Volume 226, number 2, 331-336 FEB 05458 January 1988

A quenched-flow study of a receptor-triggered second messenger response: cyclic AMP burst elicited by isoproterenol in C6 glioma cell membranes

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Received 12 November 1987

Fast kinetic studies of cAMP accumulation in C6 cell membranes show a burst of cAMP after β-adrenergic receptor stimulation by isoproterenol. This burst is no longer observed when the ATP present in membrane preparations is hydrolyzed, but can be restored by their preincubation in the presence of ATP-Mg. The size of the burst is much larger than the number of β-adrenergic receptors and is of the same order of magnitude as the value reported for G proteins. Further characterization of the burst will allow studies of the functional interaction of receptor-adenylate cyclase components in C6 membranes.

β-Adrenergic receptor; cyclic AMP synthesis; Kinetics; Pulsed quenched-flow; (C6 glioma)

1. INTRODUCTION

The recent cloning of several β-adrenergic receptors and G proteins has allowed one to clarify molecular aspects of receptor coupling to adenylate cyclase [1]. Until now, the description of such systems has been based mainly on what could be learned from the isolation and the purification of their components and from their functional reconstitution in natural or artificial membranes [2,3]. Little, however, is known concerning the stoichiometry of the interaction between the receptors and the other components (the G protein and the cyclase) in the cell membrane, and the kinetics and amplitudes of the early second messenger responses. Such characteristics can be obtained from measurements of the cyclic AMP accumulation using fast-mixing techniques which allow one to perform analyses of enzymatic reactions in the second-to-minute time range [4]. This work describes a pulsed quenched-flow study of cyclic AMP synthesis triggered by β-adrenergic receptor stimulation using C6 glioma cell membranes. The existence of a rapid burst of the second messenger produced at the expense of either a preferential pool of ATP, or a yet unidentified intermediate, is demonstrated.

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Abbreviations: [^{3}H]CGP 12177, 4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one; IBMX, isobutylmethylxanthine; OPNT, *o*-phenanthroline; PMSF, phenylmethylsulfonyl fluoride; RIA, radioimmunoassay; STI, soybean trypsin inhibitor

2. MATERIALS AND METHODS

Dulbecco's modified Eagle medium and antibiotic–antimycotic solution (penicillin, strep-

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00145793/88/$3.50

tomycin and fungizone) were from Gibco (USA) and fetal calf serum from Flow Labs (England). STI, aprotinin, PMSF, OPNT, EDTA, ATP, creatine phosphate, creatine kinase, cAMP, 2′-*O*-succinyl-cAMP-tyrosine methyl ester, IBMX, isoproterenol, alprenolol, luciferin and luciferase were purchased from Sigma (England). Forskolin was from Calbiochem (USA), carrier-free $Na^{125}I$ from NEN (FRG) and [^{3}H]CGP-12177 from Amersham (England). Antibodies against cyclic AMP were a gift from Dr Cailla (Centre d'Immunologie, INSERM-CNRS, Luminy, France).

The C6 rat glioma clone was obtained through the courtesy of Dr Legault (Collège de France, Paris). Cells were cultured at 37°C in Dulbecco's modified Eagle medium, supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), fungizone (0.25 mg/ml) and 5% fetal calf serum, in 5% CO_2-air. Preparations were carried out with confluent cells, 6 days after plating.

Membranes from cells were prepared using a modification of the method of Lucas and Bockaert [5]. Briefly, about 3×10^8 cells were washed twice with ice-cold 0.145 M NaCl, suspended in 60 ml of lysis buffer (25 mM Tris-HCl, pH 8; 5 mM EDTA; 0.1 mM PMSF; 1 mM OPNT; 0.1 μg/ml STI; 15 μg/ml aprotinin) and homogenized with a Dounce homogenizer. The suspension was centrifuged for 10 min at $1000 \times g$. The resulting supernatant was spun for 30 min at $5000 \times g$. The $5000 \times g$ membrane pellet (about 0.5 ml) was suspended in 1 ml of 10 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA. The overall dilution of the cytosolic cell content was of the order of 500-fold, assuming a volume of 1 μl for 10^6 cells. The membrane preparation was aliquoted, rapidly frozen in liquid nitrogen, and stored at −80°C. Under these conditions, no loss of isoproterenol-stimulated adenylate cyclase activity was observed for several months. The number of receptor sites of the membrane preparation, as determined with the hydrophilic antagonist [^{3}H]CGP-12177 [6], according to Voisin et al. [7], was generally close to 180–200 fmol/mg of protein. The endogenous cyclic AMP content of the preparation, as measured by the RIA method of Cailla et al. [8], was never greater than 1.5 pmol/mg of protein. The ATP content of membrane preparations was determined by the light emitted in the presence of luciferin-luciferase [9] with a LKB-Wallach 1250 luminometer. Protein content was determined according to Lowry et al. [10], using bovine serum albumin as a standard.

Adenylate cyclase activity was measured by the cAMP RIA [8], with the addition of an alumina adsorption step, as proposed by Volker et al. [11], to eliminate the cross-reactivities of ATP and the nucleotides derived from ATP. The sensitivity of this RIA, under the conditions described below for the adenylate cyclase assay, was 100 fmol. The kinetics of cyclic AMP appearance were studied with a pulsed quenched-flow apparatus, constructed in the laboratory according to Ghelis [12]. It was thermostated at 25°C, and adapted to mix 50 μl samples. Unless otherwise stated, the following final conditions were used: 1 mM ATP, 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), 50 μM isoproterenol (when needed) and 2–5 mg/ml membrane protein. Since the membrane preparation was diluted twice in the quenched-flow apparatus, the final mixture also contained EDTA and Tris. The reaction quencher was perchloric acid (1 M final concentration). All the values appearing in the figures are the means of triplicate determinations. The standard deviations, between 7 and 13%, are not represented.

3. RESULTS

Fig.1A shows the kinetics of cyclic AMP accumulation between 0.5 and 60 s after mixing freshly thawed C6 membranes in a quenched-flow apparatus with either ATP-Mg and isoproterenol (curve 1), or ATP-Mg alone (curve 2). These kinetics show the existence of a burst of cAMP that precedes the steady-state accumulation in the presence of the β-adrenergic agonist. Alprenolol prevented the isoproterenol-elicited burst (fig.1B). The size of the burst, as extrapolated from the linear portion of the curve back to zero time, was dependent upon the isoproterenol concentration (fig.1B). The EC_{50}, close to 50 nM, was as observed for the steady-state cAMP synthesis stimulated by the agonist in C6 membranes [13]. These facts revealed the true β-adrenergic character of the cAMP burst. The amplitude of the burst, close to 10 pmol/mg of protein, was much larger than the density of the β-receptors in the C6

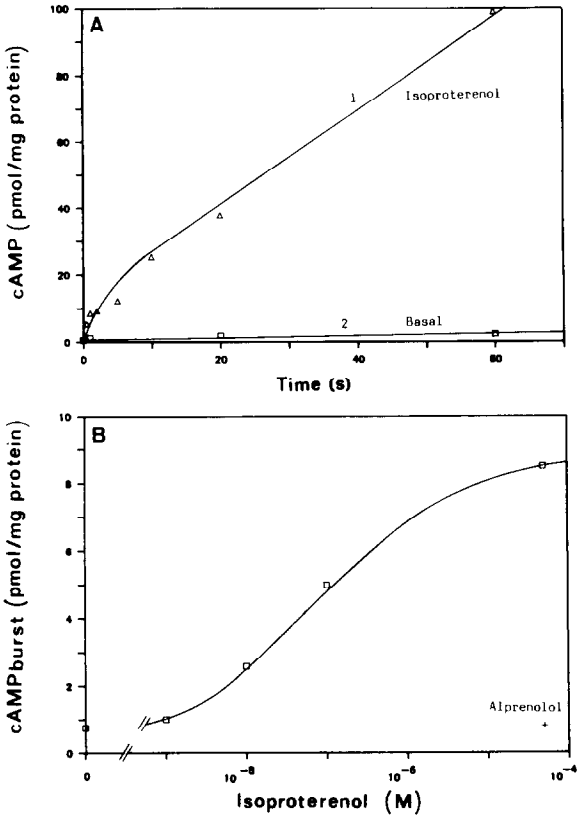

Fig.1. Quenched-flow study of isoproterenol-stimulated cAMP synthesis by C6 membrane preparations. (A) At time 0, 50 μl of the membrane preparation (2 mg/ml of protein in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA) were mixed in the quenched-flow apparatus, with 50 μl of either ATP-Mg and isoproterenol (curve 1), or ATP-Mg (curve 2). After mixing, the final concentrations of the added components were: 1 mM ATP, 6.25 mM $MgCl_2$, 50 μM isoproterenol. The concentrations of other components were as stated in section 2. cAMP was determined by RIA after quenching the reaction with perchloric acid. The amplitude of the burst could be determined by extrapolating the linear portion of each curve to zero time. (B) Same conditions as in A, except that variable final isoproterenol concentrations were used. The amplitude of the burst was determined as explained in A. For (+) 100 μM alprenolol (final concentration) was added at the same time as isoproterenol.

glioma cell membrane preparation (close to 200 fmol/mg of protein). Furthermore, a similar burst was observed when adenylate cyclase was directly stimulated by forskolin (fig.2). These

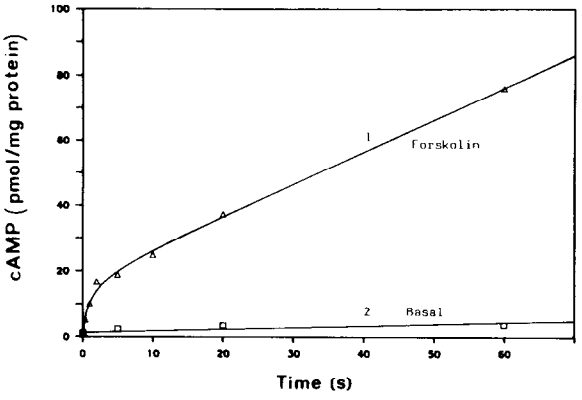

Fig.2. Quenched-flow study of forskolin-stimulated cAMP synthesis by C6 membrane preparations. Same legend as in fig.1A, except that isoproterenol was replaced by 50 μM forskolin (final concentration).

observations suggested that the burst could originate from an accumulated precursor. In order to investigate the existence of such a precursor, experiments were performed in the absence of added ATP (fig.3, curve 1). Although the steady-state phase was very slow, a burst of cAMP was still observed in the same time range and with an amplitude (9 ± 0.9 pmol/mg of protein) similar to that observed in the presence of ATP (10 ± 1 pmol/mg of protein, fig.3, curve 2). When tested with different membrane preparations, the amplitude of the burst ranged from 9 to 12 pmol/mg of protein and was similar whether measured in the absence, or in the presence of ATP.

The existence of significant cAMP synthesis after stimulation of the receptor, in the absence of added ATP, suggested that there could either be some ATP remaining in the membrane preparation, bound to membrane proteins for instance, or some other accumulated intermediate that could give rise to this rapid cyclic nucleotide synthesis. The ATP content of membrane preparations was determined. It was usually of the order of 350–400 pmol/mg of protein, much larger than the amplitude of the burst, but in good agreement with a mean cell ATP concentration of 2 mM, when the dilutions carried out during the preparation are taken into account. No decrease of the ATP content was observed when freshly thawed membranes were kept at room temperature (fig.4, curve 1). The persistence of ATP in the membrane

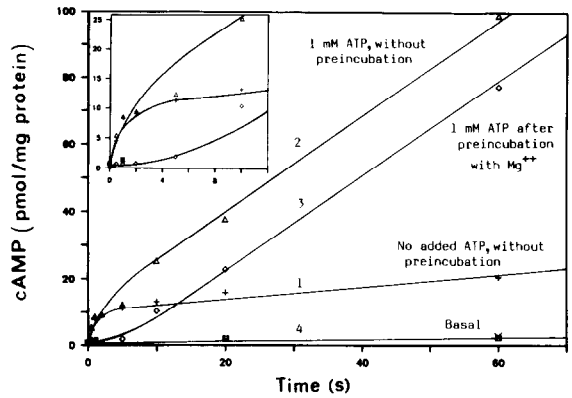

Fig.3. Isoproterenol-elicited burst of cAMP synthesis: effect of the presence of ATP and influence of the membrane preincubation with $MgCl_2$. Same legend as in fig.1A. After mixing, the final concentrations of the added components were: (1) 6.25 mM $MgCl_2$ and 50 μM isoproterenol; (2) 1 mM ATP, 6.25 mM $MgCl_2$ and 50 μM isoproterenol; (3) as in 2, except that the membrane preparation was preincubated for 40 min, before mixing, in the presence of 2 mM $MgCl_2$; (4) basal activities without preincubation: (×) no ATP; (□) 1 mM ATP. Inset: Expanded representations of the first 20 s of the reactions.

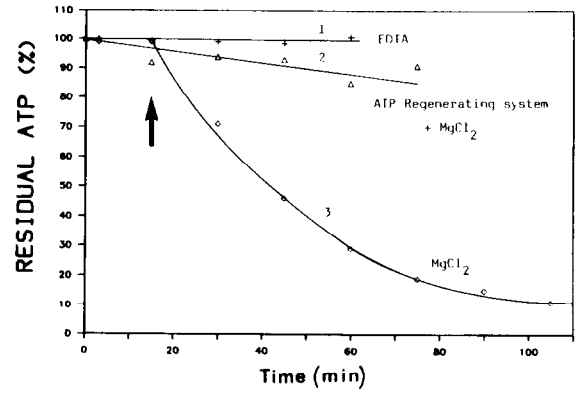

Fig.4. Endogenous ATP hydrolysis by C6 membrane preparations. The membrane preparation (about 1 mg/ml protein, in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 400 pmol/mg endogenous ATP) was incubated at room temperature under different conditions: (1) no addition, i.e. with EDTA; (2) 15 min with no addition, then 2 mM $MgCl_2$ at the arrow; (3) 2 mM $MgCl_2$, 0.2 mg/ml creatine kinase and 20 mM creatine phosphate.

preparations was presumably due to the presence of EDTA in the preparation buffers. Indeed, as soon as an excess of $MgCl_2$ was added to the membranes, the ATP concentration decreased (fig.4, curve 2). This decrease was almost completely prevented by the presence of an ATP-regenerating system (fig.4, curve 3), which demonstrated that it originated mainly from the consumption of ATP by ATPases, kinases, etc., yielding ADP.

The kinetics of cAMP appearance upon mixing with isoproterenol membranes, maintained at room temperature for up to 1 h in the presence of EDTA, were similar to those observed with the freshly thawed membranes when tested in the presence, or in the absence of ATP. One could wonder whether the preincubation of the membranes in the presence of Mg^{2+} so as to ensure ATP consumption would modify these kinetics. When measured in the absence of ATP, no significant accumulation of cAMP was observed. Upon addition of 1 mM ATP (fig.3, curve 3), no burst was observed. Instead, a lag phase occurred before the steady-state synthesis. However, the isoproterenol-elicited burst could be restored when the membranes, after a first preincubation in the presence of Mg^{2+} for 40 min, were equilibrated for 10 min with 1 mM ATP-Mg before the addition of isoproterenol.

4. DISCUSSION

Very little is known about the rates of ligand-receptor interactions and the primary rates of transduction in the case of enzyme-linked receptors, although electrophysiological techniques demonstrate that these events can take place within a few seconds. The occurrence of a finite burst of cAMP within 1–2 s of stimulation of the β-adrenergic receptor of C6 glioma cell membranes, corroborates the rapidity of the association of the agonist with the receptor, and of the subsequent interactions between the various components of the system (receptor, G_s protein, cyclase). The amplitude of the burst shows that a significant concentration of the second messenger can be built up within a few seconds in response to a stimulation of the cell receptor. The resulting concentration is within the range of the cAMP-dependent kinase activation constant [14].

To explain the origin of the burst, two hypotheses can be proposed: (i) the existence of an

endogenous ATP pool; (ii) the existence of a preexisting chemical intermediate of the cyclisation reaction. The demonstration of the existence of a specific pool of ATP, either membrane-bound or cyclase-bound, is technically difficult. The size of this pool, as can be estimated from the amplitude of the cAMP burst, would correspond to only 2–2.5% of the total amount of ATP present in the preparation. Evidence for the existence of such an ATP pool has already been obtained in the case of human red cell membranes [15].

The interpretation of the experimental results in terms of the second hypothesis implies that the preexisting intermediate, which is not cAMP, is preserved from destruction as long as the membrane preparation is maintained in the presence of EDTA, that it disappears upon Mg^{2+} addition, and that it can be regenerated in the presence of ATP-Mg. The cAMP burst would originate from the rapid utilization of this intermediate as a result of the stimulation of the cyclase by either G_s or forskolin. The slower steady-state rate of cAMP appearance would then correspond to the rate of the resynthesis of the intermediate, which would be the limiting step of the overall reaction.

The chemical nature of such an intermediate is not deducible from the present data, but the simplest explanation is that it is a derivative of the cyclase itself. Until now, no titration of adenylate cyclase has been reported. It was suggested that forskolin sites could correspond to adenylate cyclase sites [16]. Two classes of binding sites for forskolin are usually found [17]. The high-affinity sites would correspond to adenylate cyclase in interaction with G_s, while the low-affinity sites would correspond to the enzyme that can only be stimulated by forskolin [17]. The size of the burst observed in C6 membranes is similar, whether it is elicited by G_s activation with isoproterenol, or directly by forskolin and corresponds to the density of low-affinity forskolin sites in different membranes [17]. However, the assignment of the low-affinity forskolin binding sites to adenylate cyclase has not yet been fully demonstrated. The size of the cAMP burst that we observe could give a lower estimate of the adenylate cyclase sites in C6 cell membranes. Its magnitude is effectively comparable to the total concentration of G proteins in these membranes, as detected by the concentration of their common β-subunit [18]. It is possible that the interaction of adenylate cyclase with G_s in C6 cells is different from that occurring in other cells, insofar as stoichiometry and affinity are concerned, accounting for the equivalent burst observed in the presence of isoproterenol, or forskolin.

The knowledge of the stoichiometry of the adenylate cyclase system components, in natural membranes, and of the different receptors linked to them cannot rely only on indirect methods such as those developed in the present work. Information will probably be obtained through the cloning of their genes and the study of their expression. The present approach could help to understand the molecular mechanism of the cyclase activation by the receptor, via G_s, or by forskolin, and thus provide data concerning the functional stoichiometry of receptors, G proteins, and the enzyme.

ACKNOWLEDGEMENTS

This work shows partial results of the PhD thesis of H.V. We wish to acknowledge the critical reading of B. Labouesse and M. Merle.

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