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Cyclic AMP does not inhibit collagen-induced platelet signal transduction

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The adhesion of platelets to collagen and their activation is the primary event in haemostasis. Following adhesion, platelet aggregation mediated by ADP, thromboxane A_2 and thrombin leads to the formation of a platelet plug. It is known that platelet activation by each of these agonists involves an increase in the cytosolic free Ca^{2+} concentration, and this has been thought to be controlled by cyclic AMP. However, we report here that while signal transduction induced by ADP plus a thromboxane mimetic (U46619), or by thrombin, is inhibited by stimulators of adenylate cyclase such as a prostaglandin I_2 (PGI_2) analogue (Iloprost), PGD_2 and forskolin, elevation of cyclic AMP does not inhibit either platelet adhesion to collagen or the associated Ca^{2+} mobilization, phosphatidic acid formation or 5-hydroxytryptamine secretion. Furthermore, collagen did not lower elevated levels of cyclic AMP in platelets measured in the presence of both a thromboxane antagonist and an ADP-removing system. The present results are discussed in the context of previous findings.

INTRODUCTION

Many investigators have studied the effects of agents that elevate cyclic AMP on platelet adhesion to collagen and have come to varying conclusions. For example, Higgs *et al.* [1] and Weiss & Turrito [2] studied the effect of prostaglandin I_2 (PGI_2) on the adhesion of platelets to rabbit aortic subendothelium, and concluded that it prevented the formation of thrombi at very low concentrations and partially inhibited platelet adhesion at high concentrations. Cazenave *et al.* [3] studied platelet adhesion in the presence of apyrase to remove released ADP, and found that PGI_2 inhibited the attachment of ^{51}Cr-labelled platelets to a collagen-coated glass rod by up to 45%. Karniguan *et al.* [4] concluded that PGI_2 almost completely inhibited the adhesion of platelets to suspended collagen fibrils. On the other hand, in subsequent studies both Shadle & Barondes [5] and Santoro [6] concluded that PGE_1 had no effect on platelet adhesion to collagen immobilized on plastic or polystyrene dishes. More recently, based on their own results and analysis of the literature, Mazurov *et al.* [7] concluded that agents that increase cyclic AMP have no effect on the initial attachment of platelets to collagen, but do affect subsequent responses such as spreading and aggregation.

Cyclic AMP is thought to prevent nearly all aspects of platelet activation. Another second messenger, Ca^{2+}, on the other hand, is thought to be an important signal involved in the agonist-induced activation of platelets to aggregate, secrete and generate thromboxane A_2. Stimulation of platelets with ADP, the thromboxane mimetic U46619 or thrombin results in a rapid increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [8,9]. Since prior addition of adenylate cyclase stimulators such as PGD_2, PGI_2 or forskolin inhibited the rise in $[Ca^{2+}]_i$ induced by thrombin or ADP and caused a rapid fall in $[Ca^{2+}]_i$ back to the resting level when added after thrombin, it was suggested that cyclic AMP-dependent reactions maintain low levels of $[Ca^{2+}]_i$ by promoting transport and/or binding of Ca^{2+} [9,10]. We have recently developed a novel suspension assay to study the adhesion of platelets to collagen in the presence of antagonists of secondary mediators [11], and have determined by use of this assay that facile adhesion requires metabolic energy and actin polymerization, and evokes an increase in $[Ca^{2+}]_i$ [12]. Therefore we decided to re-investigate the effect of cyclic AMP on the collagen–platelet interaction. We report here the unexpected finding that elevation of cyclic AMP does not inhibit collagen-induced signal transduction.

EXPERIMENTAL

Human blood was obtained from healthy volunteers who denied taking any drug during the previous week. The blood was anticoagulated with citric acid/citrate/dextrose [13] and centrifuged at 180 *g* for 15 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 800 *g* for 20 min at room temperature to obtain a platelet pellet. For adhesion measurements, the pellet was resuspended in 5 ml of autologous plasma and incubated for 1 h with [9,10-^{3}H(n)]oleic acid (5 μCi/ml; 8.9 Ci/mmol) at 37 °C. In most experiments, fura-2 acetoxymethyl ester (3 μM) also was included. Following incubation, the platelets were separated from unincorporated radiolabel by gel filtration on a Sepharose 2B column using a Ca^{2+}-free Tyrode's buffer supplemented with 0.2% fatty acid-free BSA [14]. The platelets were adjusted to 2×10^8 cells/ml. Samples of 1 ml were stirred at 800 rev./min at 37 °C for 1 min before the addition of feedback inhibitors and collagen as described elsewhere [12]. Adhesion was determined after vacuum filtration through a 10 μm nylon mesh. Blanks, included to control for non-specific trapping of the platelets on the mesh, gave results of between 1 and 3% of the radioactivity passed through the filter. Addition of 50 μg of collagen/ml to platelet suspensions altered the pH by less than 0.1 unit.

Fura-2 fluorescence was monitored continuously using settings of 340 nm (excitation) and 510 nm (emission). Fura-2 signals were calibrated as described elsewhere [15]. $F_{max.}$ was determined by lysing the cells with 40 μM-digitonin in the presence of saturating $CaCl_2$. $F_{min.}$ was determined by the addition of 2 mM-EGTA and 20 mM-Tris base.

Phosphatic acid (PtdOH) was measured in platelets that were prelabelled in plasma with [^{32}P]P_i (carrier-free, 0.25 mCi/ml, 1 h, 37 °C) before gel filtration. After incubation with the agonist, reactions were stopped with chloroform/methanol (1:2, v/v) and extracted lipids were anaysed by t.l.c. on Silica Gel G plates

Abbreviations used: PG, prostaglandin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PtdOH, phosphatidic acid.

using water-saturated ethyl acetate/iso-octane/acetic acid (9:5:2, by vol.) as solvent. A PtdOH standard was used to identify its location on the plate.

For measurement of 5-hydroxytryptamine secretion, platelet-rich plasma was incubated for 30 min at 37 °C with 1 μM-[G-^{3}H]5-hydroxytryptamine (8 Ci/mmol) before gel filtration. Secretion was stopped in formaldehyde/EDTA according to the method of Costa & Murphy [16], and samples of the supernatants were added to scintillation fluid. In each set of experiments the total 5-hydroxytryptamine content of the platelets was measured by adding platelet suspension to the stopping solution, from which a sample was transferred to the counting solution. Release of 5-hydroxytryptamine was expressed as a percentage of the total 5-hydroxytryptamine content. For the experiments shown in Figs. 2 and 3, platelets were simultaneously preincubated with fura-2 acetoxymethyl ester, [^{32}P]P_i and [^{3}H]5-hydroxytryptamine.

Cyclic AMP formation in intact platelets was measured by incubating platelet rich-plasma for 1 h with 2 μCi of [^{3}H]adenine (31 Ci/mmol) prior to gel filtration. Aliquots of labelled cells were challenged with collagen or other agonists in the absence and presence of Arg-Gly-Asp-Ser (RGDS, 220 μM), phosphocreatine (20 mM), creatine kinase (50 units/ml) and SQ 29,548 (6 μM) for 5 min prior to addition of 0.5 ml of a stopping solution containing 0.6 M-$HClO_4$ and 2% SDS, with [^{14}C]cyclic AMP (44 mCi/mmol) as a recovery standard. The cyclic AMP content of each sample was determined by the method of Salomon [17]. Results are expressed as percentages of total radioactivity appearing as cyclic AMP.

Radiolabelled adenine, 5-hydroxytryptamine and oleic acid were from New England Nuclear, and [^{32}P]P_i was from ICN. Collagen was from Horm-Chemie, Munich, Germany, and RGDS was from Calbiochem, La Jolla, CA, U.S.A. The antagonist SQ 29,548 was a gift from D. Harris of Bristol-Myers Squibb. The stable PGI_2 analogue Iloprost was from Berlex Laboratories Inc., Cedar Knolls, NJ, U.S.A. Fura-2 acetoxymethyl ester was from Molecular Probes, Junction City, OR, U.S.A., and the nylon mesh was from Small Parts Inc., Miami, FL, U.S.A.

Statistical comparisons were made using Dunnett's t test.

RESULTS

Fig. 1 shows recordings of changes in fluorescence in fura-2-loaded platelets suspended in nominally Ca^{2+}-free medium and challenged with collagen (50 μg/ml). The numbers at the end of each trace represent the percentage of platelets adhering to the collagen at the end of the experiment. In the control tracing (Fig. 1a), collagen caused an increase in $[Ca^{2+}]_i$, even when added after an ADP-removing system consisting of phosphocreatine/creatine kinase, the thromboxane antagonist SQ 29,548 and RGDS. The increase in $[Ca^{2+}]_i$ peaked at approx. 60 s and then declined slowly. This increase was accompanied by the binding of 30.0±8.8% of the platelets to the collagen fibrils. The other three tracings show the effects of preincubation of the platelets with the adenylate cyclase stimulators Iloprost (Fig. 1b), PGD_2 (c) and forskolin (d). The adhesion of the pretreated platelets to collagen was significantly greater than for the control ($P < 0.01$), with mean values for adhesion ranging between 43.3 and 45.7%. In addition, the rate of increase in $[Ca^{2+}]_i$ in the treated cells was 2.5-fold greater than in the control cells ($P < 0.01$), the maximal level was of a slightly greater magnitude, and the Ca^{2+} transient declined 3.5-fold more rapidly ($P < 0.01$).

Fig. 2 shows the effects of Iloprost and PGD_2 on increases in $[Ca^{2+}]_i$ induced by the combination of ADP and U46619 or by thrombin. The combination of ADP and U46619 caused the $[Ca^{2+}]_i$ to increase within 10 s, from a basal level of 65 nM to over 1 μM. This increase, however, was short-lived. Very little change in $[Ca^{2+}]_i$ was induced by this combination of agonists in the

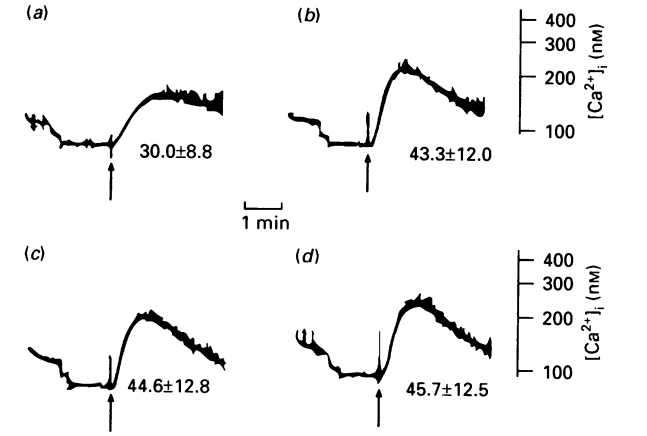

Fig. 1. Ca^{2+} mobilization induced by a collagen suspension (50 μg/ml) in the presence of phosphocreatine/creatine kinase, SQ 29,548 and RGDS

(a) Control cells. Other cells were preincubated for 2 min with the adenylate cyclase stimulators Iloprost (28 nM) (b), PGD_2 (2.8 μM) (c) or forskolin (10 μM) (d). The numbers associated with each trace indicate the means±S.D. (four determinations in each of three experiments) of the percentage of the platelets adhering to collagen at the end of the experiment. The values for adhesion were significantly increased by treatment with the adenylate cyclase stimulators ($P < 0.01$).

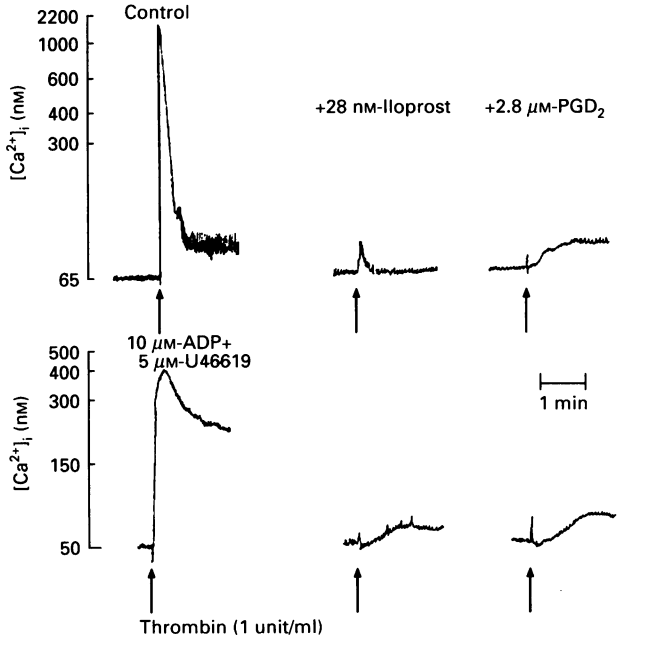

Fig. 2. Effects of incubation for 2 min with the adenylate cyclase stimulators Iloprost (28 nM) and PGD_2 (2.8 μM) on Ca^{2+} mobilization induced by the combination of ADP (10 μM) and U46619 (5 μM) or by thrombin (1 unit/ml)

The effects of thrombin were studied in the presence of phosphocreatine/creatine kinase, SQ 29,548 and RGDS. The platelets were prelabelled simultaneously with fura-2 for the measurement of $[Ca^{2+}]_i$ and with [^{32}P]P_i and [^{3}H]5-hydroxytryptamine for the measurement of PtdOH formation and 5-hydroxytryptamine secretion respectively. In four experiments, the maximal value of $[Ca^{2+}]_i$ observed after ADP plus U46619 was 1720±808 nM, and after thrombin it was 508±143 nM (means±S.D.).

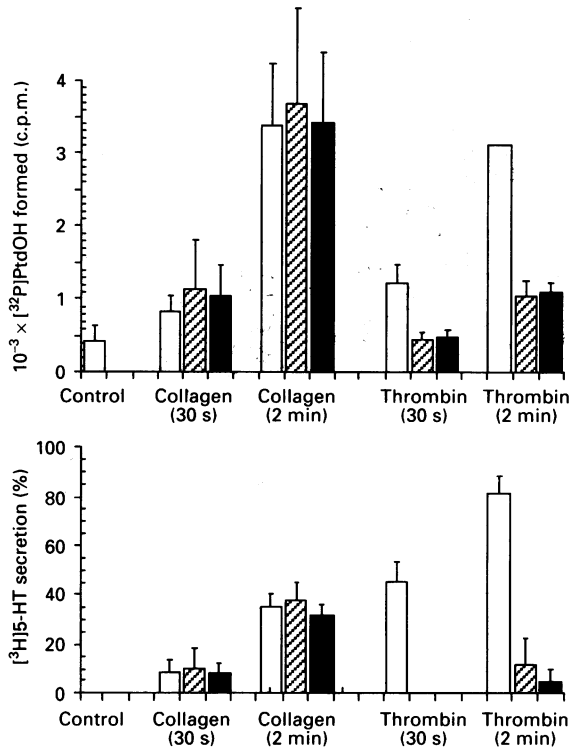

Fig. 3. Measurement of PtdOH formation (*a*) and 5-hydroxytryptamine (5-HT) secretion (*b*) in response to collagen suspension or thrombin

□, Control (agonist alone); ▨, plus Iloprost; ■, plus PGD_2. The results shown are means ± S.E.M. of three experiments (six determinations).

Table 1. Measurements of cyclic AMP in platelets prelabelled with [^{3}H]adenine

The results shown are means ± S.E.M. of triplicate determinations of cyclic AMP as a percentage of total [^{3}H]adenine nucleotides from one experiment representative of three. The first column shows cyclic AMP in control platelets, in platelets incubated for 25 min at room temperature with Iloprost (28 nM), and in platelets incubated for 20 min at room temperature with Iloprost (28 nM) followed by 5 min preincubation with adrenaline (100 μM), ADP (5 μM) or collagen suspension (50 μg/ml). The second column shows the effects on cyclic AMP of Iloprost alone or in combination with adrenaline, ADP or collagen in platelet suspensions containing the feedback inhibitors phosphocreatine/creatine kinase, SQ 29,548 and RGDS.

Treatment	Cyclic AMP (%)	
	−Feedback inhibitors	+Feedback inhibitors
Basal	0.02 ± 0.01	—
Iloprost	0.69 ± 0.03	0.79 ± 0.02
Iloprost + adrenaline	0.12 ± 0.01	0.19 ± 0.01
Iloprost + ADP	0.13 ± 0.01	0.83 ± 0.03
Iloprost + collagen	0.23 ± 0.01	0.97 ± 0.07

presence of either Iloprost or PGD_2. Thrombin also caused the $[Ca^{2+}]_i$ to increase rapidly to approx. 400 nM, and this increase decayed more slowly than that caused by the combination of ADP and U46619. The effect of thrombin also was markedly inhibited by either Iloprost or PGD_2.

Fig. 3. compares the effects of Iloprost and PGD_2 on PtdOH formation and 5-hydroxytryptamine secretion induced by collagen and thrombin. Both agonists caused increases in PtdOH and secretion which increased progressively between 30 s and 2 min. However, while thrombin-induced responses were markedly inhibited by the adenylate cyclase stimulators, collagen-induced responses were unaffected. PtdOH formation induced by the combination of ADP and U46619 was approximately twice as great at 30 s as it was at 2 min (results not shown). This was decreased by over 60% by either Iloprost or PGD_2. The combination of ADP and U46619 elicited the release of 52.3% of the total 5-hydroxytryptamine at 30 s and 60.9% at 2 min. This effect was essentially abolished by Iloprost or PGD_2.

Table 1 shows measurements of cyclic AMP in [^{3}H]adenine-labelled platelets incubated with Iloprost alone or with the subsequent addition of adrenaline, ADP or collagen in the absence or the presence of the feedback inhibitors phosphocreatine/creatine kinase, SQ 29548 and RGDS. In the absence of the feedback inhibitors, adrenaline, ADP and collagen all lowered the level of cyclic AMP below that produced by Iloprost alone. However, in the presence of the feedback inhibitors, only adrenaline was able to lower cyclic AMP, indicating that the cyclic AMP-lowering effect of ADP is abolished by the feedback inhibitors and that the effect of collagen is due to secreted products.

DISCUSSION

We have previously suggested that experiments studying the interaction of platelets with collagen should concurrently measure adhesion and stimulus–response coupling, should distinguish the primary response to the agonist collagen from the secondary response induced by released ADP and thromboxane A_2, and should be conducted at a sufficiently high concentration of collagen to elicit signal transduction in a significant number of platelets [12]. For example, under conditions where positive feedback by released agonists is blocked, there is little change in $[Ca^{2+}]_i$ at 10 μg of collagen/ml where only 10% of the platelets adhere, but an appreciable change in $[Ca^{2+}]_i$ (220 nM above basal) at 100 μg of collagen/ml where 60% of the platelets adhere [11]. In the present studies, we show that the response to the primary agonist collagen is not inhibited by elevating the cyclic AMP level and, in fact, is somewhat accelerated.

In 1979, Haslam and co-workers [18] reported that collagen selectively increased the phosphorylation of polypeptides with M_r values of 47000 and 20000, and that pretreatment of platelets with the adenylate cyclase stimulator PGE_1 decreased both this phosphorylation and the release of 5-hydroxytryptamine. They speculated that, as the phosphorylation reactions and secretion are probably caused by an increase in $[Ca^{2+}]_i$, the PGE_1 may initiate processes that decrease $[Ca^{2+}]_i$, so inhibiting the Ca^{2+}-dependent phosphorylation reactions and secretion. Subsequently, Feinstein *et al.* [9] reported that thrombin produces a rapid increase in $[Ca^{2+}]_i$ to 1–2 μM, and that this rise is inhibited by the prior addition of PGD_2, PGE_1 or forskolin. Moreover, when added at the peak of the maximal response, adenylate cyclase stimulators caused a fall in $[Ca^{2+}]_i$ back to the original resting level, and this effect coincided with the reversal of thrombin-induced, Ca^{2+}-dependent protein phosphorylation. They too suggested that cyclic AMP-dependent reactions maintain low levels of $[Ca^{2+}]_i$, perhaps by promoting transport and/or binding of Ca^{2+}. Vasopressin, platelet-activating factor, the thromboxane mimetic U46619 and ADP have also been shown to evoke rapid increases in $[Ca^{2+}]_i$, and these increases were also inhibited by cyclic AMP [8,19]. The present studies indicate that the inhibitory effect of cyclic AMP on collagen-induced responses observed by Haslam *et al.* [18] was in fact due to inhibition of

increases in $[Ca^{2+}]_i$ induced by released ADP and thromboxane A_2, rather than to inhibition of the primary response to collagen. In additional experiments we have observed that collagen itself does induce the phosphorylation of 20000- and 47000-M_r polypeptides, but that this primary response to collagen is not decreased by cyclic AMP (J. B. Smith, unpublished work). On the other hand, cyclic AMP did inhibit the increase in $[Ca^{2+}]_i$ induced by thrombin even when it was studied in the presence of inhibitors of feedback pathways (Fig. 2). Interestingly, the elevation of $[Ca^{2+}]_i$ by thrombin was somewhat less than that reported previously [8], reflecting inhibition of the autocrine stimulation by ADP and thromboxane A_2.

Mazurov *et al.* [7] observed that some collagen preparations lowered elevated levels of cyclic AMP in platelets, while others did not. The ability to lower cyclic AMP seemed to correlate with the efficacy with which the collagen induced platelet aggregation and secretion. These experiments were performed in the presence of both indomethacin and phosphocreatine/creatine kinase. However, the decrease in cyclic AMP was minor and it was not demonstrated that the ability of ADP to lower cyclic AMP [20] was overcome by their phosphocreatine/creatine kinase combination. We speculate that the lowering of cyclic AMP observed by Mazurov *et al.* [7] was actually due to released ADP. The amount of creatine kinase used by these authors was five times less than that used in the present studies, and we have noticed that at lower concentrations of this enzyme the effects of ADP are not completely suppressed.

The fact that collagen can increase $[Ca^{2+}]_i$ even when cyclic AMP levels are elevated argues against the hypothesis that the major mechanism by which cyclic AMP acts is by promoting reactions that maintain low levels of $[Ca^{2+}]_i$. In this respect, collagen appears to be unique among the platelet agonists that have been studied. On the other hand, while increases in $[Ca^{2+}]_i$ induced by collagen were somewhat greater in the presence of the adenylate cyclase stimulators than in their absence, elevated $[Ca^{2+}]_i$ did return to basal levels significantly faster in cells with elevated cyclic AMP. The accelerated increase in $[Ca^{2+}]_i$ correlates with the enhanced adhesion that was observed, while the faster decrease may reflect an enhanced resequestration mechanism. The proposal that cyclic AMP-dependent reactions may act by suppressing phospholipase C [21] also does not seem valid for collagen, as elevated cyclic AMP did not suppress collagen-induced PtdOH formation in the present studies (Fig. 3). It is known that after thrombin stimulation the majority of this PtdOH contains stearic and arachidonic acids, indicating that it is formed by phosphorylation of diacylglycerol produced in the phospholipase C pathway [22]. Of course, it is possible that PtdOH formed in response to collagen may originate directly by a phospholipase D-type reaction. Furthermore, it is possible that different receptors are coupled to different phospholipase C isoenzymes, only some of which are substrates for cyclic AMP-dependent protein kinase. Alternate hypotheses [23] that may explain the ability of cyclic AMP-dependent reactions to suppress increases in $[Ca^{2+}]_i$ induced by thrombin, ADP and U46619, but not those induced by collagen, relate to receptors or guanine-nucleotide-regulatory proteins. In this regard, Lerea *et al.* [24] have shown that elevation of cyclic AMP decreases the number of high-affinity binding sites for thrombin, suggesting that the thrombin receptor may be down-regulated by phosphorylation mediated by protein kinase A. A possible reason for the discriminatory effects of cyclic AMP could be that the collagen receptor is thought to be a heterodimeric integrin [25], while those for thrombin [24] and thromboxane A_2 [26] possess seven putative transmembrane domains.

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REFERENCES

1. Higgs, G. A., Moncada, S., Vane, J. R., Caen, J. P., Michel, H. & Tobelem, G. (1978) Prostaglandins **16**, 17–22
2. Weiss, H. J. & Turrito, V. T. (1979) Blood **53**, 244–250
3. Cazenave, J. P., Dejana, E., Kinlough-Rathbone, R. L., Richardson, M., Packham, M. A. & Mustard, J. F. (1979) Thromb. Res. **15**, 273–285
4. Karniguan, A., Simmons, P., Legrand, Y. J., Moncada, S. & Caen, J. P. (1982) Prostaglandins **24**, 827–836
5. Shadle, P. A. & Barondes, S. H. (1982) J. Cell Biol. **95**, 361–365
6. Santoro, S. A. (1986) Cell **46**, 913–920
7. Mazurov, A. V., Misselwitz, F., Hoffman, U., Leytin, V. L. & Repin, V. S. (1988) Prostaglandins **35**, 51–65
8. Sage, S. O. & Rink, T. J. (1987) J. Biol. Chem. **262**, 16364–16369
9. Feinstein, M. B., Egan, J. J., Sha'afi, R. I. & White, J. (1983) Biochem. Biophys. Res. Commun. **113**, 598–604
10. Yamanishi, J., Kawahara, Y. & Fukuzaki, H. (1983) Thromb. Res. **32**, 183–188
11. Smith, J. B. & Dangelmaier, C. (1990) Anal. Biochem. **187**, 173–178
12. Smith, J. B., Dangelmaier, C., Selak, M. A. & Daniel, J. D. (1991) J. Cell Biochem. **47**, 54–61
13. Aster, R. H. & Jandl, J. H. (1964) J. Clin. Invest. **43**, 843–855
14. Lages, B., Scrutton, M. C. & Holmsen, H. (1975) J. Lab. Clin. Med. **85**, 811–825
15. Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) Biochem. J. **235**, 869–877
16. Costa, J. J. & Murphy, D. L. (1975) Nature (London) **255**, 405–407
17. Salomon, Y. (1979) Adv. Cyclic Nucleotide Res. **10**, 35–55
18. Haslam, R. J., Lynham, J. A. & Fox, J. E. B. (1979) Biochem. J. **178**, 397–406
19. Bushfield, M., McNichol, A. & MacIntyre, D. E. (1985) Biochem. J. **232**, 267–271
20. Macfarlane, D. E. & Mills, D. C. B. (1981) J. Cyclic Nucleotide Res. **7**, 1–11
21. Rink, T. J. & Sanchez, A. (1984) Biochem. J. **222**, 833–836
22. Mauco, G., Dangelmaier, C. A. & Smith, J. B. (1984) Biochem. J. **224**, 933–940
23. Rink, T. J. & Sage, S. O. (1990) Annu. Review Physiol. **52**, 431–449
24. Lerea, K. M., Glomset, J. A. & Krebs, E. G. (1987) J. Biol. Chem. **262**, 282–288
25. Staaz, W. D., Rajpara, S. M., Wayner, E. A., Carter, W. G. & Santoro, S. A. (1989) J. Cell Biol. **108**, 1917–1924
26. Vu, T. H., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. (1991) Cell **64**, 1059–1068
27. Hirata, H., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S. & Narumiya, S. (1991) Nature (London) **349**, 617–620

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