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Model of Prostaglandin-Regulated Cyclic AMP Metabolism in Intact Platelets: Examination of Time-Dependent Effects on Adenylate Cyclase and Phosphodiesterase Activities

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SUMMARY

The kinetics of prostaglandin-regulated cyclic AMP formation by intact human platelets were studied in the presence and absence of phosphodiesterase inhibitors. In the case of iloprost, a chemically stable analogue of prostaglandin I_2, the shape of the time course varied with prostaglandin concentration. In the presence of phosphodiesterase inhibitors, low concentrations of iloprost gave a linear rate of cyclic AMP formation. At higher concentrations of iloprost, the initial rate increased as a saturable function of prostaglandin concentration but the curves decayed with time to give new linear rates of cyclic AMP formation with a different prostaglandin concentration dependence from the initial rates. Time courses were simulated using KINSIM [*Anal. Biochem.* **130**:134–145 (1983)], a kinetic simulation program that employs numerical integration, over a wide range of iloprost concentration (3 nM to 30 μM) by use of a simple model involving rapid activation of adenylate cyclase, followed by slow reversible transition of adenylate cyclase to an inactive form (desensitization) through a distinct inhibitory receptor. The model requires that the affinity for prostaglandins of both the stimulatory and inhibitory receptors declines with prostaglandin concentration, which may be related to the existence of high and low affinity receptor forms depending on the activation state of the appropriate GTP-binding protein. The same two-receptor model can be used to describe cyclic AMP metabolism in the absence of phosphodiesterase inhibitors, giving rise to characteristic peak and plateau effects in the time courses. The putative inhibitory receptor has an apparent affinity for prostaglandin lower than the stimulatory receptor in the case of iloprost and a higher affinity than the stimulatory receptor in the case of prostaglandin E_1. The contribution of phosphodiesterase activation to the time course of cyclic AMP formation through phosphorylation by cyclic AMP-dependent protein kinase was assessed. It was shown that phosphodiesterase activation must be rapid. A plausible and perhaps complete description of prostaglandin-regulated cyclic AMP metabolism in platelets is presented.

In intact cells, cyclic AMP is formed through the action of adenylate cyclase and removed by various phosphodiesterase activities. In the continuous presence of a stimulatory agonist, the cyclic AMP level might be expected to rise to a plateau, representing a steady state between formation and disappearance of the compound. In fact, the time course of cyclic AMP formation in most cells is generally observed as a rise to a peak followed by a decline to a stable plateau level (1). This phenomenon reflects desensitization, because a second challenge with the same agonist results in a blunted response. Desensitization may result from time-dependent inhibition of adenylate cyclase or time-dependent activation of phosphodiesterase activity (2). We have previously suggested a novel mechanism of desensitization involving separate stimulatory and inhibitory prostaglandin receptors linked to regulation of platelet adenylate cyclase (3, 4). This conclusion was based on the pattern of cyclic AMP formation by platelet lysates and intact platelet in the absence and presence of phosphodiesterase inhibitors; prostaglandins induced a time-dependent inhibition of their own rapid activation of platelet adenylate cyclase that showed a different prostaglandin concentration dependence from activation. The idea of separate receptors is supported by direct radioligand binding studies from other laboratories that show two distinct binding sites for both PGE_1 and PGI_2 on platelet membranes (5–7).

Receptor-linked inhibition of adenylate cyclase implies the involvement of the guanine nucleotide-binding regulatory protein G_i. In most cells, the involvement of G_i can be examined by use of pertussis toxin; however, the toxin is not effecitve against intact platelets, which apparently lack the appropriate gangliosides for toxin entry. Jakobs and co-workers (8–10) have shown that phorbol ester treatment of platelets leads to protein kinase C-mediated phosphorylation of G_i and impairment of its function. We have shown that phorbol ester treatment also

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ABBREVIATIONS: PGE_1, prostaglandin E_1; G_s, stimulatory GTP-binding protein; G_i, inhibitory GTP-binding protein; PGI_2, prostaglandin I_2; IBMX, 3-isobutyl-1-methylxanthine; G protein, GTP-binding protein.

abolishes time-dependent inhibition induced by prostaglandins (4), allowing us to conclude that the putative inhibitory receptor acts through G_i.

The two-site model was quantitated to some extent using an integrated rate equation for desensitization derived by Barber *et al.* (1, 11) that describes the time course of cyclic AMP formation in terms of rapid activation of adenylate cyclase followed by agonist-induced, slow, reversible transition of adenylate cyclase to an inactive form. We modified the equation to include independent saturable effects of prostaglandins on stimulation and desensitization and obtained EC_{50} values for the two effects, which in the case of iloprost, a stable analog of PGI_2, differed by more than 1 order of magnitude (4). However, the model was not a complete description of cyclic AMP metabolism because there were marked discrepancies between constants obtained in the absence and presence of phosphodiesterase inhibitors, suggesting that time-dependent effects on phosphodiesterase activity also play a role in regulation of cyclic AMP metabolism. Indeed, Macphee *et al.* (12) and Grant *et al.* (13) have described a 1.5- to 11-fold increase in the activity of the major low K_m phosphodiesterase in platelets, evoked by prostaglandins or forskolin, that is apparently linked to a cyclic AMP-dependent phosphorylation of the enzyme. Time-dependent activation of phosphodiesterase activity would lead to a time-dependent fall in cyclic AMP level that may contribute to the time courses observed in the absence of phosphodiesterase inhibitors. We also pointed out that, in the absence of phosphodiesterase inhibitors, the integrated rate equation can only represent an approximation of the actual time course, because it requires the limiting assumption that the intracellular levels of cyclic AMP never exceed the K_m of platelet phosphodiesterases, whereas in fact the levels of cyclic AMP generated in prostaglandin-stimulated platelets exceed the K_m values of the known platelet phosphodiesterases; this limitation could contribute to the discrepancies observed in the absence and presence of phosphodiesterase inhibitors.

In the present work we have examined cyclic AMP metabolism by intact platelets, as a function of prostaglandin concentration, by use of KINSIM (14), a powerful kinetic simulation program that employs numerical integration. KINSIM was developed in the laboratory of Dr. Carl Frieden at Washington University in St. Louis and can be used to simulate mechanisms with as many as 40 individual kinetic steps. Use of numerical integration allows simulation without the simplifying assumptions used to write explicit integrated rate equations. Use of KINSIM also permits more flexibility in modeling time courses. In the present case we obtained better fits to the data by including steps in which the affinity of the stimulatory and inhibitory receptors for prostaglandins decreased with increasing prostaglandin concentration, in a manner suggesting negatively cooperative agonist binding to the two receptors. Apparent negative cooperativity is commonly observed in ligand binding to both stimulatory and inhibitory receptors in membrane preparations and reflects the fact that receptor-mediated activation of G proteins leads to dissociation of the receptor-G protein complex with a concomitant fall in receptor affinity for agonists.

In addition to simulations involving time-dependent inhibition of adenylate cyclase through a separate prostaglandin receptor, we have also simulated the effect of a time-dependent increase in phosphodiesterase activity engendered by phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. The present work is designed to assess the contribution of prostaglandin regulation of adenylate cyclase and phosphodiesterase activities to the time dependence of cyclic AMP metabolism.

Experimental Procedures

Materials

[^{3}H]Adenine (31 Ci/mmol) and [^{14}C]cyclic AMP (44 Ci/mmol) were obtained from ICN Biomedicals (Costa Mesa, CA). Iloprost was a gift of Berlex Laboratories, Inc. (Cedar Knolls, NJ). Forskolin was obtained from Calbiochem (La Jolla, CA). PGE_1, IBMX, and most other chemicals were from Sigma Chemical Co (St. Louis, MO).

Preparation of Platelets

Platelets were isolated from fresh human blood collected into acid/citrate/dextrose anticoagulant. All operations were performed at room temperature. Platelet-rich plasma was obtained as the supernatant after centrifugation at 100 × *g* for 15 min and was treated with 1 mM aspirin to prevent interference from endogenously produced prostaglandins and thromboxanes. Platelet-rich plasma was made 2 mM in EDTA and centrifuged at 100 × *g* for 10 min to remove contaminating red and white cells. The platelets in the supernatant were pelleted by centrifugation at 1000 × *g* for 15 min and washed by resuspension in Tris-buffered saline (40 mM Tris·HCl, 7.4, with 0.15 M sodium chloride) containing 2 mM EDTA, followed by centrifugation. Platelets were suspended in the same buffer at a concentration of 3×10^8 platelets/ml. Platelet count was determined on a Coulter counter.

Measurement of Cyclic AMP Formation

Cyclic AMP formation in intact platelets was measured by incubating platelet-rich plasma for 1 hr with 2 μCi of [^{3}H]adenine before washing. Time courses were generated at 25° by addition of prostaglandins or forskolin to a suspension of radiolabeled washed platelets, withdrawing 0.5-ml samples at appropriate times into 0.5 ml of stopping solution containing 0.6 M $HClO_4$ and 2% sodium dodecyl sulfate, with [^{14}C]cyclic AMP as a recovery standard. The cyclic AMP content of each sample was determined by the method of Salomon (15). Results are expressed as percentage of total radioactivity appearing as cyclic AMP. Where indicated, IBMX was added as a 500 mM solution dissolved in dimethyl sulfoxide. As indicated previously (4), little cyclic AMP leaked from the cells during the time course of the experiments reported here.

Computer Modeling of cyclic AMP Time Courses

Kinetic analysis by use of an integrated rate expression. The time course of cyclic AMP formation in the absence of desensitization can be described by the integrated rate equation:

$$[\text{cAMP}] = k_s/k_d(1 - e^{-k_d \cdot t}) \quad (1)$$

where k_s is the zero-order rate constant for cyclic AMP synthesis and k_d is the first-order rate constant for decay of cyclic AMP through phosphodiesterase activity. Eq. 1 assumes that [ATP] is always saturating with respect to adenylate cyclase and the level of cyclic AMP is always lower than the Michaelis constant of the phosphodiesterase.

Barber *et al.* (1, 11) modified Eq. 1 to include reversible, time-dependent, transformation of adenylate cyclase to an inactive form, according to the equilibrium:

$$\text{adenylate cyclase: active} \underset{k_n}{\overset{k_m}{\rightleftharpoons}} \text{adenylate cyclase: inactive}$$

where k_m is a rate constant for conversion to the inactive form and k_n is the rate constant for reversal of inactivation.

The time-course of cyclic AMP formation can then be described by the expression:

$$[\text{cAMP}] = k_s\left[\frac{k_n(1 - e^{-k_d \cdot t})}{(k_m + k_n)k_d} + \frac{k_m[e^{-(k_m + k_n)\cdot t} - e^{-k_d \cdot t}]}{(k_m + k_n)(k_d - k_m - k_n)}\right] \quad (2)$$

Eq. 2 was derived on the assumption that hormonal concentration is saturating for the desensitization process. We modified the expression for the case in which hormone is not saturating and for the case in which stimulation of adenylate cyclase and desensitization are mediated by separate receptors (4). In the modified expression, assuming rapid binding of prostaglandin to the stimulatory and inhibitory receptors, k_s and k_m are saturable functions of prostaglandin concentration, hence:

$$k_s = k_{s_{max}} \cdot P/(EC_{50_s} + P) \quad (3)$$

$$k_m = k_{m_{max}} \cdot P/(EC_{50_m} + P) \quad (4)$$

where $k_{s_{max}}$ and $k_{m_{max}}$ are the maximum values of k_s and k_m when the stimulatory and inhibitory sites are saturated, EC_{50_s} and EC_{50_m} are the EC_{50} values of the stimulatory and inhibitory prostaglandin site, respectively, and P is the concentration of prostaglandin. By analyzing the data with Eq. 2, we were able to determine values for the rate constants and EC_{50} values of the stimulatory and inhibitory processes.

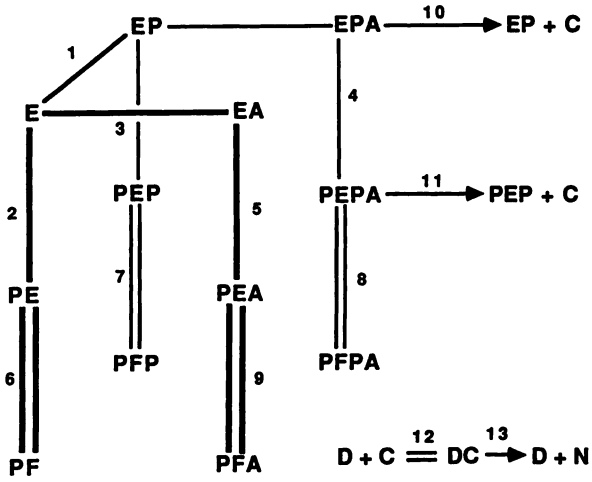
The description of cyclic AMP formation by Eq. 2 is incomplete because of the limiting assumptions used in the derivation of Eq. 1 that (a) the intracellular level of cyclic AMP is always well below the K_m for the phosphodiesterase and (b) there is only one phosphodiesterase activity. In fact, the intracellular level of cyclic AMP can rise to almost 2% of total adenine nucleotides in the presence of iloprost, corresponding to a concentration of around 150 μM, based on an estimate of about 7.5 mM for the concentration of total adenine nucleotides in the cytoplasmic pool of the platelet (16), while platelets possess at least two phosphodiesterases with K_m values of 0.18 and 50 μM. Consequently, Eq. 1 should be obtained from the expression:

$$\frac{d[\text{cAMP}]}{dt} = k_s - \frac{V_H[\text{cAMP}]}{K_H + [\text{cAMP}]} - \frac{V_L[\text{cAMP}]}{K_L + [\text{cAMP}]} \quad (5)$$

where V_H and V_L and K_H and K_L are the maximum velocities and K_m values of the high and low K_m phosphodiesterase activities. Eq. 5 cannot be explicitly integrated and, similarly, under these conditions it is impossible to obtain an integrated rate equation that includes desensitization. For this reason we have used computer simulation by numerical integration to obtain a more realistic description of cyclic AMP turnover.

Computer simulation of time courses by numerical integration. Simulations were performed on a Micro VAX computer (Digital Equipment Corporation) using KINSIM (14), a program written in FORTRAN, that was kindly provided by Dr. Carl Frieden, Washington University School of Medicine (St. Louis, MO). The mechanism is written as a series of chemical reactions represented either by a dissociation constant for rapid equilibrium steps or by forward and backward rate constants in kinetically determined steps. The computer generates a table of differential equations and performs numerical integration to yield the concentration of the designated species as a function of time.

Time-dependent inhibition of adenylate cyclase. Computer simulations involving time-dependent inhibition of adenylate cyclase were based on a model identical to that described for the integrated rate expression, except that the Michaelis constant for phosphodiesterase could be set at any value and other constants could be varied with respect to each other in a more flexible fashion. Hence, again the model involves rapid activation of adenylate cyclase followed by slow reversible transformation of adenylate cyclase to an inactive form in the presence of prostaglandins. The model is indicated in the scheme in Fig. 1. For simplicity, we treated the receptor/G protein/adenylate cyclase complex as a tightly coupled entity represented as a single enzyme species E. The enzyme E has a binding site for substrate ATP



E = Adenylate cyclase/receptor complex
A = ATP
C = cAMP
P = Prostaglandin
EP = Prostaglandin bound to a stimulatory site
PE = Prostaglandin bound to a slow-acting inhibitory site
F = Desensitized adenylate cyclase

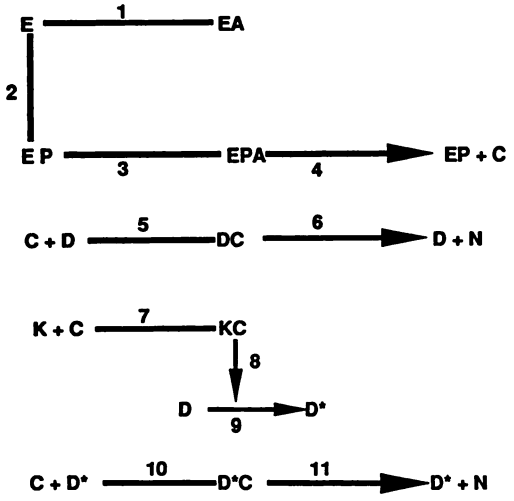
Fig. 1. Equilibria describing stimulation and desensitization of platelet adenylate cyclase in terms of two prostaglandin receptors. *Single lines* represent rapid equilibrium steps with values as dissociation constants, whereas *double lines* represent kinetically determined steps with forward and backward rate constants.

(A) as well as two distinct binding sites for prostaglandin (P). The species EA represents the enzyme substrate complex, which has negligible activity; binding of P to a stimulatory binding site gives rise to the species EP and subsequently to EPA, which is active, generating EP and cyclic AMP, represented as C. P can also bind to a separate inhibitory binding site to give the species PE. PE can slowly and reversibly convert to PF, generating the inactive enzyme species F. Once formed, cyclic AMP (C) is removed by phosphodiesterase (D) through the enzyme-substrate complex DC.

Time-dependent activation of phosphodiesterase. Computer simulations of a mechanism involving time-dependent activation of phosphodiesterase were based on the scheme in Fig. 2. Again, enzyme E can bind ATP (A) to give the nonproductive complex EA. Binding of prostaglandin P to E activates the enzyme to generate cyclic AMP, represented as C. Cyclic AMP can bind to phosphodiesterase (D) to be converted to AMP (N) and it can bind to cyclic AMP-dependent kinase (K) to give the active form of the kinase, KC. The activated kinase converts D to a more active form D^*, which acts on cyclic AMP with a greater V_{max} than D. In this mechanism, the time dependence stems from the time taken for the kinase to phosphorylate and activate the phosphodiesterase.

Results

Iloprost-regulated cyclic AMP formation in intact platelets in the presence of a phosphodiesterase inhibitor. Despite our ability to model cyclic AMP metabolism involving both adenylate cyclase and phosphodiesterase activities, we began by examining time courses of cyclic AMP formation in the presence of high concentrations of the phosphodiesterase inhibitor IBMX. These experiments eliminate both instantaneous and possible time-dependent effects of phosphodiesterase activity on the time courses and reduce the number of constants to be determined. Hence, in the presence



E = Adenylate cyclase/receptor complex
A = ATP
P = Prostaglandin
C = Cyclic AMP
D = Phosphodiesterase
D* = Phosphorylated PDE
K = cAMP Kinase
N = AMP

Fig. 2. Time-dependent activation of phosphodiesterase through a simplified mechanism representing phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. See text for details.

of phosphodiesterase inhibitors, k_d tends to zero and Eq. 2 reduces to:

$$[\text{cAMP}] = k_s\left[\frac{k_n t}{k_m + k_n} + \frac{k_m[e^{-(k_m + k_n)\cdot t} - 1]}{(k_m + k_n)(-k_m - k_n)}\right] \quad (6)$$

Eq. 6 represents a unique solution to the simple model proposed by Barber *et al.* (1, 11) in which rapid stimulation of adenylate cyclase is followed by slow reversible conversion of adenylate cyclase to an inactive form. Combined with Eq. 3 and 4, this equation can be used to express the prostaglandin concentration dependence of the time courses, assuming two classes of noninteracting sites, giving rise to the series of curves presented in Fig. 3a. For convenience, curves in Fig. 3a were generated using KINSIM with the constants given in Table 1A, under the conditions described, KINSIM and the integrated rate equation give identical sets of curves.

The simulated curves can be compared with real data presented in Fig. 3b, which shows actual time courses of cyclic AMP formation obtained at various concentrations of iloprost, a chemically stable analogue of PGI_2, using platelets incubated in the presence of 5 mM IBMX. The two sets of curves share several common features. (a) The rate of cyclic AMP is linear for many minutes at low concentrations of the prostaglandin. (b) The initial rate increases as a saturable function of iloprost concentration but higher concentrations induce a progressively greater degree of time-dependent inhibition of the rate of cyclic AMP formation. However, the simulated and real families of curves differ markedly in the apparent prostaglandin concentration dependence of the time-dependent inhibition; when the values of constants are chosen to give a reasonable fit at mid-range concentrations of prostaglandins (3.0 μM), then curves simulated at lower concentrations of prostaglandins show too

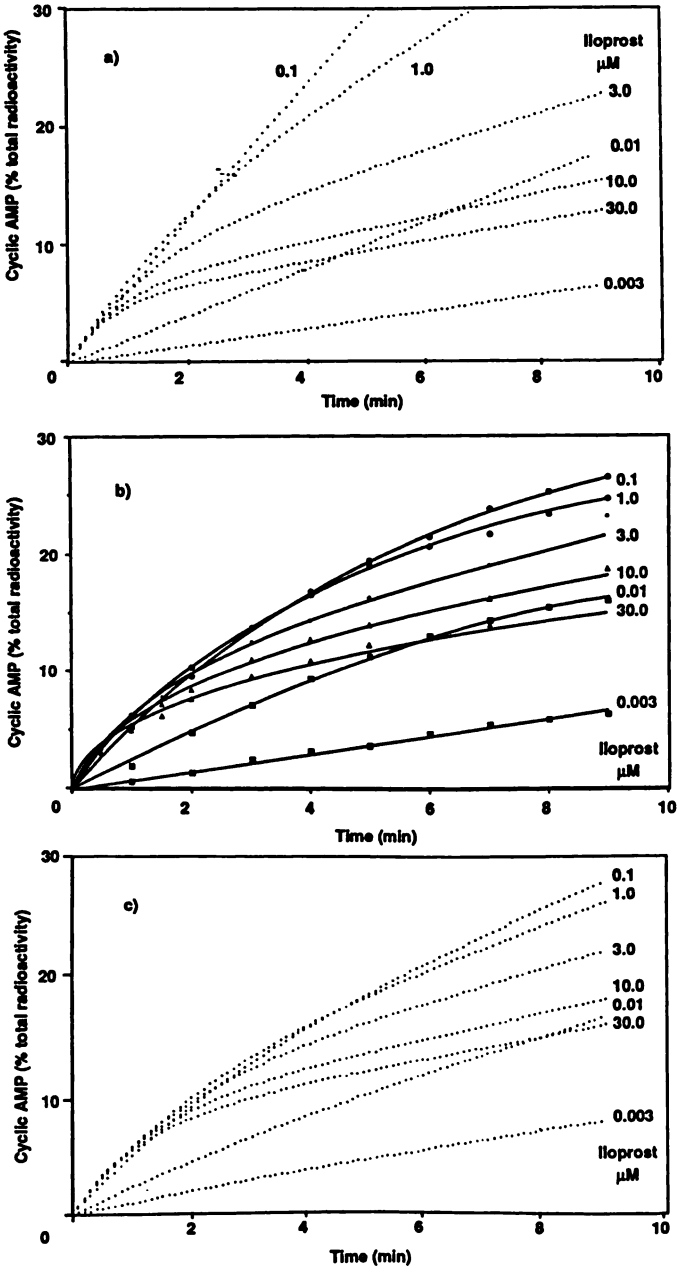


Fig. 3. Simulated and actual time courses of cyclic AMP formation in intact platelets as a function of iloprost concentration in the presence of 5 mM IBMX. The data in b were obtained as described under Experimental Procedures following treatment of platelets with 5 mM IBMX nominal concentration. Constants used in simulations in a and c using the scheme in Fig. 1 are given in Table 1A.

little inhibition, whereas curves at high prostaglandin concentration show too much inhibition (Fig. 3a).

Much better fits to the real data can be obtained by altering the values of the equilibrium constants governing Steps 2 and 5 by some factor with respect to Steps 3 and 4, as indicated in Table 1A. In other words, binding of the prostaglandin to the inhibitory site appears to weaken binding to the stimulatory site and vice versa. (To maintain thermodynamic equilibrium, the apparent dissociation constant for the step $PE + P = PEP$ would alter by the same factor with respect to $E + P = EP$.) The family of curves that can be obtained by this addition to the model (Fig. 3c) is virtually superimposable on the real data

TABLE 1

Constants used to simulate curves in Figs. 3 and 4

The step numbers in A are keyed to those in the scheme in Fig. 1. Single equal signs signify rapid equilibrium steps; double equal signs signify kinetically determined steps, where the first number refers to the forward rate constant and the second number refers to the reverse rate constant. All rate constants are in units of min^{-1}. Values given in units of percentage represent cyclic AMP concentrations expressed as a percentage of total adenine nucleotides, so that simulations were directly comparable to real data. ATP level was maintained at 100%, simulating the ability of the cell to replenish ATP through cellular metabolism. The K_m of the cyclase (i.e., the constant governing the steps $E + A = EA$ and $EP + A = EPA$) was set at 0.1%, a rather arbitrary value, the crucial point being that the cyclase is always saturated in substrate and cyclic AMP is generated in a zero-order manner. The concentration of adenylate cyclase (E) was set at 1×10^{-6} μM so that depletion of free A or P by binding to E was negligible; the rate of cyclic AMP formation is the product of E and the rate constant so that the latter was multiplied by 10^6 to compensate for the enzyme concentration. Similarly, the concentration of phosphodiesterase was set at 1×10^{-6} μM. In Fig. 3a, the apparent K_m of the phosphodiesterase is set at a very high value (1000%; approximately 75 mM) so that the simulation is directly comparable to that generated from Eq. 2. The simulations in Figs. 3a and 4a could also be performed using Eq. 6 or Eq. 2 with the constants shown in B.

A.	Step	Fig. 3a	Fig. 3c	Fig. 4a	Fig. 4c
1	$E + P = EP$	0.03 μM	0.021 μM	0.05 μM	0.017 μM
2	$E + P = PE$	4.0 μM	0.07 μM	4.0 μM	0.07 μM
3	$EP + P = PEP$	4.0 μM	5.0 μM	4.0 μM	5.0 μM
4	$EPA + P = PEPA$	4.0 μM	5.0 μM	4.0 μM	5.0 μM
5	$EA + P = PEA$	4.0 μM	0.07 μM	4.0 μM	0.07 μM
6	$PE == PF$	1.4; 0.15	0.95; 0.12	1.8; 0.1	0.95; 0.16
7	$PEP == PFP$	1.4; 0.15	0.95; 0.12	1.8; 0.1	0.95; 0.16
8	$PEPA == PFPA$	1.4; 0.15	0.95; 0.12	1.8; 0.1	0.95; 0.16
9	$PEA == PFA$	1.4; 0.15	0.95; 0.12	1.8; 0.1	0.95; 0.16
10	$EPA == EP + C$	4×10^6; 0	4×10^6; 0	2.4×10^6; 0	2.8×10^6; 0
11	$PEPA == PEP + C$	4×10^6; 0	4×10^6; 0	2.4×10^6; 0	2.8×10^6; 0
12	$D + C = DC$			1000%	1%
13	$DC == D + N$			8.5×10^6; 0	2.2×10^6; 0

B.	Constant	Fig. 3a	Fig. 4a
	EC_{50a}	0.03	0.05
	EC_{50m}	4.0	4.0
	k_{amax}	4.0	2.4
	k_{mmax}	1.4	1.8
	k_n	0.15	0.1
	k_d		0.85

over 4 orders of magnitude of prostaglandin concentration (3 nM to 30 μM).

At first sight the idea that agonist affinity decreases with increasing agonist concentration would seem to imply negative cooperativity between the stimulatory and inhibitory receptors; this is apparent from the way in which the model is presented. In fact, the same results would be obtained if the apparent negative cooperativity for both the stimulatory and inhibitory receptors were independent events. In this regard, it is generally observed in cell membrane preparations that agonist binding to both stimulatory and inhibitory receptors linked to adenylate cyclase exhibits apparent negative cooperativity, which is abolished in the presence of GTP, so that the binding curves shift to a low affinity form characterized by an apparent Hill coefficient of unity (17). The explanation for this phenomenon is that the receptor-G protein complex exhibits a high affinity for agonist whereas activation of the G protein upon binding GTP dissociates the complex to yield a receptor form with low affinity for agonist (17).

Cyclic AMP metabolism in intact platelets in response to iloprost. In the absence of phosphodiesterase inhibitors, cyclic AMP is metabolized to AMP and the shape of the time course changes. It is possible to model this situation by adding phosphodiesterase activity, so that time courses can be described by Eq. 2. Combining Eq. 2 with Eqs. 3 and 4 generates the family of curves shown in Fig. 4a, which can be compared

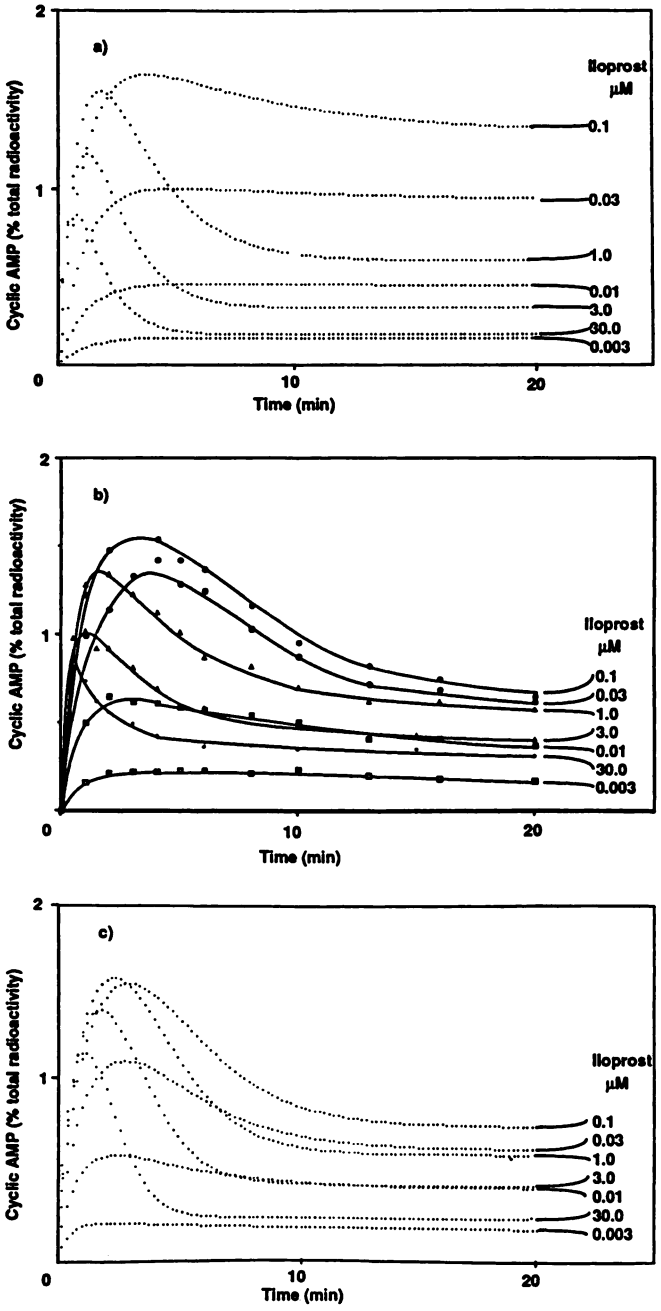


Fig. 4. Simulated and actual time courses of cyclic AMP formation in intact platelets as a function of iloprost concentration in the absence of IBMX. Constants used in simulations in a and c are given in Table 1A. The data in b were obtained as described under Experimental Procedures.

with real data in Fig. 4b. (Again, for convenience, simulations were performed using KINSIM under conditions that duplicate the integrated rate equation, using values shown in Table 1A.) The simulated curves clearly contain many of the features of the real data. (a) The shape of the time course varies with prostaglandin concentration. (b) At low concentrations of iloprost, the levels of cyclic AMP rise to a plateau and show little decline over 20 min. At higher concentrations of iloprost, the initial rate of cyclic AMP formation is faster than at low concentrations but is followed by a marked decline in cyclic AMP level to a stable plateau. (c) Peak and plateau effects increase with prostaglandin concentration to the point that the

curves cross. However, in the simulations, low concentrations of iloprost again give too little time-dependent inhibition while high concentrations give too much inhibition. The fits were not improved by lowering the K_m for phosphodiesterase activity to more realistic values.

Better fits again can be obtained by inserting apparent negative cooperativity into the agonist binding profile of both the stimulatory and inhibitory receptors. In addition, the K_m of the phosphodiesterase activity can be set at values that approximate those observed in platelets. In this case, the K_m was set at 1% of total platelet nucleotides, corresponding to about 75 μM. These changes in the values of the constants give the family of curves shown in Fig. 4c, which are a much closer approximation to the real data than the simulations presented in Fig. 4a. Note that we have not attempted to add a second phosphodiesterase activity, because we do not know the relative proportions of low and high K_m activities in the platelet.

Simulation of time-dependent phosphodiesterase activation and measurement of forskolin-stimulated cyclic AMP metabolism in intact platelets. Despite our demonstration that time-dependent inhibition of cyclic AMP formation takes place in the presence of phosphodiesterase inhibitors, indicating that a fall in adenylate cyclase activity is responsible, we also examined the effect of time-dependent activation of phosphodiesterase activity on the time-course of cyclic AMP metabolism. Macphee *et al.* (12) and Grant *et al.* (13) have shown that a low K_m platelet phosphodiesterase can be phosphorylated and activated 1.5-fold by prostaglandins (12, 13) and 11-fold by forskolin (13), generating cyclic AMP and activating cyclic AMP-dependent protein kinase. Time-dependent activation would presumably arise from the time taken to phosphorylate and activate the phosphodiesterase. A simple version of such a mechanism is shown in Fig. 2. Simulations performed using the scheme in Fig. 2 are presented in Fig. 5a, with time-dependent changes in cyclic AMP levels resulting from a 1.5-fold activation of phosphodiesterase activity, as indicated in Table 2. Values of constants were chosen so that the curves approximate the shape of the time courses observed with iloprost, although clearly this mechanism cannot account for all of the time-dependent fall in cyclic AMP levels because a time-dependent fall is observed in the presence of phosphodiesterase inhibitors. It also can be seen that, although it is possible to obtain a family of curves showing an increase in peak and plateau behavior with increasing prostaglandin concentration, the curves do not cross, so that again this mechanism cannot account for the pattern observed in Fig. 4b.

To determine the actual contribution of this mechanism to the shape of the time courses, we examined the time courses of cyclic AMP formation in intact platelets in response to various concentrations of forskolin (Fig. 5b). Forskolin is thought to act directly on the catalytic unit of adenylate cyclase and would be unlikely to act at stimulatory and inhibitory sites in the same manner as prostaglandins, so that a fall in cyclic AMP levels would probably reflect activation of phosphodiesterase. The time courses showed a rise to a plateau of cyclic AMP, with no subsequent fall in cyclic AMP levels, at all concentrations of forskolin tested. The results indicate that there is no time-dependent inhibition (desensitization) apparent with forskolin. Hence, although other investigators have shown that forskolin can induce phosphorylation and activation of platelet phosphodiesterase in the same manner as prostaglandins, any activation must be rapid, otherwise it would result in peak and plateau effects in the cyclic AMP time course similar to those apparent in the simulations in Fig. 5a.

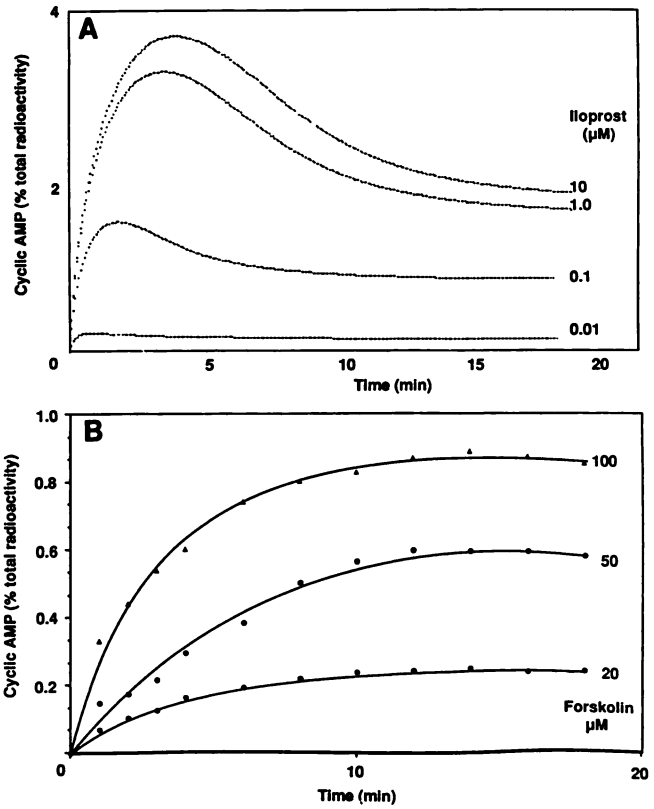


Fig. 5. Simulation of time-dependent phosphodiesterase activation and time course obtained with forskolin. Simulations in a were performed using the scheme in Fig. 2 and the constants shown in Table 2. The concentrations of adenylate cyclase, phosphodiesterase, and cyclic AMP-dependent protein kinase were set at 10^{-6} μM. Data in b were obtained as described in Experimental Procedures at the forskolin concentrations indicated.

TABLE 2

Constants used to simulate the curves in Fig. 3a

The step numbers are keyed to those in the scheme in Fig. 2. Single equal signs signify rapid equilibrium steps expressed as dissociation constants; double equal signs signify kinetically determined steps, where the first number refers to the forward rate constant and the second number the reverse rate constant. Values given in units of percentage represent cyclic AMP concentrations expressed as a percentage of total adenine nucleotides.

Step	Constant
1 $E + A = EA$	0.1%
2 $E + P = EP$	0.05 μM
3 $A + EP = EPA$	0.1%
4 $EPA == EP + C$	2.5×10^6; 0 min^{-1}
5 $C + D = CD$	1%
6 $DC == D + N$	3×10^6; 0 min^{-1}
7 $K + C = KC$	0.1%
8 $KC + D = KCD$	1×10^{-5}
9 $KCD == KC + D^*$	10; 0 min^{-1}
10 $C + D^* = D^*C$	1%
11 $D^*C == D^* + N$	4.5×10^6; 0 min^{-1}

Discussion

The aim of the present work is to provide a detailed model of prostaglandin-regulated cyclic AMP in intact platelets, based on a less exact model that we have previously proposed (3, 4).

In the current study, we have built our model from the simplest case and added to it with minimal requirements necessary to incorporate all the features of the actual data. Hence, at low concentrations of iloprost, in the presence of a high concentration of a phosphodiesterase inhibitor, the formation of cyclic AMP is linear and can be described by a single zero-order rate constant representing prostaglandin-stimulated adenylate cyclase activity at saturating substrate concentration. It is clear that the initial rate of cyclic AMP formation increases as a saturable function of prostaglandin concentration, reflecting saturation of a stimulatory prostaglandin receptor. However, high concentrations of prostaglandin cause a time-dependent decrease in the rate of cyclic AMP formation to reach a new linear rate. The decrease in rate can be attributed to a time-dependent decrease in adenylate cyclase activity, because phosphodiesterase activity is inhibited, and it can be described as desensitization, because a second challenge with prostaglandins results in an attenuated response (18).

The extent of inhibition increases with iloprost with a different concentration dependence from stimulation of cyclic AMP formation. The simplest way to account for the difference in prostaglandin concentration dependence of stimulation and inhibition is to propose separate receptors. If there are two receptors, then stimulation and inhibition may be described by different EC_{50} values. However, it was apparent that treating stimulation and inhibition as simple, independent, saturable processes was not sufficient to describe the experimental data in anything other than general terms. A much more precise fit to the data was obtained by assuming that the equilibria governing the concentration dependence of stimulation and inhibition exhibit apparent negative cooperativity. At first sight, this appears to be a major addition to the model, implying that the stimulatory and inhibitory sites interact with one another. However, the apparent negative cooperativity at each receptor can also be treated as independent events and perhaps can be explained in terms of current models of agonist/receptor/G protein interaction. Hence, apparent negative cooperativity has been observed in agonist binding to both stimulatory and inhibitory receptors in many membrane systems (17). In the absence of GTP, the binding curve is shallow, with an apparent Hill number less than 1; addition of GTP converts the curve to a weak binding form with a Hill number of 1. The explanation for this phenomenon is that the receptor-G protein complex binds agonist tightly, whereas the receptor uncoupled from G protein binds agonist weakly; activation of the G protein by exchange of GDP for GTP results in dissociation of the receptor-G protein complex and a fall in agonist affinity (17). In intact cells, high and low affinity states have been defined for certain agonists; in some cases the transition between the two states occurs with time and not agonist concentration (19, 20) and in other systems multiple affinity states are apparent as cooperativity binding (21). Overall, it seems reasonable to propose that G protein interaction is responsible for the apparent fall in affinity of both stimulatory and inhibitory receptors observed in the current work, reflecting the steady state concentration of the receptor-G protein complex responsible for effects on adenylate cyclase activity.

The model of cyclic AMP formation can be extended to include phosphodiesterase activity, yielding peak and plateau effects similar to those actually observed. By use of numerical integration with KINSIM, the K_m value of phosphodiesterase activity may be set to values closer to those observed *in vivo*. Although it is possible to include more than one phosphodiesterase activity in the simulation, the situation is too unwieldy to obtain meaningful values. Although other authors (12, 13) have shown that a platelet phosphodiesterase can be activated by phosphorylation due to prostaglandin- or forskolin-mediated activation of cyclic AMP-dependent kinase, we show here that such activation must be rapid and cannot contribute markedly to the time-dependent fall in cyclic AMP levels observed in intact platelets. Indeed, direct measurements of prostaglandin-mediated activation of phosphodiesterase indicate that it is essential complete within 1 min (22). However, phosphodiesterase activity may well vary with prostaglandin concentration in a rapid fashion and may account for the fact that the fits in Fig. 4c are not superimposable on the experimental data in Fig. 4b. It would be a simple matter to combine the schemes in Figs. 1 and 2 to account for changes in phosphodiesterase activity but, because we do not have precise values of the relevant constants, the simulations would be rather meaningless.

We have previously shown that PGE_1 gives marked peak and plateau effects in cyclic AMP formation in intact platelets. In contrast to iloprost, the shape of the time course did not change with prostaglandin concentration, although the initial rate, peak maximum, and final steady state levels all increased as a saturable function of PGE_1 concentration. Analysis of the time courses in terms of a two-receptor model showed that the EC_{50} and maximal extent of stimulation by PGE_1 were similar to those for iloprost, whereas the EC_{50} for inhibition was lower than for stimulation (4). Based on a simple two-site model, in which saturation of stimulatory and inhibitory receptors by either prostaglandin results in the same final extent of activation and inhibition, it is possible to predict that saturating inhibition, levels of iloprost should give rise to the same time course as saturating levels of PGE_2. This prediction is borne out in Fig. 6. Hence, curves obtained at 0.03 μM PGE_1 or iloprost bear no resemblance to each other, whereas curves obtained at 0.3 μM are more similar and curves at 30 μM are virtually superimposable.

Taken together, all of our findings can be summarized in the model shown in Fig. 7. In this model, PGE_1 and iloprost (or authentic PGI_2) bind equally well to a stimulatory receptor coupled to G_s, leading to rapid activation of adenylate cyclase. PGE_1 binds very tightly to an inhibitory receptor, whereas iloprost binds weakly to the inhibitory receptor. Activation of the inhibitory receptor results in slow inhibition of adenylate cyclase. We suspect that slow inhibition results from a slow

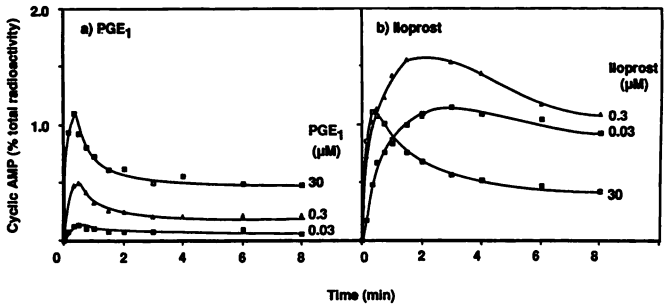


Fig. 6. Time course of cyclic AMP formation in intact platelets challenged with various concentrations of PGE_1 or iloprost. Time courses were obtained as described in Experimental Procedures at the prostaglandin concentrations indicated.

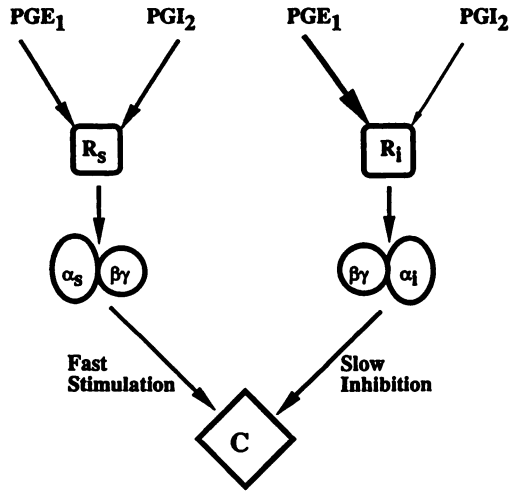


Fig. 7. Model of prostaglandin regulation of platelet adenylate cyclase. Prostaglandins bind to stimulatory and inhibitory receptors with relative affinities indicated by the *thickness of the arrows*. Authentic PGI_2 gave identical results to iloprost in kinetic studies and is, therefore, indicated in this scheme.

rearrangement of the equilibrium between the protein components of the adenylate cyclase system, including the α and $\beta\gamma$ subunits of G_s and G_i, rather than a phosphorylation event. Support for this mechanism comes from studies with platelet lysates in which we showed that time-dependent inhibition was only apparent in the presence of nonhydrolyzable GTP analogues (3). In addition, Katada *et al.* (23) have shown that the purified β-subunit of G_i can cause slow inhibition of adenylate cyclase in platelet membranes.

Support for the two-receptor model comes from our studies (24) with the compound pinane thromboxane A_2. This compound, which is a putative thromboxane antagonist, acts also as an agonist to inhibit platelet adenylate cyclase in a time-dependent fashion and appears to act exclusively at the putative prostaglandin inhibitory site, with little or no overlap at the stimulatory site. In addition, Sonnenburg and Smith (25) have proposed that there are distinct stimulatory and inhibitory prostaglandin receptors on renal tubules. Their proposal was not based on mathematical modeling but on the physiological observation that low concentrations of PGE_1 inhibit arginine-vasopressin-induced water reabsorption through G_i-mediated inhibition of adenylate cyclase, whereas higher concentrations of PGE_1 cause G_s-mediated activation of adenylate cyclase, presumably causing water reabsorption. These findings also argue for a receptor-linked mechanism for G_i activation that clearly differs in agonist affinity from G_s activation.

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