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Storage of Biogenic Amines in Intact Blood Platelets of Man

Dependence on a Proton Gradient

HUBERT AFFOLTER AND ALFRED PLETSCHER
Department of Research, Kantonsspital, CH-4031 Basel, Switzerland

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SUMMARY

The actions of ionophores with different ion specificities and of thrombin on the release of ^{14}C-labeled 5-hydroxytryptamine, [^{3}H]noradrenaline, and endogenous ATP were measured in human platelets suspended in media with various K^+ and Na^+ concentrations. Besides thrombin, those ionophores [monensin, nigericin, and the combination of carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) with nonactin and/or valinomycin] which cause a rapid collapse of H^+ gradients induced a fast and virtually total release of ^{14}C-labeled 5-hydroxytryptamine and [^{3}H]noradrenaline into the various media. FCCP alone, which causes an inversion of the membrane potential to inside negative values, induced a considerably slower amine release. Changes in the K^+ and Na^+ gradients did not lead to amine release, nor did interference with energy transduction by antimycin A with or without glycolysis inhibitors. Monensin and FCCP did not release ATP, whereas thrombin, added before or after incubation of platelets with FCCP and monensin, caused a marked liberation of the nucleotide. It is concluded that in intact human platelets (a) the intragranular storage of 5-hydroxytryptamine and noradrenaline mainly depends on the proton gradient across the granular membrane, and (b) ionophores causing a collapse of H^+ gradients induce non-exocytotic release of 5-hydroxytryptamine and noradrenaline from intracellular storage granules.

INTRODUCTION

The accumulation of biogenic amines in blood platelets depends on various mechanisms. The uptake of 5-HT[1] at the plasma membrane is governed by a specific, carrier-mediated, Na^+- and Cl^--dependent process which also cotransports some dopamine and possibly some NA (1-3). Within the platelet most of the 5-HT and the catecholamines [whose amounts are several orders of magnitude lower than those of 5-HT (4)] are accumulated in specific subcellular organelles, the 5-HT storage granules (or dense bodies) which also contain ATP (5). The rest is localized at extragranular sites, the nature of which is unknown. At the level of the dense bodies at least two processes are responsible for the storage of 5-HT and probably also of catecholamines, i.e., an uptake at the granular membrane and a reversible binding to intragranular constituents such as ATP (3).

The transport of biogenic amines across the granular membrane of platelets has not been fully elucidated. It is possibly similar to that of adrenal chromaffin granules, where it seems to be coupled to a proton gradient generated by ATP hydrolysis (6, 7) and to the membrane potential. Indeed, the intragranular space of 5-HT organelles (8), like that of adrenal chromaffin granules (6), has been found to be more acidic than the surrounding cytoplasm. Also, the accumulation of 5-HT by isolated membrane vesicles of storage organelles of platelets occurs only in the presence of a pH gradient across the granular membrane (9-11). Furthermore, a Mg^{2+}-, Ca^{2+}-dependent ATPase is present in membranes of 5-HT organelles (12). In addition, it has been postulated that in whole platelets the cytoplasmatic ratio of Na^+ to K^+ affects the stability of 5-HT storage within the granules (13).

In the present experiments, ion gradients and membrane potentials were altered by ionophores, which transport specific cations across membranes by forming lipid-soluble, reversible complexes with these polar ions. The experiments indicate that in blood platelets of man the intracellular storage of 5-HT and NA mainly depends on the proton gradient across the granular membrane and is not influenced by the Na^+:K^+ ratio in the cytoplasm. However, some influence of the granular membrane potential cannot be excluded. The experiments were carried out with those radioactive amines whose intracellular distributions in platelets have been shown to be similar to those of endogenous amines (4, 14). The following

[1] The abbreviations used are: 5-HT, 5-hydroxytryptamine; NA, noradrenaline (norepinephrine); FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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ionophores were used: FCCP (transporting protons), valinomycin (K^+), nonactin (K^+ and Na^+), monensin, and nigericin (exchanging Na^+, K^+, and H^+ against each other). (For review of the properties and mode of action of ionophores see ref. 15.)

MATERIALS AND METHODS

Materials. 5-Hydroxy[*side chain*-2-^{14}C]tryptamine creatinine sulfate (specific activity 2.15×10^{12} Bq/mole), [L-7,8-^{3}H]NA (specific activity 1.85×10^{15} Bq/mole, and [^{3}H]inulin (specific activity 4.5×10^{13} Bq/mole) were obtained from Amersham International Ltd. The following substances were purchased from commercial sources: ATP-Na_2 $H_2 \cdot 3H_2O$, ATP-bioluminescence CLS (test combination), antimycin A, and FCCP from Boehringer Mannheim AG (Cham, Switzerland); nigericin and monensin from Calbiochem AG (Luzern, Switzerland); valinomycin, nonactin, deoxyglucose, and glucono-δ-lactone from Fluka AG (Buchs, Switzerland); dextran T-10 from Pharmacia-pdf AG (Zurich, Switzerland); Instagel from Packard IC (Zurich, Switzerland). Bovine thrombin (70 units/mg) was a gift from Dr. R. Strässle, F. Hoffmann-La Roche & Company Ltd. (Basel, Switzerland).

Labeling and isolation of platelets. Blood was obtained from healthy volunteers by puncturing the cubital vein and collecting into a plastic vial containing 0.1 volume of trisodium citrate (final concentration 1.3 mM). Platelet-rich plasma was prepared by centrifugation of the whole blood for 10 min at 600 × *g* and 22°. The platelets were then double-labeled by incubation of the platelet-rich plasma at 37° for 90 min with [^{14}C]5-HT (10^{-7} M) and [^{3}H]NA (5×10^{-8} M). These concentrations were chosen so that enough radioactivity entered the platelets for the measurements. Portions of plasma (20 ml) were then layered on an ice-cold gradient consisting of 4 ml of 20% dextran and 7 ml of 10% dextran. For the preparation of the dextran solutions a stock solution of dextran T-10 (30%, w/w) in a medium containing bovine serum albumin (2.25%, w/w), 4.5 mM glucose, and 2 mM EGTA (final pH 7.4 at 22°) was used. The stock solution was diluted with the Na^+ medium, which contained 155 mM NaCl, 6.1 mM KCl, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 2 mM EGTA (final pH 7.4 at 22°). After centrifugation at 5800 × *g* for 10 min at 4°, the platelets which had banded between the two layers were removed by puncturing the tube from the bottom and diluted about 10 times with ice-cold Na^+ medium. The suspension was centrifuged at 180 × *g* for 10 min at 4° and the pellet was resuspended in 1 ml of Na^+ medium. After counting in a Thrombocounter the concentrated suspension (average concentration 3.8×10^9 platelets per milliliter) was stored on ice in order to prevent loss of amine (stock suspension) (16).

Release experiments. For the incubations, Na^+ medium (described above), K^+ medium (in which all Na^+ of the Na^+ medium was replaced by K^+), or Na^+/K^+ medium (containing 40 mM Na^+ and 90 mM K^+) was used. In the experiments in which glycolysis was inhibited, the medium consisted of 124.5 mM NaCl, 4.8 mM KCl, 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM EGTA, 30 mM deoxyglucose, and 10 mM glucono-δ-lactone (pH 7.4). The latter was added immediately prior to use. The pH in the media did not change more than 0.15 unit during an experiment.

Aliquots of 0.1 ml of the stock suspension of double-labeled platelets were each diluted with 2.4 ml of the various media which had been prewarmed for 10 min at 37° and to which the ionophores had been added as necessary. After incubation with gentle shaking at 37° for various periods, the suspensions were cooled in ice-water and centrifuged at 3500 × *g* and 4° for 10 min. All tubes were stored on ice and centrifuged together with the other tubes of the test series. An aliquot of the supernatant (2 ml) was put into counting vials. The rest of the supernatant was decanted, the walls of the tubes were cleaned with a cotton stick, and the platelet pellets were resuspended in a mixture of 0.1 ml of Na^+ medium and 0.1 ml of Triton X-100 (1%). The suspension was placed in counting vials, the tubes were rinsed twice with 0.9 ml of Na^+ buffer, and the liquids were added to the vials. After the addition of 10 ml of Instagel to the counting vials and vigorous shaking, the samples were counted in a refrigerated scintillation counter (Type MR 300 DPM; Kontron AG, Zurich, Switzerland).

ATP release experiments were performed under identical conditions (including preloading with unlabeled amines). ATP was determined in 80 μl of supernatant after centrifugation of the suspension at 9500 × *g* for 30 sec in a desk-top centrifuge. The luciferin/luciferase reaction of the firefly *Photins pyralis* (ATP-bioluminescence CLS test combination) in an Aminco-Bowman spectrofluorophotometer was used for the assay. The concentrations chosen for monensin (15 μM) and FCCP (16 μM) corresponded to 5 times the concentration causing maximal [^{14}C]5-HT release within 5 min. Antimycin A in the concentration used (5 μM) has been shown to block mitochondrial respiration in human platelets (17).

Calculations. The counted values from the dual-labeled samples were corrected for channel spillover, for quenching, and for counting efficiency by the $^3H/^{14}C$ program of the scintillation counter. Contamination of the pellets by supernatant fluid was determined in parallel experiments by means of [^{3}H]inulin, yielding a mean contamination of 1.2 ± 0.2%. The data were corrected accordingly (see below). Results are given as percentage radioactivity in the pellet, taking the value for $t = 0$ as 100%, according to the following formula:

$$\%\ \mathrm{dpm'_{sed}} = 100 \cdot \frac{\mathrm{dpm_{sed}} - k \cdot \mathrm{dpm_{sup}}}{\mathrm{dpm_{tot}}} \cdot \frac{100}{\%\ \mathrm{dpm'_{t=0}}}$$

where % $\mathrm{dpm'_{sed}}$ = corrected value (percentage) of the radioactivity in the sediment, $\mathrm{dpm_{sed}}$ = disintegration per minute in the sediment, $\mathrm{dpm_{sup}}$ = disintegration per minute in the supernatant, k = correction constant for the mean contamination of sediment by supernatant ($k = 0.012$), $\mathrm{dpm_{tot}}$ = total amount of radioactivity in the sample, and % $\mathrm{dpm'_{t=0}}$ = corrected value (percentage of the total amount of radioactivity) in the sediment at time $t = 0$.

Statistical analyses. Statistical analyses were performed with the distribution-free Wilcoxon rank test (one sided, not paired, $\alpha \leq 0.05$). In the legends to the figures, significance is indicated only where it is not evident.

RESULTS

Absolute values. Before incubation with ionophores, 10^8 platelets contained 38 ± 3 pmoles and 0.62 ± 0.04 pmoles of [^{14}C]5-HT and [^{3}H]NA, respectively. With 0.12 unit of thrombin per milliliter, 62 ± 4 pmoles ATP per 10^8 platelets were released.

Release of [^{14}C]5-HT, K^+ medium. Suspension of platelets in K^+ medium alone (controls); with antimycin A (Fig. 1A), valinomycin, or nonactin (data not shown); or with a combination of valinomycin and nonactin (Fig. 1B) did not result in any release of [^{14}C]5-HT. In contrast, FCCP induced a fast release of the amine which was enhanced by a factor of 1.5 when nonactin and valinomycin were added (Fig. 1B). Monensin also caused a release of [^{14}C]5-HT which was about equal to that induced by FCCP together with valinomycin and nonactin. Nigericin showed the same effect as monensin (data not shown). Thrombin had a faster action than the ionophores, the effect reaching a maximum (release of about 90% of [^{14}C]5-HT) after 5 min as compared with 20–30 min for the ionophores (Fig. 1). There was no secondary rise of [^{14}C]5-HT in the thrombin-treated platelets. The monensin-induced [^{14}C]5-HT release was very sensitive to temperature. At 22° the release was minimal; at 30° it was more marked than at 22°, but still considerably less than at 37° (Fig. 2A).

Release of [^{14}C]5-HT, Na^+ medium. In Na^+ medium there was no spontaneous release of [^{14}C]5-HT, nor did antimycin A or valinomycin alone or combined with nonactin liberate the amine. Monensin, nigericin (data not shown), and thrombin released [^{14}C]5-HT in the same way as in K^+ medium (Fig. 3). The [^{14}C]5-HT release induced by FCCP was less rapid and marked than in K^+ medium, but was enhanced by valinomycin alone and more so by valinomycin plus nonactin. However, even in the latter combination the [^{14}C]5-HT release was slower than that due to thrombin and monensin. As expected, the [^{14}C]5-HT content of the platelets, after its maximal depletion by thrombin, showed a slight secondary rise due to reuptake of [^{14}C]5-HT (16).

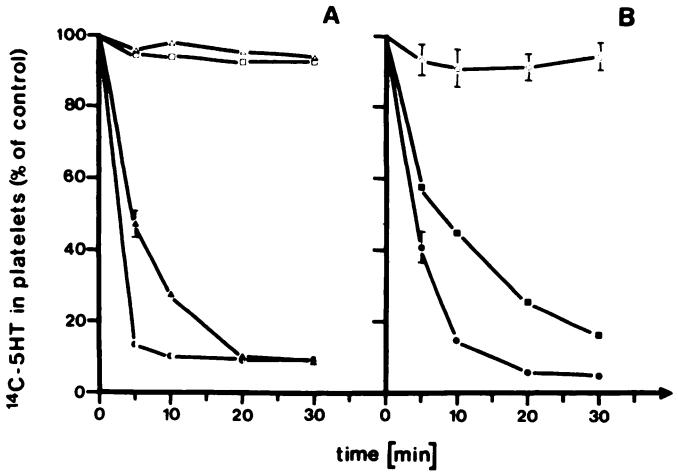

FIG. 1. *Effects of ionophores on the release of [^{14}C]5-HT in K^+ medium at 37°*

The [^{14}C]5-HT content of the platelets is given as a percentage of that present before incubation (at time 0). □, No addition (10); ◐, thrombin, 0.12 unit/ml (6); ■, 16 μM FCCP (6); ●, 16 μM FCCP + 4 μM valinomycin + 10 μM nonactin (6); ○, 4 μM valinomycin + 10 μM nonactin (6); ▲, 15 μM monensin (12); △, 5 μM antimycin A (6). Numbers of experiments are indicated in parentheses. The standard error of the mean, if more than 3%, is indicated. The difference between the values for FCCP and those for FCCP + valinomycin + nonactin was significant ($\alpha \leq 0.05$) at all times.

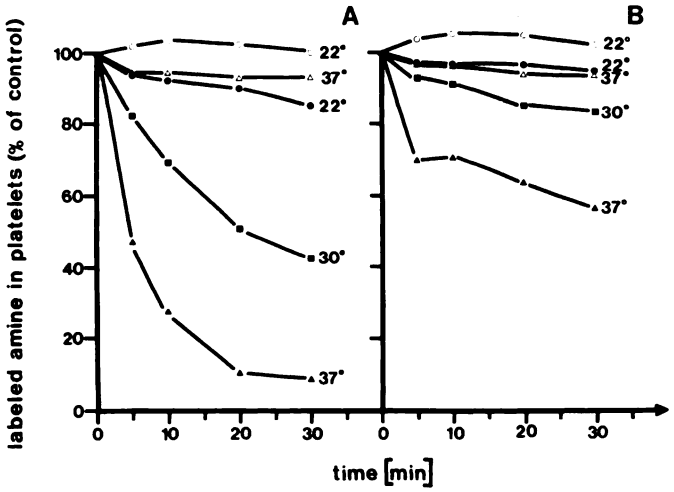

FIG. 2. *Temperature dependence of [^{14}C]5-HT (A) and [^{3}H]NA (B) release caused by monensin in K^+ medium*

The content of labeled amines in the platelets is given as a percentage of that present before incubation (averages ± standard error of the mean of eight experiments). The [^{3}H]NA values at 37° and the [^{14}C]5-HT values at 37° and 30° were significantly different from the respective control values ($\alpha \leq 0.05$) at all times. *Open symbols*, controls; *closed symbols*, with 15 μM monensin.

Release of [^{3}H]NA, K^+ medium. The actions of the

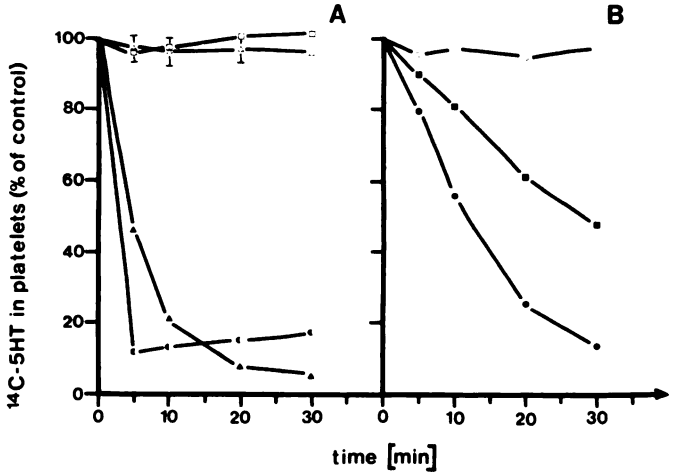

FIG. 3. *Effects of ionophores on the release of [^{14}C]5-HT in Na^+ medium*

The [^{14}C]5-HT content of the platelets is given as a percentage of that present before incubation (time 0; averages ± standard error of the mean of six experiments). For explanation of symbols see legend to Fig. 1. The thrombin values at 5 min were significantly different from those at 20 and 30 min ($\alpha \leq 0.05$).

ionophores on [^{3}H]NA in principle were similar to those on [^{14}C]5-HT, but considerably less pronounced (data not shown). The release never exceeded 40% of the amine initially present in the platelets. The release of [^{3}H]NA by monensin was also temperature-sensitive (Fig. 2B).

Thrombin caused a fast and marked release of [^{3}H]NA which had a time course similar to that of [^{14}C]5-HT (Fig. 1A). However, the maximal release of [^{3}H]NA (about 80% after 30 min) was somewhat less than that of [^{14}C]5-HT.

Release of [^{3}H]NA, Na^+ medium. Results were the same as for the K^+ medium, except that monensin had a more pronounced effect (Fig. 4).

Effect of antimycin A alone and in combination with inhibitors of glycolysis. Antimycin A alone (5 μM) had no effect on the content of [^{14}C]5-HT or [^{3}H]NA of platelets. Antimycin A in combination with the glycolysis inhibitors deoxyglucose (30 mM) and glucono-δ-lactone (10 mM) caused a slow release of [^{14}C]5-HT (23 ± 2% after 30 min) and of [^{3}H]NA (13 ± 1%), significantly ($\alpha \leq 0.05$) less than that induced by FCCP (52 ± 6% for [^{14}C]5-HT and 31 ± 2% for [^{3}H]NA; Figs. 3 and 4). Antimycin A, with or without the inhibitors of glycolysis, did not modify the amine-releasing effect of FCCP (data not shown).

Release of ATP, Na^+ medium. Neither monensin nor FCCP plus valinomycin and nonactin caused a release of ATP differing from that in platelets incubated in the medium alone. In contrast, thrombin given before or after incubation with the ionophores (30 min) had a fast and marked ATP-releasing action (Fig. 5).

DISCUSSION

Our results show that ionophores which cause a collapse of H^+ gradients induced a fast and virtually total release of [^{14}C]5-HT and [^{3}H]NA from human platelets. There are various possible mechanisms for this release.

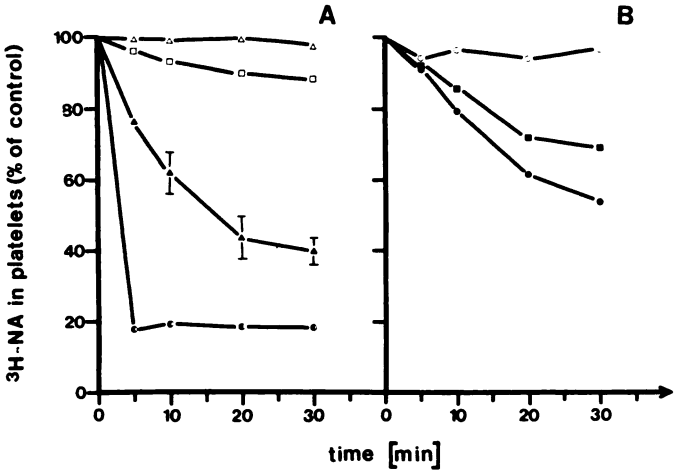

FIG. 4. *Effects of ionophores on the release of [^{3}H]NA in Na^+ medium*

The [^{3}H]NA content of the platelets is given as a percentage of that present before incubation (time 0; averages ± standard error of the mean of six experiments). For explanation of symbols see legend to Fig. 1. Significant differences ($\alpha \leq 0.05$): control versus FCCP (20 and 30 min), control versus FCCP + valinomycin + nonactin (10–30 min), FCCP versus FCCP + valinomycin + nonactin (20 and 30 min).

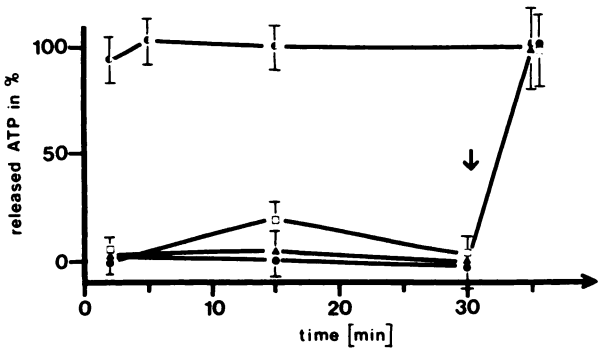

FIG. 5. *Release of ATP into supernatant of platelet suspensions*

Platelets were diluted with Na^+ medium (final concentration 0.82 × 10^8/ml) alone (controls, □) or containing thrombin 0.12 unit/ml (◐); 15 μM monensin (▲); or 16 μM FCCP + 4 μM valinomycin + 10 μM nonactin (●). The difference after a 15-min incubation at 37° between the ATP concentration in the supernatant of controls (time 0) and of thrombin-treated platelets was taken as 100%. After a 30-min incubation, thrombin (0.12 unit/ml) was added to the suspensions containing the ionophores as marked by the *arrow* (averages ± standard error of the mean of seven experiments).

The first possibility, exocytosis, can be excluded because [^{14}C]5-HT, [^{3}H]NA, and ATP, which are stored together in the same granules (3, 14), were released to different degrees. In contrast, thrombin, an agent known to act by exocytosis, caused a fast and marked release of all three constituents.

Second, the ionophores might act by inducing a disturbance of energy transduction. This is unlikely, as the results with antimycin A and inhibitors of glycolysis show. In human platelets, antimycin A lowers cytosolic ATP and in combination with glycolysis inhibitors it completely depletes this ATP pool (17). However, antimycin A, which inhibits mitochondrial ATP production at least as strongly as FCCP (18), did not influence the amine content or the amine-releasing effect of FCCP. Even a complete reduction of cytosolic ATP by antimycin A plus glycolysis inhibitors had no marked action on the amine content. This indicates that the releasing action of ionophores is independent of changes in cytosolic ATP. Furthermore, valinomycin and nonactin, two ionophores which—like FCCP—uncouple oxidative phosphorylation in mitochondria (15), did not cause amine release.

Third, an apparent release of amine might be brought about by a mere inhibition of the uptake mechanism at the plasma membrane. However, when labeled platelets were incubated in a Na^+-poor K^+ medium (less than 4.5 mM Na^+), in which no active 5-HT uptake is possible (9), there was no release of [^{14}C]5-HT.

It can therefore be concluded that the properties possessed by monensin, nigericin and FCCP of abolishing the proton gradient of the granular membrane, or of changing its potential, are responsible for the release of the amines. In the case of monensin (and nigericin), abolition of the proton gradient is the main factor; the membrane potential is probably not affected, because monensin causes an electro-neutral exchange of H^+, Na^+, and K^+ against each other, and the H^+ conductance of the granular membrane is very low (8).

The effect of FCCP is more complex. It has been shown

with isolated storage organelles of pig platelets (11) that FCCP on its own causes a slow reduction of proton gradients and a marked inversion of membrane potentials from inside positive to negative, which inhibits the further efflux of protons. The amine-releasing action of FCCP was less marked than that of monensin; therefore, even if the effects of FCCP were due exclusively to the alteration in membrane potential, it would mean that this mechanism of releasing amines is less effective than reduction of the proton gradient. The action of FCCP plus nonactin also shows that a proton gradient is important for amine storage. This combination, which causes a more rapid reduction of the proton gradients than FCCP alone (because charge compensation can occur faster), also induced a more rapid amine release. At the same time the change in membrane potential was probably smaller than with FCCP alone.

A major influence of K^+ and Na^+ gradients on amine storage is unlikely, because the combination of valinomycin and nonactin, which eliminates K^+ and Na^+ gradients (13) without affecting H^+ gradients, did not cause any amine release. In addition, incubation of platelets in media containing different Na^+ and K^+ concentrations, which influence intracellular Na^+:K^+ ratios (13), did not interfere with the amine content (experiments without ionophores) or markedly change the effect of ionophores.

The release of [^{3}H]NA occurs more slowly than that of [^{14}C]5-HT, but this cannot be the consequence of more catecholamines being stored at extragranular sites. Thus, thrombin, which acts on granular amines (causing exocytosis) induced a release of [^{3}H]NA which was as fast and almost as marked as that of [^{14}C]5-HT (80% and 90% release, respectively). The slowness of the [^{3}H]NA release may be related to the relatively poor permeability for NA (compared with 5-HT) of the granular membrane or with the fact that no specific transport system for NA exists at the plasma membrane.

To summarize our findings on the factors influencing amine storage in intact platelets, a proton gradient is of primary importance. In the case of 5-HT this confirms earlier findings with 5-HT vesicles (9–11). Also, some influence of granular membrane potentials cannot be excluded. On the other hand, intracellular Na^+ and/or K^+ gradients do not play a role. In part this finding is at variance with previous findings (13). The discrepancies may have arisen because the earlier experiments used ionophores (monensin and nigericin) which influenced Na^+ and K^+ as well as H^+ gradients. In addition, the marked temperature dependence of the amine outflow (very little at 22°) was not taken into account. Preliminary experiments indicate that in brain synaptosomes a proton gradient also plays a role in the storage of 5-HT and NA. Interference with the proton gradient across the granular membrane may be a new principle for designing drugs which release neurotransmitters without causing exocytosis.

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Send reprint requests to: Prof. A. Pletscher, Department of Research, Kantonsspital, Hebelstrasse 20, CH-4031 Basel, Switzerland.