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The role of Na^+/H^+ exchanger in serotonin secretion from porcine blood platelets

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This study was undertaken to evaluate whether a link exists between the activation of protein kinase C (PKC), operation of Na^+/H^+ exchanger (NHE), cell swelling and serotonin (5-HT) secretion in porcine platelets. Activation of platelets by thrombin or phorbol 12-myristate 13-acetate (PMA), a PKC activator, initiated a rapid rise in the activity of Na^+/H^+ exchanger and secretion of 5-HT. Both thrombin- and PMA-evoked activation of Na^+/H^+ exchanger was less pronounced in the presence of ethyl-isopropyl-amiloride (EIPA), an NHE inhibitor, and by GF 109203X, a PKC inhibitor. Monensin (simulating the action of NHE) caused a dose-dependent release of 5-HT that was not abolished by GF 109203X or EGTA. Lack of Na^+ in the suspending medium reduced thrombin-, PMA-, and monensin-evoked 5-HT secretion. GF 109203X nearly completely inhibited 5-HT release induced by PMA-, partly that induced by thrombin, and had no effect on 5-HT release induced by monensin. EIPA partly inhibited 5-HT release induced by thrombin and nearly totally that evoked by PMA. Electronic cell sizing measurements showed an increase in mean platelet volume upon treatment of cells with monensin, PMA or thrombin. The PMA- and thrombin-evoked rise in mean platelet volume was strongly reduced in the presence of EIPA. As judged by optical swelling assay monensin and PMA produced a rapid rise in platelet volume. The swelling elicited by PMA was inhibited by EIPA and its kinetics was similar to that observed in the presence of monensin. Hypoosmotically evoked platelet swelling did not affect platelet aggregation but significantly potentiated thrombin-evoked release of 5-HT and ATP. Taken together, these results show that in porcine platelets PKC may promote 5-HT secretion through the activation of NHE. It is hypothesized that enhanced Na^+/H^+ antiport may result in a rise in cell membrane tension (due to cell swelling) which in turn facilitates fusion of secretory granules with the plasma membrane leading to 5-HT secretion.

Keywords: platelets, Na^+/H^+ exchanger, platelet secretion, platelet swelling, serotonin release

Platelets are circulating secretory cells that respond to substances appearing at the site of damage to the vascular endothelium (e.g., thrombin, collagen) (Kroll & Schafer, 1996; Flaumenhaft, 2003). Platelets possess three types of secretory organelles known as α granules (contain, e.g., platelet factor IV and β-thromboglobulin), dense granules (contain ATP, serotonin, Ca^{2+}) and lysosomes (contain acid hydrolases) (Harrison & Cramer, 1993; McNicol, 1999; Flaumenhaft, 2003). Following platelet activation by specific stimulators, the contents of these granules are released into the extraplatelet environment, without cell lysis, by regulated exocytosis (White, 1999; Reed *et al.*, 2000; Flaumenhaft, 2003).

Although signal transduction pathways in activated platelets have been studied extensively, the molecular mechanism of platelet secretion is still unclear. However, it has been established that in human platelets a synergistic action of elevated cytosolic Ca^{2+} levels and enhanced protein kinase C activity is necessary for a maximal platelet secretory response (McNicol, 1999). It is agreed that in acti-

Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; GF 109203X, bisindolylmaleimide; 5-HT, serotonin; Me_2SO, dimethyl sulfoxide; NHE, Na^+/H^+ exchanger; NMDG, *N*-methyl--D-glucamine; OCS, open canalicular system; PgE_1, prostaglandin E_1; pH_i, cytosolic pH; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PRP, platelet-rich plasma; SBFI, sodium-binding benzofuran isophtalate; SERT, serotonin transporter.

vated human platelets PKC is involved in both Ca^{2+}-dependent and -independent granule secretion (Rink *et al.*, 1993; Yashioka *et al.*, 2001).

Activated PKC has been proposed to promote secretion through its downstream effectors (Reed *et al.*, 2000; Flaumenhaft, 2003). The list of potential downstream PKC effectors comprises SNARE proteins, MARCKS (myristoylated alanine-rich C kinase substrate) and PIPKII (type II phosphatidylinositol 5-phosphate 4-kinase) (Flaumenhaft, 2003). The precise role of these effectors in platelet secretion is not yet established but all of them have been proposed to be involved in platelet membrane fusion (Flaumenhaft, 2003). The list of PKC downstream effectors involved in secretion in platelets is not closed and further studies are needed for identification of their full complement.

The Na^+/H^+ exchanger seems to be an important PKC effector with potential function in platelet exocytosis (Rosskopf, 1999). The Na^+/H^+ exchanger is a plasma membrane glycoprotein that mediates the electroneutral exchange of intracellular H^+ for extracellular Na^+ (Putney *et al.*, 2002). Compared with erythrocytes and lymphocytes, rather high levels of the NHE1 protein have been observed in platelets (Borin & Siffert, 1990; Rosskopf, 1999).

An involvement of the Na^+/H^+ exchanger in the platelet secretion process is likely since physiological stimulators (e.g. thrombin) enhance its activity in human platelets (Borin & Siffert, 1990; Rosskopf, 1999). Rapid Na^+ influx observed in platelets activated by thrombin (due to operation of the Na^+/H^+ exchanger) is expected to produce platelet swelling. Studies performed on cells other than platelets have revealed that cell swelling by itself may evoke an immediate secretory burst of substances stored in secretory vesicles, with dynamics indistinguishable from those induced by specific secretagogues (Lang *et al.*, 1998; Strbak & Greer, 2000; Straub, 2002).

The aim of this study was to estimate whether a link exists between the activation of PKC, operation of the Na^+/H^+ exchanger, cell swelling and serotonin secretion in porcine platelets.

The obtained results indicate that in porcine platelets PKC may promote serotonin secretion through the activation of the Na^+/H^+ exchanger. It is hypothesized that the resulting rise in cytosolic Na^+ concentration may produce platelet swelling and increased plasma membrane tension which in turn may facilitate fusion of secretory granules with the plasma membrane leading to serotonin secretion.

MATERIALS AND METHODS

Chemicals. Monensin, Ca^{2+} ionophore A23187, phorbol 12-myristate-13-acetate (PMA), Hepes, EGTA, imipramine, reserpine, dimethyl sulfoxide (Me_2SO), apyrase, *N*-methyl-D-glucamine (NMDG), bovine serum albumin (BSA), prostaglandin E_1 (PgE_1), 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), bisindolylmaleimide (GF 109203X), 2,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) were purchased from Sigma Chemical Co. (St. Louis MO, USA). Thrombin (human) was from La Roche (Basel, Switzerland). Aggrastat^{TM} (tirofiban hydrochloride) was from Merck Scharp & Dohme B.V. (Holland). [γ-^{3}H]5-hydroxy tryptamine ([^{3}H]serotonin) with a specific activity of 8.6 Ci/mmol was purchased from the Radiochemical Center (Amersham, England). Monensin and EIPA were dissolved in ethanol and methanol, respectively. PMA was dissolved in Me_2SO.

Preparation of platelet-rich plasma. Blood was collected in a local slaughterhouse from adult pigs. The blood was withdrawn by direct carotid catheterization and collected into 3.8% (w/v) sodium citrate, one volume per nine volumes of blood. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 200 × ***g*** for 20 min at room temperature. The upper two-thirds of the supernatant was used for the preparation of either [^{3}H]serotonin- or BCECF-loaded platelets.

Measurement of platelet secretion. Secretion was determined by the release of [^{3}H]serotonin essentially as described by Holmsen and Dangelmaier (1989). To load platelets with serotonin freshly prepared PRP was incubated with [^{3}H]serotonin (1 μCi/ml) for 45 min at 37°C. After acidification to pH 6.5 of the PRP with 1 M citric acid, the suspension was centrifuged at 1500 × ***g*** for 20 min to obtain a pellet which was resuspended in a Tyrode/Hepes buffer containing: 139 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM glucose, 10 μM EGTA, 10 mM Hepes, pH 7.4, albumin (3.5 mg/ml) and apyrase (2 U/ml). The platelets were washed once and suspended in the same buffer without apyrase and EGTA. In some experiments platelets were washed and suspended in a Ca^{2+}-free Tyrode/Hepes buffer in which sodium ions were replaced by isoosmotic concentrations of NMDG. Imipramine was added to the solution at 3 μM in order to prevent re-uptake of secreted [^{3}H]serotonin. Secretion was estimated at 37°C in stirred (800 r.p.m.) 1 ml aliquots of [^{3}H]serotonin-loaded platelets by the addition of an appropriate stimulus. Aliquots of 0.2 ml were taken from the incubation mixture at time 0–5 min and mixed with 80 μl of ice-cold stop solution containing 3 mM EDTA and 1.5% (w/v) formaldehyde. The aliquots were immediately mixed and centrifuged at 11000 × ***g*** for 1.5 min. [^{3}H]Serotonin released from the platelets was determined by counting the supernatant in a liquid scintillation counter using Instagel (Canberra Packard) as a scintillant.

Preparation of reserpinized [^{3}H]serotonin-loaded platelets. Platelets were loaded with [^{3}H]serotonin as described above, by adding the labeled compound to PRP pre-incubated for 10 min with 10 μM reserpine. Reserpinized [^{3}H]serotonin-loaded platelets were then resuspended in a Ca^{2+}-free Tyrode/Hepes buffer supplemented with 10 μM reserpine and immediately used for experiments.

Measurement of intracellular pH. Cytosolic pH (pH_i) was determined using the intracellularly trappable fluorescent pH indicator BCECF, essentially as described by Siffert *et al.* (1989). PRP (20 ml) was acidified to pH 6.5 with 1 M citric acid and centrifuged at 1500 × *g* for 20 min at room temperature to obtain a pellet, which was then resuspended in 2 ml of supernatant plasma. BECF/AM (final conc. 10 μM) was added and the cells were incubated for 30 min at 37°C. Thereafter 10 μM PgE_1 was added and the platelets were washed twice in Tyrode/Hepes buffer, pH 6.5, by repeated centrifugation at 1500 × *g* for 20 min and resuspension at room temperature. At the last washing step, the final pellet was taken up into Tyrode/Hepes buffer, pH 7.4, at a concentration of 5×10^9 cells/ml. Samples of these suspensions (30–40 μl) were then transferred to a plastic cuvette containing 2 ml of the Tyrode/Hepes buffer, pH 7.4, without EDTA, PgE_1, apyrase and albumin, and prewarmed to 37°C for 5 min. In some experiments the Tyrode/Hepes buffer was replaced by NMDG medium (142 mM *N*-methyl D-glucamine, 0.8 mM $MgCl_2$, 5.6 mM glucose and 10 mM Hepes buffer, pH 7.4). Measurements of the BCECF fluorescence were performed in a Nova 2 spectrofluorimeter (Baird Atomic Ltd, England) connected with a recorder and equipped with a thermostated cell holder and a stirring device. The monochromator settings were 495 nm for excitation and 530 nm for emission, respectively. The calibration of the fluorescence signals was carried out by lysis of platelets with 50 μM digitonin followed by adjustment of the pH to defined values.

Simultaneous measurement of platelet aggregation and secretion. The release of dense granule ATP from aggregating platelets was monitored simultaneously with optical aggregation using a lumiaggregometer (Chrono-log Corp., Havertown, PA, USA) connected with a dual channel recorder. All procedures were conducted as described previously (Tomasiak *et al.*, 2004). The results of the chemiluminescence light signal were compared with an ATP standard curve and converted to nanomoles per 10^8 cells.

Measurement of mean platelet volume. Changes in platelet volume were determined by electronic cell sizing using a hematological analyzer (Coulter Electronic GmbH). Changes of platelet volume produced by PMA and thrombin were measured in the presence of tirofiban. Tirofiban is known to block the final step of platelet activation: the binding of fibrinogen to GPIIb/IIIa receptors and formation of platelet aggregates (Bednar *et al.*, 1998). In porcine platelets treated with thrombin this drug strongly inhibits aggregation, slightly affects (prolongs) shape change and is without any effect on calcium signal (not shown here). To prevent pairing of platelets and formation of microaggregates, relatively high (8 mM) concentrations of aggrastat were selected (Storey *et al.*, 1998). Inefficient inhibition of microaggregate formation may result in an overestimation of the mean platelet volume determined with a Coulter-type counter.

Measurement of changes in platelet volume. Changes in platelet volume (swelling) were followed spectrophotometrically by recording optical density (OD) at 680 nm in diluted (to prevent aggregation) cell suspensions, essentially as described by Rosskopf *et al.* (1991). A drop in OD reflects a rise in cell volume. The changes in OD were measured at 37°C in plastic cuvettes (light path 10 mm) without stirring. Aliquots of PRP (140 μl) were incubated for 2 min at 37°C in the cuvette of the instrument, then 860 μl of Tyrode/Hepes buffer (pH 7.4, osmolality 340 mOsm, previously prewarmed to 37°C) and 1–5 μl of the tested substances (or vehiculum) were added immediately. The suspension was carefully mixed for exactly 10 s and the changes in OD were recorded for 2 min at 10 s intervals using a sensitive spectrophotometer (Helios gamma, Unicam) connected with a personal computer.

Data analysis. Data reported in this paper are the mean (± S.D.) of the number of determinations indicated (n). Statistical analysis was performed by the Student's *t*-test and elaboration of experimental data by the use of Slide Write plus (Advanced Graphics Software, Inc, Carlsbad, CA, USA.)

RESULTS

To estimate whether in porcine platelets the Na^+/H^+ exchanger may be controlled by PKC, the effects of PMA (a PKC activator) and thrombin (produces Ca^{2+} signal and activates PKC) on the intracellular pH were studied and compared with that exerted by monensin. The results of these experiments are shown in Fig. 1. Monensin is an ionophore known to mediate the exchange of intracellular H^+ for extracellular Na^+ and alkalizing the cytosol thus mimicking the operation of the Na^+/H^+ exchanger (Mollenhauer *et al.*, 1990). Alkalization of the cytosol reflects a rise in the Na^+/H^+ exchanger activity (Rosskopf, 1999; Putney *et al.*, 2002). In contrast to thrombin and PMA, monensin (up to 30 μM) is unable to evoke aggregation of porcine platelets (not shown). Panels A, B and C show that thrombin (0.3 U/ml), PMA (300 nM), and monensin (30 μM) pro-

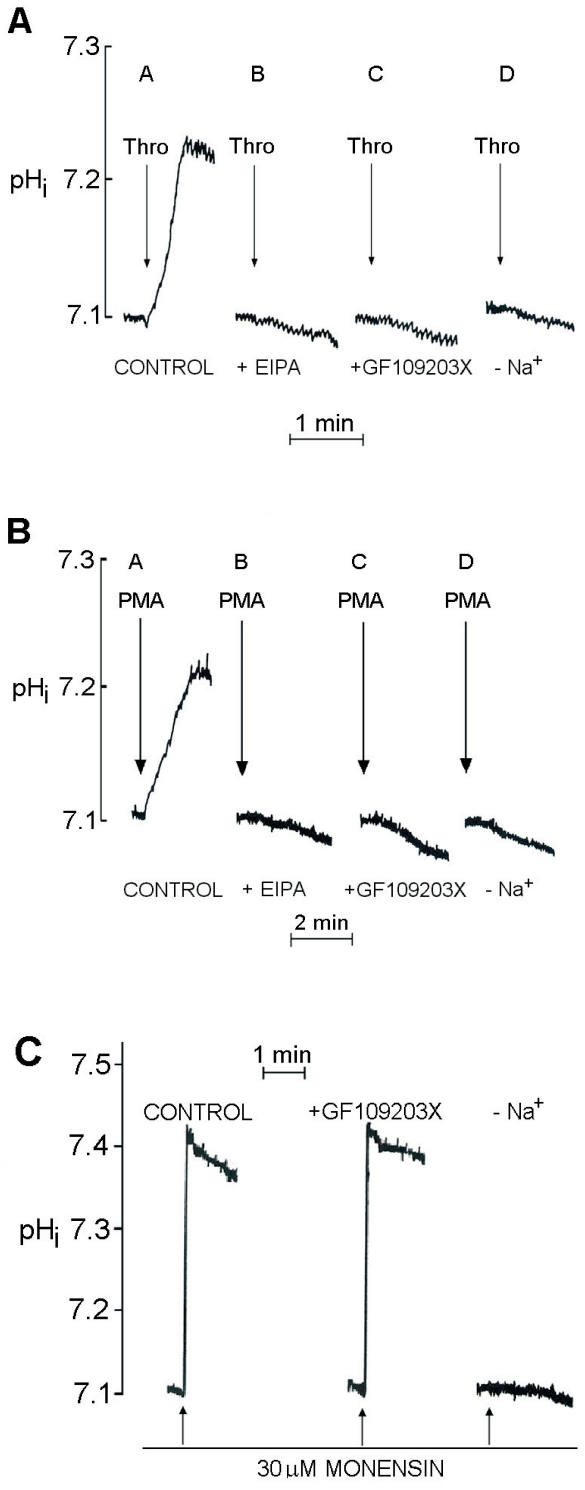

Figure 1. Effect of thrombin, PMA and monensin on platelet cytosolic pH (pH$_i$).
BCECF-2-loaded platelets suspended in normal Tyrode/Hepes buffer or in sodium-free medium (–Na$^+$) were treated at times indicated by arrows at 37°C with 0.3 U/ml of thrombin (panel A), 300 nM PMA (panel B) or 30 μM monensin (panel C). As indicated, EIPA (200 μM final conc.) or GF 109203X (7 μM final conc.) were added 30 s before the stimulus. pH$_i$ was estimated as described in Materials and Methods. Each trace is representative of at least six determinations performed in three different platelet preparations.

duce, after 1 min, an increase of pH$_i$ by about 0.15, 0.075 and 0.25 units, respectively. The thrombin- and PMA-evoked cytosol alkalization was strongly reduced in the absence of extracellular sodium or in the presence of EIPA, a specific inhibitor of the Na$^+$/H$^+$ exchanger (Sweatt *et al.*, 1985), or GF 109203XGX, a specific inhibitor of PKC (Toullec *et al.*, 1991). Monensin-evoked cytosol alkalization was not affected by GF 109203XGX but was inhibited in the absence of sodium in the suspending medium. These results suggest that in porcine platelets the activity of the Na$^+$/H$^+$ exchanger may be at least in part controlled by PKC.

To establish whether in porcine platelets the Na$^+$/H$^+$ exchanger is involved in a secretory process, thrombin-evoked serotonin secretion from platelets suspended in sodium-free medium (to inhibit the Na$^+$/H$^+$ exchanger) was studied. Since platelet secretory response depends on the strength of the stimulus, experiments were performed using threshold, suprathreshold and superthreshold concentrations of thrombin. Threshold concentration of thrombin is defined as the minimum amount of the stimulus that produces, within 3 min, at least a 70% increase in light transmission of platelet suspension stirred at 37°C (in the cuvette of the optical aggregometer) platelet suspension. Figure 2 shows that thrombin (0.1–0.8 U/ml) in both a time- and dose-dependent manner, induces serotonin secretion from porcine platelets. Blocking of the Na$^+$/H$^+$ exchanger by replacing sodium in the suspension medium by NMDG distinctly reduces thrombin-evoked serotonin release (panel B). The degree of this reduction depends on the strength of the stimulus. Thus, secretion produced by a 3-min incubation of platelets with a subthreshold (0.1 U/ml), threshold (0.3 U/ml) and suprathreshold (0.8 U/ml) concentration of thrombin was reduced by 33%, 50% and 77%, respectively.

As it is seen from Fig. 3, preincubation of platelets with GF 109203X or EIPA reduced the serotonin secretion produced by threshold concentrations of thrombin by about 50%. These results indicate the involvement of PKC and the Na$^+$/H$^+$ exchanger in the thrombin-evoked serotonin release.

Figure 4 illustrates the effect of monensin-evoked Na$^+$/H$^+$ exchange on serotonin secretion. Monensin (30 μM) produces serotonin release in a time-dependent manner. Serotonin secretion observed following a 3-min incubation of platelets with monensin was similar to that produced by threshold concentrations of thrombin (compare Fig. 2, panel A, and Fig. 4). Monensin-evoked serotonin secretion was not affected after the inhibition of protein kinase C by GF 109203X and following diminishing of the concentration of extracellular calcium (by EGTA). However, it was strongly reduced in the absence of sodium ions in the suspending medium. Monensin

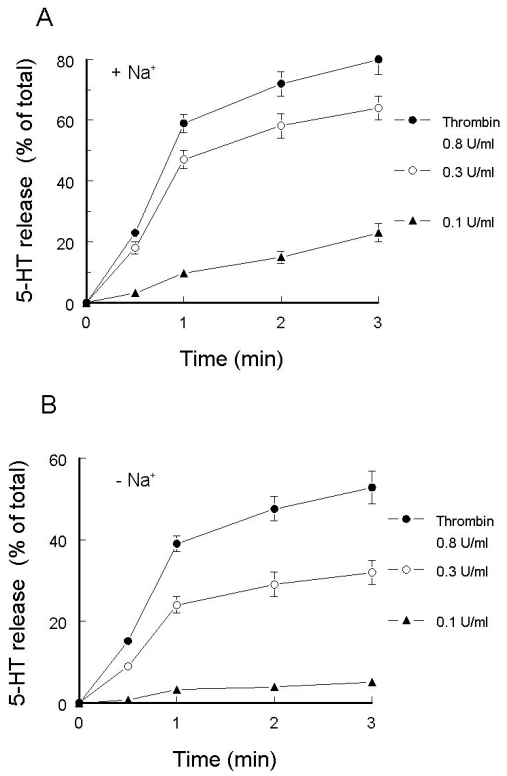

Figure 2. Time course of thrombin-evoked serotonin release in the presence (panel A) or absence (panel B) of sodium in suspension medium.
Aliquots of [^{3}H]serotonin-loaded platelets suspended in normal Tyrode/Hepes buffer (+Na^+) or in sodium-free medium (–Na^+) were incubated for 0.5–3 min at 37°C with thrombin added (at time 0) to the final concentration as indicated. The amount of [^{3}H]serotonin released within 0.5–3 min was estimated. Results are expressed as the percentage of the total amount of [^{3}H]serotonin contained in unstimulated platelets and are presented as means± S.D. All experiments were performed at least in quadruplicate using five different platelet preparations.

(30 μM, 3 min) did not cause lysis of porcine platelets. Monensin-induced LDH release was negligible (below 3.5%), similar to that produced by collagen (not shown). These data indicate that enhanced Na^+/H^+ exchange may result in serotonin release.

Platelets are known to take up serotonin from plasma and accumulate it in storage granules (Holmsen, 1982; Da Prada *et al.*, 1988; McNicol & Israels 1999). Transport of the amine from the extra-cellular compartment into the cytosol is mediated by a highly selective, imipramine-sensitive plasma membrane serotonin transporter, SERT (Da Prada *et al.*, 1988; Lesch *et al.*, 1993). A second carrier located in the membranes of storage granules, i.e. vesicular monoamine transporter, which is specifically inhibited by reserpine, then enables accumulation of serotonin in dense granules (Da Prada *et al.*, 1988). Recently, Turetta *et al.* (2004) reported that in human platelets monensin-evoked rise in the intracellular sodium concentration might initiate transport of serotonin from the cytosol to the extracellular space

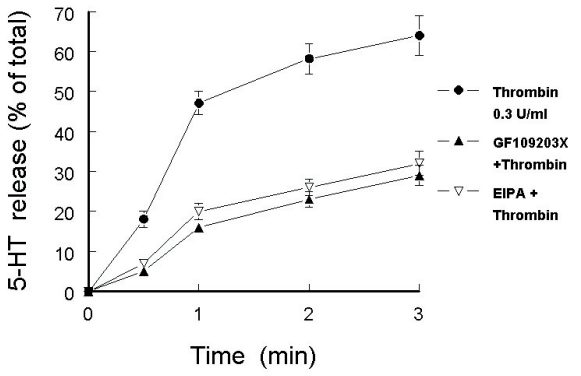

Figure 3. The effect of GF 109203X and EIPA on thrombin-evoked serotonin release.
Aliquots of [^{3}H]serotonin-loaded platelets suspended in normal Tyrode/Hepes buffer were incubated for 0.5–3 min at 37°C without or with GF 109203X or EIPA added 30 s before stimulus, to the final concentration of 7 μM or 300 μM, respectively. Secretion was initiated by the addition of thrombin to the final concentration of 0.3 U/ml. The amount of [^{3}H]serotonin released within 0.5–3 min was estimated. Further details as in the legend to Fig. 2.

through the putative SERT operating in reverse mode.

Experiments presented in Fig. 5 were performed to establish whether in porcine platelets monensin is also able to produce such non-exocytotic efflux of serotonin from the cytosol to the extracellular milieu. Platelets were pre-loaded with [^{3}H]serotonin in the absence or in the presence of reserpine. Since reserpine is known to block transport of serotonin from the cytosol to granules all [^{3}H]serotonin accumulated in reserpinized platelets is expected to be present in the cytosol, whereas in non-reserpinized cells the bulk of [^{3}H]serotonin is

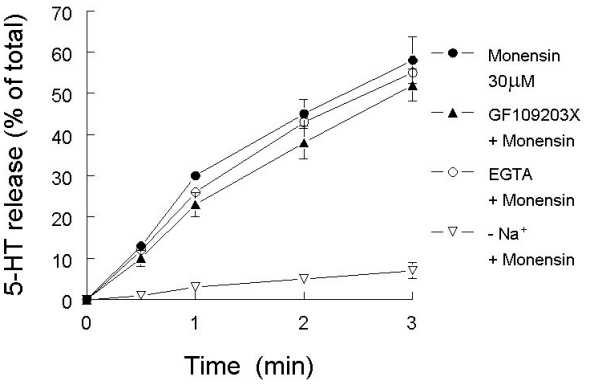

Figure 4. Time course of monensin-induced serotonin release.
Aliquots of [^{3}H]serotonin-loaded platelets suspended in normal Tyrode/Hepes buffer were incubated at 37°C for 0.5–3 min with 30 μM monensin or with combination of 30 μM monensin + 7 μM GF 109203X or 1 mM EGTA. In the experiment designated as –Na^+ 30 μM monensin was added to platelets suspended in sodium-free medium. The amount of [^{3}H]serotonin released within 0.5–3 min was estimated. Further details as in the legend to Fig. 2.

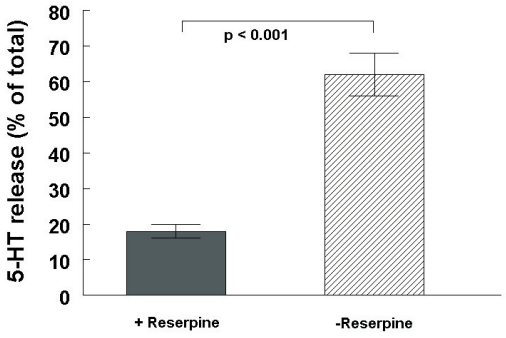

Figure 5. Monensin-evoked serotonin release from normal and reserpine-treated platelets.
Reserpinized [^{3}H]serotonin-loaded platelets were prepared as described under Materials and Methods. Aliquots of normal or reserpinized [^{3}H]serotonin-loaded platelets isolated from the same donor were incubated with stirring (800 r.p.m.) for 3 min at 37°C. Secretion was initiated by the addition of monensin to the final concentration of 30 μM. The amount of [^{3}H]serotonin released within 3 min after the stimulus was estimated. Results are expressed as a percentage of the total amount of [^{3}H]serotonin contained in unstimulated platelets and are presented as means ±S.D. All experiments were performed at least in quadruplicate using four different platelet preparations.

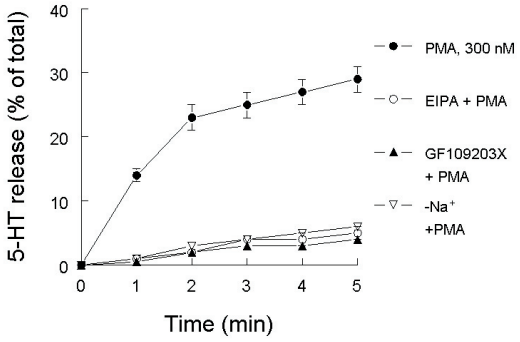

Figure 6. Time course of phorbol 12-myristate 13-acetate (PMA)-induced serotonin release.
Aliquots of [^{3}H]serotonin-loaded platelets suspended in normal Tyrode/Hepes buffer were incubated at 37°C for 1–5 min with: 300 nM PMA or with combination of 300 nM PMA + 200 μM EIPA or 7 μM GF 109203X. In the experiment designated as –Na^+ 300 nM PMA was added to platelets suspended in sodium-free medium. The amount of [^{3}H]serotonin released within 1–5 min was estimated. Further details as in the legend to Fig. 2.

believed to be stored in dense granules (Holmsen, 1982; McNicol & Israels 1999).

As seen in Fig. 5, a 3-min treatment of reserpinized platelets with 30 μM monensin produces an 18% release of serotonin accumulated in platelet cytosol. By comparison, the same monensin concentration releases after 3 min about 65% of serotonin accumulated in dense granules of non-reserpinized cells.

Experiments shown in Fig. 6 were performed to establish whether the PMA-evoked serotonin release is mediated by the Na^+/H^+ exchanger. Activation of human platelets by PMA results in a rise in PKC activity and serotonin secretion (Nishizuka, 1986; Elzagallaai *et al.*, 1998). It has been found that in human and in porcine platelets PMA produces a continuous rise in the intracellular Na^+ concentration (Borin & Siffert, 1991; Stelmach *et al.*, 2002). It was also reported that in activated platelets PKC was able to accelerate Na^+-H^+ antiport (Rosskopf, 1999). Thus, activation of PKC, a rise in the activity of the Na^+/H^+ exchanger, and sodium influx are expected in platelets after their activation by PMA. Our results show that PMA (300 nM) induced serotonin secretion from porcine platelets in a time-dependent manner. A five-minute incubation of platelets with 300 nM PMA produced 25% serotonin secretion. Inhibition of the Na^+/H^+ exchanger by EIPA or of PKC by GF 109203X, an inhibitor of the catalytic PKC domain, or a lack of sodium ions in the medium significantly reduced PMA-evoked serotonin release. These data indicate that in porcine platelets the Na^+/H^+ exchanger may be involved in PKC-dependent serotonin release.

Experiments shown in Figs. 7 and 8 were performed to assess whether the Na^+-H^+ antiport evoked by monensin, PMA or thrombin produces a rise in platelet volume. A Coulter-type counter (Fig. 7) and optical swelling assay (Fig. 8) were employed to measure changes in platelet volume. The optical method allows recording of the kinetics of cell swelling, but it cannot be used to measure the platelet volume changes eventually produced by thrombin due to the interference of the optical perturbances produced by platelet shape change. The Coulter counter cannot follow the kinetics of swelling but it allows measuring the platelet volume following addition of thrombin or PMA as long as

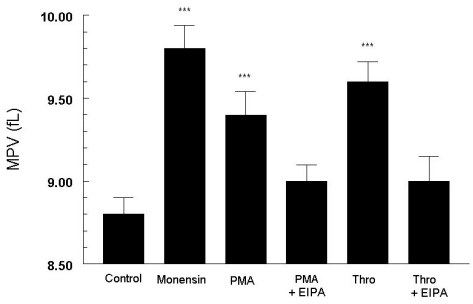

Figure 7. Platelet volume changes following monensin, PMA, and thrombin treatment.
Aliquots of platelets suspended in normal Tyrode/Hepes buffer were incubated at 37°C for 1 min with no additions (control) or with: 30 μM monensin, 300 nM PMA or 0.2 U/ml of thrombin. When indicated EIPA was added to 200 μM 1 min before the stimulus. To avoid platelet aggregation in experiments with thrombin and PMA aggrastat (8 mM) was added 30 s before the stimulus. Mean platelet volume was measured using electronic counting technique. The data represent mean values (±S.D.) of four experiments, each performed on a separate platelet preparation (n = 16). Pair Student's *t*-test was used to calculate *P*-values. *** $P < 0.005$.

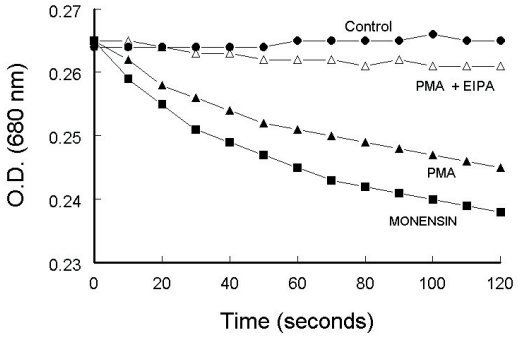

Figure 8. Kinetics of platelet swelling following addition of phorbol 12-myristate 13-acetate (PMA) or monensin.
At time 0 suspensions of washed platelets were supplemented with PMA or monensin added to the final concentrations of 300 nM or 30 μM, respectively. When indicated EIPA was added to 200 μM 1 min before the stimulus. In the control vehiculum (ethanol) was added instead. Changes in platelet volume (swelling) were followed at 37°C by recording optical density as described in Materials and Methods. Each trace is representative of at least 15 determinations performed in five different platelet preparations.

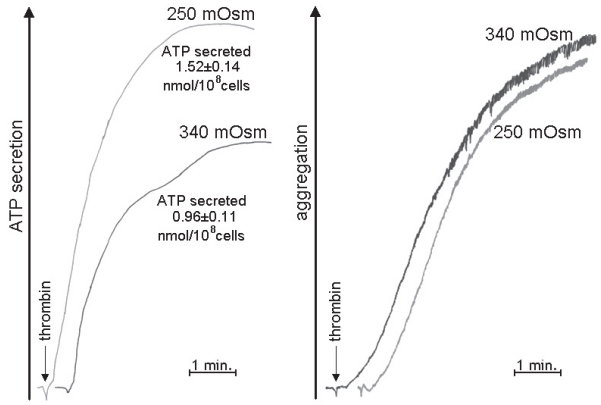

Figure 9. The effect of hypoosmotic shock on platelet aggregation and secretion evoked by thrombin.
Prewarmed (37°C) samples of washed platelets (0.375 ml) suspended in Tyrode/Hepes buffer (osmolality 340 mOsm) were incubated with 25 μl of luciferin/luciferase reagent in the cuvette of a lumiaggregometer at 37°C for 30 s with stirring (800 r.p.m.). Then 100 μl of prewarmed Ca^{2+}-free Tyrode/Hepes buffer (osmolality 340 mOsm) or deionized water were added to the stirring suspension to obtain the final osmolality of 340 and 250 mOsm, respectively. Platelets were activated by the addition of thrombin to the final concentration of 0.2 U/ml. Release reaction (panel A) and optical aggregation (panel B) were monitored simultaneously as described in Materials and Methods. Five-minute incubation of the cells in hypotonic medium (250 mOsm) without stimulus did not result in the appearance of significant amounts of ATP outside platelets. Results of one representative experiment (out of five) are presented. Numbers in the left-hand panel express mean ±S.D. of ATP released (nanomoles ATP/10^8 cells) following five-minute incubation of platelets with thrombin.

microaggregate formation is prevented. Figure 7 shows that artificially-evoked (monensin treatment) Na^+-H^+ antiport, thrombin or PMA, produce a rapid increase in platelet volume. One-minute treatment with 30 μM monensin, 300 nM PMA or thrombin (0.2 U/ml) resulted in a rise in the mean platelet volume by about 1.0, 0.6 and 0.8 femtoliters, respectively. The rise in the mean platelet volume evoked by PMA or thrombin was less pronounced in the presence of EIPA. As seen in Fig. 8, PMA (300 nM) produced platelet swelling. The kinetics of PMA-evoked swelling was similar to that observed following the addition of monensin (30 μM). The PMA-induced platelet swelling was strongly reduced by EIPA. These observations indicate that platelet activation is associated with a rise in cell volume which may be produced by activated Na^+/H^+ exchanger.

We subsequently studied how the rise in platelet volume produced by hypoosmolarity affects platelet aggregation and secretion. Aggregation and secretion were recorded simultaneously using a lumiaggregometer. The instrument detects ATP released from platelet dense granules (Holmsen, 1981). Since serotonin and ATP have been established to be stored in platelet dense granules, the release of ATP reflects serotonin secretion (Holmsen, 1987). As shown in Fig. 9, reduction of osmolality of the suspending medium from 340 mOsm to 250 mOsm results in a significant (about 50%) potentiation of ATP secretion induced by subthreshold concentrations of thrombin and is without any effect on aggregation. The potentiation of ATP secretion was not a result of damage to the plasma membrane, eventually occurring due to the platelet swelling, since a 5-min

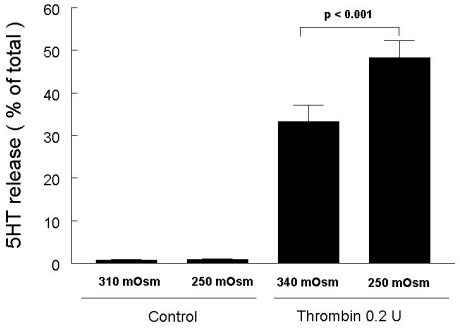

Figure 10. The effect of hypoosmotic shock on thrombin-evoked serotonin release.
Aliquots of [^{3}H]serotonin-loaded platelets suspended in normal Tyrode/Hepes buffer (osmolality 340 mOsm) or in medium with reduced osmolality (250 mOsm) were incubated at 37°C for 3 min with stirring (800 r.p.m) without (control) or with 0.2 U/ml thrombin. The amount of [^{3}H]serotonin released within 3 min after the addition of the stimulus was estimated. Results are expressed as a percentage of the total amount of [^{3}H]serotonin contained in unstimulated platelets and are presented as means ±S.D. All experiments were performed at least in quadruplicate using five different platelet preparations.

incubation of the cells in a hypotonic medium without a stimulus did not result in the appearance of significant amounts of ATP outside the platelets.

Figure 10 shows that the reduction of osmolality of the suspending medium from 340 mOsm to 250 mOsm produced a significant potentiation of serotonin secretion induced by subthreshold concentrations of thrombin. The potentiation of serotonin secretion was not a result of a damage to the plasma membrane, since the hypoosmotic shock alone did not result in significant serotonin secretion. These observations indicate that hypoosmotically-evoked platelet swelling may facilitate serotonin release.

DISCUSSION

The present results show that activation of porcine platelets by thrombin initiates a rapid rise in the activity of the Na^+/H^+ exchanger. In porcine platelets, the enhancement of the Na^+/H^+ exchanger activity may be produced by the activated protein kinase C but involvement of $[Ca^{2+}]_i$ cannot be excluded. This is based on the observation that in porcine platelets alkalization of the cytosol (a measure of the Na^+/H^+ activity) evoked by thrombin (produces both a rise in $[Ca^{2+}]_i$ and in PKC activity) is significantly faster than that elicited by PMA (only produces permanent activation of PKC). In this respect porcine platelets are similar to human platelets, which have been proposed to possess the NHE1 isoform of the Na^+/H^+ exchanger (Rosskopf, 1999) known to be controlled by PKC- and Ca^{2+}-dependent mechanisms (Putney *et al.*, 2002). Acceleration of the Na^+/H^+ exchange in thrombin-activated platelets is expected to evoke a rise in intracellular $[Na^+]$ (Lang *et al.*, 1998). In fact, it has been reported that human and porcine platelets upon stimulation by thrombin, collagen or PMA take up sodium from the surrounding medium and that this sodium influx may be mediated by the activated Na^+/H^+ exchanger (Borin & Siffert, 1991; Samson *et al.*, 2001; Stelmach, 2002).

The rise in the intracellular Na^+ concentration is expected to induce an uptake of water and concomitant cell swelling (Lang *et al.*, 1998; Wehner & Tinel, 1998). In rat hepatocytes the Na^+/H^+ exchanger has been reported to be involved in the regulatory volume increase (Wehner & Tinel, 1998). The present findings indicate that also porcine platelets stimulated by PMA or thrombin augment their volume (swell) due to a rise in the Na^+/H^+ exchanger activity.

As it is reported here, in porcine platelets activation of the Na^+/H^+ exchanger may result in serotonin secretion. The resulting serotonin release cannot be explained solely by the rise in $[Ca^{2+}]_i$ eventually produced by the Ca^{2+}/Na^+ exchanger operating in a reverse mode (Blaustein & Lederer, 1999). This is because artificially evoked Na^+-H^+ antiport elicits serotonin secretion in the absence of extracellular calcium.

Here we hypothesize that serotonin secretion produced by facilitated Na^+/H^+ exchange may be mediated by platelet swelling. This is based on the finding that the rise in cell volume and secretion may occur in platelets simultaneously, due to activation of the Na^+/H^+ exchanger. This is additionally confirmed by the observation that the thrombin-evoked serotonin and ATP secretion can be significantly accelerated by the rise in platelet volume induced by hypoosmotic shock. Thus, in porcine platelets a link may exist between cell swelling and the secretory process.

Platelet swelling is expected to be associated with an extension of the plasma membrane (Strbak & Greer, 2000; Straub, 2002). This in turn may result in a rise in membrane tension. Alternatively, the extension of the platelet plasma membrane may occur in activated platelets due to cytoskeletal rearrangements. It has been established that stimulated platelets undergo a series of morphological rearrangements that include the formation of pseudopods and centralization of secretory granules (Fritz *et al.*, 1994; White, 1999; Reed *et al.*, 2000; Flaumenhaft, 2003). During the exocytosis secretory granules fuse with the plasma membrane (Fritz *et al.*, 1994) or with the channels of the open canalicular system (OCS) (Flaumenhaft, 2003; White, 1999). OCS channels are formed by the invaginations of platelet plasma membrane (White & Clawson, 1980; White & Escolar, 1991; White, 1999). Following platelet stimulation channels of the OCS become dilated or may even undergo evagination during platelet spreading on collagen-coated surfaces (White & Escolar, 1991; White, 1999). Both the dilation and/or evagination of the OCS membranes allow the platelet to enlarge its surface necessary for the formation of pseudopods. The increase of platelet surface is likely to be accompanied by a rise in the plasma membrane tension (Morris & Homann, 2001). Thus, in activated platelets cell swelling and/or cytoskeletal reorganization may result in a rise in plasma membrane tension.

How can the membrane tension affect platelet secretion? One explanation would be that an increase in cell membrane tension, as it occurs during cell swelling, might trigger fusion of secretory granules with the plasma membrane. Information about the relationship between platelet membrane tension and granule secretion is not available. Experiments performed on model systems and cells other than platelets have shown that a rise in plasma membrane tension may significantly accelerate exocytosis by facilitating fusion of the secretory granule membranes with the plasma membrane (Lang *et al.*, 1998; Strbak & Greer, 2000; Hamill & Martinac, 2001; Apodaca, 2002; Straub *et al.*, 2002).

It has been found that membrane fusion, crucial for exocytosis, requires an input of energy to overcome the energetic barrier that normally blocks it (Zimmerberg *et al.*, 1993; Flaumenhaft, 2003). The mechanisms reducing the energetic barrier critical for membrane fusion in activated platelets are not known with certainty. A prominent role in reducing this energetic barrier was recently proposed to be played by SNAREs and their chaperon proteins, but an involvement of the physical forces generated by an extended membrane cannot be excluded (Apodaca, 2002; Flaumenhaft, 2003).

Membrane tension has been proposed to generate physical perturbations which enable the input of energy necessary for the reduction of the energy barrier preventing fusion of the membranes (Straub *et al.*, 2002). Alternatively, the physical changes occurring in an extended membrane may reduce the total energy input required for exocytosis to take place. Such an input of energy was recently proposed to be responsible for the Ca^{2+} independent hypotonic stimulation of insulin exocytosis in βHC9 cells (Straub *et al.*, 2002).

All these data taken together strongly suggest that in porcine platelets a link may exists between the activation of protein kinase C, operation of the Na^+/H^+ exchanger, cell swelling, and serotonin secretion. We suggest that in activated platelets protein kinase C may cause the stimulation of the Na^+/H^+ exchanger. The resulting sustained rise in intracellular [Na^+] may cause platelet swelling and a rise in cell membrane tension, which in turn may facilitate fusion of granule membranes with the plasma membrane leading to serotonin secretion.

Studies on the role of the Na^+/H^+ exchanger in serotonin secretion from human platelets have produced inconsistent results. Although some observations indicated for its involvement in platelet secretion induced by thrombin, ADP and adrenalin (Connolly & Limbird, 1983; Sweatt *et al.*, 1985; Joseph *et al.*, 1990), its role in collagen-mediated platelet activation was questioned (Joseph *et al.*, 1990). Results of early studies indicated that stimulation of human platelets by collagen was not associated with activation of the Na^+/H^+ exchanger and that blocking of the antiport by removing extracellular sodium potentiated collagen-induced serotonin secretion (Joseph *et al.*, 1990). In contrast, recently published results indicate that collagen-evoked platelet activation is associated with an EIPA-sensitive rise in pH (measured with BCECF) and a simultaneous sodium influx (measured by SBFI), which clearly indicates activation of the Na^+/H^+ exchanger (Roberts *et al.*, 2004). The rational explanation for this apparent discrepancy would be that only at high collagen concentrations stimulation of platelets is associated with a rise in phospholipase C (PLCγ2) activity and subsequent activation of PKC (Roberts *et al.*, 2004). At lower concentrations PLCγ2 may remain unactivated and many of the effects of collagen are enhanced by its production of thromboxane A_2 (TxA_2) (McNicol & Nickoaychuk, 1995; Roberts *et al.*, 2004). Since the Na^+/H^+ exchanger activity in platelets has been proposed to be controlled by PKC (Rosskopf 1989; Livne *et al.*, 1991) rather than by TxA_2, this means that at lower concentrations of collagen the antiporter cannot be activated and does not play any role in cell stimulation (or secretion). But even in platelets activated by high collagen concentrations the role of the Na^+/H^+ exchanger in platelet secretion seems to be of lesser importance than in cells stimulated by high thrombin concentrations. This is so because at high collagen concentrations PKC activity (measured as phosphorylation of p47, a PKC substrate) has been reported to be by about 40% lower than that observed following stimulation of platelets by high thrombin concentrations (Joseph *et al.*, 1990). Consistent with this is that in human platelets collagen is a much less potent inducer of platelet secretion than thrombin (Holmsen, 1982), and that in porcine platelets the extent of serotonin secretion is positively correlated with thrombin concentration (Fig. 2.)

Studies on the role of the Na^+/H^+ exchanger in PKC-mediated serotonin release from human platelets have also yielded inconsistent results. In 1988 Krishnamurti and coworkers reported that in human platelets serotonin secretion induced by very low concentrations of PKC activators (PMA, diacylglycerol) was significantly enhancened after replacing the extracellular sodium with choline. Since a lack of sodium in the extracellular space prevents the Na^+/H^+ exchange, that observation was interpreted to mean that the Na^+/H^+ exchanger inhibited rather than promoted secretory processes in platelets. In contrast, the results reported by Joseph *et al.* (1990) and by Connolly and Limbrid (1983) showed that serotonin secretion in response to thrombin, a strong PKC activator (Siess, 1989), was significantly reduced in sodium-free medium, thus indicating a prosecretory role of Na^+/H^+ exchanger in platelets. In those experiments NMDG was the substitute for the extracellular sodium. The inconsistent results might be explained, at least in part, by the procedures used to eliminate Na^+/H^+ exchange. In the experiments performed by Krishnamurti *et al.* (1989) choline was used to block the Na^+/H^+ exchanger. It is likely that the enhancement of phosphorylation of a 20 kDa protein (apparently myosin chain kinase) seen in platelets suspended in a choline-containing medium (Krishnamurti *et al.*, 1989) might have been responsible for the stimulation of serotonin secretion rather than the Na^+/H^+ exchanger itself. It is commonly accepted that Ca^{2+}-calmodulin-dependent myosin light chain kinase-mediated phosphorylation is necessary for promoting the secretory process in platelets (Flaumenhaft, 2003). A recent finding

that in chromaffin cells choline is able to develop a calcium signal and to cause catecholamine release additionally supports its prosecretory properties (Fuentealba *et al.*, 2004).

In conclusion, both the above-mentioned reports and the results presented here indicate an involvement of the Na^+/H^+ exchanger in serotonin release induced by strong platelet stimulators like thrombin and high collagen concentrations.

The results presented here indicate that in porcine blood platelets the Na^+/H^+ antiport evoked by monensin may result in serotonin release. This observation could be interpreted to mean that in platelets activation of the Na^+/H^+ exchanger may be sufficient to trigger secretion. This is, however, not certain.

Monensin, besides its well established ability to mediate Na^+/H^+ exchange (Mollenhauer, 1990), has been proposed to induce secretion by triggering cytoskeletal rearrangements. It has been reported that in rat atrial myocytes monensin turns on microtubule-associated translocation of secretory granules from the center of the cell to the plasma membrane by modifying the interaction between microtubules and secretory granules (Lida *et al.*, 1988). Such action of monensin has never been reported in platelets, but cannot be excluded. An investigation using atomic force microscopy of living platelets indicates that following activation secretory granules move from the interior of the cell towards extended plasma membrane where they disappear, most probably due to fusion (Fritz *et al.*, 1994). Movement of secretory granules in activated platelets is likely to be associated with cytoskeletal rearrangements in which microtubules may be involved (Flaumenhaft, 2003). Although the role of the cytoskeleton in platelet secretion is not yet completely clear, one study demonstrated that inhibition of tubulin using monoclonal antibodies diminished platelet granule secretion (Berry *et al.*, 1989). It is therefore likely that the prosecretory action of monensin in platelets may be associated not only with the extending of the plasma membrane (due to swelling) but also with its concomitant interaction with microtubules which results in granule movement towards the extended plasma membrane where they undergo exocytosis.

Another option could be that monensin mediates the release of serotonin from platelets by a mechanism that differs from exocytosis of dense granules. It has been recently proposed that in human platelets a rise in the intracellular sodium concentration (produced by monensin) may activate a putative plasma membrane serotonin transporter (SERT) mediating translocation of serotonin from the cytosol to the extracellular space (Turetta *et al.*, 2004). The key evidence for the existence of such a type of transporter is the observation that the rise in the intracellular calcium and sodium in reserpine-treated human platelets results in an efflux of serotonin from the cytosol to the extracellular space (Turetta *et al.*, 2004). We have performed similar experiments with porcine platelets and found that 30 μM monensin is able to release (within 3 min) only about 18% of serotonin accumulated in the cytosol of reserpinized platelets (Fig. 5). By comparison, similar monensin concentrations liberate (within 3 min) about 65% of serotonin accumulated in platelet granules (i.e. in the absence of reserpine). However, non-exocytotic release of serotonin (possibly mediated by SERT operating in a reverse mode) must proceed by its translocation from granules to the cytosol. Whether monensin is able to discharge the granule contents directly to the cytosol remains an open question. Further studies assessing the detailed mechanism of monensin action on platelets may shed some light on this issue.

In summary, although a large part of the prosecretory effect of monensin in platelets seems to be due to the Na^+/H^+ antiport we believe that the role of the Na^+/H^+ exchanger in porcine platelet secretion is rather accessory than causative.

It is not known yet how the proposed mechanism is relevant to the situation *in vivo*. Understanding the role of Na^+/H^+ exchanger in platelet physiology is of importance since an enhancement of its activity in blood cells of patients with essential hypertension and with diabetic nephropathy has been described by various investigators (Siffert & Dusing, 1996; Aviv, 1992). In platelets from patients suffering from hypertension a higher Na^+/H^+ exchange rate has been found to correlate with an increase in PKC activity and with a decrease in the amount of serotonin stored in platelet granules (Le Quan Sang *et al.*, 1987; Weiner, 1987; Siffert & Dusing, 1996). Therefore, the results presented in this report further support the hypothesis assuming an involvement of PKC, the Na^+/H^+ exchanger and serotonin in the development of hypertension, preeclampsia and insulin resistance (Aviv, 1992)

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