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STIMULATION OF VASCULAR PROSTACYCLIN BY SKF525-A (PROADIFEN) AND RELATED COMPOUNDS*

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Abstract—SKF 525-A (proadifen), a well-known inhibitor of drug metabolism and cytochrome P-450 activity, stimulated the release of prostacyclin (PGI_2) from the rabbit aorta *in vitro*. The threshold concentration producing a detectable effect was 20 μM; the time course of SKF 525-A action exhibited particular features—progressive onset, long duration and slow reversibility—distinct from those of other stimuli (ADP, ionophore A23187 f.i.). The PGI_2-stimulating activity of SKF 525-A was characterized by specific structural requirements: activity was abolished by the deletion of the terminal propyl group and increased by its elongation into an isobutyl group; chlorination of the phenyl groups increased the potency. SKF 525-A increased the production of PGI_2 by cultured endothelial cells from bovine aorta and human umbilical vein, but had no effect on cultured smooth muscle from the bovine aortic media. Stimulation of PGI_2 release could be explained by an increased availability of free arachidonic acid, which was probably independent from cytochrome P-450 inhibition. In human platelets, SKF 525-A inhibited prostaglandin and thromboxane production induced by A23187, thrombin and ADP. Simultaneous stimulation of endothelial PGI_2 and inhibition of platelet TxA_2 represents an original pharmacological profile: SKF 525-A might thus constitute the prototype of a new class of antiplatelet drugs.

Oxidation by liver and kidney cortex cytochrome P-450 constitutes a novel pathway of arachidonic acid metabolism, which generates *inter alia* epoxides and vicinal dihydroxy acids [1–3]. Similar products of arachidonic acid have been detected in canine polymorphonuclear leukocytes [4] and in the thick ascending limb of the loop of Henle [5], where their biosynthesis is inhibited by β-diethylaminoethyl-2,2-diphenylpentanoate (SKF 525-A, proadifen), a known inhibitor of cytochrome P-450 [6]. The observation that SKF 525-A inhibits the endothelium-dependent relaxation of the rabbit aorta induced by acetylcholine and arachidonic acid led to the hypothesis that this relaxation could be mediated by a cytochrome P-450 product of arachidonic acid [7]. The presence of a cytochrome P-450 activity has indeed been detected in some blood vessels, including the rabbit aorta [8–12], but its involvement in arachidonic acid metabolism has not been demonstrated. If oxidation by cytochrome P-450 represented a significant pathway of arachidonic acid metabolism in blood vessels, it could be expected that its inhibition by SKF 525-A would increase the amount of arachidonic acid available for the cyclooxygenase and thus enhance the biosynthesis of prostacyclin (PGI_2).‡ We therefore investigated the effect of SKF 525-A on the vascular production of PGI_2.

MATERIALS AND METHODS

Preparation and incubation of washed human platelets. Blood from volunteers, who had not taken antiinflammatory drugs during the last 2 weeks, was collected with 1/10 vol. EDTA (0.077 M) and centrifuged for 15 min at 200 *g* at room temperature. The resulting platelet rich plasma was centrifuged at 100 *g* for 15 min. The platelet pellet was resuspended in buffer (NaCl, 139 mM; Tris HCl pH 7.4, 10 mM; glucose, 5 mM; EDTA, 1.5 mM). The platelets were washed once with the same buffer and were resuspended in a medium of the following composition: NaCl, 139 mM; Tris HCl pH 7.4, 10 mM; glucose 5 mM. The platelet count was around 800,000/μl. Incubations were performed for 10 min at 37° after which the platelets were rapidly centrifuged (30 sec in an Eppendorf centrifuge model 5412). The supernates were collected for the assay of thromboxane B2 (TxB_2) and prostaglandin E_2 (PGE_2).

Prostaglandin and thromboxane radioimmunoassay (RIA). The production of PGI_2 was estimated by the RIA of its stable degradation product 6-K-$PGF_{1\alpha}$. All the assays were performed directly in the incubation media, without extraction and chromatography. Antisera were raised in rabbits against 6-K-$PGF_{1\alpha}$ or TxB_2 coupled to bovine serum albumin, as described [13]. For 6-K-$PGF_{1\alpha}$, the limit of detection was 16 pg and the cross-reactions were 1.2% with $PGF_{2\alpha}$, 0.3% with PGE_2 and less than 0.1% with TxB_2. For TxB_2, the limit of detection was 4 pg and the cross-reactions were 0.1% with $PGF_{2\alpha}$ and

* Part of this study was presented at the Symposium "New Mediators in Tissue Reactions" held in Brussels on December 7, 1985.

‡ Abbreviations used: PGI_2, prostacyclin; 6-K-$PGF_{1\alpha}$, prostaglandin 6-keto-$F_{1\alpha}$; PGE_2, prostaglandin E_2; TxB_2, thromboxane B_2; BSA, bovine serum albumin; RIA, radioimmunoassay; DMEM, Dulbecco's modification Eagle's medium; GLC, gas liquid chromatography; ECD, electron capture detection; RP-HPLC, reversed-phase-high-performance liquid chromatography.

less than 0.1% with PGE_2 and 6-K-$PGF_{1\alpha}$. Aliquots of incubation media, ^3H-labeled tracers of 6-K-$PGF_{1\alpha}$ or TxB_2 (11,000 dpm), antiserum (final dilution: 10^{-4} for 6-K-$PGF_{1\alpha}$, 6×10^{-5} for TxB_2) and bovine gamma globulins (0.25 g/dl) in Tris–HCl buffer (pH 7.4, 50 mM) were incubated in a total volume of 0.4 ml for 60 min at room temperature. Then 0.4 ml of a cold 25% (w/w) solution of polyethylene glycol was added to separate bound and free antigen. The pellet was dissolved in 0.1 ml 0.1 M NaOH and counted in liquid scintillation. PGE_2 was assayed by a similar procedure. In order to validate the direct RIA procedure, 6-K-$PGF_{1\alpha}$ assay was repeated after purification of some incubation media samples by reversed-phase-high-performance liquid chromatography (RP-HPLC), as described [14].

Metabolism of ^{14}C-arachidonic acid. Confluent monolayers of bovine aortic endothelial cells were incubated for 18 hr in a culture medium containing DMEM (75%), Ham F_{12} (25%), glutamine (2 mM) and [1-^{14}C] arachidonic acid (0.25 μCi/ml, 1.4 μg/ml) with or without SKF 525-A (100 μM). At the end of this incubation period, the media were collected and aliquots were directly analyzed by RP-HPLC on a C_{18} ultrasphere ODS column (5 μ particles: Altex). The injector (model U6K) and the pumps (model 600) were from Waters Associates. Elution was performed sequentially with water–acetic acid (99:1, v/v, 10 min), water–acetonitrile–acetic acid (70:30:0.1, v/v; 25 min), water–methanol–acetic acid (21:79:0.01, v/v; 25 min) and pure methanol (20 min), at a flow rate of 1 ml/min. One-millilitre fractions were collected and counted by liquid scintillation.

The dissection and incubation of vessel rings, the preparation and culture of endothelial cells [15–17] and bovine aortic media explants and the assay of free arachidonic acid release [18] were performed as described in detail elsewhere [19].

Materials. SKF 525-A and related compounds were provided by Smith Kline & French Laboratories. Indomethacin was given by Merck Sharp & Dohme. ADP, bovine thrombin, arachidonic acid, docosahexaenoic acid and diisopropylethylamine were purchased from Sigma Chem. Co. Ionophore A23187 was purchased from Boehringer Mannheim. [^3H]-6-K-$PGF_{1\alpha}$, [^3H] TxB_2, [^3H] PGE_2 and [1-^{14}C] arachidonic acid were obtained from Amersham. Anti-PGE_2-BSA antiserum was purchased from Institut Pasteur, Paris. BSA (fatty acid poor) and pentafluorobenzylbromide were obtained respectively from Calbiochem-Behring Corp and Pierce Chemical Co. DMEM, Ham F_{12}, glutamine, penicillin, streptomycin, amphotericin B and collagenase type II were purchased from Flow Laboratory. Fetal calf serum was obtained from Gibco. Solvents for HPLC were purchased from Burdick-Jackson.

RESULTS

SKF 525-A stimulated the production of PGI_2 in rings of rabbit aorta as measured by the release of its stable degradation product, 6-K-$PGF_{1\alpha}$. The dose-dependency and time-course of this effect are depicted in Fig. 1. The onset of SKF 525-A action was progressive: the amplitude of the stimulation

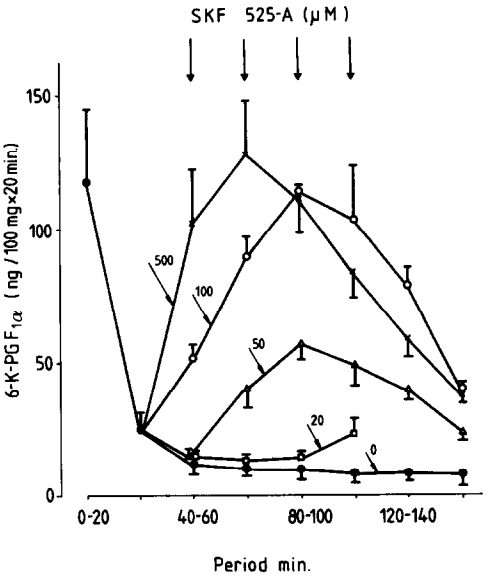

Fig. 1. Time course and concentration-dependency of the stimulatory effect of SKF 525-A on the release of PGI_2 from the rabbit aorta. Rings of rabbit aorta were incubated for eight periods of 20 min: the medium was collected and replaced at the end of each period. As indicated by the arrows, SKF 525-A was added during periods 3 to 6. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium (mean ± SD of six measurements: duplicate determinations in 3 separate experiments).

induced by 100 μM SKF 525-A rose from 2.6 ± 0.3-fold during the 1st 30 min exposure to the drug to 8.8 ± 1.2-fold during a second 30 min period (mean ± SE, 12 experiments). At concentrations below 100 μM, the stimulatory effect of SKF 525-A appeared after a concentration-dependent delay (Fig. 1): 20 μM was the minimal concentration to produce a detectable stimulation. Once established, the stimulatory effect of the drug was sustained for at least 60 min and slowly decreased following its removal (Fig. 1). Indomethacin abolished the release of PGI_2 induced by SKF 525-A (data not shown). This release was not diminished by cycloheximide (5 or 25 μg/ml: data not shown). To confirm that the product measured under stimulation by SKF 525-A was authentic 6-K-$PGF_{1\alpha}$, RIA was repeated after RP-HPLC purification of several samples: all the immunoreactivity was indeed recovered in the fractions where a standard of 6-K-$PGF_{1\alpha}$ was eluted (data not shown).

In order to characterize the chemical specificity of SKF 525-A action, several analogs and structurally related compounds were tested (Fig. 2, Tables 1 and 2). Procaine, which has the same polar head as SKF 525-A, was completely inactive, even at 0.5 mM (Table 1); on the other hand, SKF 2314, which corresponds to the hydrophobic tail of SKF 525-A, was also inactive (Table 1). The secondary amine analog SKF 8742-A stimulated PGI_2 release, although less than SKF 525-A itself (Table 1). Removal of the terminal propyl chain (SKF 962-A) abolished the PGI_2-stimulating activity, whereas its replacement by an isobutyl chain (SKF 490)

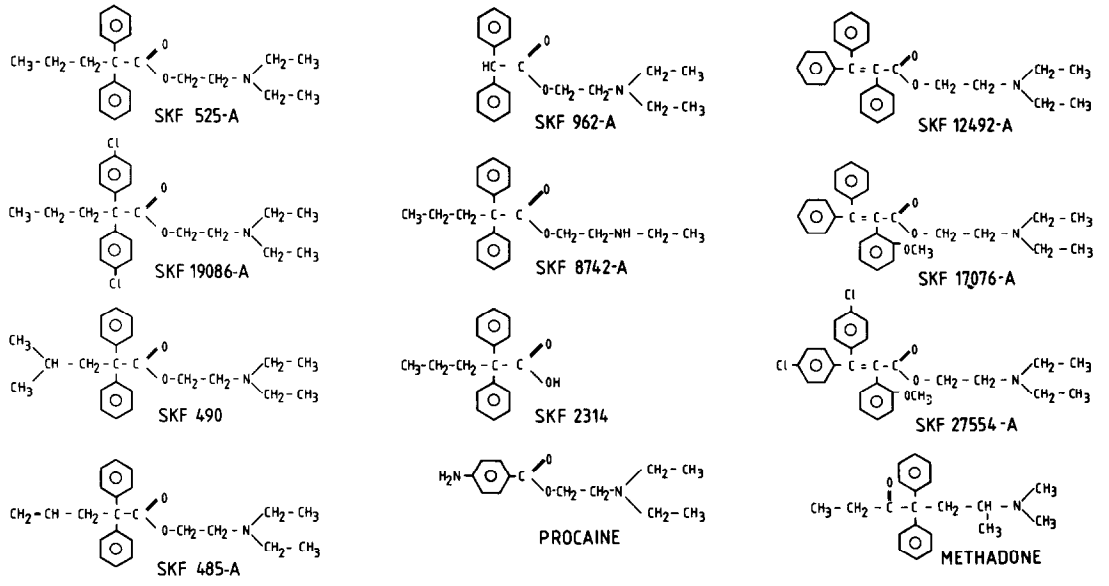

Fig. 2. Structure of the various analogs of SKF 525-A tested in this study.

increased it (Table 1). The crucial role of this part of the SKF 525-A molecule was underscored by the decreased activity of SKF 485-A, in which the propyl group was replaced by an acryl group (Table 1). The dichloro-derivatives SKF 19086-A was the most potent analog of SKF 525-A among those that we tested (Table 2): at 10 μM, a stimulatory effect became apparent after 30 min. At low concentrations, SKF 19086-A was more active than SKF 490, but its maximal effect was smaller (Table 2). Compounds containing an additional aromatic ring were less effective than SKF 525-A itself: the stimulatory effects of SKF 12492-A, SKF 17076-A and SKF 27554-A represented respectively 33%, 44% and 50% of the stimulation induced by SKF 525-A itself. Methadone, which shares some common structural

Table 1. Comparative study of various SKF 525-A analogs on the release of PGI_2 from rings of rabbit aorta

ng 6-K-$PGF_{1\alpha}$/100 mg × 30 min			
—	12 ± 2	—	11 ± 3
SKF 962-A	10 ± 6	SKF 485-A	34 ± 7
SKF 490	213 ± 58	SKF 525-A	112 ± 16
SKF 525-A	77 ± 22		
		—	3 ± 1
—	12 ± 6	Procaine	4 ± 2
SKF 2314	8 ± 5	SKF 525-A	39 ± 9
SKF 8742-A	40 ± 14		
SKF 525-A	122 ± 19	—	5 ± 2
		Methadone	6 ± 2
		SKF 525-A	140 ± 42

The rings were incubated for three periods of 30 min in the presence of these drugs (at a 100 μM concentration). Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the incubation medium at the end of the second period (mean ± SD of 9 measurements: triplicate determinations in three separate experiments). Each part of the table corresponds to experiments performed with the aorta of different animals.

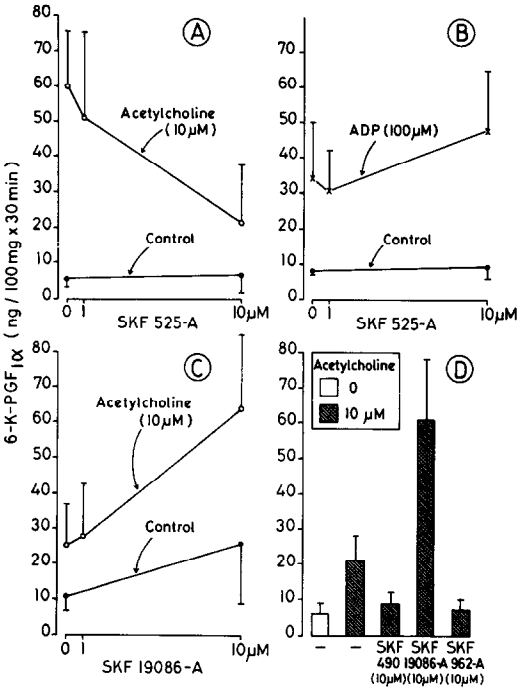

Fig. 3. Interaction of SKF 525-A and its analogs with the PGI_2-stimulating agents, acetylcholine and ADP, in the rabbit aorta. Rings of rabbit aorta were incubated for two periods of 30 min and the medium was changed between the two periods. SKF 525-A and its analogs were present during the two periods, whereas acetylcholine or ADP were added only during the second one. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of this second period (mean ± SD of 9 measurements: triplicate determinations in three separate experiments, for each part of the figure).

Table 2. PGI_2-stimulating activity of the dichloro-derivative SKF 19086-A in the rabbit aorta

ng 6-K-$PGF_{1\alpha}$/100 mg × 30 min			
0	7 ± 1	0	9 ± 5
2	8 ± 1	SKF 490 (20 μM)	26 ± 4
10	16 ± 5	SKF 19086-A (20 μM)	40 ± 5
20	45 ± 13	SKF 490 (100 μM)	112 ± 20
50	58 ± 17	SKF 19086-A (100 μM)	54 ± 3
SKF 19086-A			

The rings were incubated for three periods of 30 min in the presence of the drugs at various concentrations. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the incubation medium at the end of the second period (mean ± SD of 6—left part, or 9—right part measurements: triplicate determinations in separate experiments).

features with SKF 525-A (Fig. 2), was completely inactive at 100 μM (Table 1) and even 500 μM.

At concentrations (1, 10 μM) below those which *per se* increased PGI_2 release from the rabbit aorta, SKF 525-A inhibited the stimulation of this release induced by acetylcholine [20], but had no effect on the response to ADP (Figs 3, A and B). The structural requirements of this inhibitory action were completely different from those characterizing the PGI_2-stimulating activity: SKF 962-A produced the same inhibition as SKF 490, whereas potentiation rather than inhibition was obtained with SKF 19086-A (Figs 3, C and D).

Since both the endothelial cells and the smooth muscle cells of the aorta generate PGI_2 [21], experiments were performed to determine which of these cells are stimulated by SKF 525-A. The responsiveness to the drug was lost following the mechanical removal of the endothelium from the rabbit aorta strips (data not shown). SKF 525-A (not shown) and

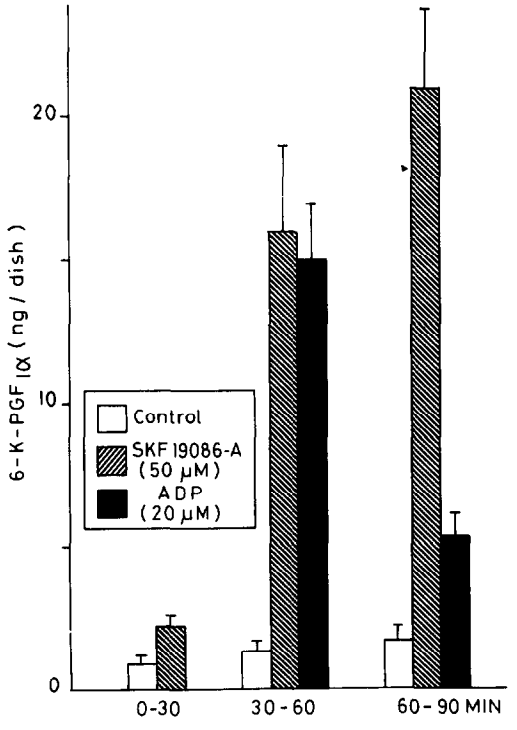

Fig. 4. Stimulation by SKF 19086-A of PGI_2 release from bovine aortic endothelial cells: comparison with the stimulatory effect of ADP. The cells were incubated for three periods of 30 min: the medium was collected and changed at the end of each period: SKF 19086-A was present throughout the experiment, whereas ADP was added only during the second and third periods. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium (mean ± SD of six measurements: triplicate determinations in two separate experiments).

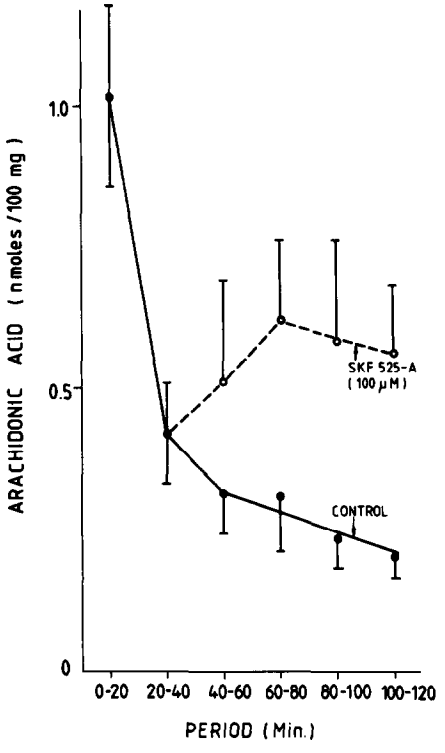

Fig. 5. Time course of free arachidonic acid mobilization in the rabbit aorta stimulated by SKF 525-A. Rings of rabbit aorta were incubated for six periods of 20 min in a medium containing BSA (1 mg/ml) and indomethacin (1 μg/ml): the medium was collected and replaced at the end of each period. SKF 525-A was added in the last four periods. Arachidonic acid was measured by GLC-ECD, as described in Materials and Methods. Results represent the mean ± SD of 6 measurements (duplicate determinations in three separate experiments): —●—: no SKF 525-A; --○--: SKF 525-A (100 μM).

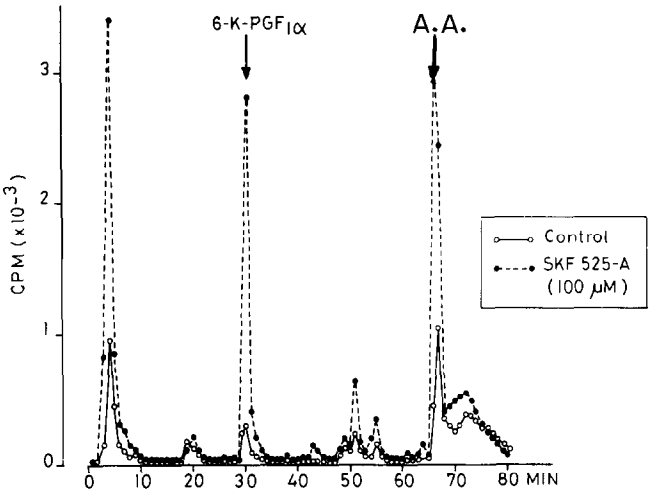

Fig. 6. Effect of SKF 525-A on the metabolism of [^{14}C]arachidonic acid in bovine aortic endothelial cells. The cells were incubated in the presence of [^{14}C]arachidonic acid (0.25 μCi/ml), with or without SKF 525-A (100 μM). After 18 hr, the media were collected and 1 ml aliquots were analyzed by RP-HPLC, as described in Materials and Methods. The figure depicts typical chromatograms, representative of 2 experiments performed with cells from different aortas (duplicate dishes in each experiment). A.A.: arachidonic acid.

SKF 19086-A (Fig. 4) stimulated the release of PGI_2 from bovine aortic endothelial cells, with the same kinetic features as in the whole rabbit aorta: the response to SKF 19086-A was delayed and sustained, whereas the effect of ADP was immediate and transient. SKF 19086-A was also active on endothelial cells cultured from human umbilical veins (not shown). Cultured explants of bovine aortic media, a preparation which contains exclusively smooth muscle cells, are stimulated to produce PGI_2 by serotonin [22]: SKF 525-A and SKF 19086-A had no effect in that system (data not shown).

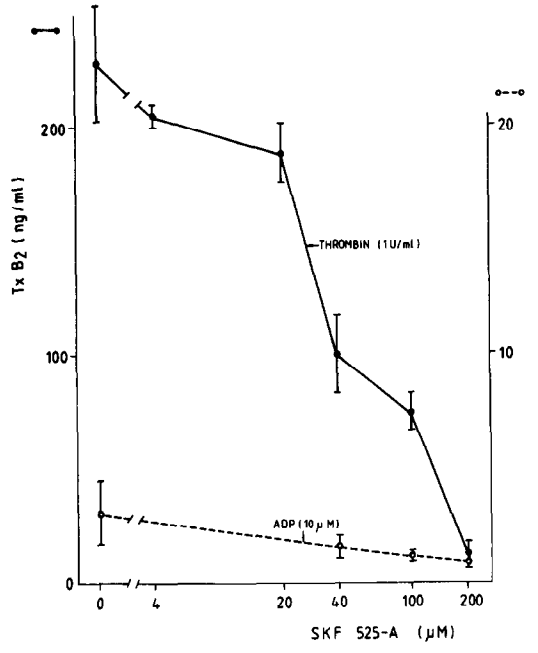

Fig. 7. Inhibition by SKS 525-A of TxB_2 release from washed human platelets stimulated by thrombin and ADP. After a 5 min preincubation in the presence of SKF 525-A, bovine thrombin (1 U/ml: –●–) or ADP (10 μM: --○--) were added and the incubation was continued for another 5 min. Results represent the mean ± SD of four measurements (duplicate determinations in two separate experiments).

SKF 525-A increased the level of free arachidonic acid in rabbit aorta rings, with a time course similar to that of PGI_2 release stimulation (Fig. 5). Two experiments indicated that, contrary to our initial hypothesis, this increased availability of free arachidonic acid was not likely to result from cytochrome P-450 inhibition. The stimulatory effect of SKF 525-A on PGI_2 release from rabbit aorta rings was not mimicked by another cytochrome P-450 inhibitor, metyrapone (data not shown). RP-HPLC analysis of the incubation media of bovine aortic endothelial cells labeled with [^{14}C] arachidonic acid resolved several compounds other than arachidonic acid itself and 6-K-$PGF_{1\alpha}$; none of these peaks was inhibited by SKF 525-A (Fig. 6).

SKF 525-A did not stimulate the release of TxB_2 from washed human platelets: furthermore, it inhibited the stimulation of this release induced by thrombin (Fig. 7), ADP (Fig. 7) or ionophore A23187 (not shown), but had no effect on the conversion of exogenous arachidonic acid into TxB_2 (not shown).

DISCUSSION

SKF 525-A was developed more than 30 years ago: it is a compound of minimal pharmacological activity of its own, but which can markedly prolong the duration of action of a diversity of drugs, including hypnotics, analgesics and central nervous system stimulants [23]. This prolongation results from the inhibition of various pathways of drug metabolism: for instance, SKF 525-A inhibits procaine esterase, formation of morphine glucuronide and reduction

of nitro compounds [24]. The *N*-demethylation of ethylmorphine by liver microsomes is competitively inhibited by SKF 525-A, which acts as an alternate substrate [25]. The binding of SKF 525-A to one species of hepatic cytochrome P-450 has been demonstrated by spectrophotometric and EPR measurements [6, 26]. Limited studies of SKF 525-A have been performed in humans with the purpose to prolong the action of hypnotics and anticonvulsivants, but no clinically-significant potentiation was observed (Schlosser, personal communication). SKF 525-A has remained useful as an experimental tool for studying drug metabolism and the role of cytochrome P-450. Recently SKF 525-A has been shown to inhibit one pathway of arachidonic acid metabolism in canine polymorphonuclear leukocytes [4] and in the thick ascending limb of the loop of Henle [5]. Inhibition of drug metabolism and cytochrome P-450 activity is the most studied, but not the only biological effect of SKF 525-A. This drug and its quaternary ammonium analog, meproadifen, are local anesthetics and non-competitive antagonists of the nicotinic receptor [27, 28]: they enhance the agonist-induced desensitization of this receptor [29, 30]. SKF 525-A exerts an oestrogenic effect on the rat uterus *in vivo* [31], which can be explained by a direct interaction with the oestradiol receptor [32]. SKF 525-A produces a rapid, sustained and reversible lowering of plasma cholesterol in various animals: a direct inhibition of cholesterol biosynthesis by SKF 525-A has been observed in liver homogenates [33]. In this paper, we have reported a new pharmacological activity of SKF 525-A: the stimulation of PGI_2 release from the vascular endothelium.

Stimulation of vascular PGI_2 by SKF 525-A appears as a general phenomenon observed with vascular preparations from rabbit, bovine and human origin. The stimulation takes place selectively in the endothelium, the aortic smooth muscle exhibiting complete unresponsiveness. The concentrations of SKF 525-A required to induce PGI_2 synthesis are high, but in the same range as those which produce other effects, like inhibition of drug metabolism in liver microsomes ($K_I = 10\ \mu M$: 24) or displacement of oestradiol from its receptor ($K_I = 100\ \mu M$: 31). The stimulatory action of SKF 525-A is characterized by its progressive onset and long duration: these features are in sharp contrast with repeated observations that stimuli of endothelial PGI_2, like ADP [14], thrombin or ionophore A23187 [34] and arachidonic acid itself [35, 36] induce immediate and short-lived effects, followed by a period of refractoriness. Our study with SKF 525-A indicates that it is possible to produce a sustained stimulation of endothelial PGI_2. The comparison of SKF 525-A with several chemical congeners has demonstrated that the PGI_2-stimulating activity is characterized by specific structural requirements. The most striking one involves the terminal propyl group: activity is decreased following its replacement by an acryl group, abolished by its deletion and on the contrary enhanced by its elongation into an isobutyl group. Increased potency is obtained after chlorination of the phenyl rings. Another action of SKF 525-A, that we have detected in this study, is the inhibition of the cholinergic stimulation of PGI_2 release. This effect, which might result from a direct interference with the muscarinic receptors, is obtained at concentrations lower than those required for a direct stimulation of PGI_2 by the drug. It is characterized by entirely different structural requirements: it is unaffected by the propyl group deletion, but becomes undetectable following chlorination. These observations suggest that the stimulation of endothelial PGI_2 synthesis by SKF 525-A does not result from non-specific interactions of this amphiphilic molecule with plasma membrane lipids. It is also unlikely that this stimulation results from the inhibition of a cytochrome P-450 pathway of arachidonic acid metabolism leading to an increased substrate availability for the cyclooxygenase. This hypothesis, which was the starting point of our study, is contradicted by two arguments. The lack of a similar stimulation by metyrapone is not a strong argument, because of the existence of multiple forms of cytochrome P-450, which are selectively inhibited either by SKF 525-A or by metyrapone [6]. More convincingly, we were unable to detect a single product of arachidonic acid in bovine aortic endothelial cells which was inhibited by SKF 525-A. Of course, we cannot exclude the possibility that, even after a prolonged incubation, exogenous arachidonic acid does not gain access to a pool of endogenous arachidonic acid selectively coupled with cytochrome P-450. The enhanced production of PGI_2 by the endothelium in response to SKF 525-A can be explained by the increased mobilization of arachidonic acid, that we have observed: the mechanism of this last effect remains thus to be elucidated. In the same range of concentrations which stimulate endothelial PGI_2, SKF 525-A inhibits TxB_2 release from platelets challenged with ADP or thrombin: this effect cannot be explained by a direct inhibition of TxA_2 synthase or cyclooxygenase, since the conversion of exogenous arachidonic acid is not prevented. Further studies are needed to characterize and understand the action of SKF 525-A on platelets.

PGI_2 is a potent inhibitor of platelet aggregation, but its therapeutic use is limited by its chemical and metabolic instability [37]. Agents increasing the endogenous production of PGI_2 by blood vessels might constitute a new class of antiplatelet drugs, potentially superior to existing ones. So far, essentially three drugs have been reported to increase the vascular production of PGI_2: nitroglycerin [38], dipyridamole [39, 40] and nafazatrom [41]. The initially reported effect of nitroglycerin could not be reproduced later [42]. The antithrombotic agent nafazatrom is a radical scavenger which can protect cyclooxygenase and PGI_2 synthase from oxidative inactivation [43]: its action is rather to prolong the synthesis of PGI_2 stimulated by another agent than to induce it *per se* [44]. The mechanism of dipyridamole action could be similar to that of nafazatrom [43]: we recently observed that dipyridamole does not stimulate the release of PGI_2 from endothelial cells (bovine aorta and human umbilical vein), but prolongs the transient release of PGI_2 induced by removing the endothelium from the rabbit aorta [45]. SKF 525-A appears therefore as a unique prototype of PGI_2-stimulating drug: the simultaneous inhibition of platelet TxA_2 constitutes an additional feature of

potential benefit. Since high concentrations of SKF 525-A are required to stimulate *in vitro* PGI_2 synthesis and since its chronic administration to animals produces a reversible fatty infiltration of the liver [33], it seems unlikely that this drug itself could ever be used clinically to increase vascular PGI_2. However, our study has shown that subtle modifications of SKF 525-A structure produce striking effects on the amplitude and potency of PGI_2 stimulation. These observations might constitute a starting point for the design of more effective and specific PGI_2-stimulating agents.

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