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Pre-Steady State Study of β-Adrenergic and Purinergic Receptor Interaction in C6 Cell Membranes: Undelayed Balance between Positive and Negative Coupling to Adenylyl Cyclase

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SUMMARY

Interactions between β-adrenergic and ADP purinergic receptors in C6 glioma cell membrane preparations were investigated under steady state and then pre-steady state conditions of adenylyl cyclase (EC 4.6.1.1) activity, in order to determine how fast the second receptor antagonizes the transduction mechanism of the first. Cell membranes were washed to deplete them as thoroughly as possible of low molecular weight compounds, especially ATP and ADP, and to ensure better control of both substrate and agonist nucleotide concentrations. ATP concentrations were kept constant with the use of an ATP-regenerating system; the C6 cell line exhibited very active ectonucleotidases. The purinergic agonist ADP was replaced by its nonhydrolyzable congener adenosine 5′-*O*-(2-thio)diphosphate (ADPβS), which was demonstrated, like ADP, to inhibit isoproterenol-stimulated adenylyl cyclase activity in intact cells (IC_{50} for ADP, 0.5 ± 0.1 μM; IC_{50} for ADPβS, 25 ± 2 μM) and in membrane preparations (IC_{50} for ADPβS, 79 ± 20 μM). In the case of membrane preparations, ADPβS did not compete with ATP, the substrate of the cyclase-catalyzed reaction, and behaved apparently as a noncompetitive inhibitor of the enzyme. The pre-steady state kinetics of isoproterenol-stimulated adenylyl cyclase activity measured with a pulsed quenched-flow apparatus have previously been shown to include two steps, the first very rapid (taking place within 1–2 sec) and giving rise to a burst of cAMP synthesis and the second much slower and corresponding to the steady state reaction. ADPβS inhibited the occurrence of both steps with comparable IC_{50} values (mean value, 55 ± 20 μM). In the presence of increasing concentrations of the purinergic receptor agonist, the time constant of the exponential burst reaction was not affected, but its amplitude progressively decreased to zero. These results showed that the extinction of the β receptor cAMP response by the purinergic ADP receptor occurred within the dead-time of the pulsed quenched-flow apparatus, which was 50 msec. Such a rapid inhibition of cAMP production excluded modulation of isoproterenol-stimulated adenylyl cyclase activity by the ADP receptor by a pathway other than its direct negative coupling to the cyclase via a G_i protein. In this respect, the P_2 purinergic ADP receptor of the C6 glioma cell line appears comparable to the P_{2t} receptor of platelets.

The molecular mechanisms of receptor cross-talk are of major interest in cellular biology. On the other hand, the growing body of knowledge concerning this aspect of cell regulation has not yet included much precise evidence on how fast a cell can modulate the signal due to activation of a receptor, resulting in a significant intracellular second messenger concentration. Experiments with live cells are usually carried out on the time scale of minutes, whereas seconds could be enough to reduce such responses. Still, when a cell population can yield cell membrane preparations in which good coupling of receptor transduction mechanisms are maintained, the time scale of seconds can be more easily explored by using fast-mixing techniques. The rat C6 glioma is such a cell line, from which cell membrane preparations can be obtained with a low basal adenylyl cyclase activity and an efficient coupling of the enzyme to β-adrenergic receptors. Such membranes, which are essentially cell ghosts, are exploitable for studying receptor interactions on the time scale of seconds by examining pre-steady state cAMP responses, for instance to isoproterenol combined with any other receptor agonist.

Studies of pre-steady state kinetics of complex enzymatic reactions using fast-mixing devices, developed for soluble enzymes (1), have been used for the last 10 years in the investigation of membrane systems such as ATPases or nucleotide transport systems (2) and more or less abundant receptors (3–5). Using a pulsed quenched-flow apparatus, we have shown in a former work (6) that stimulation of the β-adrenergic receptor by isoproterenol in C6 cell membranes triggers a pre-steady state response of adenylyl cyclase, corresponding to a rapid burst of cAMP synthesis, that precedes the slower steady

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; ADPβS, adenosine 5′-*O*-(2-thio)diphosphate; Gpp(NH)p, guanylylimidodiphosphate; IBMX, 3-isobutylmethylxanthine.

production of the second messenger. This finding demonstrated that in these cells adenylyl cyclase activation by a receptor occurs rather rapidly. This activation process implies a series of noncovalent interactions between several molecules and macromolecules (an agonist, a protein receptor, a heterotrimeric G_s protein, GDP, GTP, and the cyclase) but in principle no enzymatic step involving a covalent bond, be it either formation or breakage. The amount of cAMP produced in the initial rapid step was found to be high enough to activate protein kinase A and lead, within 4–5 sec, to the phosphorylation of a reproducible set of membrane proteins (7).

In the present work, knowing that in C6 cells adenylyl cyclase can be activated within 1 sec by β-adrenergic receptors, we use the same pre-steady state approach to determine how fast the enzyme can be uncoupled, i.e., driven from an active to an inactive (or less active) state, when another receptor exerting an opposing action is stimulated at the same time. Advantage is taken of the presence of receptors for ADP, which depress adenylyl cyclase response to isoproterenol in living C6 cells (8), to address this question. The results of the present study, based on the pre-steady state kinetics of cAMP production, lead to better understanding of the transduction mechanism of these purinergic receptors that originally were presumed to be of the P_2 type. Pianet *et al.* (8) effectively showed that the response to ADP was abolished by pertussis toxin treatment of the cells, suggesting the involvement of a G protein of the type G_i, coupling the receptor negatively to adenylyl cyclase. In astrocytes, however, P_2 purinoceptors have been shown to be coupled to phosphoinositide hydrolysis (9), which may regulate the β receptor response by an indirect protein kinase-linked pathway. Furthermore, because pertussis toxin action is not restricted to $G_{i\alpha}$, the negative coupling of P_2 receptors to adenylyl cyclase in C6 cells required investigation.

These cells and their membrane preparations hydrolyze both ATP and ADP very actively (6, 8). The use of the ADP analog ADPβS was, therefore, considered, because it is less metabolizable. Its action was shown to be comparable to that of ADP on the isoproterenol-stimulated cAMP response of live C6 cells, thereby justifying its use with membrane preparations for the steady state and pre-steady state analysis of adenylyl cyclase activity during the β-adrenergic and purinergic ADP receptor interaction.

Experimental Procedures

Materials. Sources of materials and drugs for cell culture, membrane preparation, adenylyl cyclase assay, and cAMP radioimmunoassay were as described before (6, 8). Gpp(NH)p, heparin, and the protein kinase inhibitor were purchased from Sigma (L'isle d'Abeau, France). (−)-3-[^{125}I]Iodocyanopindolol (74 TBq/mmol) and [^{3}H]CGP 12177 [4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one] (1.1–1.8 TBq/mmol) were from Amersham (Les Vlis, France). ADPβS was from Boehringer Mannheim (Maylan, France).

Cell culture and membrane preparation. Cells were cultured and cell membranes were prepared, frozen in liquid nitrogen, and stored at −80°, as described in Valeins *et al.* (6). Because it has been shown that such membrane preparations contain ATP, the thawed membrane sample was taken up before each experiment in 20 volumes of a cold buffer containing 10 mM Tris·HCl, pH 7.4, and 1 mM EDTA. The suspension was gently shaken and then centrifuged for 10 min at 10,000 × *g* and 4°, and the resulting pellet was resuspended in the starting sample volume of the same buffer. When needed, the membrane preparation was diluted in the same buffer.

cAMP and adenylate cyclase activity determinations. cAMP production in intact cells was determined as described by Pianet *et al.* (8), by a cAMP radioimmunoassay (10). Adenylyl cyclase activity was measured as described by Volker *et al.* (11), also by the cAMP radioimmunoassay. Standard conditions for the steady state assay (final volume of the reaction mixture, 100 μl) were as follows: variable ATP concentration, variable GTP concentration, 6.25 mM $MgCl_2$, 0.2 mM IBMX, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 10 mM phosphate buffer, pH 7.6, 10–20 μg of membrane protein, and when necessary 50 μM isoproterenol and variable ADPβS concentrations. The reaction mixture was incubated at 25° for 10 min and then quenched with 100 μl of 2 M perchloric acid. Pre-steady state activity conditions were the same, except that the membrane protein concentration was 1–2 mg/ml in order to generate a measurable cAMP signal on the time scale of seconds. In this case, the membrane sample brought 5 mM Tris·HCl and 0.5 mM EDTA into the reaction mixture. Experiments were usually repeated two or three times.

Pulsed quenched-flow. The pulsed quenched-flow apparatus (Fig. 1) was constructed according to the method of Ghelis (12). It was adapted to mix 50-μl samples. The apparatus was placed in a Plexiglass box, where the atmosphere was thermostatted at 25 ± 1°. The reservoirs were maintained under a controlled pressure of 0.7 bar of nitrogen, with no leakage of the electric valves when not activated. Upon activation of a valve, the gas pressure pushed the liquid through the valve. The reactant volume delivered from the reservoir depended on the specification of the valve but was also controlled by the programmed length of its opening. The relationship between the time during which the valve was open and the delivered volume was linear above 30 msec. An opening time of 50 msec corresponded to 50 μl of the reactants (EV_2 and EV_3) and 100 μl of the quencher (EV_1). The dead-time was 50 msec.

Fifty microliters of the membrane preparation and the same volume of the substrate and effector mixture were delivered to the reaction tube (RT) through the mixing chamber by simultaneous activation of valves EV_2 and EV_3. With a delay corresponding to the desired reaction time, 100 μl of the quencher (2 M perchloric acid) were added by the programmed opening of valve EV_1. When the protein concentration of the membrane sample was high (above 1 mg/ml), the viscosity of the sample led to a decrease in the volume delivered during a 50-msec opening of valve EV_3. In this case, the volume delivered in relation to the membrane sample concentration was calibrated, and the substrate and effector concentrations were adjusted so as to take into account

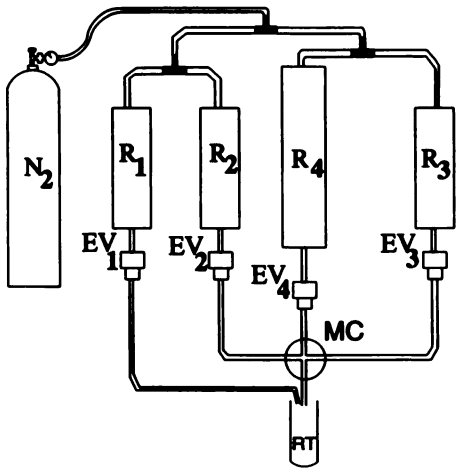

Fig. 1. Scheme of pulsed quenched-flow apparatus. N_2, nitrogen gas tank; R_1, quencher (2 M perchloric acid) reservoir; R_2, membrane preparation reservoir; R_3, substrate and effector reservoir; R_4, water reservoir for washings; EV_1, EV_2, EV_3, and EV_4, electric valves controlled by a microprocessor; *MC*, mixing chamber; *RT*, reaction tube. For operation details, see Experimental Procedures.

this volume variation and to run the assays under the initially defined conditions.

ATP and ADP contents and β-receptor sites of membrane preparations. ATP and ADP contents of membrane preparations were determined according to the method of Pianet *et al.* (8). The number of β receptor sites in membrane preparations was measured at equilibrium according to the method of Voisin *et al.* (13), using either [^{125}I]iodocyanopindolol or [^{3}H]CGP 12177; both antagonists gave comparable results.

Results

Effect of ADPβS on β-Adrenergic cAMP Response in Intact C6 Cells

It has previously been shown that ADP is a potent inhibitor of cAMP production in intact isoproterenol-stimulated C6 glioma cells (8). It has also been observed that these cells carry ectopic enzymes that hydrolyze ATP and ADP (8). Because cell membrane preparations also do (6, 14), we chose to use a less metabolizable analog of ADP in order to establish altogether reproducible ATP and purinergic agonist concentrations. ADPβS has been proposed by Goody *et al.* (15) as such an ADP congener. We tested it on intact cells and compared it with ADP. Fig. 2 shows the dose-response for inhibition by ADP and ADPβS of the isoproterenol-stimulated cAMP production in living cells. As previously described, ADP inhibited up to 50% of cAMP production with an IC_{50} value of 0.5 ± 0.1 μM, in good agreement with the previously published value (8). The nonhydrolyzable analog ADPβS led to an even larger inhibition of the response, up to 60%, with an IC_{50} of 25 ± 2 μM. This derivative thus behaved as a P_2 agonist (although less potent than ADP by nearly 2 orders of magnitude) and was used to study the ADP receptor transduction mechanism in cell membranes, in the presence of the ATP-regenerating system.

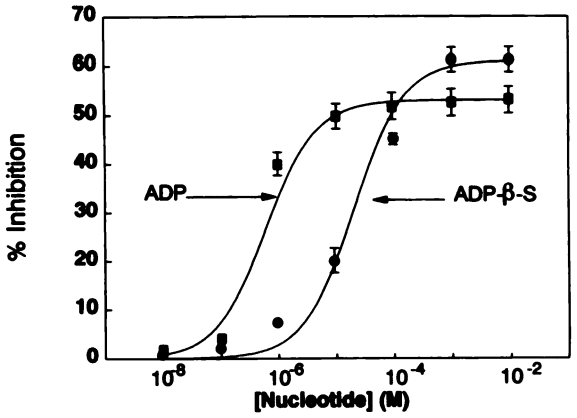

Fig. 2. Concentration dependence of inhibition by ADP and ADPβS of isoproterenol-stimulated cAMP production in intact C6 cells. At time 0, cells received either 50 μM isoproterenol or 50 μM isoproterenol and various concentrations of ADP (■) or ADPβS (●). The reaction was stopped 1 min later by addition of perchloric acid, and the cAMP content was determined by radioimmunoassay, as described in Experimental Procedures. The percentage of inhibition was calculated by taking as reference the maximal control response (251.0 ± 13.6 pmol/mg/min), measured in the presence of isoproterenol alone. Basal activity (14.0 ± 0.6 pmol/mg/min), in the absence of isoproterenol, did not vary significantly with increasing concentrations of nucleotides and was subtracted from all of the isoproterenol-stimulated responses. Data points represent the mean ± standard error for triplicate determinations. Standard error bars that are not shown are within the symbols. Data were analyzed and the IC_{50} values were calculated as described previously (8).

Depletion of Nucleotides in Membrane Preparations by Washing

Cell membrane preparations used in our previous work were demonstrated to contain ATP (6). As described in Experimental Procedures, additional washing of the preparation was performed before each experiment, in order to eliminate low molecular weight molecules. Table 1 shows that membranes prepared according to our previous method contained not only ATP but also ADP in large amounts. The washing step eliminated nearly all of both nucleotides present in the samples. The drop in their concentrations could not be explained only by their dilution but also was the result of their dissociation from numerous membrane enzymatic systems. Because the washed preparations were also depleted of other compounds and especially those necessary for maximal isoproterenol-stimulated adenylyl cyclase activity, as seen in Table 1, the latter became dependent on GTP supplementation.

Washing of the membrane supension led to about a 60% protein loss (mean value) and a 30% decrease (mean value) of the β-adrenergic receptor density. Loss of β-adrenergic sites was difficult to interpret. One possible explanation is that during the washing step some denaturation or some inside-out inversion occured. Specific adenylyl cyclase activity, after GTP supplementation, was in some cases also slightly lower than before washing. The amplitude of the cAMP burst observed under pre-steady state conditions, in these latter cases, was also lower.

Effect of ADPβS on Isoproterenol-Stimulated Adenylyl Cyclase Activity of C6 Cell Membranes under Steady State Conditions

Fig. 3 shows the ADPβS concentration dependence of the inhibition of cAMP production by washed C6 cell membranes at three ATP concentrations. The ATP dependence of the isoproterenol-stimulated adenylyl cyclase activity of nonwashed preparations has been measured by Volker (16) under conditions very close to those used in this work. He determined for ATP a K_m value of 30 μM. Washed membranes exhibited a very similar ATP concentration dependence, with the activity measured in the absence of ADPβS at 30 μM ATP being half of that measured at 1 mM ATP. In the presence of ADPβS, inhibition was observed without competition towards ATP; the ADPβS concentration dependence was very similar at the three tested ATP concentrations (30 μM, 0.1 mM, and 1 mM). The IC_{50} values for this inhibition by ADPβS at the three tested ATP concentrations were quite comparable. The mean value was in the range of 75 μM (79 ± 20 μM). Nearly full inhibition was observed at all ATP concentrations.

Effect of ADPβS on Isoproterenol-Stimulated Adenylyl Cyclase Activity of C6 Cell Membranes under Pre-Steady State Conditions

Effect of ATP concentration on amplitude of cAMP burst. The effect of ATP concentration on the pre-steady state cAMP response to isoproterenol in washed membranes was investigated with pulsed quenched-flow technique. Fig. 4 shows that an isoproterenol-elicited burst was observed only in the presence of added ATP, in contrast to what has been observed with nonwashed preparations where enough ATP was present to allow some adenylyl cyclase activity in the absence of ATP supplementation (6). The amplitude of the burst was of about

TABLE 1

Effect of depletion of low molecular weight molecules on properties of C6 cell membranes

Thawed membrane samples were suspended in 20 volumes of cold 10 mM Tris·HCl, pH 7.4, buffer containing 1 mM EDTA and were then centrifuged for 10 min at 10,000 × *g*, and the pellet was resuspended in the starting volume of the same buffer. As an illustration of the effect of this washing procedure, the properties before and after treatment of two typical preparations are presented. ADP and ATP contents were determined as described in Experimental Procedures. Isoproterenol-stimulated adenylyl cyclase activity was measured under steady state conditions (1 mM ATP, 50 μM isoproterenol) either in the presence or in the absence of 100 μM GTP. Density of β receptor sites was determined by the binding of the antagonist [^{125}I]iodocyanopindolol to membranes, as described in Experimental Procedures. Experimental data are expressed as mean ± standard error for triplicate determinations.

	Preparation 1		Preparation 2	
	Nonwashed	Washed	Nonwashed	Washed
ATP (pmol/mg)	365 ± 5	0.5 ± 0.1	500 ± 10	1.0 ± 0.1
ADP (pmol/mg)	3015 ± 20	6 ± 1	4125 ± 22	10 ± 2
Adenylyl cyclase activity (pmol/min/mg)				
−GTP	30 ± 2	≤0.1	28 ± 1	≤0.1
+GTP	45 ± 2	40 ± 1	40 ± 1	35 ± 1
β receptor sites (fmol/mg)	245 ± 15	185 ± 20	300 ± 2	180 ± 20
Protein (mg/ml)	11.0 ± 0.5	5.0 ± 0.1	15.0 ± 1.0	6.0 ± 1.0

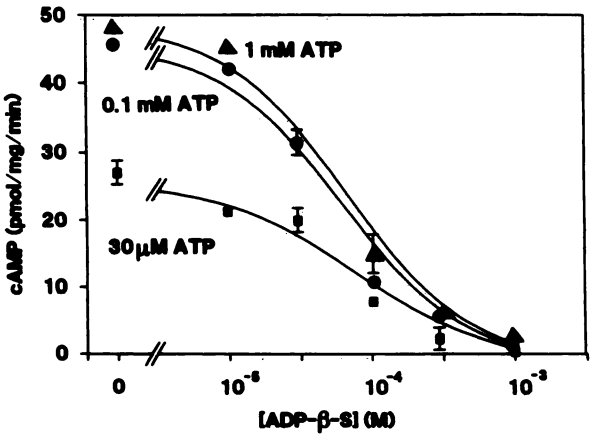

Fig. 3. Concentration dependence of inhibition by ADPβS of isoproterenol-stimulated adenylyl cyclase activity of C6 cell membranes under steady state conditions. The adenylyl cyclase activity of the membrane preparations was measured under steady state conditions, in the presence of either 50 μM isoproterenol or 50 μM isoproterenol and increasing concentrations of ADPβS, at increasing ATP concentrations, 30 μM (■), 0.1 mM (●), and 1 mM (▲), as described in Experimental Procedures. cAMP was measured by radioimmunoassay. Data points are mean ± standard error for triplicate determinations. Standard error bars that are not shown are within the symbols. Basal activity (5.0 ± 0.8 pmol/mg/min) did not vary with increasing ADPβS concentrations and was subtracted from the isoproterenol-stimulated values. Data were analyzed according to an equation for noncompetitive inhibition: rate = $V(1 + (ADP\beta S)/IC_{50})$, with V corresponding to the rate in the absence of ADPβS. The curves correspond to the following values of rates V and IC_{50} for ADPβS: at 30 μM, 0.1 mM, and 1 mM ATP concentrations V = 25, 47, and 50 pmol/mg/min and IC_{50} = 103, 64, and 69 μM, respectively.

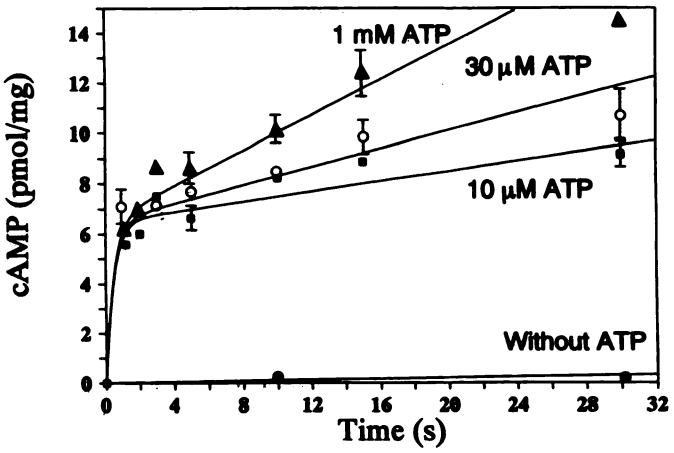

Fig. 4. Pre-steady state kinetics of isoproterenol-stimulated adenylyl cyclase activity of C6 cell membranes at increasing ATP concentrations. Adenylyl cyclase activity was measured with the pulsed quenched-flow apparatus in the presence of 50 μM isoproterenol and either in the absence of ATP (●) or in the presence of increasing ATP concentrations, 10 μM (■), 30 μM (○), and 1 mM (▲), as described in the legend to Fig. 3 and in Experimental Procedures. Basal activity (2.5 ± 0.5 pmol/mg/min) was subtracted from the determined values. Data points represent mean ± standard error for triplicate determinations. Standard error bars that are not shown are within the symbols. Data were analyzed using eq. 1. The curves correspond to the following parameter values: π = 6.5 pmol/mg, k = 2 sec^{-1}, and $k_{cat}E_o$ = 6, 10.8, and 21 pmol/mg/min at 10 μM, 30 μM, and 1 mM ATP concentrations, respectively.

the same order of magnitude at 10 μM, 30 μM, or 1 mM ATP. The rate of the slower steady phase of the kinetics was ATP concentration dependent, increasing 2-fold between 30 μM and 1 mM ATP. For a given membrane preparation, at all ATP concentrations steady state rates measured with the pulsed quenched-flow, over tenths of seconds, were equal to rates measured under steady state conditions, over a reaction time of 5 or 10 min.

The existence of a burst of synthesis of one of the products is well known for reactions catalyzed by enzymes where a covalent intermediate is accumulated owing to a subsequent rate-limiting step (17). This is, for instance, the case in the hydrolysis of ester substrate by chymotrypsin, where an acyl-enzyme intermediate has been isolated and for which a burst of alcohol production is well established (17). The burst π is governed in such a situation by an equation of the form:

$$P = k_{cat}\, E_o t + \pi(1 - e^{-kt}) \qquad (1)$$

where P is the product that is determined, k_{cat} is the rate constant of the steady state phase, E_o is the total enzyme concentration, π is the maximum product burst, corresponding to the maximum intermediary complex that can be formed, k is the constant of the exponential pre-steady step of the complex formation, and t is time.

The first term of the equation corresponds to the product formed during the steady state phase of the reaction and the second to the initial burst of synthesis. According to eq. 1, the value of P is closest to π when the first term is much smaller than the second, i.e., when the amount of product formed during the steady state phase is the smallest possible, i.e., when its rate ($k_{cat}E_o$) is the slowest possible.

It is not yet known whether the activation of adenylyl cyclase activity implies the existence of a covalent intermediate of the enzyme. Fig. 4 suggests, however, that this activation occurs in at least two steps, with the second being much slower than the first. In order to substantiate the kinetic significance of these two steps, it was verified that under the same pre-steady state conditions no significant cAMP hydrolysis occurred in the presence or in the absence of 0.2 mM IBMX (data not shown). The absence of cAMP degradation, on the time scale of seconds, excluded the hypothesis that the slowing down of cAMP accumulation during the second step was due to hydrolysis by phosphodiesterases. It was also checked that this slowing down of cAMP production was not due to β receptor desensitization; the same kinetics were observed (data not shown) in the presence and in the absence of either 1 μM heparin or 1 μM protein kinase inhibitor, β-adrenergic receptor kinase and protein kinase A (the two kinases known to be responsible for the onset of desensitization) (18) inhibitors, respectively.

The slow step of cAMP production proved to be ATP concentration dependent (K_m close to 30 μM), whereas the preceding one, more rapid with a half-life of <0.5 sec, was apparently ATP concentration independent, at least at 5 mM free Mg^{2+} and in the range of tested substrate concentrations. Possibly, the time resolution of the pulsed quenched-flow apparatus used in this study did not allow the concentration dependence of the rapid first step to be observed. Assuming that activation of adenylyl cyclase can be described phenomenologically by eq. 1, we kept the ATP concentration the lowest possible (10 μM) to minimize the contribution of the first term to cAMP production and to measure the cAMP burst and its changes as accurately as possible.

Effect of GTP and Gpp(NH)p on cAMP burst. Because maximal adenylyl cyclase responses to isoproterenol of washed membrane preparations were shown to need GTP supplementation, the GTP concentration dependence of the pre-steady state kinetics of cAMP production was investigated. Fig. 5A shows the isoproterenol-elicited cAMP burst amplitude changes seen with increasing GTP concentration, measured at 10 μM ATP, compared with those of the steady state rate of isoproterenol-stimulated activity, measured at 1 mM ATP. Both parameters increased with increasing nucleotide concentrations with comparable EC_{50} values, around 0.1 μM. Activation of adenylyl cyclase by 10 μM GTP or 10 μM Gpp(NH)p in the presence of 50 μM isoproterenol was also compared under pre-steady state conditions. Fig. 5B shows that the cAMP burst was also elicited by the nonhydrolyzable nucleotide.

Effect of ADPβS on cAMP burst. Pulsed quenched-flow study of the inhibition by ADPβS of isoproterenol-stimulated cAMP production, using 10 μM ATP, is presented in Fig. 6. Both the burst amplitude and the steady state rate decreased in the presence of increasing concentrations of the ADP analog. Fig. 7 gives the ADPβS concentration dependence for the decrease of burst amplitude and steady state rate. The inhibition of both parameters went nearly to completion with very comparable IC_{50} values, corresponding to a mean value of 55 ± 20 μM. However, the time course of the burst did not change with increasing purinergic agonist concentrations; the first-order rate constant remained close to a mean value of 1.25 sec^{-1}, corresponding to a half-life on the order of 0.5 sec.

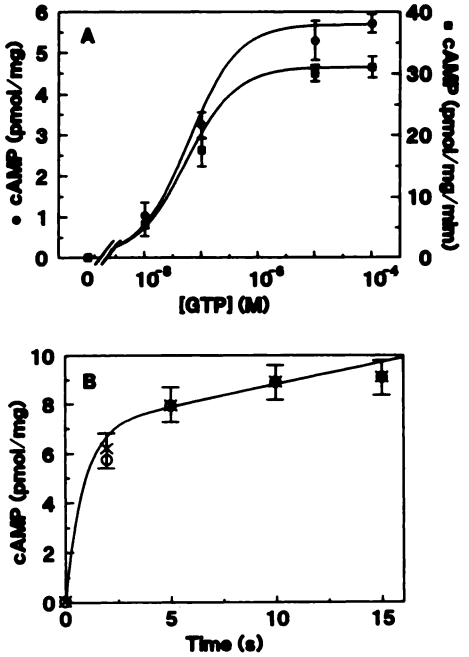

Fig. 5. Effect of GTP and Gpp(NH)p on cAMP burst amplitude. A, GTP concentration dependence of cAMP burst amplitude and steady state rate. ●, cAMP burst; ■, steady state rate. B, Comparison of pre-steady state cAMP accumulation in the presence of either 10 μM GTP (○) or 10 μM Gpp(NH)p (×). Adenylyl cyclase activity was measured with the pulsed quenched-flow apparatus, as described in the legend to Fig. 4, at 10 μM ATP for the burst and at 1 mM ATP for the steady state rate, in the presence of 50 μM isoproterenol. Basal activity (2.3 ± 0.6 pmol/mg/min in A, 2.8 ± 0.6 pmol/mg/min in B at 10 μM ATP and 5.2 ± 0.6 pmol/mg/min at 1 mM ATP) was subtracted from the data points, which are mean ± standard error of triplicate determinations. Membrane preparations were different in A and B.

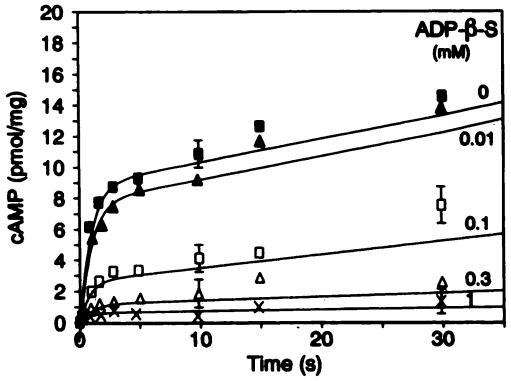

Fig. 6. Pre-steady state kinetics of isoproterenol-stimulated adenylyl cyclase activity of C6 cell membranes in the presence of increasing concentrations of ADPβS. Adenylyl cyclase activity was measured with the pulsed quenched-flow apparatus in the presence of 10 μM ATP and 50 μM isoproterenol, either in the absence (■) or in the presence of increasing concentrations of ADPβS, 0.01 mM (▲), 0.1 mM (□), 0.3 mM (△), and 1 mM (×). Data points were determined, analyzed, and represented as described in the legend to Fig. 4. Basal activity (2.8 ± 0.4 pmol/mg/min) was subtracted from the determined values. Resulting bursts (π) and rates V (corresponding to $k_{cat}E_o$ of eq. 1 were used to plot the curves in Fig. 7. The determined first-order rate constants (k) of the bursts at increasing ADPβS concentrations were between the values of 1.05 and 1.55 sec^{-1}, with a mean value of 1.25 ± 0.3 sec^{-1}.

Effect of Pertussis Toxin on ADPβS Inhibition of cAMP Responses in Cell Membranes

Inhibition by ADP of the intact cell cAMP response to

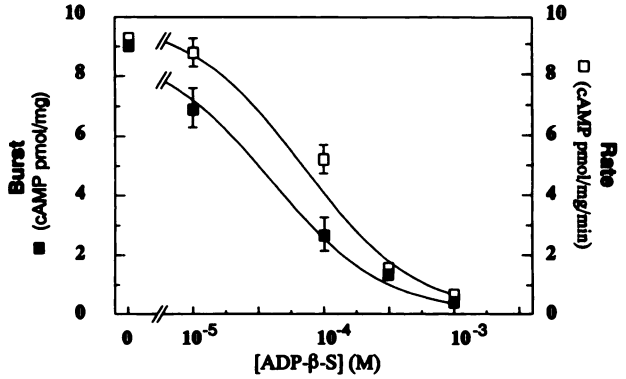

Fig. 7. ADPβS concentration dependence of isoproterenol-stimulated burst and steady state production of cAMP in C6 cell membranes, measured with the pulsed quenched-flow apparatus. Data of Fig. 6 were analyzed as described in the legend to Fig. 3. The represented curves correspond to the following parameter values: ■, Burst, π, in the absence of ADPβS, 9 pmol/mg; ADPβS IC_{50}, 40 μM. □ Rate, V, in the absence of ADPβS, 9 pmol/min/mg; ADPβS IC_{50}, 70 μM.

TABLE 2

Abolishing effect of pertussis toxin cell treatment on inhibition by ADPβS of isoproterenol-stimulated adenylyl cyclase activity of C6 cell membranes

C6 cells were treated with 100 ng/ml pertussis toxin in the culture medium for 24 hr before harvesting and membrane preparation. Adenylyl cyclase activities of resulting membranes were determined under steady state conditions, in the presence of 50 μM isoproterenol, and either in the presence or in the absence of 1 mM ADPβS at two ATP concentrations, as described in the legend to Fig. 3. Basal activity was measured in the absence of isoproterenol and ADPβS. Experimental data are mean ± standard error for triplicate determinations.

	Adenylyl cyclase activity		
	Basal	Isoproterenol	Isoproterenol + ADPβS
	pmol/min/mg		
Control cells			
10 μM ATP	3.2 ± 0.2	13.2 ± 1.0	2.5 ± 0.2
1 mM ATP	2.5 ± 0.1	30.8 ± 1.9	2.8 ± 0.3
Treated cells			
10 μM ATP	1.9 ± 0.2	13.7 ± 1.0	16.0 ± 1.2
1 mM ATP	2.3 ± 0.1	29.1 ± 2.0	27.3 ± 2.1

isoproterenol stimulation has previously been shown to be abolished by treatment of the cells with pertussis toxin (8). This toxin action on cells was also tested on cAMP responses of cell membranes. A culture was treated by pertussis toxin 24 hr before cell harvesting, and membranes were prepared from control and treated cells. Table 2 demonstrates that inhibition by ADPβS of the isoproterenol-stimulated adenylyl cyclase activity was abolished in the membrane preparation from treated cells, when measured at both 10 μM and 1 mM ATP concentrations.

Effect of ADP on Isoproterenol-Stimulated cAMP Responses in Cell Membranes

In spite of ATP and ADP hydrolysis by membrane preparations, inhibition by ADP of the stimulated cAMP response could be demonstrated by using a large excess of both ADP and ATP in the absence of the ATP-regenerating system. Under such conditions, neither ATP nor ADP concentrations were controlled, and no burst and rate values could meaningfully be determined. Fig. 8 shows, however, that under such conditions an initial cAMP burst was observed and could be inhibited in the presence of 1 mM initial ADP concentration. This shows

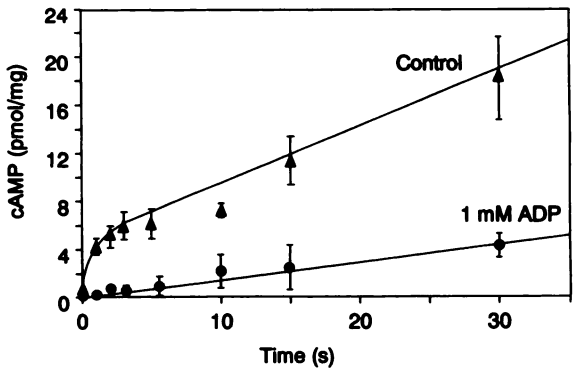

Fig. 8. Effect of ADP on pre-steady state isoproterenol-stimulated adenylyl cyclase activity of C6 membranes in the absence of an ATP-regenerating system. Adenylyl cyclase activity was measured in the presence of 1 mM initial ATP concentration and 50 μM isoproterenol, either in the absence (control, ▲), or in the presence (●) of 1 mM initial ADP concentration, as described in the legend to Fig. 4. Creatine kinase and creatine phosphate were omitted from the reaction mixture. Basal activity (2.5 ± 0.4 pmol/mg/min) was subtracted from data points, which are mean ± standard error for triplicate determinations.

that ADPβS mimicked ADP action in cell membranes, as it did in intact cells.

Discussion

P_2 receptors as well as P_1 receptors seem to be able to activate several transduction mechanisms in various cells (19). In the case of the C6 cell line, the action of pertussis toxin on cAMP response in intact cells has suggested the involvement of a G_i protein in adenylyl cyclase inhibitory ADP action (8). A few years ago, the discovery that $G_{\beta\gamma}$ subunits are interchangeable between G_s and G_i proteins led to the concept that negative coupling corresponds to a shift in the position of the equilibrium between $G_{s\alpha}$, $G_{i\alpha}$, and $G_{\beta\gamma}$ subunits and the cyclase. This modification has been postulated to occur with (20) or without (21) total dissociation of G_α subunit from the corresponding heterotrimer. Hence, one of the questions that may be raised by the decrease in activity of an effector enzyme is how direct the mechanism of its switch-off by activation of a given inhibitory receptor is. Does the enzyme activity decrease because of the modification of protein interactions within the cell membrane, as suggested above, or does the enzyme respond to the activation of another transduction system with the involvement of another second messenger and the stimulation of the phosphorylation of some protein? In other words, is the pathway direct or indirect? Many experimental data have shown that receptor cross-talk may occur via the phosphorylation of one of the members of the receptor-transducer-effector systems by a second messenger-activated protein kinase. The present work attempts to provide experimental data for estimating the time necessary for inhibiting β-adrenergic activation of adenylyl cyclase when ADP receptors are stimulated at the same time. Such evidence could confirm or refute the existence of covalent steps in the mechanism of inactivation of the effector enzyme (22). For instance, electrophysiological techniques allow an estimation of the time necessary for a change from the open to the closed state (or vice versa) of an ion channel in response to its second messenger-induced phosphorylation. Several studies have indeed shown a time lapse of several seconds between the moment when a G protein-linked, second messenger-producing

receptor is stimulated and the moment when modification of membrane ionic permeability occurs (23, 24).

The inhibitory action of ADP on adenylyl cyclase at presumed P_2 receptors was first demonstrated in live C6 cells (8). ADP has been shown to inhibit cAMP production in isoproterenol-stimulated cells with an IC_{50} value close to 0.5 μM. ATP added to the cells in the presence of an ATP-regenerating system, in order to preclude ADP accumulation, and the nonhydrolyzable ATP analog adenosine (β,γ-methylene)triphosphate were shown to be 1000-fold less potent in this respect (8). However, in C6 cells ATP and the nonhydrolyzable nucleotide were found to behave as agonists, and not as antagonists, of ADP receptors, in contrast to what has been shown in the case of platelets carrying typical P_2 receptors, which have long been known to be negatively coupled to adenylyl cyclase (25). In these latter cells, P_2 purinoceptors have recently been recognized as a definite pharmacological subtype, classified as P_{2t} (26). P_2 purinoceptors have also been found in various other cell types, for instance, hepatocytes (27) and astrocytes (9). In the latter cell type, P_2 purinergic receptors are sensitive to both ATP and ADP and are linked to phosphoinositide hydrolysis. Mahaut-Smith *et al.* (28) have also recently described another transduction mechanism for ADP receptors in platelets, i.e., activation of the opening of ionic channels, suggesting that ADP could promote platelet aggregation through modulation of cell membrane permeability to cations. Their finding would explain experimental data that support the concept that ADP-induced platelet aggregation and ADP-mediated adenylyl cyclase inhibition are two independent events in these cells (29).

The difficulty in using adenine nucleotides as agonists or antagonists has often been stressed (30). It lies in the susceptibility of these metabolites to hydrolysis by cell nucleotidases and their utilization in various enzymatic reactions. In the case of the C6 cell line, this drawback is even worse because of the tumoral character of these cells, a state that is accompanied by the presence of very active ectonucleotidases (31). P_2 sites have, however, been determined in some cases by binding of various radioactive ADP and ATP analogs. Macfarlane *et al.* (32) have used 2-methylthio-[β-^{32}P]ADP to measure P_2 receptor density in human platelets. Cooper *et al.* (33) have reported the determination of P_{2y} sites in turkey erythrocyte membranes using the ADP analog [^{35}S]ADPβS. The use of the same analog, ADPβS, which is poorly reactive toward enzymes (15), is instrumental in the present study of membrane responses to ADP receptor activation.

Before its use with cell membranes, the P_2 agonist action of ADPβS toward intact cells was ascertained by comparing it with that of ADP. This ADP congener decreased the isoproterenol-stimulated cAMP response of cells as did ADP, with an IC_{50} value close to 25 μM (Fig. 2), >3 orders of magnitude larger than the K_d value measured by Cooper *et al.* (33) in erythrocytes for the binding of this compound at P_{2y} sites. Inhibition of the cell isoproterenol-stimulated cAMP response by ADP and ADPβS was not complete (60%). With cell membrane preparations, the inhibition due to ADPβS went to completion. Examples of incomplete inhibition of β-adrenergic responses in whole cells by inhibitory receptors can be found in the literature (34). Difference in the extents of inhibition between live cells and membrane preparations, in the case of C6 cells, may be explained by altogether different adenylyl cyclase activity conditions, i.e., ATP and GTP concentrations, G_s to G_i ratios, etc. When related to the number of β-adrenergic sites, the isoproterenol-stimulated activity of whole C6 cells is usually about 5 times higher than that of membrane preparations (measured at 1 mM ATP and 100 μM GTP) (16), a difference that could be the consequence of a loss of G_s during cell disruption and subsequent steps of membrane manipulation. Nonequilvalent losses of G_s and G_i from membranes could occur and lead to different percentages of inhibition in intact cells and membrane preparations.

The IC_{50} value determined for ADPβS in the inhibition of the steady state isoproterenol-stimulated adenylyl cyclase activity of membranes was in the range of 75 μM (mean value, 79 ± 20 μM), at ATP concentrations varying between K_m and saturation (Fig. 3). The data of Fig. 3 could be analyzed in terms of noncompetitive inhibition. Absence of competition between ADPβS and ATP suggested that the nucleotide action did not occur by inhibition of the binding of the substrate of adenylyl cyclase. Inhibition of adenylyl cyclase at the so-called "P site" is also known to give rise to noncompetitive inhibition (34). Both a P site effect and competition at the substrate ATP binding site had to be excluded because inhibition by ADPβS was abolished in membrane preparations by pertussis toxin treatment of the cells (Table 2). Therefore, the inhibitory effect of the ADP analog on isoproterenol-stimulated adenylyl cyclase activity could only be receptor mediated, the consequence of its binding to the receptor, resulting in an inhibition of the enzyme. This inhibition of a noncompetitive type reduced the apparent steady state amount of active enzyme. Investigation of ADPβS action under pre-steady state conditions demonstrated that it induced a decrease of both the amplitude of the cAMP burst and the rate of the slower step of cAMP production, with comparable IC_{50} values for both parameters, close to 50 μM (mean value, 55 ± 20 μM), when analyzed according to noncompetitive inhibition (Figs. 6 and 7). Here, the binding of the analog to the receptor not only reduced the amount of active cyclase, as measured under steady state conditions, but also prevented the occurrence of the initial rapid step of enzyme activation. Thus, in both steps, the ADP analog concentration appeared to control the existence of active cyclase or its activatability.

Unlike the steady state rate, the time constant of the cAMP burst was not affected by the presence of the ADP analog (Fig. 6); the first-order rate constant remained close to 1.25 sec^{-1} at increasing ADPβS concentrations. Because the two agonists of the two interacting receptors, isoproterenol and ADPβS, were mixed at the same time with the membrane preparation (maintaining membranes in the presence of isoproterenol was avoided to exclude any possible desensitization phenomenon), the equilibrium allowing only a fraction of adenylyl cyclase activity to be expressed was clearly reached within the dead-time of the apparatus, a time shorter than the burst time course. Hence, as discussed above, a time course shorter than 1 sec reasonably excludes the occurrence of steps involving covalent bond formation, such as the production of another second messenger (for instance, inositol trisphosphate and/or diacylglycerol) and/or the induced phosphorylation of one of the proteins of the β-receptor transduction system. The concomitant stimulation of the two receptors by their respective agonists resulted, therefore, in the reshuffling of the interactions between them, $G_{s\alpha}$, adenylyl cyclase, $G_{\beta\gamma}$, and $G_{i\alpha}$, with all of these events occurring in the plane of the membrane or in its neighborhood. Half-lives

of <1 sec are compatible with the time constants of lateral diffusion of proteins in the plane of a membrane (35). This estimation of the time necessary to balance the interactions of β-adrenergic and purinergic receptors, resulting finally in the inhibition of adenylyl cyclase, gives supplementary credit to a real involvement of a G_i protein in the ADP receptor transduction mechanism and to a direct negative coupling of this receptor to the enzyme. Such a result supports the classification of these receptors of C6 cells as a P_2 subtype, analogous to the P_{2t} subtype of platelets, that one could name for instance P_{2tg}, because they are expressed in a rat glioma.

Using a rapid-mix quench method very comparable to ours, Thomsen and Neubig (5) have studied in platelet membranes the transient kinetics of the inhibition of forskolin-stimulated adenylyl cyclase activity mediated by the α_2-adrenergic agonist epinephrine. They observed at 1 μM GTP a slower onset of the adenylyl cyclase inhibition by epinephrine (half-life close to 6 sec) than occurred with ADPβS in isoproterenol-stimulated C6 cell membranes. However, in the presence of 100 mM NaCl and 100 μM GTP, this rate increased 5-fold. With the same membrane preparation, the authors noted no burst of cAMP production upon activation of adenylyl cyclase by either PGE_1 or forskolin (5). It would of course be of great interest to interpret the molecular meaning of the cAMP burst observed in C6 cell membranes upon β-adrenergic receptor stimulation. Its amplitude exceeds by far the number of β-adrenergic sites in these membranes (200–300 fmol/mg of protein). The number of adenylyl cyclase molecules has not yet been determined directly in any membrane with molecular probes. The enzyme has been estimated to represent 0.01–0.001% of membrane protein content in synapses (36), which would correspond to a density of 5–50 fmol/mg of protein, a value much smaller than the size of the burst. The $G_{s\alpha}$ protein content of cell membranes is somewhat better known. It is closer to, and sometimes even larger than, the amplitude of the cAMP burst (20, 37). Because stimulation of ADP receptors reduces the apparent amount of active adenylyl cyclase, the amplitude of the initial step of cAMP synthesis, in the absence of ADP, may well correspond to the number of $G_{s\alpha}$ molecules involved in the activation of the enzyme during this initial step of cAMP synthesis. GTP concentration dependence of the cAMP burst amplitude is compatible with this hypothesis. In the presence of ADP or its analog, the number of $G_{s\alpha}$ molecules interacting with the cyclase could progressively decrease with the involvement of G_i, which would induce a shift of the equilibrium between $G_{s\alpha}$, $G_{\beta\gamma}$, and the heterotrimer G_s toward its undissociated form. Such a hypothesis is of course compatible only with the existence in the β-adrenergic receptor-mediated activation of adenylyl cyclase of a distal rate-limiting step, as has already been suggested for some other receptor systems linked via G proteins to effector enzymes (5, 38, 39).

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