Biochemical Pharmacology, Vol. 37, No. 12, pp. 2315–2320, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

EFFECTS OF SK&F 94120, AN INHIBITOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE TYPE III, ON HUMAN PLATELETS

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(*Received* 22 *June* 1987; *accepted* 11 *November* 1987)

Abstract—Elevation of cyclic AMP concentrations in platelets inhibits agonist-induced responses. Pharmacological interventions which could increase the levels of platelet cyclic AMP include activation of the synthesis of cyclic AMP or inhibition of its breakdown. In this study we have investigated the effects of SK&F 94120 on human platelet phosphodiesterase (PDE) activities separated by ion-exchange chromatography, and studied the effects of this agent on platelet responses caused by the agonists collagen, U44069 and ADP.

Four PDE activities were identified from human platelet preparations. The PDE activities found comprised a cyclic GMP selective PDE, a Ca^{2+}/calmodulin stimulated PDE, a cyclic GMP stimulated PDE and a "low K_m" PDE activity called PDE III by analogy with activities described in other tissues. SK&F 94120 was found selectively to inhibit the "low K_m" PDE III activity with an IC_{50} of 10.8 μM, which is consistent with the effects of this compound on cardiac ventricle PDE activities. Exposure of human platelets to SK&F 94120 produced concentration dependent increases in cyclic AMP, showing that inhibition of PDE III activity alone can cause an increase in the level of platelet cyclic AMP. SK&F 94120 also caused an inhibition of platelet responses to collagen, U44069 and ADP. However, SK&F 94120 was much less effective as an inhibitor of aggregation induced by ADP ($IC_{50} > 100$ μM) than by collagen ($IC_{50} = 24.1$ μM) or by U44069 ($IC_{50} = 1.7$ μM). Isobutylmethylxanthine (IBMX), a non-selective PDE inhibitor, was less effective than SK&F 94120 as an inhibitor of platelet responses for the same measured increase in cyclic AMP levels. M&B 22948 and rolipram, inhibitors of PDE I and PDE IV respectively, had no significant effect on platelet responses.

These data suggest that selective inhibition of PDE III is the primary mechanism of action of SK&F 94120 as an inhibitor of agonist-induced platelet responses, and that increased cyclic AMP in the pool controlled by PDE III has important consequences on platelet responses. Moreover, these data suggest that some form of compartmentalization of cyclic AMP and/or PDE activity exists in human platelets.

Elevation of cyclic AMP concentration in blood platelets inhibits agonist-induced responses [1–4]. Prostaglandins I_2 and E_1 exert their inhibitory effects by activating adenylate cyclase and thus increasing cyclic AMP production [4, 5]. Agents which inhibit breakdown of cyclic AMP by cyclic nucleotide phosphodiesterases can also raise cyclic AMP concentrations and reduce platelet responsiveness [1, 2]. Like many other cells, platelets contain several types of cyclic nucleotide phosphodiesterase which can be separated by ion exchange chromatography, have different affinities for cyclic AMP and cyclic GMP and differential responsiveness to Ca^{2+}-calmodulin [6].

Until recently, most PDE inhibitors were non-selective, and investigations of the role of individual PDEs in physiological responses, using such compounds, was not practical because of the difficulties of interpretation. However, with the recognition of a range of selective inhibitors of PDE, studies of this nature have become possible. Investigations of the effects of these compounds on a variety of physiological phenomena have been of interest, including their effects on cardiac function [7] and platelet responses [8].

SK&F 94120 (5-(4-acetimidophenyl)pyrazin-(1H)-one) is a novel pyridazinone originally identified as an inotrope-vasodilator and now known to be a specific inhibitor of PDE III, the so-called "low K_m" isoenzyme [9, 10]. Here we report (i) the effect of the drug on different PDE fractions separated from human platelets, and (ii) the changes in platelet responsiveness and cyclic AMP concentrations produced by SK&F 94120 compared with those produced by the non-selective agent IBMX and by selective inhibitors of other types of PDE namely rolipram, which inhibits the newly described PDE IV or cyclic AMP specific PDE [11] and M&B 22948, which inhibits PDE I the cyclic GMP specific isoenzyme [12].

MATERIALS AND METHODS

Preparation of platelet rich plasma (PRP) and washed platelets. Platelet rich plasma (PRP) was prepared from whole blood, freshly drawn from healthy volunteers who gave informed consent, anticoagulated with 1/10 volume of acid–citrate dextrose containing: 7 mM citric acid, 93 mM sodium citrate and 139 mM dextrose. The citrated blood was centri-

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fuged at 700 *g* for 5 min and the PRP removed. The remaining red cells and plasma were centrifuged for a further 15 min at 900 *g* and the platelet poor plasma (PPP) removed. This was then mixed with the PRP giving a final platelet count of 2–3 × 10^8 cells/ml.

Washed platelets were prepared from whole blood anticoagulated with 1/6 volume of acid–citrate dextrose containing: 65 mM citric acid, 85 mM sodium citrate and 110 mM dextrose and centrifuged for 5 min at 700 *g*. The PRP was removed and then centrifuged at 350 *g* for 20 min and resuspended in *N*-2-hydroxyethyl piperazine-*N*-2 sulphonic acid (Hepes)-buffered physiological saline also containing 10 mM glucose, and 0.05 U/ml hirudin.

Preparation and ion-exchange chromatography of platelet PDE. Washed human platelets (obtained from Addenbrookes Hospital Blood Bank) were stored at −40° until homogenization and chromatography. All subsequent procedures were carried out at 4°. 11.5 ml of suspension were thawed on ice and homogenised in 35 ml of buffer containing of 20 mM bis-Tris, 5 mM 2-mercapto-ethanol, 2 mM benzamidine, 2 mM ethylenediaminetetracetic acid (EDTA), 50 mM sodium acetate, pH 6.5 in a Polytron® with a PT 10 probe for 10 sec, setting 6. Phenylmethanesulphonyl fluoride was dissolved in propan-2-ol and added to the buffer prior to homogenization to a final concentration of 50 μM (1 μl/l). The homogenate (40 ml) was then centrifuged for 20 min at 25,000 *g* and the supernatant applied to the DEAE-Sepharose CL-6B column (17 × 1.5 cm) pre-equilibrated in homogenization buffer. A flow rate of 80 ml/hr was used throughout the ion-exchange chromatography procedures. The column was washed with 170 ml of homogenisation buffer and PDE activities eluted with a linear 400 ml, 50–1000 mM sodium acetate gradient. 7.5 ml fractions were collected. Fractions were stored at 4° until assay for PDE activity and determination of initial kinetic parameters. Subsequently ethylene glycol was added to a final concentration of 30% (v/v) and fractions stored at −20°. Activity was stable for several weeks under these conditions.

Assay of phosphodiesterase activity. PDE activity was determined using the method of Davis and Daly [13] but with the modifications described by Reeves *et al.* [11]. Kinetic parameters K_m and V_{max} were determined by increasing the amount of unlabelled cyclic AMP (or cyclic GMP) in assays. A correction for the concurrent reduction in specific activity of the substrate was made and data analysed by non-linear least squares regression analysis to obtain values for K_m, V_{max} and Hill coefficients.

Measurement of aggregation and secretion. Aggregation was measured either alone in a twin channel HU aggregometer or simultaneously with secretion in a Chronolog Lumiaggrometer. 0.5 ml aliquots of PRP were equilibriated to 37° before being placed in the sample chamber. The aliquots were incubated for 2 min with varying concentrations of the PDE inhibitor or with dimethylsulphoxide (DMSO) and methanol vehicle. DMSO or methanol vehicle never exceeded 0.20%. After the addition of the agonist, aggregation was determined by the change in absorbance monitored for 4 min. Aggregation was then expressed as a percentage of control absorbance, each control having the same [DMSO] or [methanol] as the experimental sample. For simultaneous measurement of ATP secretion in the Lumiaggrometer, 10 μl of Chronolume reagent (Coulter) was added to each aliquot prior to the addition of the PDE inhibitor or vehicle.

After the addition of the agonist the luminescence signal was monitored for 4 min and then 1 mM ATP was added. ATP secretion was calibrated as the ratio of the luminescence signal to that produced by the addition of 1 μM ATP. Aggregation and secretion were then expressed as a percentage of the control.

Measurement of cAMP in washed platelets. Aspirin-treated washed platelets were incubated at 37° with the appropriate agents and then quenched with 6% TCA. The tubes were vortexed and centrifuged at 14,000 *g* for 2 min and 1.1 ml of the supernatant removed and stored at −20°. Cyclic AMP was separated from 1 ml of the sample on Dowex 50W-x8 (H^+) columns, recovery being checked by adding 0.1 pmol of [^{3}H]-cyclic AMP. The relevant fractions were pooled, dried down, and then reconstituted in 1 ml Na acetate buffer pH 6.2 and the cyclic AMP assayed with DuPont RIANDEN cyclic AMP [^{125}I] kit. The results are reported as pmol/10^8 cells (approximately equivalent to μM cyclic AMP).

Materials and solutions. [8-^{3}H] Adenosine 3′,5′-cyclic phosphate (26.5 Ci/mmol), [8-^{3}H]guanosine 3′,5′-cyclic phosphate (15 Ci/mmol), [U-^{14}C]-adenosine 5′-monophosphate (507 mCi/mmol) and [U-^{14}C]guanosine 5′-monophosphate (526 mCi/mmol) were obtained from Amersham Int. (Amersham, Bucks. U.K.). DEAE-Sepharose® CL-6B was obtained from Pharmacia Fine Chemicals. Affi-Gel® 601 was obtained from Bio-Rad Laboratories. Chronolume (luciferin-luciferase reagent) came from Coulter; ATP from Boehringer-Mannheim, apyrase, aspirin, hirudin and fibrinogen from Sigma; collagen from Hormon-Chemie; U44069 from Upjohn; and SK&F 94120 and zaprinast (M&B 22948) were synthesised in the Department of Medicinal Chemistry at SK&F. Rolipram was a gift from Schering Ag.

The composition of platelet buffer was 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose and 10 mM Hepes pH 7.4 at 37°. For cell suspensions 10 mM glucose was added. ACD[1] was 7 mM citric acid, 93 mM Na_3 citrate, 139 mM glucose pH 6.5—ACD[2] was 65 mM citric acid, 85 mM Na_3 citrate, 110 mM glucose.

Analysis of data. Concentration inhibition curves were fitted to the logistic equation by computer using the ALLFIT program [14]. The points are means ± SE. Errors quoted for EC_{50} and IC_{50} values are approximate standard errors derived from this fitting procedure. Differences between values were analysed in a paired *t*-test.

RESULTS

Influence of SK&F 94120 on PDE activity

The profile of PDE activities obtained from chromatography of human platelet homogenates is shown in Fig. 1. Three major peaks could be distinguished. Only the third major peak of activity was inhibited by SK&F 94120—a fraction called PDE

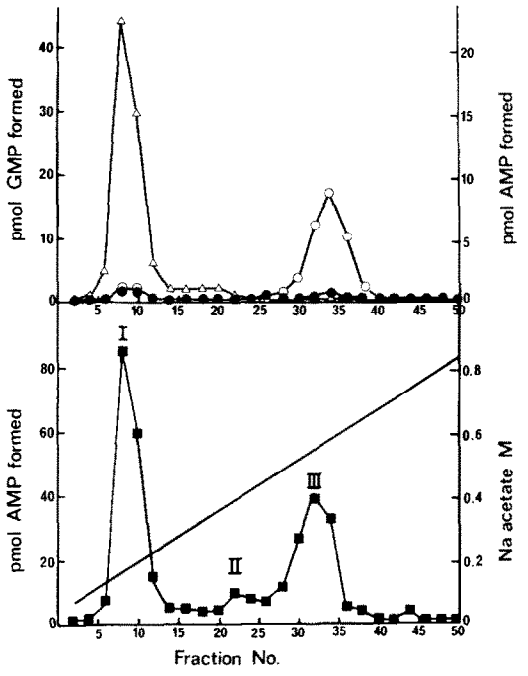

Fig. 1. Profile of elution of PDE activities from human platelets after DEAE-Sepharose chromatography. An extract of human platelets was prepared and applied to a column of DEAE-Sepharose equilibrated as described in the Materials and Methods section. The column was eluted with a sodium acetate gradient (——), 7.5 ml fractions being collected. Activity was assayed with 1 μM cyclic AMP (○), 1 μM cyclic AMP + 100 μM SK&F 94120 (●), 50 μM cyclic AMP (■) or 1 μM cyclic GMP (△).

III by analogy with nomenclature in other tissues [11, 15]. This profile is similar to that reported by other workers in human platelet [6]. The kinetic properties of these activities are summarised in Table 1. The first peak corresponds to the cyclic GMP specific PDE also found in smooth muscle [12] and lung tissues [16]. The second peak probably contained two enzymes, the calmodulin stimulated PDE and the cyclic GMP stimulated PDE, since both these agents caused a stimulation of cyclic AMP hydrolysis by this activity. The third peak of activity had properties typical of PDE III from other tissues [11, 17] and SK&F 94120 produced a concentration-dependent inhibition of platelet PDE III with a IC_{50} of 10.8 μM. This compares well with IC_{50}s of 11.0 μM and 11.6 μM for inhibition of PDE III from guinea-pig and cat heart [11, 18]. SK&F 94120 thus shows potent and selective inhibition of human platelet PDE III.

Effects of SK&F 94120 and IBMX on platelet cAMP concentrations

In unstimulated, washed platelets SK&F 94120 produced a concentration-dependent increase in the measured cyclic AMP as shown in Fig. 2. Mean basal cyclic AMP was 3.92 ± 0.7 pmol/10^8 cells (mean ± SEM, N = 16). The lowest concentration to give a significant increase was 3 μM, and 100 μM SK&F 94120 produced a 77.6 ± 12.5% increase in cyclic AMP (N = 8). The non-selective PDE inhibitor IBMX produced a similar concentration-dependent rise in cyclic AMP, 100 μM IBMX giving a 79 ± 15% (N = 8) increase in cyclic AMP over control. We also examined the influence of maximally effective concentrations of U44069 a stable prostaglandin endoperoxide [19] and ADP on the alterations in cyclic AMP produced by SK&F 94120.

2 μM U44069 further increased the concentration of cyclic AMP reached after treatment with 100 μM SK&F 94120 by 45 ± 9% (N = 5). This is a significant increase over control cells treated with 100 μM SK&F 94120 ($P < 0.05$). In cells treated with 100 μM SK&F 94120, 20 μM ADP may have slightly reduced the cyclic AMP concentration by 10 ± 5% (N = 6), compared with that seen with SK&F 94120 alone, but the difference is not significantly different. In control cells U44069 and ADP did not produce significant changes in cyclic AMP concentrations.

Effects of SK&F 94120 and IBMX on platelet aggregation and secretion

Figure 3 shows the inhibitory effects of SK&F 94120 and IBMX on aggregation and secretion evoked by 5 μg/ml collagen in PRP. SK&F 94120 produced a concentration-dependent inhibition of both aggregation and secretion with calculated IC_{50}s of 36 ± 5 μM and 37 ± 9 μM (N = 8). 100 μM SK&F 94120, the highest concentration tested, inhibited collagen evoked aggregation by 74 ± 3% and secretion by 72.5 ± 4.0%. In contrast IBMX was surprisingly ineffective; 100 μM IBMX produced only a 13.5 ± 4.0% reduction in aggregation, and a 23 ± 4.0% reduction in secretion, even though both compounds had apparently similar effects on cyclic AMP as seen in Fig. 2.

Table 1. Kinetic properties of human platelet PDE activities

PDE activity	K_m for cyclic AMP (μM)	K_m for cyclic GMP (μM)	Activator
Peak I	>100	1.77	none
Peak II	* n.d.	* n.d.	calmodulin, cyclic GMP
Peak III	0.35	0.36	none

* K_m could not be determined because of the presence of two activities but positive cooperativity was observed with cyclic AMP as substrate.

PDE activities were separated from human platelets as shown in Fig. 1. Kinetic constants were determined as described in the Materials and Methods section. Abbreviation: n.d., not determined.

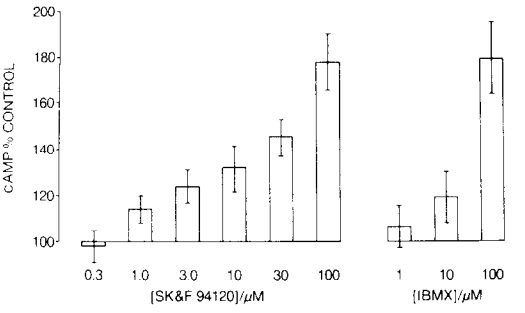

Fig. 2. The effects of SK&F 94120 and IBMX on cyclic AMP levels in washed human platelets after a 2 min incubation. Increases in cyclic AMP are expressed as a percentage of cyclic AMP in control cells which were incubated with vehicle only. Values are means ± SEM, N = 4–12. The increase in cyclic AMP at 3 and 10 μM SK&F 94120 is significant with $P < 0.05$, and of 30 μM and 100 μM SK&F 94120 with $P < 0.001$.

The effects of 100 μM SK&F 94120 and 100 μM IBMX were also compared under the same conditions in which the cyclic AMP measurements were made, i.e. in washed platelets (where differential protein binding would not be an issue). Here, 100 μM SK&F 94120 inhibited collagen-evoked aggregation by 83.5 ± 4.5%; 100 μM IBMX inhibited aggregation by only 48 ± 12.5% (N = 4). IBMX might be less effective because it interfered with transduction of elevated cyclic AMP into inhibition of responses. Therefore, in a separate experiment, the effects of 100 μM SK&F 94120 alone, 100 μM IBMX alone, or 100 μM IBMX and 100 μM SK&F 94120 together, on collagen-evoked aggregation in PRP were compared.

In the presence of 100 μM SK&F 94120 aggregation was reduced to 10 ± 2% of control; with 100 μM IBMX aggregation was 89 ± 4% of control and with both agents together aggregation was reduced to 19 ± 4.4% of control. Therefore the presence of 100 μM IBMX did not appear to prevent SK&F 94120 having its inhibitory effect. Also shown in Fig. 3 is the effect of 10 μM indomethacin (sufficient to suppress responses to arachidonic acid) on collagen-evoked aggregation and secretion; aggregation was inhibited by 56 ± 9% and secretion by 37 ± 6% (N = 8), i.e. rather less than by 100 μM SK&F 94120.

Effects of SK&F 94120 on responses to collagen. U44069 and ADP in the presence of cyclooxygenase inhibitors

Figure 4 shows that SK&F 94120 has effects after inhibition of thromboxane formation. In Fig. 4A a

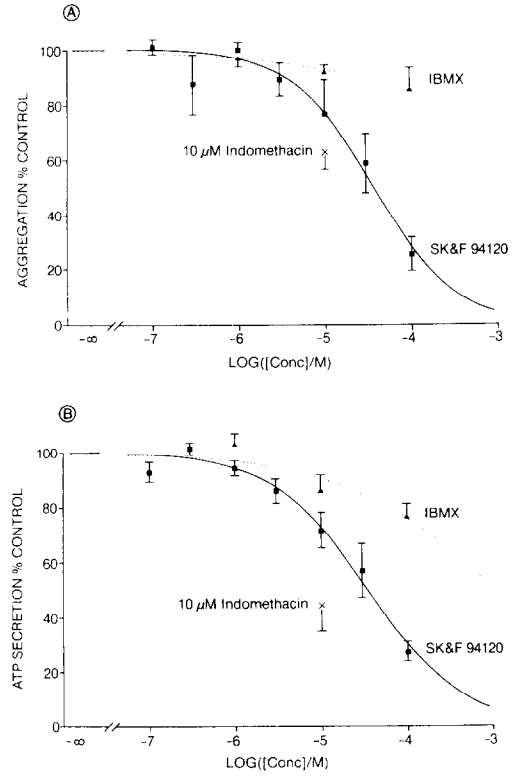

Fig. 3. (A) Inhibition of collagen (5 μg/ml) evoked aggregation and (B) ATP secretion in human PRP by SK&F 94120 (■) and IBMX (▲). Also shown is the inhibition of aggregation and secretion brought about by inhibition of thromboxane synthesis using 10 μM indomethacin (×). Values are means ± SEM, N = see main text.

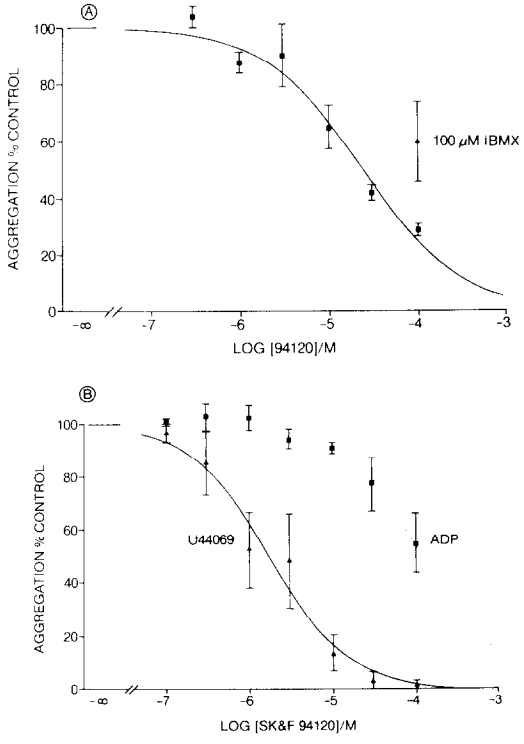

Fig. 4. Effects of SK&F 94120 on collagen, U44069 and ADP evoked aggregation in the presence of cyclooxygenase inhibitors. (A) Inhibition of collagen (5 μg/ml) evoked aggregation by SK&F 94120 (■) and 100 μM IBMX (▲) in indomethacin (10 μM) treated PRP. (B) Inhibition of 10 μM U44069 (▲) and 10 μM ADP (■) evoked aggregation by SK&F 94120 in aspirin (100 μM) treated PRP. Values are means ± SEM, N = see main text.

marked inhibition of collagen evoked aggregation is observed in indomethacin-treated PRP. The IC_{50} for this inhibition was $24.1 \pm 4.6\,\mu M$ SK&F 94120. SK&F 94120 thus inhibits responses evoked in the absence of thromboxane formation. Figure 4B shows the effect of SK&F 94120 on aggregation evoked by $10\,\mu M$ U44069 (a stable endoperoxide which acts at the thromboxane receptor [19]) in aspirin-treated PRP. The IC_{50} for this inhibition was $1.7 \pm 0.5\,\mu M$ which is lower than that for collagen under similar conditions. Also shown in Fig. 4B is the effect of SK&F 94120 on ADP evoked aggregation in aspirin-treated cells. ADP evoked aggregation was less readily inhibited; $100\,\mu M$ SK&F 94120 inhibited aggregation by $45 \pm 11\%$. Under the same conditions the selective PDE I and PDE IV inhibitors, M&B 22948 and rolipram had little inhibitory effect on U44069- or ADP- evoked aggregation. With $100\,\mu M$ M&B 22948, U44069 evoked aggregation was $78.7 \pm 12.0\%$ of control, and ADP evoked aggregation was $81.6 \pm 6.3\%$ of control ($N = 3$). In platelets treated with $100\,\mu M$ rolipram these values were 91.3 ± 0.6 and $102.6 \pm 3.8\%$ of control ($N = 4$).

DISCUSSION

The data in Fig. 1 show that SK&F 94120 selectively inhibited PDE III in human platelets, and had little effect on the other sub-types at concentrations up to $100\,\mu M$. The IC_{50} of $10.8\,\mu M$ was similar to that previously reported for this compound against PDE III from guinea-pig heart and cat [11, 18]. SK&F 94120 was capable of inhibiting cyclic AMP breakdown in intact platelets since it produced a concentration-dependent increase of platelet cyclic AMP content and a concentration-dependent inhibition of aggregation and secretion evoked by collagen, U44069 and ADP. The ability of SK&F 94120 to inhibit responses of platelets incapable of producing cyclo-oxygenase products shows that suppression of thromboxane production could not account for the action of the compound.

Our results suggest that the inhibitory effects of SK&F 94120 may be mediated through an increase in the cyclic AMP concentration. The proposal implies: (1) that in control conditions that cyclic AMP is generated at a rate of at least 2 pmol per 10^8 cells per min since the content rose this much in the presence of $100\,\mu M$ SK&F 94120; and (2) that PDE III plays a major role in regulating the functionally important pool of cyclic AMP in human platlets. The relative ineffectiveness of specific inhibitors of PDE I or PDE IV (M&B 22948 and rolipram) tends to support this conclusion. Some of our results are, however, not easily fitted to this simple model. It is not obvious why SK&F 94120 should have such different apparent potencies for the inhibition of responses to different agonists; for instance why are functional responses to U44069 so much more susceptible than those to ADP? One partial explanation is that U44069 may stimulate adenylate cyclase acting as a partial agonist at a prostaglandin receptor [5]. Measurement of cyclic AMP in platelets exposed first to $100\,\mu M$ SK&F 94120 and then to U44069 showed cyclic AMP concentration altered in the direction predicted by this explanation. Whatever the mechanism, these findings suggest that studies of *ex vivo* platelet responsiveness in animals or humans dosed with this class of drugs may give very different indications of functional inhibition depending on the agonist examined.

Another unexpected finding was the relative ineffectiveness of IBMX. This was surprising, since the measured elevation of cyclic AMP with IBMX was similar to that produced by SK&F 94120. The possibility that IBMX produces another effect, e.g. an elevation of cyclic GMP that counteracts the effect of cyclic AMP, seems unlikely, since simultaneous application of IBMX and SK&F 94120 had an inhibitory effect very similar to that of SK&F 94120 alone. Models can be constructed based on different compartments of cyclic AMP, which would require that IBMX mainly acts on other PDE isoenzymes which control a pool of cyclic AMP that is functionally less relevant to aggregation than that regulated by PDE III. This kind of model has been proposed to explain the finding that isoprenaline produces a much larger cardiac inotrope effect than PGE_1, but a smaller measured elevation of cyclic AMP concentration [20]. It may be that measurements of total tissue cyclic AMP are inappropriate as an index of second messenger function, as are measurements of total cell Ca^{2+}.

Despite the complications arising from a detailed analysis of these results we can conclude that; SK&F 94120 is an inhibitor of PDE III in human platelets; it elevates cyclic AMP content; it inhibits functional responses to collagen, U44069 and ADP; and much, if not all, the inhibitory effect can be attributed to an elevation of the cyclic AMP concentration. Compounds of this class being developed as vasodilator/inotropes for treatment of cardiac failure are likely to have some antiplatelet activity, the significance of which will require evaluation by long-term clinical observation.

Acknowledgements—We thank Drs P. J. England, T. J. Hallam and R. W. Gristwood for helpful advice, Ms B. Leigh for editorial assistance, Mrs M. Bowden for typing the manuscript, and Mr Tim Williams for help with the cyclic AMP assays.

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