{max}$ and 3 min at $600g_{max}$. Using this preparation, the platelet fraction remains above or near the BSA–plasma interface, while contaminating erythrocytes and leukocytes migrate well into the BSA layer. The platelet fraction is removed with a siliconized pipet, the cells are pelleted by centrifugation at $600g_{max}$ for 4 min, and the pellet is resuspended in Buffer B to $5–8 \times 10^8$ cells/ml. All experiments were carried out in Buffer B except for the "pH drop" experiment (Fig. 2), in which case the platelets were suspended in Buffer C to give 3×10^9 cells/ml.

Assay of 5-HT release. Platelets were suspended in Buffer B to 8×10^8 cells/ml, then incubated with 3×10^{-7} M 5-hydroxytryptamine creatinine sulfate (8.75 μCi/ml) for 30 min at 25°C. The cells were pelleted by centrifugation at $600g_{max}$ for 2 min, then gently resuspended in Buffer B to give 3×10^9 platelets/ml. Assay for 5-HT release was initiated by adding 20 μl of the platelet suspension to 180 μl of a solution containing the indicated treatment (Con A with or without Ca^{2+}) in Buffer B. The pH of this treatment solution had previously been adjusted to the desired approximate pH with either NaOH or HCl. Ten-minute incubations (25°C) were terminated by centrifugation at $11{,}000g_{max}$ for 15 s, and aliquots of the supernatant fluid (25 μl) were removed and assayed for released 5-HT by liquid scintillation counting. The exact pH of the remaining supernatant fluid was then measured. Efficiency of release was determined by comparison of cpm released into the supernatant fluid to cpm in a platelet suspension solubilized by the addition of 1% sodium dodecyl sulfate. Released ^3H was verified to comigrate with serotonin using high-performance liquid chromatography.

Assay of shape change. A modification of the turbidimetric method of Born (5) was used for shape change analysis. Platelets were suspended to a cellular concentration of 2×10^8/ml in Buffer B which had been previously adjusted to the approximate desired pH. This suspension was then placed in siliconized quartz cuvettes and a baseline 600-nm absorbance established using a dual-beam recording spectrophotometer (Perkin–Elmer 124D). Into the 400-μl platelet sample, 100 μl of Con A solution was rapidly injected, giving a final lectin concentration of 50 μg/ml. The initial response to this treatment is a decrease in absorbance due to dilution, followed by an increase due to the altered light-scattering properties of spherical as compared to discoid platelets (5, 10). If aggregation were occurring under these conditions, it would be detectable by this method as an apparent decrease in absorbance. Con A, however, induces no measurable aggregation of platelets, as has been reported by others (12, 16). In addition, Con A-induced shape change occurs without requirements for stirring or for the presence of extracellular Ca^{2+} (8, 10, 14). Therefore, in order to further reduce the possibility of aggregation, we neither stir nor add Ca^{2+} to the platelets in shape change experiments.

Binding of ^3H-Con A to platelets. Platelets in Buffer B which had been preadjusted to the approximate desired pH with either NaOH or HCl were incubated with ^3H-Con A (50 μg/ml; 0.58 mCi/mg) for 5 min at 25°C. The samples were divided into two aliquots, one for pH determination (as described previously) and the other for assay of Con A binding. The suspensions were filtered by suction through Bio-Rad Uni-Pore (0.4-μm) polycarbonate filters. The filters were washed three times with 3 ml of Buffer A. After drying, the filters were counted in OCS (Amersham) in a Packard scintillation counter. Samples from which platelets had been omitted were used as controls for binding of Con A to the filters, and the cpm obtained from these samples were subtracted from the corresponding platelet-containing samples. This background level was usually about 20% of the level bound by platelets.

Assay of protein phosphorylation. A modification (8) of the method of Haslam and Lynham (7) was used to assay for protein phosphorylation while obtaining a simultaneous measurement of 5-HT release. Labeling conditions were essentially the same as for 5-HT release, except that ^{32}P-orthophosphate was added to the platelet suspension (final activity = 1 mCi/ml) 30 min prior to the addition of ^3H-5-HT. After labeling, the cells were pelleted, resuspended, and treated as described previously. After dilution into treatment medium, 150-μl aliquots were removed and, 10 min after the initiation of treatment, were assayed for 5-HT release and pH. To the remaining 50-μl aliquot, 50 μl of SDS sample buffer was added, giving a final concentration of 0.2% SDS, 6.3×10^{-2} M Tris–HCl, pH 6.8, 10% glycerol, and 1×10^{-2} M β-mercaptoethanol. The samples were heated at 50°C for 10 min, then loaded onto 7.5–20% gradient polyacrylamide slab gels containing 0.1% SDS (11). After staining for protein, the gels were dried, then exposed to X-ray film, and the autoradiographic images were scanned by

densitometry. Phosphorylation of proteins is expressed as percentage of total phosphorylation, so that changes in a given protein are relative to the other phosphoproteins in that sample. As shown previously (8), the 20K and 40K proteins are the only peptides affected by Con A treatment. No proteins showed either a selective or a nonspecific loss of ^{32}P with increasing alkalinity.

"pH drop" experiment. ^3H-5-HT-labeled platelets were resuspended in Buffer C, pH 7.6, to 3×10^9 cells/ml, then divided into two aliquots. To one, sufficient HCl was added to lower the pH to 6.8; an equal volume of Buffer C, pH 7.6, was added to the other. One group of samples was treated with Con A at pH 6.8, the other group at pH 7.6. After 2 min of Con A treatment at 25°C, the pH 7.6 samples were acidified with an amount of HCl predetermined to yield pH 6.8, and Ca^{2+} (to give 0.5 mM) was added where designated. Final cell concentration was 3×10^8/ml. Ten minutes after the addition of Ca^{2+}, the samples were assayed for ^3H-5-HT release as described previously, and the exact pH of each supernatant was verified.

Reagents. Con A (25 mg/ml, 5% sterile glucose solution), bovine serum albumin (30% sterile solution), and Hepes were obtained from Calbiochem. Phosphocreatine, CPK, Mes, and Tris were from Sigma. ^3H-(G)-Con A and ^3H-5-hydroxytryptamine creatinine sulfate were from New England Nuclear, and ^{32}P-orthophosphate was from ICN. All other reagents were analytical grade.

RESULTS

Effect of pH on Ca^{2+}-dependent 5-HT release. The incubations used for these studies were carried out at a lower temperature and for a longer time than are commonly used for studies of platelet release. The conditions were chosen in an attempt to produce maximum levels of release without interference by reuptake. We have found that under these conditions, reuptake of ^3H-5-HT is minimal (≤10%) and that Ca^{2+} has no detectable influence on reuptake. As mentioned in the Introduction these studies use concanavalin A (Con A) as a secretagogue, with creatine phosphate/creatine phosphokinase being included in the incubation medium as an ADP-reducing antiaggregant (13). Figure 1 shows that Con A alone causes only marginal release of preloaded ^3H-serotonin, but that in the presence of 0.5 mM Ca^{2+} it produces a marked stimulation of secretion. This Ca^{2+} dependence is not due merely to the Ca^{2+}-facilitated binding of Con A to platelets since ^3H-Con A binds to platelets equally well in the presence or absence of Ca^{2+} (9). Further, neither Con A-induced shape change (10, 14) nor Con A-induced protein phosphorylation (8) displays a requirement for extracellular calcium. Calcium-dependent release has been observed in previous studies (9) in which secretion was shown to be linear at 25°C for at least the first 5 min following the addition of calcium. Furthermore, it has been reported that the Con A (plus calcium)-induced release is not accompanied by platelet aggregation (10, 12) and does not require stirring (14); we have confirmed this (also see Fig. 3). Figure 1 demonstrates that calcium-dependent release is particularly sensitive to pH between the values of 6.8 and 8.3. The shape of the curve thus obtained resembles the data reported by

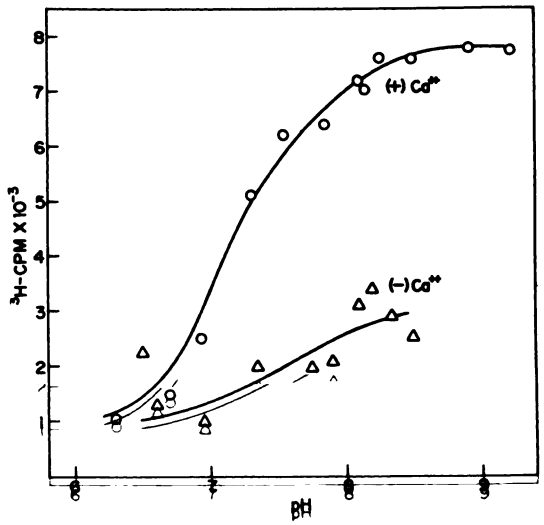

FIG. 1. *^3H-5-Hydroxytryptamine release by platelets as a function of pH*

Platelets were incubated with 50 μg/ml Con A ± 0.5 mM Ca^{2+}, in Buffer B at the indicated pH. Maximal release (pH 8.5, +Ca^{2+}) represents 25% of the total platelet ^3H-5-HT content. No backgrounds have been subtracted from the data—in this system centrifugation does not induce significant levels of release. This experiment has been repeated at least six times with virtually identical results.

Pollard *et al.* (4) using thrombin as stimulant, with one exception. Those authors reported a marked decrease in secretion over the pH range 8.0–9.2, whereas our data show no significant decrement over that range. It seems likely that their results were influenced by alkali inactivation of thrombin activity (15), which would explain the apparent discrepancy. Since such a problem could arise with any secretagogue and since a test of the "OH^- as permeant anion" hypothesis requires that the pH dependence of release be a property intrinsic to the cells rather than to the secretagogue, we have assayed Con A binding to platelets over the same pH range.

Binding of ^3H-Con A to platelets. A polycarbonate filter binding assay (9), which traps platelets and platelet-bound Con A, was used to determine the pH component of the Con A–platelet interaction. ^3H-Con A was incubated with platelets at the same concentration and buffer conditions used in secretion experiments. Following incubation, binding of ^3H to polycarbonate (0.4-μm-pore) filters was measured. Our findings (Table 1) indicate that binding of Con A to platelets is essentially independent of pH over the range used in these experiments. Although we have not assessed whether binding of Con A represents activation of specific platelet receptors, it is apparent that the attraction between Con A and the platelet surface is not strongly affected by pH. Therefore, effects of hydroxyl ions on platelets most likely occur subsequent to Con A binding, i.e., the effects of pH we report are probably intrinsic to the cells.

5-HT release at pH 6.8 following Con A treatment at pH 7.6. If pH were producing its effects on release simply by determining the concentration of "permeant hydroxyl ions" in the medium, then the amount of secretion should be absolutely dependent upon pH *at the time of exocytosis*. Alternatively, if pH operates on some other component of the stimulus–secretion sequence, it should be

TABLE 1

Con A binding to platelets: Influence of pH

Platelet samples were incubated in duplicate with 50 μg/ml ^{3}H-Con A and the assay was carried out as described in Methods. The values obtained were converted to ng Con A/10^8 platelets using a specific activity of 0.58 mCi/mg Con A; 1 ng/10^8 platelets represents 6000 cpm bound to a filter at the platelet concentrations used. Blank values representing ^{3}H-Con A bound to filters in the absence of platelets have been subtracted. These blank values were consistently less than 25% of the amounts bound in the presence of platelets. α-Methyl mannoside at a concentration of 0.1 M reduces platelet-bound Con A by approximately 90%.

pH	ng Con A bound/10^8 platelets
6.5	2.0
6.7	2.2
7.2	2.2
8.3	1.9

possible to obtain release in an acidic medium provided that the other stages of the incubation were carried out under more favorable pH conditions. To test this, we incubated platelets at pH 7.6 with Con A but no Ca^{2+} (hence no significant release) and then lowered the pH of the medium to 6.8. Upon the addition of calcium (Fig. 2), a robust secretion of serotonin occurred, a result which would not be anticipated if hydroxyl ions were acting solely upon exocytosis. This result suggests that hydroxyl ions are active at some stage antecedent to the Ca^{2+} component of release, and it led us to investigate pH dependency of two other Con A-initiated effects which are thought to be involved in platelet secretion: shape change and protein phosphorylation.

pH effects on platelet "shape change." A preliminary and essential component of the general platelet response is shape change from a discoid to a more spherical form with numerous pseudopodia, a process which can be measured turbidimetrically as an increase in light absorbance at 600 nm (5). Figure 3 shows the platelets' response to Con A treatment at various pH levels. It should be noted that unlike the case for release, the effects of Con A on shape change are essentially independent of extracellular calcium (10, 12, 14). It can be seen from Fig. 3 that alkaline media conditions support faster and more extensive shape changes than do more acidic conditions, and below pH 7.4 shape change is substantially inhibited.

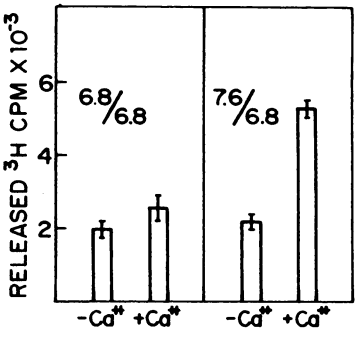

FIG. 2. *"pH drop experiment"*

Platelets in Buffer C at either pH 6.8 or pH 7.6 were treated with 50 μg/ml Con A. After 2 min at 25°C, the pH of the alkaline samples was lowered to 6.8 by the addition of a precalibrated amount of HCl, and $CaCl_2$ was added where indicated to give a final concentration 0.5 mM. Release of ^{3}H-5-HT was measured, as described in Methods, 10 min after Ca^{2+} addition. The upper term in the ratio is the pH of Con A treatment; the lower number is the pH at which Ca^{2+} was added. Maximal release represents 20% of the total ^{3}H-5-HT content. This experiment was carried out in triplicate, and the error bars indicate ±SD. The entire experiment has been repeated four times, with essentially the same result.

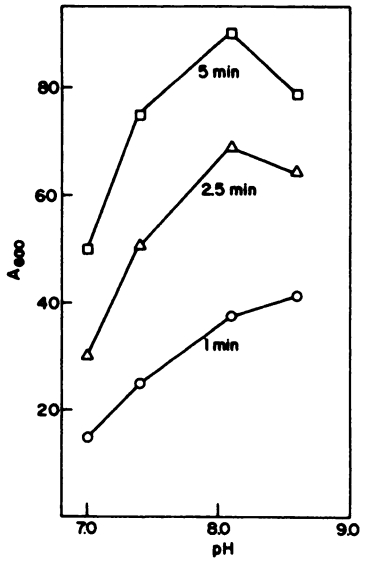

FIG. 3. *Influence of pH on platelet shape change*

Platelets were diluted into Buffer B at the indicated pH as described in Methods. Con A was added by rapid injection to give a final lectin concentration of 50 μg/ml, and the change in A_{600} was measured (5). The units of the scale are A/1000, such that the full scale is 0.1 A_{600}.

Effect of pH on protein phosphorylation. Platelets treated with collagen (7) or thrombin (6) exhibit a highly specific phosphorylation of two proteins of 20,000 and 40,000 daltons. Recent work has shown that Con A will also produce phosphorylation of the two proteins and that this effect is independent of extracellular calcium—as anticipated, under these low-Ca^{2+} conditions Con A-induced phosphorylation was not accompanied by release (8). We have examined the influence of pH on phosphorylation of these proteins by prelabeling platelets with ^{32}P-orthophosphate and ^{3}H-serotonin, then measuring the effects of Con A plus Ca^{2+} on serotonin release and protein phosphorylation across the pH range 6–9. The data in Fig. 4 indicate that the 20,000- and 40,000-dalton proteins are phosphorylated as a function of the alkalinity of the solution across essentially the same range as that which defines the pH dependence of release. Although the exact role of protein phosphorylation in secretion is unknown, it is clear that in addition to modulation of secretion and shape change, pH also influences the phosphorylation of these two proteins. Taken together, the data suggest a site of pH influence in the exocytotic sequence earlier than that proposed by Pollard *et al.* (4).

DISCUSSION

The data presented in this paper confirm the observation that serotonin secretion by blood platelets is most

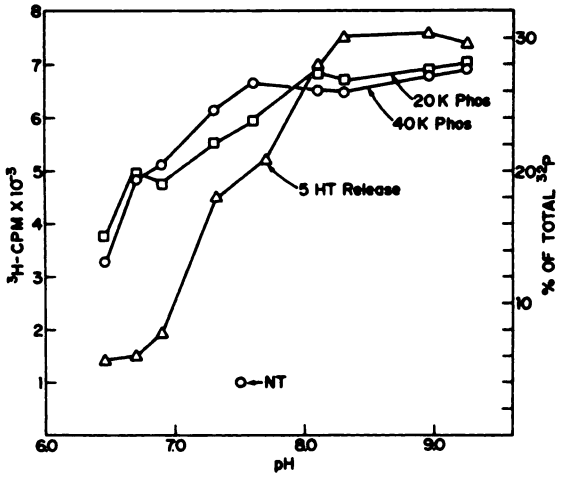

FIG. 4. *^{3}H-5-HT release and protein phosphorylation as a function of pH*

$^{32}PO_4^{3-}$- and ^{3}H-5-HT-labeled platelets were treated with 50 μg/ml Con A + 0.5 mM Ca^{2+}, and analysis was carried out as described in Methods. The phosphorylation data represent changes in the percentage of the total ^{32}P in protein in that sample. NT = the level of phosphorylation of the 40,000 MW protein in platelets not treated with Con A or Ca^{2+}. Efficiency of release with Ca^{2+} at pH 8.5 = 40%.

efficient in an alkaline medium. This, combined with the fact that anion transport inhibitors also inhibit release (4), implies that hydroxyl ions are involved in some process which ultimately results in secretion. However, our findings indicate that hydroxyl ions influence an earlier stage in exocytosis than that proposed by Pollard *et al.* (4). This conclusion follows from the observation that 5-HT secretion *can* occur at a low pH, provided that the platelets are preincubated with the secretagogue under alkaline, low-Ca^{2+} conditions (optimal for shape change and protein phosphorylation). This result differs from a prediction of the model and implies that the pH of the *treatment* medium is a more important determinant for secretion than the pH of the medium into which the cells release. The mechanics by which pH influences secretion remain undetermined but the aforementioned experiments clearly demonstrate that both shape change and specific protein phosphorylation are markedly influenced by the alkalinity of the incubation medium. Since these two responses to Con A invariably precede secretion, but can occur in the absence of extracellular calcium (and hence in the absence of significant levels of release), it seems reasonable to suppose that they are prerequisites to secretion. This idea is consistent with earlier work suggesting a linkage between shape change and release (see Ref. 1) and between protein phosphorylation and release (6–8). At a minimum, the shape change and phosphorylation experiments indicate that the effects of hydroxyl ions are not restricted to release, and this alone would raise questions about the permeant hydroxyl ion hypothesis.

We do not mean to imply that other physiological or biochemical mechanisms of the platelets are not affected by pH; rather, we are suggesting that if earlier stages of the stimulus–exocytosis sequence are also pH sensitive in the same approximate range as is secretion, then the regulatory function of OH^- in platelet granule secretion needs to be examined more critically. If, in fact, hydroxyl ions are the permeant anions which trigger release, then our data suggest a revision of the model in which OH^- enters the serotonergic granule directly. More consistent with our data would be a model in which OH^- enters the cytosol and triggers some pH-dependent biochemical process which then ultimately leads to release. For example, intracellular pH might modulate the release or sequestration of a second messenger such as Ca^{2+} or a cyclic nucleotide. Alternatively, some component of the extragranular secretory mechanism might be directly activated by alkaline conditions. Although the relationship between intracellular and extracellular pH in platelets is unclear, it is possible that a protein kinase is activated by intracellular hydroxyl ions. The best-known alkali-activated protein kinase is phosphorylase B kinase (PBK), which exhibits 20-fold more activity at pH 8.2 than at pH 6.8 (17). It is noteworthy in this regard that brain synaptic membranes contain a PBK substrate whose behavior on SDS gels is quite similar to that of the 40,000 MW platelet protein (18). Recent studies have suggested that this synaptic membrane protein is phosphorylated during repetitive synaptic transmission (19). If a PBK-like enzyme were the attendant kinase of the platelet 40,000 MW protein, it would be expected to display a pH dependence like that observed in Con A-stimulated platelets.

There is another distinct alternative to the hydroxyl ion model, which is that some other ion is actually the permeant species, and the ion channel itself is pH sensitive. Ample evidence exists for pH-sensitive ion channels, for example, anion and cation channels isolated from sarcoplasmic reticulum are responsive to external pH, both being more efficient under alkaline conditions (20). Furthermore, isolated voltage-dependent anion channels from *Torpedo californica* display maximal Cl^- conductance at pH 7.3 and virtually no Cl^- transport at pH 6.5 (21; also see Refs. 22–25). If the anion channels of platelets were similar to those just mentioned, then OH^- would exert its effects outside the cell rather than inside, and the activities ascribed to OH^- in the model could be carried out by some other ionic species while still generating the observed pH dependence for serotonin release.

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