figure 1. From the semilog plot of reactivation a value of 0.039 min^{-1} for k_3 was calculated.

The effect of kallikrein and benzoyl kallikrein on kinin liberation in vivo was followed by the drop in blood pressure in rabbits (an example is shown in fig. 2). Kallikrein administered intravenously causes a quick transient drop in systemic blood pressure. Besides sympathetic contraregulation and degradation of the pharmacologically active kinins, the inhibition of the administered kallikrein by natural plasma inhibitors may be of importance for the fast normalisation of blood pressure[10]. Compared to the situation with the non-acylated enzyme, the duration of the decrease in blood pressure following a bolus injection of acyl-kallikrein is longer and equals the time of reactivation (fig. 1). The time-course of the decrease in blood pressure corresponds to that of an infusion of the same dose of kallikrein within 40 min. The results indicate that acylated kallikrein is protected from being inactivated by plasma inhibitors up to restoration of enzymatic activity.

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Dissociation between inhibition of phospholipid methylation and production of PAF-acether by rabbit platelets

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Summary. Platelet-activating-factor (PAF-acether, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) is formed by and released from rabbit platelets stimulated with thrombin, with the ionophore A23187, with collagen and with the platelet-stimulating glycoprotein convulxin. We here show that 3-deazaadenosine (C_3ado) and L-homocysteine (HCy), two inhibitors of platelet methylation, reduced the formation of PAF-acether and of its deacetylated product lyso-PAF-acether by rabbit platelets challenged with thrombin, under conditions where the accompanying aggregation was not significantly modified. In contrast, platelet aggregation induced by convulxin was completely and irreversibly blocked when C_3ado and HCy were associated. Aggregation by thrombin was not affected by the methylation inhibitors even when ADP was scavenged and thromboxane formation was suppressed. Our results indicate that phospholipid methylation, thrombin-induced platelet aggregation and formation of PAF-acether can be dissociated. The formation of PAF-acether by rabbit platelets can be blocked by mechanisms other than inhibition of phospholipase A2, since the latter is not affected by C_3ado and/or HCy.

PAF-acether is a phospholipid mediator released from various cell types upon appropriate stimulation, which induces platelet aggregation and the secretion of their granular constituents. Since platelet aggregation by PAF-acether does not require ADP release nor formation of thromboxane A2, PAF-acether was proposed as the mediator of a 3rd pathway of platelet aggregation[1-3]. 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine is the presently accepted formula of this mediator[4-6] which is also formed by and released from rabbit platelets stimulated with the calcium ionophore A23187[1], with collagen, thrombin[2] or with convulxin, a platelet-stimulating glycoprotein extracted from the venom of *Crotalus durissus cascavella*[7-9]. Platelet phospholipid methyl transferases, which catalyse the formation of phosphatidylcholine (PC) from phosphatidylethanolamine (PE)[10,11], can be blocked by 3-deazaadenosine (C_3ado) and/or L-homocysteine (HCy)[12,13]. These reagents also suppress collagen- and convulxin-induced platelet activation, but do not reduce significantly aggregation due to ADP, to thrombin, to trypsin or to A23187[12-14].

Since it has been hypothesized that a PC analogue (1 alkyl-2-acyl-sn-glycero-3-phosphorylcholine) may be a precursor of PAF-acether[15] and of its deacetylated analogue lyso-PAF-acether in platelets[16], we decided to investigate whether the inhibition of the formation of PC from PE and of their respective l-alkyl analogues by methyl transferases would interfere with the formation of PAF-acether and how this would correlate with platelet aggregation.

Materials and methods. Measurement of phospholipid methylation. Blood was collected from the central ear artery of adult New Zealand white rabbits on a mixture of disodium and tetrasodium salts of EDTA (5 mM final concentration). Washed platelets prepared as described[2] were resuspended in tris-Tyrode buffer containing 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 2.5 mg/ml of fatty acid-free bovine serum albumin (Sigma) at pH 7.4. Platelet suspensions were incubated at room temperature with 3-deazaadenosine (C_3ado, Southern Research Institute, Birmingham, USA) and/or L-homocysteine thiolactone (HCy, Sigma) or with their solvent in the presence of 160 µCi/ml of Me-^{3}H-L-methionine (2 µM, 80 Ci/mmol, NEN), which was added to the platelets 1 h before the potential inhibitors. The time-course of the incorporation of radiolabeled methyl into phospholipids was followed. At different intervals a 0.5-ml sample was removed from the incubates and added to 1 ml of chloroform-methanol (3:1 vol) at 4 °C. The mixture was processed according to Randon et al.[12], to measure the extent of phospholipid methylation.

Determination of the formation of PAF-acether by platelets. The platelet suspensions prepared as indicated above were incubated at room temperature with the potential inhibitors (C_3ado and/or HCy) or with their solvents. At different time-

intervals (fig. 1) a 2-ml sample was collected and placed at 37 °C. After 2 min the platelets were stimulated under stirring with 2.5 U/ml of bovine thrombin (Hoffmann-La Roche, Basel) during 7 min, and the reaction was stopped with 4 vol of pure ethanol. The mixture was then processed according to Benveniste et al.[16]. The bioassay of PAF-acether was performed on plasma-free rabbit platelets pretreated with aspirin (0.1 mM) and in the presence of the ADP-scavenging system creatine-phosphate/creatine phosphokinase (CP/CPK, 0.7 mM/13.9 U/ml, respectively). The identity of PAF-acether was checked by incubating the extracts with snake venom phospholipase A_2 (from *Crotalus durissus terrificus*, Sigma), which completely removed the aggregating activity by hydrolyzing the acetate in position 2 of the skeleton of PAF-acether[4,5]. The lyso-PAF activity was measured as described[16]. In some experiments (table 2), the platelet suspensions were stimulated with thrombin (2.5 U/ml) 4 h after the introduction of the inhibitors and PAF-acether formation was determined as indicated above.

Measurement of platelet aggregation. 0.4-ml samples of the platelet preparations were collected after different intervals and challenged with thrombin or convulxin (donated by Dr G. Marlas), as indicated in figures 2 and 3. In some experiments (table 1) the platelets were pretreated with 0.1 mM aspirin in the presence or in the absence of C_3ado and/or HCy. 4 h later the platelet suspensions were incubated with CP/CPK and challenged after 2 min with thrombin or with convulxin under the conditions indicated in the table 1. Aggregation was recorded as a decrease in the percent light transmission accross the suspension using a Chrono-log aggregometer.

Results. Phospholipid methylation by rat platelets in plasma was suppressed whether the inhibitors were used alone (0.5 mM of either) or combined (0.1 mM of each)[12]. With plasma-free rabbit platelets, the same results were obtained (fig. 1). When C_3ado and HCy (0.1 mM of each) were incubated together with the platelets for different time intervals, inhibition of phospholipid methylation started rapidly and was completed within 30 min whereas the formation of PAF-acether triggered by 2.5 U/ml of bovine thrombin was reduced time-dependently, reaching after 2 h 50% of the amounts formed by the paired untreated control platelets (fig. 1). Neither C_3ado nor HCy displayed such an effect when used alone. Contrary to what would be expected if PAF-acether were generated from a PE analogue by methyl transfer, and in contrast to the rapid inhibition of phospholipid methylation, the inhibition of formation of PAF-acether showed a slow onset and reached a plateau within 2 h. These kinetics were similar to those observed when C_3ado plus Hcy was used to suppress collagen-induced platelet aggregation[12].

In confirmation, rabbit platelets incubated with C_3ado plus HCy became progressively refractory to convulxin (fig. 2), which shares with collagen the ability to release PAF-acether (Vargaftig et al., submitted). As also shown in this figure, suppression of convulxin-induced aggregation was obtained when 0.5 mM of C_3ado plus HCy were incubated with the platelets for 4 h. Under those conditions, even a 100-fold increment in the amount of convulxin needed to induce a submaximal aggregation of control platelets failed to stimulate the treated platelets (table 1). When cysteine (0.5 mM) was used in place of HCy, aggregation by convulxin persisted, irrespective of the presence of C_3ado (not shown). Nevertheless, even though the thresholds for thrombin-induced aggregation were increased by the association of C_3ado and HCy (fig. 3), aggregation per-

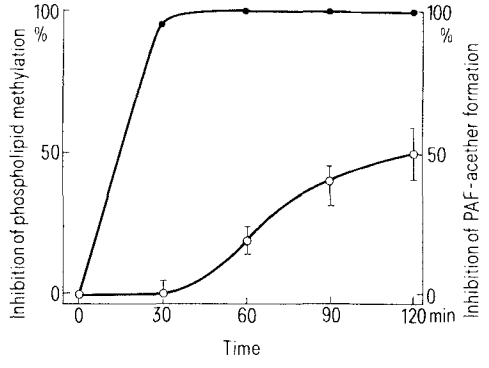

Figure 1. Time-dependent inhibition of phospholipid methylation and of formation of PAF-acether by 3-deazaadenosine and L-homocysteine thiolactone. Plasma-free rabbit platelets were incubated at room temperature with radiolabeled methionine as indicated in the text. 1 h later, the incubate was separated into 2 parts, a test tube to which C_3ado (0.1 mM) and HCy (0.1 mM) were added, and a control tube to which the solvent (saline) was added. At different time intervals, a 0.5-ml sample was removed from each tube, and added to 1 ml of chloroform:methanol (2:1 in vol) maintained at 4 °C. The mixture was processed according to Randon et al.[12] to measure the extent of phospholipid methylation (●). For the determinations of PAF-acether (○) the platelet preparations were incubated at room temperature with the combined inhibitors or with their solvent. At different time intervals a 2-ml sample was collected, and placed at 37 °C. 2 min later the platelets were stimulated with 2.5 U/ml of bovine thrombin. After 7 min the reaction was stopped with 4 vol of pure ethanol. The mixture was then processed according to Benveniste et al.[16]. Bioassay of PAF-acether was performed on plasma-free rabbit platelets pre-treated with aspirin (0.1 mM) and in presence of the ADP-scavenging system CP/CPK, as indicated in the text.

Table 1. Interference of 3-deazaadenosine and L-homocysteine thiolactone with platelet aggregation induced by thrombin and by convulxin

C_3ado (mM)	HCy (mM)	n	Threshold for triggering aggregation	
			Thrombin (U/ml ± sem)	Convulxin (nM ± sem)
0	0	4	0.19 ± 0.05	1.2 ± 0.4
0.1	0.1	4	0.31 ± 0.10	> 100
0.25	0.25	4	0.59 ± 0.16	> 100
0.5	0.5	4	0.78 ± 0.26	> 100
1.0	1.0	2	0.78	> 100
0.5	0	4	0.17 ± 0.02	1.75 ± 0.48
1.0	0	2	0.24	3.40
0	0.5	4	0.17 ± 0.02	1.47 ± 0.43
0	1.0	2	0.31	5.00

The amounts of convulxin or of thrombin needed to aggregate the platelets by 50–60% were measured. Washed platelets were treated with 0.1 mM of aspirin added to the 1st washing fluid[2]. Platelets were incubated at room temperature for 4 h with C_3ado and HCy at the concentrations indicated. Aggregation being triggered in the presence of the ADP scavenging system CP/CPK, as indicated in the text. Figures are the mean ± SEM of thrombin (U/ml) and convulxin (nM). n, Number of experiments.

Table 2. Inhibition by 3-deazaadenosine and L-homocysteine thiolactone of the formation of PAF-acether and of lyso-PAF-acether by rabbit platelets

C_3ado (mM)	HCy (mM)	n	Percent formation as compared to controls	
			PAF-acether	Lyso-PAF acether
1	1	2	0	38 (33–42,8)
1	0	2	110 (85,7–133)	137 (110–164)
1	0,1	1	24	43
0	0,1	1	100	135
0	0,5	4	44,6 ± 13	68 ± 5
0	1	2	0	49 (42,8 – 55,4)
0,5	0	4	133,6 ± 20	83 ± 6
0,5	0,5	4	17,9 ± 10	44 ± 7

Figures indicate the percent formation of PAF-acether and of lyso-PAF-acether by thrombin-stimulated platelets incubated with C_3ado and HCy, alone or combined for 4 h at room temperature. Values are given as percent of paired controls ± SEM. n, Number of experiments.

sisted for concentrations of thrombin above 0.6 U/ml. Moreover, in the presence of CP/CPK, the combination which scavenges ADP, and of aspirin used to suppress cyclooxygenase activity, the concentrations of thrombin needed to induce submaximal aggregation were raised from 0.19 ± 0.05 U/ml (mean ± SEM) for the control (aspirin + CP/CPK) platelets up to 0.78 ± 0.26 U/ml ($p < 0.05$; $n = 4$; see table 1) when C_3ado plus HCy (0.5 mM each) were also present. These amounts of C_3ado plus HCy incubated with the platelets for 4 h (the protocol which suppressed aggregation by convulxin), reduced the formation of PAF-acether and of lyso-PAF-acether triggered by thrombin (table 2). C_3ado alone was inactive, but HCy alone was partially effective in reducing the formation of PAF-acether and of lyso-PAF-acether. This contrasts with the inability of HCy alone to block aggregation by collagen and by convulxin[12], and suggested that either thrombin stimulates aggregation via additional final mediators as compared to convulxin and to collagen, or that residual PAF-acether formed by thrombin-stimulated platelets despite C_3ado plus HCy might still account for the persistence of aggregation. Further attempts were therefore performed to suppress fully the formation of PAF-acether, by increasing the concentrations of the potential inhibitors. As seen in table 2, the formation of PAF-acether and of lyso-PAF-acether was markedly reduced in the presence of HCy (1 mM) with or even without C_3ado. Nevertheless, in spite of the inhibition of the formation of PAF-acether, aggregation by concentrations of thrombin above 1 U/ml persisted, even if ADP was scavenged by CP/CPK and cyclooxygenase was inhibited with aspirin.

Discussion. Suppression of the stimulus-dependent formation of PAF-acether by rabbit platelets has been described previously for various inhibitors of phospholipase A_2[16]. Since neither C_3ado nor HCy, alone or associated, block this enzyme[17], another mechanism must account for their activity. Inhibition is not an intrinsic property of C_3ado, because it did not interfere with aggregation or with formation of PAF-acether when used alone up to 1 mM. Concentrations of 0.1–0.5 mM associated with HCy (0.1–0.5 mM) inhibited markedly the formation of PAF-acether and of lyso-PAF-acether under conditions where Hcy alone was only active in part.

As seen in table 2 a concentration of 1 mM of C_3ado associated with 0.1 mM HCy blocked 75% of the formation of PAF-acether under conditions when C_3ado 1 mM or HCy 0.1 mM used separately did not impair this formation. This confirms the potentiation of HCy by C_3ado, and strongly suggests the involvement of methylation reactions, since C_3ado inhibits S-adenosyl-L-homocysteine hydrolase, leading to the accumulation of S-adenosyl-L-homocysteine, which inhibits transmethylations[18] including that of platelet phospholipids[12].

Inhibition of phospholipid methylation occurred shortly after the addition of C_3ado and/or HCy to platelets (fig. 1), whereas inhibition of the formation of PAF-acether and of lyso-PAF-acether required a relatively prolonged incubation. Moreover, C_3ado alone suppresses phospholipid methylation[12], but now failed to interfere with the formation of PAF-acether and with convulxin-induced platelet aggregation. Since HCy alone at 0.5 mM also inhibits phospholipid methylation in platelets, but failed to block aggregation by convulxin or collagen, which are antagonized by its combination with C_3ado, we concluded that the associated inhibitors suppress platelet activation by a mechanism distinct from interference with the conversion of PE into PC[12].

C_3ado or HCy induce at least 3 effects on platelets: 1. when used alone or associated, phospholipid methylation is inhibited at once; 2. when used together, provided incubation is prolonged, they inhibit aggregation by convulxin and by collagen; 3. HCy alone, or combined with C_3ado, inhibits the formation of PAF-acether and of lyso-PAF-acether triggered by thrombin, here again if incubation with the platelets is prolonged.

It is likely that incorporation of radioactive methyl groups into the platelet phospholipids is a complex event, and that our measurements provide only a global evaluation of a dynamic process. The inhibitors used may also reach different intracellular sites at different time intervals. Furthermore, it is also conceivable that due to the different turn-over of the enzymes involved with formation of PAF-acether, enough of them may persist, and ensure its stimulus-dependent generation, before the inhibitory effects of HCy and/or C_3ado are observed. Under those conditions, the consequences of the inhibition of these enzymes, i.e., a clearcut reduction of the yields of PAF-acether, would be delayed as compared to inhibition of phospholipid methylation. Those explanations do not account for the fact that C_3ado alone failed to block the formation of PAF-acether, when inhibiting phospholipid methylation. Despite this reservation, it is important to note that the thresholds for aggregation by thrombin were raised by C_3ado plus HCy, when ADP and thromboxane-dependent mechanisms

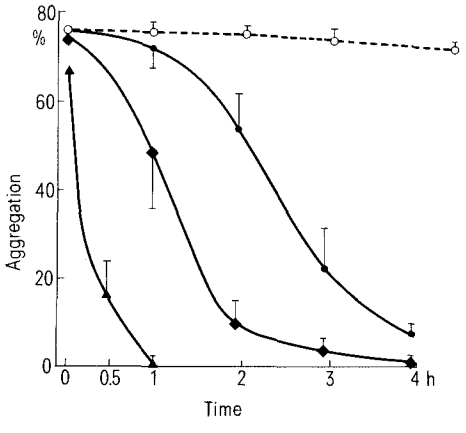

Figure 2. Time-dependent inhibition of convulxin-induced platelet aggregation by 3-deazaadenosine associated to L-homocysteine thiolactone. Plasma-free rabbit platelets were incubated at room temperature with C_3ado plus HCy (0.5 mM of each) or with their solvent (control). At different time intervals aliquots of the platelet suspension (0.4 ml) were challenged with convulxin (10 nM, ●, 2.5 nM, ◆ and 1 nM, ▲). Aggregation was recorded as a decrease in the per cent light transmission accross the suspension (vertical scale). Convulxin at 1 nM induced full aggregation of the control platelets (○) throughout the 4-h duration of the experiment.

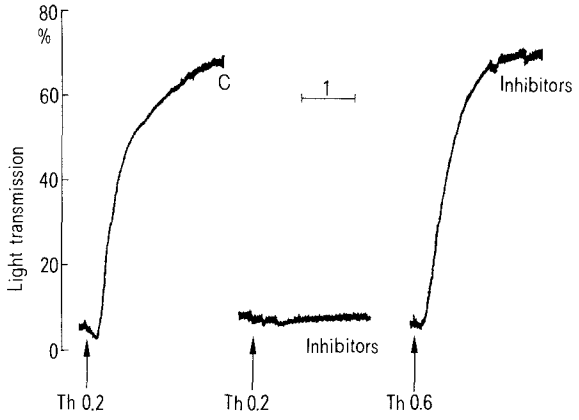

Figure 3. Inhibition by 3-deazaadenosine associated to L-homocysteine thiolactone of thrombin-induced platelet aggregation. Plasma-free rabbit platelets were washed as indicated[2] and were incubated at room temperature with C_3ado plus HCy (0.5 mM of each; indicated as inhibitors) or with their solvent (indicated as C) for 4 h. Aliquots were challenged with thrombin (0.2 and 0.6 U/ml), aggregation being recorded as described in figure 2. Time scale: 1 min.

were eliminated, suggesting a correlation between the inhibition of aggregation and that of formation of PAF-acether. Failure to suppress aggregation by thrombin, when formation of PAF-acether was abrogated, may imply that PAF-acether is not responsible for the non-ADP and non-thromboxane-dependent effects of thrombin. Alternatively, residual aggregation by thrombin may still be accounted for by formation of PAF-acether within the cell, which cannot be detected by our present bioassay.

In an attempt to eliminate this possible undetectable PAF-acether, we recently incubated C_3ado plus HCy with the platelets at 37 °C in place of 22 °C, under the assumption that at the physiological temperature inhibition would be better observed. We noted indeed that aggregation by thrombin was more markedly reduced (experiments in progress).

This is the first description of biochemical interference with the generation of PAF-acether by mechanisms other than inhibition of phospholipase A_2[16], correlating with suppression of aggregation by collagen and by convulxin, and with a significant increase in the threshold for thrombin. Further studies, particularly better-adapted protocols for the use of the inhibitors, as well as their use in other cell systems, should provide a better understanding of the role of phospholipid methylation in its formation and/or release and, overall, of its role in the activation of inflammatory cells in general.

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Species difference in sensitivity to the diabetogenic action of triphenyltin hydroxide

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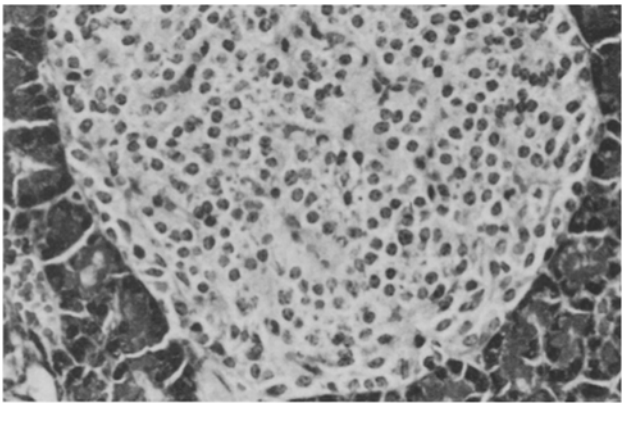
Summary. The sensitivity to the diabetogenic action of triphenyltin hydroxide (TPTOH) was investigated in 5 species of experimental animals. A single oral administration of TPTOH produced marked hyperglycemia and triglyceridemia in rabbits and hamsters, but no evidence of diabetes was found in mice, rats and guinea-pigs. No morphological abnormality was observed in islet tissue from TPTOH-treated hamsters.

Triphenyltin compounds are widely used in agricultural fungicides and antifoulants[1]. However, the potential hazard from occupational exposure to the compounds is not fully understood. Hyperglycemia has been observed in humans[2] and rabbits[3] after exposure to triphenyltin compounds. The hyperglycemia induced in rabbits was explained by an interference of the compounds with the process(es) leading to the release of insulin into the blood[3].

We found that the administration of TPTOH to rats did not produce hyperglycemia. This prompted us to determine whether there is a species variability in the induction of the diabetic state by triphenyltin.

Materials and methods. Animal studies were carried out on male Japan white rabbits (2.5–3.0 kg), male Wistar rats (140–160 g), male ddY mice (28–32 g), male Hartley guinea-pigs (230–260 g) and male golden hamsters (110–125 g). Animals were allowed commercial food (Oriental Kobo Co., Tokyo, Japan) and water ad libitum throughout the study unless otherwise stated. A single oral dose of TPTOH was administered in



Islet tissue from the pancreas of a hamster 3 days after treatment with a single oral dose of triphenyltin hydroxide (100 mg/kg). None of the islet cells showed significant changes. Hematoxylin and eosin, × 300.