0026-895X/90/010046-08$02.00/0

MOLECULAR PHARMACOLOGY, 38:46-53

Novel Mechanism of Heterologous Desensitization of Adenylate Cyclase: Prostaglandins Bind with Different Affinities to Both Stimulatory and Inhibitory Receptors on Platelets

BARRIE ASHBY

Department of Pharmacology and Thrombosis Research Center, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140

Received December 26, 1989; Accepted March 30, 1990

SUMMARY

Prostaglandins E_1, I_2, and D_2 (PGE_1, PGI_2, and PGD_2) all stimulate and desensitize platelet adenylate cyclase, giving rise to peak and plateau effects in the time course of cyclic AMP metabolism in the intact cell. The peak and plateau effects vary with prostaglandin concentration to a different extent for each prostaglandin. However, at high concentrations, all prostaglandins give rise to the same time course of cyclic AMP formation. Differences in the extent of activation and desensitization can be modeled in terms of distinct stimulatory and slow-acting inhibitory receptors that differ in affinity for each prostaglandin but lead to the same maximum extent of activation and desensitization for all prostaglandins. The affinity for the stimulatory receptor is in the order $PGI_2 > PGE_1 \gg PGD_2$; the affinity for the inhibitory receptor is in the order of $PGE_1 > PGI_2 \gg PGD_2$. In addition, the inhibitory receptor binds PGE_1 more tightly than the stimulatory receptor, whereas in the case of PGI_2 or PGD_2, the stimulatory receptor binds agonist more tightly than the inhibitory receptor. It is shown that the model gives rise to heterologous desensitization such that PGE_1 readily inhibits PGI_2- and PGD_2-stimulated cyclic AMP formation, because it has high affinity for the inhibitory receptor. At the same time, because the final steady state concentration of cyclic AMP depends on the fractional occupancy of both the stimulatory and inhibitory receptors, PGE_1 can cause either a rise or fall in cyclic AMP level, depending on the concentration of PGI_2 or PGD_2 used to challenge the platelets before PGE_1 addition. The presence of a distinct inhibitory receptor may represent a general mechanism of autocoid desensitization, buffering cellular response against transient localized increases in agonist concentration that may occur when agonists are produced close to their sites of action.

Desensitization of adenylate cyclase is commonly observed in many cell types in response to hormones or autocoids. Desensitization can be observed in intact cells in the time course of cyclic AMP formation, where it is manifested as a peak and plateau effect (1, 2) distinct from the rise to a simple plateau in the absence of desensitization that represents the steady state between cyclic AMP formation and hydrolysis. Based on observations of the time course of cyclic AMP formation in platelets, we have suggested a mechanism of desensitization involving separate stimulatory and inhibitory prostaglandin receptors (3–7). Hence, prostaglandins induce a time-dependent inhibition of their own rapid activation of platelet adenylate cyclase that shows a different prostaglandin concentration dependence from activation (3–7).

The mechanism is supported by radioligand binding studies from other laboratories that indicate that there are two classes of prostaglandin binding sites on platelet membranes (8–10). The mechanism is further supported by our ability to model cyclic AMP metabolism in intact platelets as a function of prostaglandin concentration (6) using KINSIM, a powerful kinetic simulation program (11). Time courses could be simulated over a wide range of prostaglandin concentrations by use of a simple scheme involving rapid activation of adenylate cyclase, followed by slow reversible transition of adenylate cyclase to an inactive form (desensitization) through a distinct inhibitory receptor (6). Time-dependent activation of phosphodiesterase activity is not involved in desensitization (6).

We further concluded that, whereas the stimulatory receptor is linked to adenylate cyclase through the G protein G_s, the putative inhibitory receptor acts though G_i to bring about time-dependent inhibition, possibly by time-dependent rearrangement of the equilibrium between the subunits of the G proteins and the catalytic unit of adenylate cyclase (7). In most cells, the role of G_i can be probed by use of pertussis toxin; however, the toxin is not effective against intact platelets. Jakobs and associates (12–14) showed that phorbol ester treatment of intact platelets leads to protein kinase C-mediated phosphorylation of G_i and impairment of its function. We have

This work was supported by National Institutes of Health Grant HL 36579.

ABBREVIATIONS: G protein, GTP-binding protein; G_s, stimulatory GTP-binding protein; G_i, inhibitory GTP-binding protein; PGE_1, prostaglandin E_1; PGI_2, prostaglandin I_2; PGD_2, prostaglandin D_2.

shown (4) that phorbol ester also abolishes time-dependent inhibition induced by prostaglandins, indicating the involvement of G_i.

PGE_1, PGI_2, and PGD_2 all stimulate platelet adenylate cyclase; however, the time course of cyclic AMP formation in intact platelets is different for each prostaglandin (15–17). Peak and plateau effects are observed at all concentrations of PGE_1, whereas low concentrations of PGI_2 (or its stable analog iloprost) give little desensitization and high concentrations of PGI_2 give more desensitization. PGD_2 gives a pattern of cyclic AMP formation similar to that of PGI_2, except that the highest level of cyclic AMP attained is much lower in the case of PGD_2. The two-receptor model may explain the difference in response in terms of differences in affinity of the stimulatory and inhibitory receptors for each prostaglandin.

Desensitization is generally divided into two major types; homologous desensitization refers to cases in which only stimulation to the desensitizing agonist is attenuated, whereas heterologous desensitization refers to diminished responsiveness to other agonists as well. Because our model is based on the idea that desensitization occurs through turn-on of G_i, it would be expected to be a mechanism of heterologous desensitization towards all receptors linked to stimulatory agonists, because G_i-mediated inhibition should act to inhibit all G_s-stimulated events. Hence, treatment with PGE_1 should lead to an attenuated response to both PGI_2 and to PGD_2. However, previous work by Miller and Gorman (15) indicated that this does not occur. These authors concluded that, whereas PGE_1 desensitizes towards PGI_2, it does not desensitize towards PGD_2; similarly, PGD_2 appears to desensitize towards itself but not towards PGE_1 or PGI_2. They described the phenomenon as "cross-desensitization," which is different from either homologous or heterologous desensitization, and suggested that it arises because PGI_2 and PGE_1 share a common binding site, whereas PGD_2 binds to a separate site. Similar conclusions were reached by Mills and Macfarlane (16).

In this paper, we have reexamined the question of cross-desensitization of prostaglandins in platelets and we show that PGE_1 does indeed desensitize to PGD_2 in a heterologous fashion and that high concentrations of PGD_2 can desensitize towards PGI_2. In place of PGI_2, we have used its chemically stable analog iloprost. Similar results were obtained with authentic PGI_2. Our criteria for desensitization are that either a prostaglandin leads to an attenuated response to a second prostaglandin when added some time before the second prostaglandin or the desensitizing prostaglandin causes a fall in the steady state level of cyclic AMP when added to a prostaglandin-stimulated time course already in progress. These are essentially the same criteria used by Miller and Gorman (15) to define cross-desensitization by prostaglandins. We show that the idea of separate stimulatory and inhibitory prostaglandin receptors, each with different affinities for different prostaglandins, offers a clear explanation for the discrepancy between our findings and those of Miller and Gorman (15) and Mills and Macfarlane (16) and that heterologous desensitization can be observed among all of the prostaglandins at certain concentrations predicted by the model.

Experimental Procedures

Materials. [2,8-^{3}H]Adenine (31 Ci/mmol) and [^{14}C]cAMP (44 Ci/mmol) were obtained from ICN Biomedicals (Costa Mesa, CA). The stable PGI_2 analog iloprost was a gift of Berlex Laboratories, Inc. (Cedar Knolls, NJ). PGE_1 and PGD_2 were obtained from Sigma.

Preparation of platelets. Platelets were isolated and washed as previously described (6). Platelets were isolated from fresh human blood collected into acid/citrate/dextrose anticoagulant. Platelet-rich plasma was treated with 1 mM aspirin, to prevent interference from endogenously produced prostaglandins and thromboxanes, and was made 2 mM in EDTA before centrifugation 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, pH 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 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]cAMP as a recovery standard. In some experiments, a second prostaglandin was added to the platelet suspension 2 min after addition of the first prostaglandin. Sampling was continued to examine the modified time course. Time courses obtained at different concentrations in the same experiment were obtained simultaneously by sampling from separate reaction mixtures at 10-sec intervals. The cyclic AMP content of each sample was determined by the method of Salomon (18). Results are expressed as percentage of total radioactivity appearing as cyclic AMP. As indicated previously (4), little cyclic AMP leaked from the cells during the time course of the experiments reported here.

Computer simulation of cyclic AMP time courses. Simulations were performed on a MicroVAX computer (Digital Equipment Corporation) using the program KINSIM (11), which was kindly provided by Dr. Carl Frieden, Washington University School of Medicine (St. Louis, MO).

We have previously described a model of prostaglandin regulation of platelet adenylate cyclase (6). We have extended the model to incorporate the following features.

1. Prostaglandins act through stimulatory receptors coupled to G_s to activate adenylate cyclase. All prostaglandins are full agonists, causing the same maximal extent of activation of adenylate cyclase.

2. The affinity of the stimulatory receptor for agonists varies with the prostaglandin. In order of descending affinity, PGI_2 (iloprost) > $PGE_1 \gg PGD_2$.

3. Prostaglandins act through receptors distinct from the stimulatory receptor to bring about time-dependent inhibition (desensitization) of adenylate cyclase. The inhibitory receptor is coupled to adenylate cyclase inhibition through G_i, converting adenylate cyclase to an inactive form in a reversible manner. All prostaglandins cause the same maximal extent of inhibition.

4. The affinity of the inhibitory receptor varies with the prostaglandin. In order of descending affinity, $PGE_1 \gg PGI_2$ (iloprost) > PGD_2.

5. Binding of prostaglandins to either stimulatory or inhibitory receptors shows an apparent 50-fold decrease in agonist affinity throughout the binding curve, perhaps reflecting the steady state concentration of the receptor-G protein complex responsible for effects on adenylate cyclase activity (6, 19).

6. Time-dependent activation of phosphodiesterase activity is not involved in generating the peak and plateau effect (6).

The mechanism is presented formally in the Appendix, together with a table of equilibria describing the mechanism and the values of constants used in simulations. It is important to note that, in simulating the response to the three prostaglandins, PGE_1, PGI_2 (iloprost), and PGD_2, the only parameters that are altered are the binding constants for the stimulatory and inhibitory receptors. All other constants, in-

cluding maximum rate constants for cyclic AMP formation and desensitization, are the same for all prostaglandins.

The model provides a mechanism of heterologous desensitization among the prostaglandin as follows. The ratio of active to inactive adenylate cyclase depends on the fractional occupancy of the stimulatory and inhibitory receptors as well as the time of exposure to the agonist. After a long enough period of time (several minutes), the ratio depends solely on fractional occupancy of the two receptors. At saturation of both types of receptor, all prostaglandins give rise to the same level of adenylate cyclase activity, which is reflected in the steady state level of cyclic AMP. At other concentrations of prostaglandins, the shape of the time course and the final steady state level of cyclic AMP depend on the affinity of individual prostaglandins for the stimulatory and inhibitory receptors. When two prostaglandins are added to the same reaction mixture, either at the same time or at separate times, the shape of the time course and the final steady state level of cyclic AMP represent contributions from each prostaglandin. Hence, incubation of platelets with a prostaglandin that binds tightly to the inhibitory receptor will lead to apparent desensitization to a second prostaglandin because adenylate cyclase is already inhibited. The clearest way to see the effect of combinations of prostaglandins added together or separately is through simulations using the model described in the Appendix and developed in detail in Ref. 6. The following results describe real data obtained with various combinations of prostaglandins, together with simulations based on the two-receptor model.

Results

Time course of cyclic AMP formation in response to various concentrations of PGE_1, iloprost, or PGD_2. Time courses of cyclic AMP formation at four different concentrations each of PGE_1, iloprost, or PGD_2 are shown in Fig. 1A. In the case of PGE_1, the shape of the time course was the same at all concentrations, although the initial rate, peak maximum, and final steady state level of cyclic AMP formation all increased as a saturable function of PGE_1 concentration. In the case of iloprost, the initial rate increased as a function of prostaglandin concentration but time-dependent inhibition also increased to the point that the curves crossed each other. PGD_2 showed a less marked increase in initial rate with prostaglandin concentration and a less marked increase in time-dependent inhibition. At a concentration of 30 μM of each prostaglandin, the time courses of cyclic AMP formation were similar to each other.

We have previously postulated that the difference in response to PGE_1 and iloprost reflects differences in affinity of stimulatory and inhibitory receptors for the two prostaglandins (6). We now apply the same argument to the pattern of curves obtained with PGD_2. We suggest that PGD_2 can stimulate and inhibit platelet adenylate cyclase to the same maximal extent as both PGE_1 and iloprost but the affinity of the prostaglandin receptors for PGD_2 is much weaker than for the other prostaglandins. Evidence to support this suggestion comes from the simulations in Fig. 1B, showing that the families of curves may be modeled by this mechanism. Further support comes from the observation that a high concentration of PGD_2 gave a time course similar to that of high concentrations of PGE_1 or iloprost.

The values of the stimulatory and inhibitory receptor binding constants used in the simulations for the three prostaglandins are indicated in Table 1. Other constants are given in the Appendix, in Table 2.

We examined the consequences of the model on the pattern of cyclic AMP formation obtained with combinations of prostaglandins added some minutes apart. Our findings extend the work of Miller and Gorman (15) and provide us with data to analyze within the framework of our model.

Addition of PGE_1 at various concentrations to a PGD_2-stimulated cyclic AMP progress curve. PGD_2 (1 μM) was

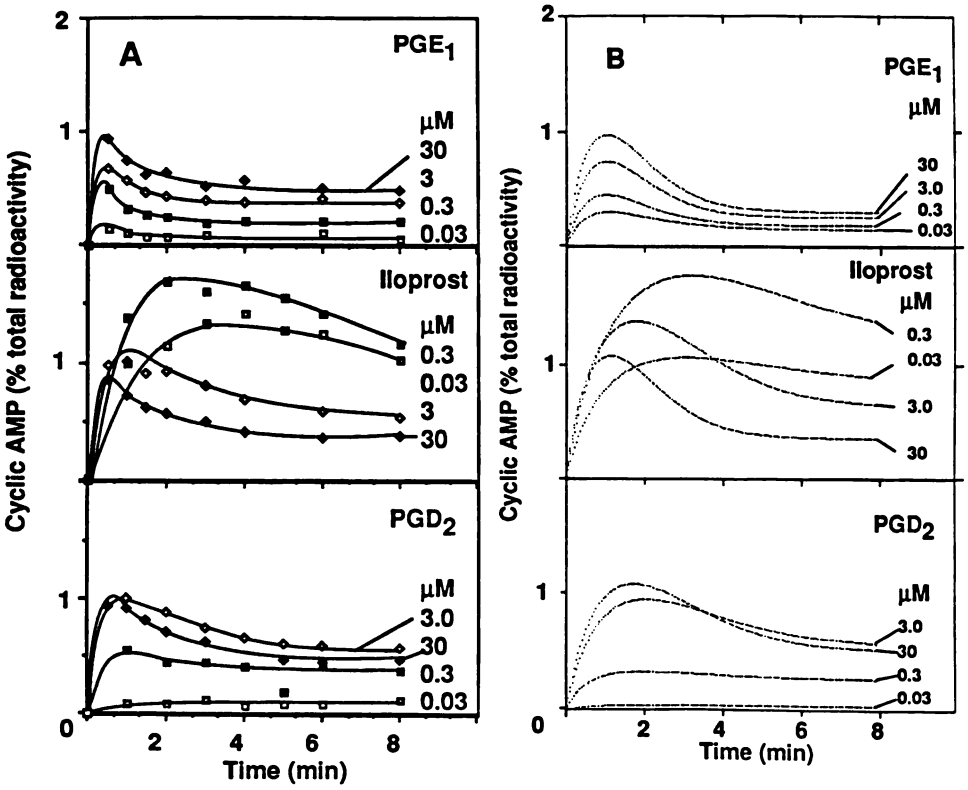

Fig. 1. Real and simulated time course of cyclic AMP formation in intact platelets challenged with various concentrations of PGE_1, iloprost, or PGD_2. A, Real data were obtained as described in Experimental Procedures at the prostaglandin concentrations indicated. B, Simulations were performed as described in the Appendix, using the constants given in Table 2 and the values of affinity constants for each prostaglandin indicated in Table 1.

TABLE 1

Apparent dissociation constants for binding of prostaglandins to the stimulatory and inhibitory receptors

The constants given in the table are apparent dissociation constants for the tight binding forms of the stimulatory and inhibitory receptors. The model elaborated in Ref. 6 requires two binding constants for each receptor. The first binding constant represents tight binding to a receptor-G protein complex; the second binding constant (about 50 times weaker binding) represents binding to the receptor uncoupled from activated G protein. The idea of high and low affinity states stems from the work of Stadel *et al.* (19). It is tempting to describe each binding constant as a true thermodynamic equilibrium constant for binding to the two receptor states, especially because the constants used in the simulations turn out to be very similar to those determined from radioligand binding studies (8–10). However, receptor coupling is a complex, multistep event with unknown stoichiometry of interaction between receptors and G proteins, so that the apparent dissociation constants represent complex constants.

Prostaglandin	Apparent dissociation constant	
	Stimulatory receptor	Inhibitory receptor
	μM	
PGE_1	0.03	0.02
Iloprost (PGI_2)	0.02	0.1
PGD_2	0.8	2.0

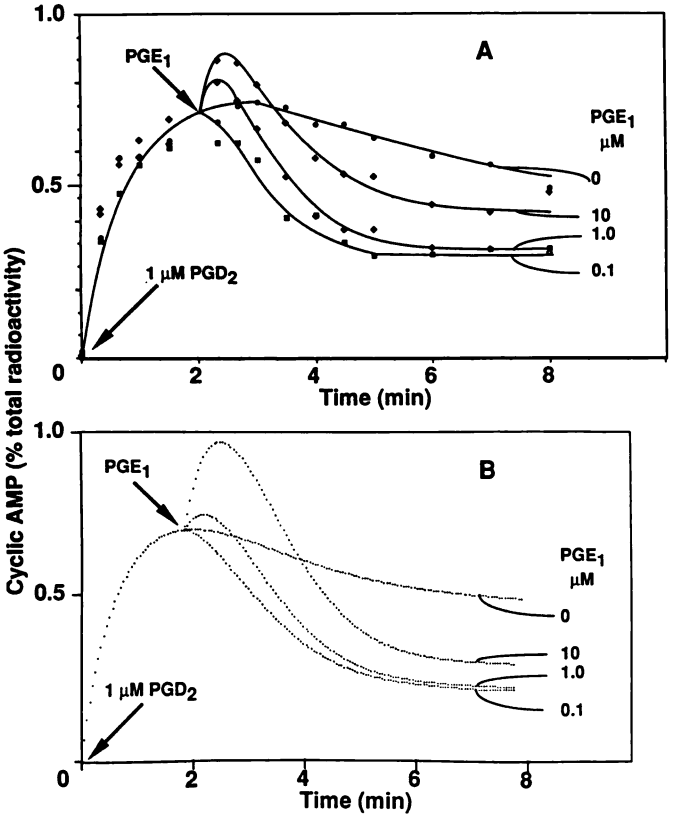

Fig. 2. Effect of addition of PGE_1 at various concentrations to a PGD_2-stimulated cyclic AMP progress curve. A, Real data were obtained as described in Experimental Procedures, with reactions being initiated by addition of 1 μM PGD_2. After 2 min, PGE_1 was added at the concentrations indicated. B, Simulations were performed as described in the Appendix, using the constants given in Tables 1 and 2.

added to radiolabeled platelets and the time course of cyclic AMP was monitored over 8 min (Fig. 2A). In parallel experiments, various concentrations of PGE_1 were added to the platelet suspension 2 min after initiation of the reaction with PGD_2 and the time course of cyclic AMP formation was again monitored (Fig. 2A). Addition of 0.1 μM PGE_1 resulted in a rapid drop in cyclic AMP to a new steady state level. Addition of 1.0 μM PGE_1 led to a transient increase in cyclic AMP followed by a fall to a steady state level above that observed with 0.1 μM PGE_1. Similarly, 10 μM PGE_1 gave a transient increase followed by a fall to a steady state level above that observed at 1.0 μM PGE_1. Clearly, PGE_1 inhibits PGD_2-stimulated cyclic AMP formation but inhibition is more pronounced at low rather than high concentrations of PGE_1. It is obvious that PGE_1 cannot be simply acting as a partial agonist at the stimulatory PGD_2 receptor, because the degree of inhibition would then increase with PGE_1 concentration. The observed result can be readily explained by tight binding of PGE_1 to a distinct inhibitory receptor to lower the cyclic AMP level, together with weaker PGE_1 binding to the stimulatory receptor to raise the steady state level. The family of curves can be simulated using the two-receptor model (Fig. 2B).

Addition of PGE_1 to an iloprost-stimulated cyclic AMP progress curve. Addition of 0.1 μM iloprost to intact platelets resulted in a rapid increase in cyclic AMP formation followed by a slow decline (Fig. 3A). Addition of 10 μM PGE_1 several minutes after 0.1 μM iloprost led to a rapid decline in the cyclic AMP level to a plateau level well below that observed in the presence of iloprost alone. In contrast, addition of 0.003 μM iloprost to platelets led to a small rise in cyclic AMP to a plateau level, whereas addition of PGE_1 after 2 min resulted in a rapid rise in the cyclic AMP level to a peak followed by a decline to a stable plateau level that was higher than that observed in the presence of 0.003 μM iloprost alone. The two sets of curves can be simulated using the two-receptor model (Fig. 3B).

Addition of PGD_2 to an iloprost-stimulated cyclic AMP progress curve. Miller and Gorman (15) indicate that, whereas PGE_1 can inhibit PGI_2 (iloprost)-stimulated cyclic AMP formation, PGD_2 cannot. We have shown here that the pattern of cyclic AMP formation reflects the affinity of separate stimulatory and inhibitory receptors for each prostaglandin. Because PGD_2 causes inhibition of its own stimulation, it would be expected to inhibit iloprost-stimulated cyclic AMP formation as well. Fig. 4A shows that high concentrations of PGD_2 do inhibit. The curves are simulated in Fig. 4B. In contrast to PGE_1 inhibition observed in Fig. 2, the extent of inhibition increased with PGD_2 concentration. This is because the stimulatory receptor is already virtually saturated with iloprost, so that PGD_2 makes little contribution to stimulation, but high concentrations of PGD_2 do bind to the inhibitory receptor.

Addition of PGD_2 to a PGE_1-stimulated cyclic AMP progress curve. Addition of PGD_2 after 2 min to a reaction mixture initiated by PGE_1 resulted in a different response to PGD_2 from that observed when PGD_2 was added alone (Fig. 5). PGD_2 added alone gave a marked rise to a peak followed by a fall to a plateau level. In contrast, PGD_2 added to the PGE_1 curve showed no peak but a rise to the same steady state level observed with PGD_2 alone. The response to PGD_2 was clearly desensitized by prior treatment with PGE_1, in terms of a reduced initial rate of PGE_1-stimulated cyclic AMP formation.

It should be noted that we cannot simulate this case using the two-receptor model as formulated in the Appendix. The reason for this follows. In order to obtain a workable model with a reasonable number of steps, we treated the receptor/G protein/adenylate cyclase complexes as a single enzyme species, with a stimulatory site and an inhibitory site for prostaglandins, so that stimulation and inhibition appear as linked equilibria. Consequently, in the simulation, addition of PGD_2 not only

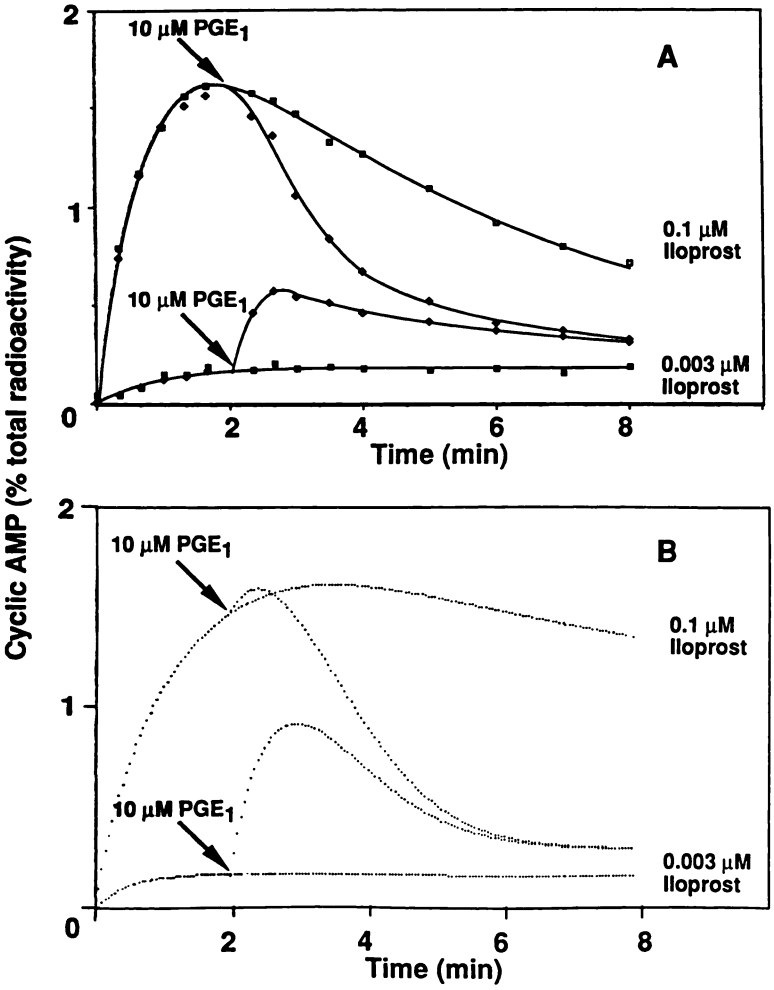

Fig. 3. Effect of addition of PGE_1 to cyclic AMP progress curves initiated by addition of iloprost. A, Real data were obtained as described in Experimental Procedures, with reactions being initiated by addition of either 0.003 or 0.1 μM iloprost. After 2 min, 10 μM PGE_1 was added. B, Simulations were performed as described in the Appendix, using the constants given in Tables 1 and 2.

gives activation through a direct effect on the stimulatory site but also shifts the equilibrium between active and inactive forms of the enzyme, leading to a time-dependent increase in active enzyme and a further increase in cyclic AMP level. In reality, the shift from inactive to active enzyme does not occur, because stimulation and inhibition represent separate G protein-mediated pathways.

Discussion

We have previously proposed a two-receptor model to explain prostaglandin regulation of platelet adenylate cyclase (6). In this paper, we have shown that the model readily explains the difference in response of platelets to various prostaglandins in terms of differences in affinity of the stimulatory and inhibitory receptors for each prostaglandin. Our studies indicate that all prostaglandins are full agonists at both the stimulatory and inhibitory receptors. Consequently, the model predicts that saturating levels of all prostaglandins should give rise to the same time course and, in fact, curves obtained at high concentrations of prostaglandins tend towards the same pattern. The putative inhibitory receptors have an apparent affinity for prostaglandins lower than that of the stimulatory receptors in the case of PGI_2 and PGD_2 and a higher affinity than that of the stimulatory receptor in the case of PGE_1. The difference in apparent effectiveness among prostaglandins arises from differences in affinity for the two receptors and the ability of PGE_1 to turn off adenylate cyclase more readily at low concentrations due to the higher affinity of the inhibitory receptor for PGE_1. The model provides an explanation for mixed agonist/antagonist behavior and explains the partial agonist behavior of PGE_1 and PGD_2 in terms of affinities for the two receptors, rather than differences in the maximum evoked effect of each agonist.

We have demonstrated the versatility of the model in explaining the complex patterns of cyclic AMP formation obtained when two prostaglandins are added consecutively, some minutes apart. It is shown that the model gives rise to heterologous desensitization among all prostaglandins. Heterologous desensitization is a manifestation of binding of prostaglandins to the inhibitory site. Hence, because PGE_1 binds most tightly to the inhibitory site, it gives rise to the most striking degree of desensitization. In contrast, PGD_2 binds weakly to the inhibitory site and gives little desensitization.

Both Miller and Gorman (15) and Mills and Macfarlane (16) have used the fact that different prostaglandins have different effects on the time course of cyclic AMP formation to argue that PGE_1 and PGI_2 bind to a common receptor on platelets, whereas PGD_2 binds to a distinct receptor. We show here that such distinctions are not sufficient to describe a separate stimulatory binding site for PGD_2 but merely reflect weak binding of PGD_2 to the putative inhibitory site. On the other hand, it is not essential for our model that any of the prostaglandins

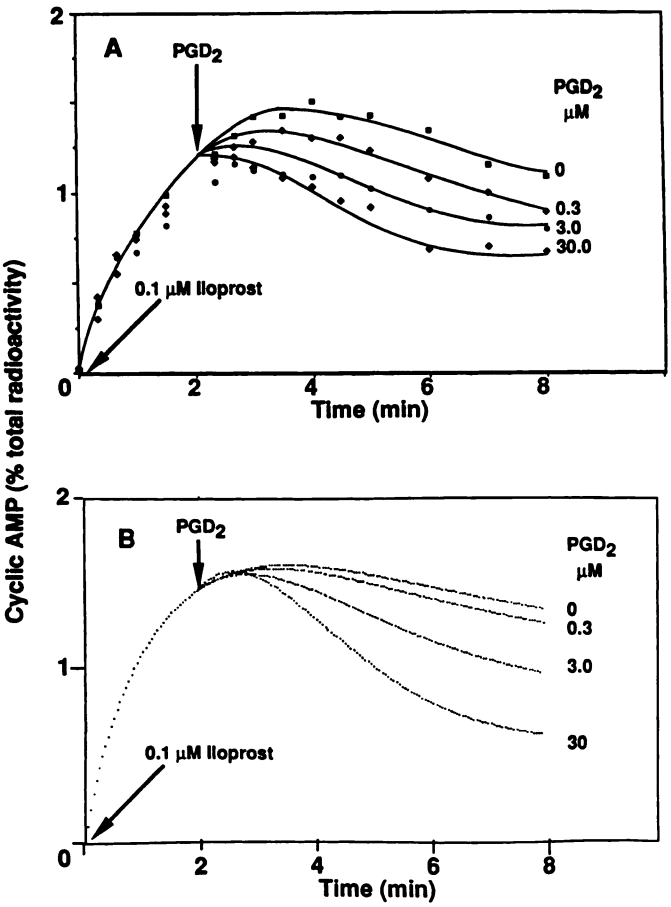

Fig. 4. Effect of addition of PGD_2 at various concentrations to an iloprost-stimulated cyclic AMP progress curve. A, Real data were obtained as described in Experimental Procedures, with reactions being initiated by addition of 0.1 μM iloprost. After 2 min, PGD_2 was added at the concentrations indicated. B, Simulations were performed as described in the Appendix, using the constants given in Tables 1 and 2.

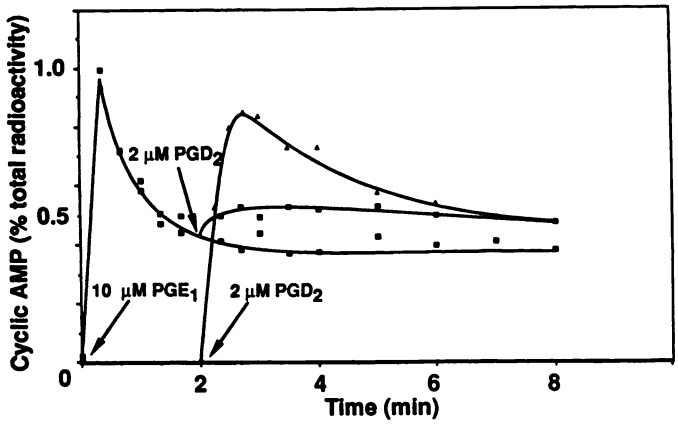

Fig. 5. Effect of addition of PGD_2 to cyclic AMP progress curves initiated by addition of PGE_1. Data were obtained as described in Experimental Procedures, with reactions being initiated by addition of 10 μM PGE_1. After 2 min, 2 μM PGD_2 was added. The experiment was repeated by addition of vehicle at zero time, instead of PGE_1, and addition of PGD_2 again after 2 min.

bind to the same receptors, merely that the prostaglandins affect G_s and G_i to the same extent. In fact, the main purpose of the work described here was to show that the two-receptor model is a generalized mechanism of heterologous desensitization, operating at the G protein level.

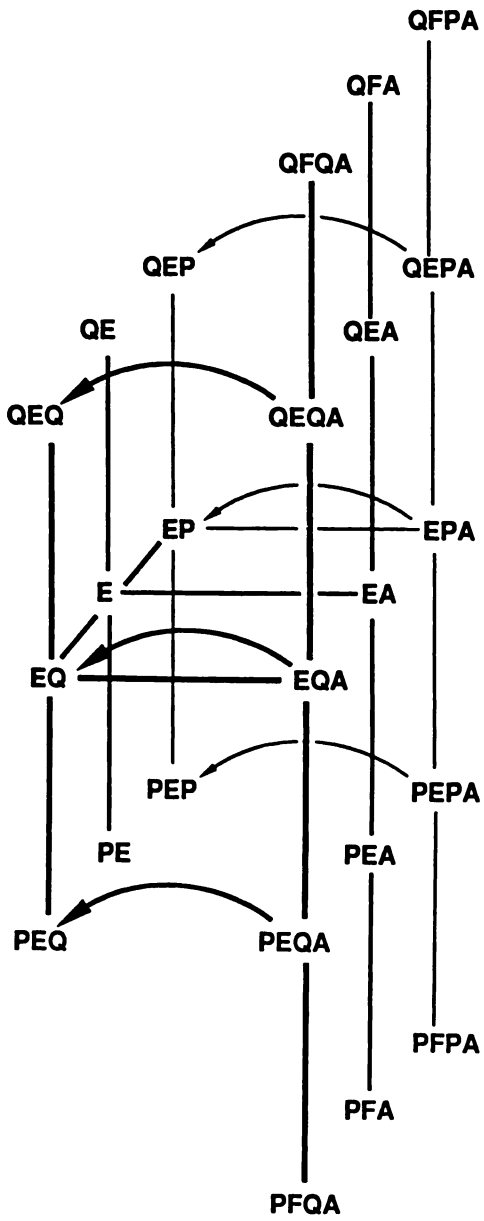

Fig. 6. Scheme describing regulation of platelet adenylate cyclase in terms of competition between two prostaglandins (*P* and *Q*) for binding to separate stimulatory and inhibitory receptors. *Curved arrows*, catalytic steps leading to the formation of cyclic AMP from ATP (*A*); *E*, Adenylate cyclase/receptor complex; *P*, first prostaglandin; *Q*, second prostaglandin; *EP* or *EQ*, prostaglandin bound to a stimulatory site; *PE* or *QE*, prostaglandin bound to an inhibitory site; *F*, desensitized adenylate cyclase.

The clearest way to distinguish between receptors for individual prostaglandins is through studies with selective antagonists. In comparing antagonist effects on the action of various prostaglandins, however, it is necessary to consider that PGD_2 binds much more weakly to both its stimulatory and inhibitory receptors than either PGE_1 or PGI_2. For this reason, appropriately high concentrations of antagonist must be used to overcome the high affinity of PGE_1 or PGI_2, in order to make a valid comparison with PGD_2 antagonism. In this regard, the compound BW A868C, described by Trist *et al.* (20), appears to fulfill the criteria of a selective PGD_2 antagonist on platelets. Overall, it seems that PGE_1 and PGI_2 may share a common stimulatory receptor, whereas PGD_2 appears to bind to a dis-

TABLE 2

Reactions and constants used to simulate curves in Figs. 1–4

=, Rapid equilibrium steps; = =, kinetically determined steps. 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 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, P, or Q 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. Once formed, cyclic AMP (C) is removed by phosphodiesterase (D) through the enzyme-substrate complex DC to give AMP (N). The concentration of phosphodiesterase was set at 1×10^{-6} μM. The K_m for phosphodiesterase activity was set at 1% of total adenine nucleotides, corresponding to a cyclic AMP concentration of about 75 μM (6). Apparent dissociation constants for binding to the stimulatory receptor (K_s) and inhibitory receptor (K_i) are given. When prostaglandins are bound to either the stimulatory or inhibitory receptors, K_s and K_i increase by about 50-fold, giving rise to apparent negative cooperativity, as discussed in detail in Ref. 6.

Equilibrium			Description of rate/equilibrium constant	Value of rate/equilibrium constant
$A + E$	=	EA	K_m for ATP	0.1%
$A + EP$	=	EPA		
$A + EQ$	=	EQA		
$E + P$	=	EP	K_s for binding of P to stimulatory receptor	See Table 1
$E + P$	=	PE	K_i for binding of P to inhibitory receptor (K_{i_P})	See Table 1
$EA + P$	=	PEA		
$EP + P$	=	PEP	K_i for binding of P to inhibitory receptor when stimulatory receptor is occupied	50 times K_{i_P}
$EPA + P$	=	$PEPA$		
$EQ + P$	=	PEQ		
$EQA + P$	=	$PEQA$		
$E + Q$	=	EQ	K_s for binding of Q to stimulatory receptor	See Table 1
$E + Q$	=	QE	K_i for binding of Q to inhibitory receptor (K_{i_Q})	See Table 1
$EA + Q$	=	QEA		
$EQ + Q$	=	QEQ	K_i for binding of Q to inhibitory receptor when stimulatory receptor is occupied	50 times K_{i_Q}
$EQA + Q$	=	$QEQA$		
$EP + Q$	=	QEP		
$EPA + Q$	=	$QEPA$		
$PEPA$	= =	$PFPA$	Forward and backward rate constants for desensitization	Forward rate, 1.0 min^{-1} Backward, 0.25 min^{-1}
PEA	= =	PFA		
QEQA	= =	QFQA		
QEA	= =	QFA		
PEQA	= =	PFQA		
QEPA	= =	QFPA		
EPA	= =	EP + C	Maximum rate constant for cyclic AMP formation	3×10^6 min^{-1} (backward rate is zero)
PEPA	= =	PEP + C		
EQA	= =	EQ + C		
QEQA	= =	QEQ + C		
PEQA	= =	PEQ + C		
QEPA	= =	QEP + C		
D + C	=	DC	K_m for phosphodiesterase	1.0%
DC	= =	$D + N$	Rate constant for phosphodiesterase	2.5×10^6 min^{-1}

tinct stimulatory receptor; at the same time, all of the prostaglandins interact with one or more inhibitory receptors to bring about heterologous desensitization, which occurs at the level of common G proteins.

The physiological relevance of dual regulation of adenylate cyclase by a single agonist acting on the same cell type perhaps can be explained in terms of the difference between endocrine and autocrine hormones (autocoids). Endocrine hormones are generally produced by cells distant from their site of action, so that they are diluted in the blood stream to an elevated but stable concentration by the time they reach their target cells. In contrast, autocoids are produced by the same cell type on which they act (or, in the case of platelets, by the intimately related endothelial cells), forming a localized feedback mechanism. Consequently, autocoids may reach transiently high levels at their sites of action. The presence of a second type of receptor that negates the action of the first receptor would tend to buffer cellular responses to transient extremes of agonist concentration. The slow onset of inhibition would also allow for time-dependent buffering, providing additional control over autocoid release and effect. The rapid desensitization of adenylate cyclase caused by prostaglandins can then be distinguished from slower and more complex forms of desensitization observed with, for example, β-adrenergic agonists (21). Hence, prostaglandin or autocoid desensitization may simply represent a relatively rapid response to localized transient concentrations

of agonist, maintaining cellular responsiveness within reasonable bounds, whereas desensitization to endocrine hormones represents true desensitization and down-regulation to long term agonist exposure.

Acknowledgments

I am grateful to Ms. Eve Wernick for technical assistance and to Dr. Carl Frieden for providing a copy of KINSIM.

Appendix

Computer simulation of cyclic AMP time courses. Simulations were performed on a MicroVAX computer (Digital Equipment Corporation) using KINSIM (11), a program written in FORTRAN, that was kindly provided by Dr. Carl Frieden, Washington University School of Medicine (St. Louis, MO). Mechanisms are written as a series of chemical reactions represented either by a dissociation constant for rapid equilibrium steps or by forward and backward rate constants for 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, either as graphical output or as numerical values.

Model of prostaglandin regulation of platelet adenylate cyclase. Our original model of desensitization (6) involved binding of a prostaglandin to a stimulatory receptor to bring about rapid activation of adenylate cyclase, together with binding to a separate inhibitory receptor to induce slow reversible transformation of the enzyme to an inactive form. The current work extends the model to include a second prostaglandin competing for both the stimulatory and inhibitory receptors. The model is indicated by the scheme in Fig. 6 and by the series of reactions presented in Table 2. The receptor/G protein/adenylate cyclase complex is represented as a single enzyme species *E*. The enzyme *E* has a binding site for substrate ATP (*A*) as well as two distinct binding sites for the first 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 *EP* and subsequently to *EPA*, which is active, generating *EP* and cyclic AMP (*C*), a reaction that is represented by the *curved arrow* in Fig 6. *P* can also bind to a separate inhibitory binding site to give *PEA*. *PEA* can slowly and reversibly convert to *PFA*, generating the inactive enzyme species *FA*. A second prostaglandin (*Q*) can compete with *P* for both the stimulatory and inhibitory binding sites, giving rise to the enzyme species indicated in Fig 6 and Table 2.

Simulations were performed as follows. In the case of a single prostaglandin, curves of cyclic AMP formation were generated by setting equilibrium and kinetic constants as indicated in Tables 1 and 2, with cyclic AMP at an initial concentration of zero and all of the enzyme in the active form *E*. When we wished to simulate the effect of a second prostaglandin added 2 min after the first prostaglandin, we stopped the simulations after 2 min and obtained numerical values for the concentration of cyclic AMP as well as the total concentration of the *E* species and the total concentration of the *F* species. We used these values to set the initial conditions for simulations involving two prostaglandins. (The total *E* species could be entered as any one of the various liganded forms, because it rapidly equilibrates among the other forms; the same is true of the *F* species.) The subsequent simulation then represented the sum of contributions from both prostaglandins following 2-min exposure to the first prostaglandin. The curves could be moved 2 min along the time axis to generate the figures used in the main body of the paper.

References

1. Barber R., R. B. Clark, L. A. Kelly, and R. W. Butcher. A model of desensitization in intact cells. *Adv. Cyclic Nucleotide Res.* **9**:507–516 (1978).
2. Barber, R., K. P. Ray, and R. W. Butcher. Turnover of adenosine 3′,5′-monophosphate in WI-38 cultured fibroblasts. *Biochemistry* **19**:2560–2567 (1980).
3. Ashby, B. Kinetic evidence indicating separate stimulatory and inhibitory prostaglandin receptors on platelet membranes. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**:291–300 (1986).
4. Ashby, B. Cyclic AMP turnover in response to prostaglandins in intact platelets: evidence for separate stimulatory and inhibitory prostaglandin receptors. *Second Messengers Phosphoproteins* **12**:45–57 (1988).
5. Ashby, B. Effect of thromboxane antagonists on prostaglandin regulation of platelet adenylate cyclase. *Second Messengers Phosphoproteins* **12**:241–250 (1988).
6. Ashby, B. Model of prostaglandin-regulated cyclic AMP metabolism in intact platelets: examination of time-dependent effects on adenylate cyclase and phosphodiesterase activities. *Mol Pharmacol.* **36**:866–873 (1989).
7. Ashby, B. Computer simulation of prostaglandin-regulated cyclic AMP metabolism in platelets: activation and desensitization of adenylate cyclase appear to be mediated through separate prostaglandin receptors coupled to G_s and G_i, in *Biology of Cellular Transducing Signals. IXth International Washington Spring Symposium* (J. Vanderhoek, ed.). Plenum Press, New York, 73–82 (1990).
8. Siegl, A. M. Receptors for PGI_2 and PGD_2 on human platelets. *Methods Enzymol.* **86**:179–192 (1982).
9. Siegl, A. M., J. B. Smith, M. J. Silver, and K. C. Nicolau. Selective binding site for [^{3}H]prostacyclin on platelets. *J. Clin. Invest.* **63**:215–220 (1979).
10. Schafer, A. I., B. Cooper, D. O'Hara, and R. I. Handin. Identification of platelet receptors for prostaglandins I_2 and D_2. *J. Biol. Chem.* **254**:2914–2917 (1979).
11. Barshop, B. A., R. F. Wrenn, and C. Frieden. Analysis of numerical methods for computer simulation of kinetic processes: development of KINSIM: a flexible, portable system. *Anal. Biochem.* **130**:134–145 (1983).
12. Jakobs, K. H., S. Bauer, and Y. Watanabe. Modulation of adenylate cyclase of human platelets by phorbol ester. *Eur. J. Biochem.* **151**:425–430 (1985).
13. Katada, T., A. G. Gilman, Y. Watanabe, S. Bauer, and K. H. Jakobs. Protein kinase C phosphorylates the inhibitory guanine nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur. J. Biochem.* **151**:431–437 (1985).
14. Watanabe, Y., F. Horn, S. Bauer, and K. H. Jakobs. Protein kinase C interferes with N_i-mediated inhibition of human platelet adenylate cyclase. *FEBS. Lett.* **192**:23–27 (1985).
15. Miller, O. G., and R. R. Gorman. Evidence for distinct prostaglandin I_2 and D_2 receptors in human platelets. *J. Pharmacol. Exp. Ther.* **210**:134–140 (1979).
16. Mills, D. C. B., and D. E. Macfarlane. Evidence for separate receptors mediating the stimulation of platelet adenylate cyclase by prostaglandin I_2 (PGI_2), PGD_2 and PGE_1. *Thrombosis Haemostasis* **42**:118 (1980).
17. Mills, D. C. B. The role of cyclic nucleotides in platelets, in *Handbook of Experimental Pharmacology* (J. W. Kebabian and J. A. Nathanson, eds.), Vol. 58/II. Springer-Verlag, Berlin, 732–761 (1982).
18. Salomon, Y. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* **10**:35–55 (1979).
19. Stadel, J. M., A. De Lean, and R. J. Lefkowitz. Molecular mechanisms of coupling in hormone receptor-adenylate cyclase systems. *Adv. Enzymol.* **53**:1–43 (1982).
20. Trist, D. G., B. A. Collins, J. Wood, M. G. Kelly, and A. D. Robertson. The antagonism by BW A868C of PGD_2 and BW245C activation of human platelet adenylate cyclase. *Br. J. Pharmacol.* **96**:301–306 (1989).
21. Sibley, D. R., and R. J. Lefkowitz. Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature* (*Lond.*) **317**:124–129 (1985).

Send reprint requests to: Barrie Ashby, Ph.D., Department of Pharmacology, Temple University Health Sciences Center, 3400 North Broad Street, Philadelphia, PA 19140.