Biochemical Pharmacology, Vol. 44, No. 2, pp. 351–358, 1992.
Printed in Great Britain.

0006-2952/92 $5.00+0.00

EFFECT OF ALBUMIN ON ADENYLATE CYCLASE RECEPTOR-RELATED SIGNAL TRANSDUCTION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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(*Received* 14 *October* 1991; *accepted* 6 *April* 1992)

Abstract—In the present study we investigated *in vitro* the effect of human serum albumin (HSA) on receptor-stimulated cAMP production in isolated human peripheral blood mononuclear cells (PBMC). The cAMP production is strongly correlated with the pH of the medium during long incubations with albumin. Adenylate cyclase is stimulated by receptor agonists like histamine, forskolin, prostaglandin E_2 and the β-adrenergic agonist isoprenaline, in the presence or absence of HSA. This protein, at concentrations above 0.1%, dose-dependently inhibits both basal and agonist-stimulated cAMP levels in PBMC. In the presence of 0.5% HSA a significant reduction of 30–60% (cell batch dependent) is induced, a reduction which is not incubation time dependent. Washing the cells after a period of incubation with 2% HSA does not reverse the HSA-induced cAMP inhibition. Oleic acid-evoked conformational changes in HSA were not capable of influencing the inhibition processes of HSA on the isoprenaline-stimulated cAMP production. Structure-controlled interactions between HSA and membrane or adenylate cyclase are therefore unlikely. Bovine serum albumin and chicken albumin had different effects upon the agonist-stimulated cAMP production as compared with HSA. At this moment no explanation for this behavior can be provided. The findings indicate that albumin may inhibit non-specifically cAMP production in PBMC and possibly influences membrane-controlled processes.

The mechanism of the signal transduction pathway in mammalian cells is quite complex. Part of the early route in this process is protein controlled. The adenylate cyclase complex consists of several proteins responsible for signal transport and amplification. Among the different types of stimulatory receptors, the β-adrenergic receptor proteins are linked to guanine nucleotide binding proteins (G-proteins‡), which in turn are coupled to adenylate cyclase [1]. The active site of the transmembrane protein adenylate cyclase is located in the cytoplasmatic region of the membrane. The adenylate cyclase complex induces variation in cAMP levels and these fluctuations are pivotal for the regulation of cell function. The ability of albumin to deregulate different cellular processes has been established [2–8]. Involvement of the regulatory pathway stimulated by cAMP has been suggested [8]. *In vitro* mechanistic research on receptors is mostly performed with straightforward model studies, omitting every possible interfering substance and consequently not studying the involvement of extracellular medium conditions. When, however, cell viability or function changes, nutritional additives often include serum. For some reason the possible effects of serum addition or omittance are frequently not investigated Moreover, the complex composition of serum prohibits detailed mechanistic studies. However, the absence or presence of serum products in nutrient buffer solutions may cause strong differences in the potencies of certain drugs [3, 4]. Although many reports show substantial evidence for interference of the major serum protein albumin with cellular events like proliferation, differentiation and transformation [2, 5, 9, 10], the role of albumin in cellular signal transduction is largely unknown.

Serum albumin has high affinity sites for fatty acids and therefore traps efficiently fatty acids released from cell stores. Arachidonic acid metabolism is linked with bovine serum albumin (BSA) in macrophages (2% BSA) and in human lung fibroblasts (0.2% BSA) [2, 3]; hormone production in Leydig cells is affected by extracellular fatty acid binding to human serum albumin (HSA) [10]. Affinity of prostaglandin receptors in rat epididymal adipocyte membranes is modulated by BSA [11] and the prostaglandin synthesis itself is additionally related to the trapping of fatty acid in fibroblasts [3]. Mammalian cell membranes are primary stores of lipids and therefore interactions with high affinity fatty acid binding proteins like albumin may induce modifications of the internal characteristics of the membranes. The high concentration of albumin in the plasma surrounding the cells makes interactions even more likely.

In this study we investigated the influence of HSA on the membrane-bound adenylate cyclase of human peripheral blood mononuclear cells (PBMC), and tried to link it with previously reported fatty acid-induced conformational changes in the overall structure of HSA. Oleic acid was used to examine

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‡ Abbreviations: HSA, human serum albumin; BSA, bovine serum albumin; PGE_2, prostaglandin E_2; PBMC, peripheral blood mononuclear cells; G-protein, guanine nucleotide binding protein; IBMX, 3-isobutyl-1-methylxanthine.

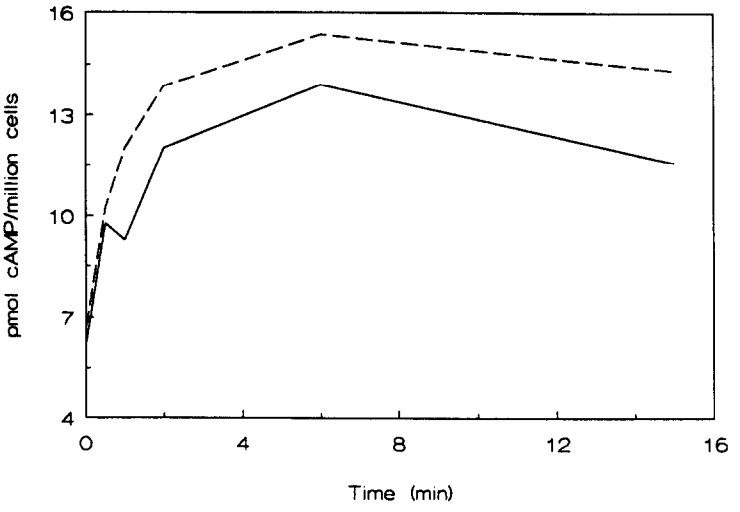

Fig. 1. The effect of stimulation time on isoprenaline (10^{-6} M)-stimulated cAMP production in PBMC in the presence of 0.5% HSA (———) or in the absence of HSA (– – –). Data are corrected for basal cAMP values in the presence or absence of HSA.

the effect of conformational transitions in HSA on cAMP production in PBMC. The receptor-mediated stimulation of the cAMP production process was studied by means of histamine, prostaglandin E_2 (PGE_2) and the β-adrenergic agonist isoprenaline. Forskolin, a direct adenylate cyclase activator, was used to bypass the receptor. Consequences of the observed behavior of HSA and the cell membrane are discussed.

MATERIALS AND METHODS

HSA was isolated from pooled human plasma according to the method described by Kremer [12]. BSA, chicken albumin and all other chemicals were of the highest analytical grade (Pharmacia, Uppsala, Sweden; Janssen Chimica, Beerse, Belgium; Sigma Chemical Co., St Louis, U.S.A.). Chemicals and albumins were dissolved in the incubation medium (RPMI-1640). Solutions prepared for incubation experiments were pH adjusted and sterilized through a 0.22 μm filter.

PBMC were isolated from buffy coats (Bloodbank, Utrecht, The Netherlands). Cells were harvested from the interface layer of a Ficoll–Hypaque gradient centrifigation (20 min, 1000 g). After three wash sequences (10 min, 500 g) with RPMI-1640 (pH 7.60) supplemented with Hepes (25 mM), sodium bicarbonate (10 mM) and gentamycine (0.005% w/v), PBMC were resuspended at 5×10^6 cells/mL. Pre-incubations with or without albumin or oleic acid were performed in a CO_2-incubator (5% CO_2/95% air) for a maximum of 20 hr. After the pre-incubation period the cells were concentrated at 2×10^7 cells/mL with or without washing and the first supernatant was used for pH control. Adenylate cyclase stimulation by histamine, isoprenaline, PGE_2, forskolin and aluminium fluoride (AlF_4^- in buffered salt solution without calcium and magnesium) was established by incubating at 37° for 6 min in a shaking water bath, in the presence of 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), in a final volume of 200 μL. Basal control experiments on both albumin pre-incubated and non-albumin pre-incubated cells were performed without stimulant. Metabolic processes were stopped and cells were lysed by heating at 100° for 6 min. Precipitated protein was pelleted by centrifugation (15 min; 1500 g) and the supernatant was used to measure accumulated cAMP by means of a competitive protein binding assay (Amersham, U.K.). Presence of HSA at maximal experimental concentration did not disturb cAMP levels in the assay.

At all times, viability was accounted for using trypan blue exclusion (>95%). Binding of agonists to albumin was measured with equilibrium dialysis as described by Bos *et al.* [13]. Binding of cAMP to albumin was verified by means of adding controlled amounts of cAMP to albumin. Results are presented as means ± standard deviation (SD) of triplicate determinations.

RESULTS

The time course of cAMP production after stimulation of PBMC with agonists in the presence of IBMX very rapidly reaches a plateau (Fig. 1). The presence of HSA only induces a constant decrease in cAMP accumulation with time. Therefore, a stimulation period of 6 min was considered to be sufficient. The high number of cells, necessary due to the limitations of the cAMP assay and the resuspension in the incubation medium prepared according to manufacturing prescription, induced a decrease in pH from 7.4 at time zero to 7.2 after 20 hr (Fig. 2A). Figure 2B shows basal and isoprenaline-stimulated cAMP production after 0 and 20 hr incubation in a comparable experimental

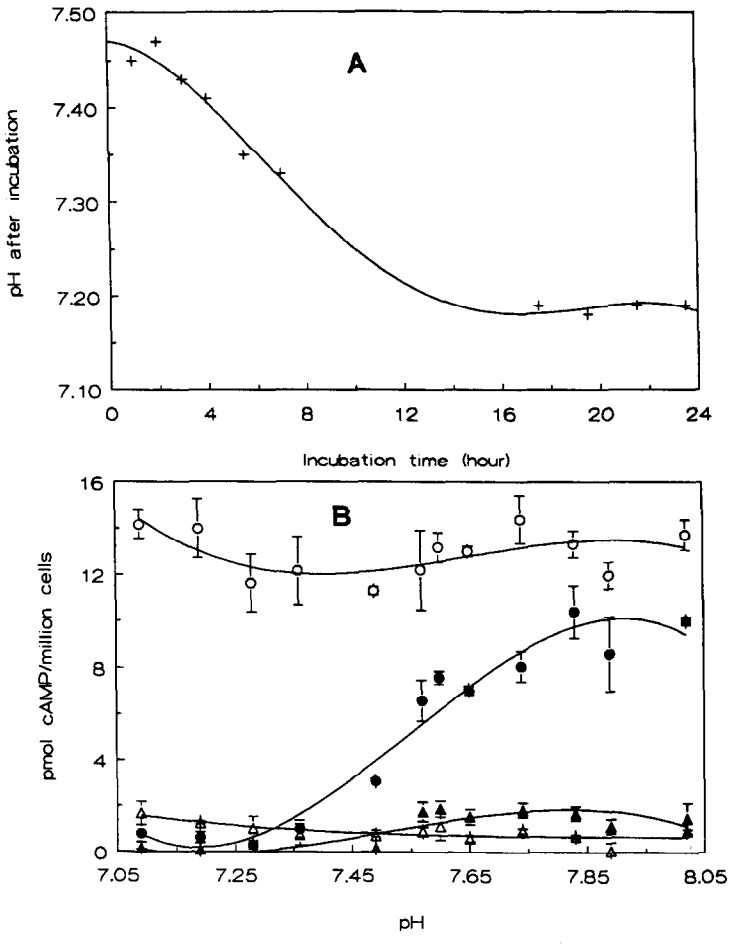

Fig. 2. Panel A: effect of incubation time of PBMC on pH in RPMI-1640 supplemented with 20 mM Hepes and 10 mM sodium bicarbonate. The initial pH was 7.40. Cell concentration was 5×10^6 cells/mL. Panel B: under similar conditions as in panel A, the graph shows the effect of variation in starting pH on isoprenaline-stimulated cAMP production in PBMC after a 0-hr (open symbols) and 20-hr (closed symbols) incubation period. Cells were concentrated at 2×10^7 cells/mL, incubated with isoprenaline (10^{-6} M) and consequently assayed for cAMP (circles). Basal values are control stimulations without isoprenaline (triangles).

setup at different starting pH values. Below pH 7.35, isoprenaline-stimulated cAMP output is drastically lowered compared with basal levels. Above the physiological pH of 7.4 production stabilizes. Therefore additional buffer was added to the medium and the pH was raised to 7.60. The pH after a 20-hr pre-incubation with the buffer never dropped below 7.35. The new conditions also prevented disturbance of pH by concentrated HSA solutions.

Increasing concentrations of HSA inhibited dose dependently isoprenaline-stimulated cAMP formation (Fig. 3). At 0.5% HSA the inhibition amounted to 40%. Fluctuation between cell batches was observed (blood donor differences); however, inhibition was never less than 20% at these HSA levels as compared with controls. Below 0.05% HSA no significant reduction in cAMP production was observed. Data were corrected for basal cAMP levels. Variation in incubation time (3 and 20 hr) did not influence the inhibition.

Direct stimulation with zero time preincubation also shows this repression (Fig. 4). Basal cAMP values were reduced by the same percentage. Above an albumin concentration of 1% denaturation of protein during cell lysis prohibited execution of the cAMP assay. Therefore, cells were washed with fresh medium after a 2% albumin preincubation of 20 hr. Similar reductions in cAMP levels were observed when PBMC were stimulated thereafter with isoprenaline and PGE_2. However, washing the cells reduced metabolic processes and viability resulting in lower cAMP values. As inhibition was not affected, washing was omitted in further experiments. Only negligible binding of cAMP to 0.5% albumin was found after the denaturation step (data not shown).

Variation in the fatty acid to albumin ratio in the presence of a constant 0.5% albumin concentration had no effect on cAMP levels (Fig. 5). Results of agonist stimulation of different receptors linked to

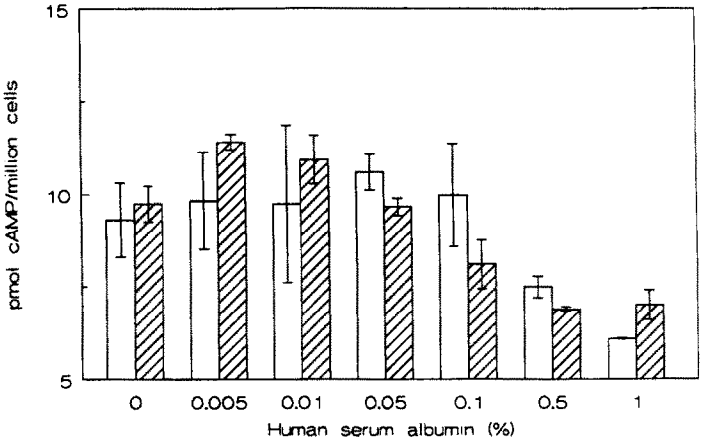

Fig. 3. Effect of various concentrations of HSA at 3 hr (open bars) or 20 hr (hatched bars) on cAMP formation after isoprenaline (10^{-6} M) stimulation of PBMC, corrected for basal cAMP. The values represent the means ± SD of triplicate determinations. The percentage inhibition at higher HSA concentrations varied between 20 and 60% as compared with controls.

the adenylate cyclase complex after exposure of PBMC to albumin are shown in Fig. 6. Direct stimulation in the absence and presence of 0.5% HSA by histamine, PGE_2 and isoprenaline reduced cAMP accumulation by 20 to 50%. Stimulation by forskolin, a direct adenylase cyclase activator, gave similar results. The cAMP production in all cases showed a dose-dependent relationship. It can thus be seen that HSA induced comparable and non-selective percentual inhibitory effects with all the agonists used. Although the reduction varied between cell batches, it amounted to 20–60%. The agonist AlF_4^- (10^{-3}–10^{-2} M NaF/10 μM $AlCl_3$), a stimulator of G-proteins, has no influence on cAMP production, either with or without albumin. Equilibrium dialysis with relevant concentrations of albumin and agonists revealed no significant binding, except for isoprenaline. Dialysis experiments in the presence of isoprenaline were impossible due to instability of the drug.

Comparison of albumin from different species (HSA, BSA and chicken albumin) at the same concentration (0.5%) showed type-dependent inhibition of cAMP (Table 1). BSA was more effective than HSA, whereas chicken albumin induced almost no reduction in cAMP. After a 20-hr incubation period the inhibitory differences almost disappeared.

DISCUSSION

Our present findings demonstrate that HSA at a concentration of one tenth of the plasma concentration inhibits *in vitro* basal and stimulated cAMP production in PBMC. Increasing the albumin concentration does not increase the inhibitory effect.

Although comparable with cAMP measurements by Van Oosterhout and Nijkamp [14], in the present study the use of high cell concentrations in combination with a large dose of HSA caused pH

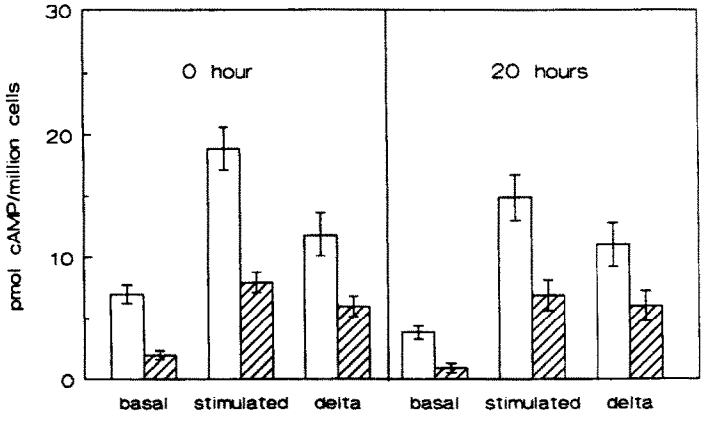

Fig. 4. Influence of HSA and incubation time on basal and isoprenaline-stimulated (10^{-6} M) cAMP production in isolated PBMC. Cells were incubated with (hatched bars) and without (open bars) 0.5% HSA for 0 and 20 hr at 37°. Basal values represent control stimulations without isopenaline. Delta symbolizes cAMP data corrected for basal values. Data are expressed as means ± SD of triplicate determinations.

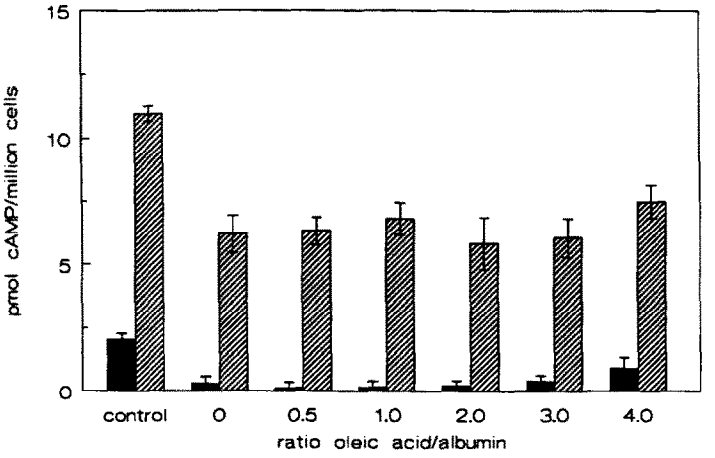

Fig. 5. Effect of variation in the fatty acid to albumin molar ratio in the presence of 0.5% HSA on isoprenaline-stimulated cAMP production. PBMC were incubated for 20 hr, concentrated by centrifugation, remaining supernatant was diluted five times with fresh medium to reduce oleic acid concentration, cells were resuspended to 2×10^7 cells/mL and thereafter stimulated with (hatched bars) or without (closed bars) isoprenaline (10^{-6} M). Data are expressed as means ± SD of triplicate determinations.

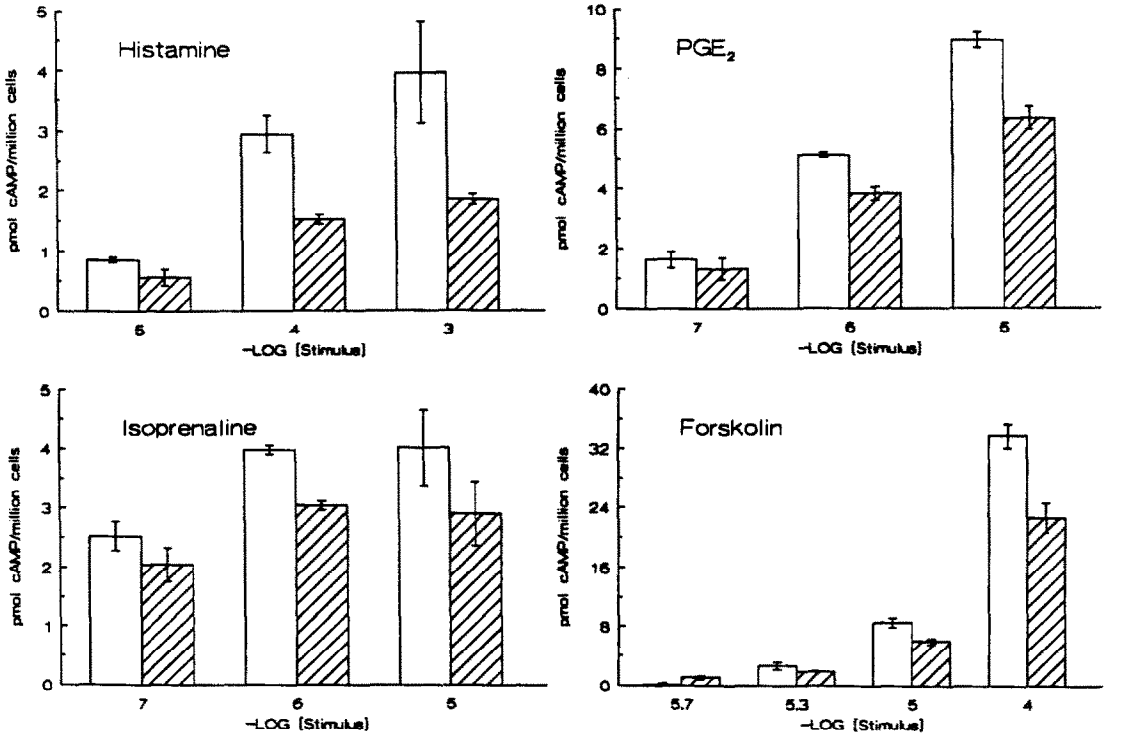

Fig. 6. The influence of HSA (0.5%; hatched bars) on histamine-, PGE_2-, isoprenaline- and forskolin-stimulated cAMP production, corrected for basal; cAMP, in freshly isolated PBMC. Basal cAMP values were 1.73 ± 0.28 and 0.35 ± 0.17 pmol cAMP/million cells in the absence or presence of HSA, respectively. Control stimulations are performed without HSA (open bars). Results are expressed as means ± SD of triplicate determinations.

Table 1. Effect of long and short preincubation of various albumins on stimulated cAMP production in PBMC

Albumin (0.5%)	Isoprenaline		Histamine		PGE_2	
	0 hr	20 hr	0 hr	20 hr	0 hr	20 hr
Control	100 ± 6.7	100 ± 10.5	100 ± 18.6	100 ± 13.2	100 ± 2.8	100 ± 9.5
HSA	41.1 ± 0.9	42.1 ± 4.4	54.8 ± 3.3	96.4 ± 3.6	52.0 ± 2.6	58.9 ± 0.6
BSA	21.4 ± 0.6	26.1 ± 0.7	21.0 ± 0.5	52.1 ± 1.2	25.2 ± 0.3	32.0 ± 2.6
Chicken	69.6 ± 3.2	26.7 ± 2.2	129 ± 7.8	42.5 ± 1.2	85.5 ± 0.8	28.3 ± 2.0

Results are expressed as percentages of cAMP production by PBMC not incubated with HSA and are means ± SD of triplicate determinations, corrected for basal values.

stabilization problems. The results show that pH is an important regulator of cell processes and should always be monitored. The modified medium provided the required experimental control of cAMP production.

Abnormal cell function in the presence of albumin has already been observed [2–8]. It has been suggested that BSA in rat tracheal epithelial cells stimulated or inhibited cell proliferation dependent on its concentration [8]. These effects were attributed to inhibition of regulatory pathways stimulated by cAMP. Moreover, impurities present in albumin were presumed to be responsible for these activities. In contrast, our results on HSA-induced cAMP reduction with different receptor agonists seem to indicate that HSA influences the early part of the signal transduction pathway, somewhere between receptor and adenylate cyclase. Albumin can bind large amounts of diverse substances and in that way can act as a transporter or trapper molecule [15]. It was suggested that impurities bound to albumin and resembling interleukin-1- and interleukin-3-like activities in lymphocytes (granulocytes and macrophages, respectively [6, 7]) were related to cellular responses. If such substances affect the cell system, then dissociation of these impurities from albumin and subsequent specific binding to a receptor will regulate subsequent signal transduction into the cell. However, if an impurity present on albumin is responsible for the observed inhibition of cAMP production, this substance should most likely interact solely with one receptor and larger differences should have been seen in the percentage reduction in the presence of albumin among the various receptor agonists used, compared with basal inhibition. However, the effects of isoprenaline, histamine and PGE_2 are inhibited similarly in the presence of HSA. This means that the inhibitory process for the studied adenylate cyclase receptors seems to evolve along a comparable non-selective mechanism. Nevertheless, albumin impurities interacting with other receptors linked to adenylate cyclase cannot be ruled out as having an indirect effect.

The inhibition process could equally well be explained if agonist concentration was reduced through binding to HSA. However, equilibrium dialysis demonstrated that binding of agonist to HSA never exceeded 5% in PBMC incubations (data not shown). Estimation of cAMP levels, based on agonist free fraction resulting from equilibrium dialysis, revealed no significant decrease in cAMP due to the presence of albumin. Therefore, mere binding of a drug to albumin probably cannot explain the experimental data. Binding studies of PGE_2 to rat epididymal adipocyte membranes performed in the presence of BSA showed concentrations of unbounded PGE_2 similar to our results [11]. However, PGE_2 binding to these membranes was enhanced and this could have quite opposite results for cAMP production.

Interestingly, the exposure time after addition of HSA to the cells has no relation with the inhibitory events (Fig. 4). The process takes place immediately after albumin is added to the cells. Disturbed cell function or viability are therefore unlikely causes of the inhibition of cAMP production. Perhaps the fast reaction induces a direct interaction of HSA with the cell membrane, possibly influencing all kinds of membrane-embedded receptors [5].

Aluminium fluoride, a well-known activator of G-proteins [16, 17], did not increase cAMP in PBMC (data not shown), either with or without albumin. The G-protein stimulator is normally used in experiments with membranous preparations or leaky cells [18]. The result demonstrates that HSA does not function as a transporter molecule of aluminium fluoride. Furthermore, in the presence of albumin, there is no indication of perturbation of the cell membrane causing aluminium fluoride to exert its well-known action on the α-subunit of the GTP-binding protein. The role of the G-proteins in the inhibitory process of HSA on adenylate cyclase activity remains unclear.

The agonists histamine, isoprenaline and PGE_2, the receptors of which are directly coupled to G-proteins trigger the same route of signal transduction. However, forskolin is able to activate directly the c-subunit of the effector enzyme adenylate cyclase [19, 20]. Although the mechanism is not fully understood, it is clear from binding studies with [^{3}H]forskolin that the complex of the activated α_s-subunit of the G_s-protein and the catalytic subunit of adenylate cyclase has a high affinity binding site for forskolin [21]. The exact location of this binding site is unknown. Most likely a conformational change is induced in the adenylate cyclase complex which controls simulated activity. Changes in membrane-bound protein environment, through the action of highly specific binding, are believed to influence membrane fluidity and lipid composition [22, 23]. *Vice versa*, alterations in the composition or fluidity of the membrane will have a major effect on the

activity of the catalytic adenylate cyclase complex [24]. A comparable process is found with supplemented polyunsaturated fatty acids in cellular systems [23]. HSA is capable of reducing the forskolin-stimulated direct route of cAMP activation. Moreover, without stimulation cAMP production is also inhibited. Therefore, albumin, a protein with high affinity for fatty acids [15], must have some interaction with the lipid bilayer of the membrane. Inhibitory effects of HSA on forskolin-stimulated cAMP production may partly prevent binding of forskolin to adenylate cyclase. This possible decrease in binding is supported by the intact inhibitory effect on cAMP production after a long incubation period with 2% HSA, followed by a thorough wash procedure. It implies that the interaction of HSA with the membrane is to some extent irreversible. However, albumin receptors have never been found *in vivo*. Therefore, the interaction will be more controlled by capacity than by affinity.

From previous work [10, 25, 26], the conformational changes in HSA are considered to be involved in activation processes across cell membranes. To determine whether the overall structure of HSA is important in the inhibition of cAMP production, we studied the interaction of different ratios of oleic acid to HSA with isoprenaline-stimulated PBMC (Fig. 5). Oleic acid has no direct effect on the β-adrenergic receptor–adenylate cyclase coupling mechanism [23]. Dröge *et al.* [27] found, with fatty acid to HSA ratios above two, conformational alterations in HSA due to the binding of fatty acid to hydrophobic cores inside the protein. The observed equivalence in cAMP production with increasing oleic acid concentration contrasts with the presumed conformational changes occurring in HSA due to higher fatty acid levels. Therefore, the interaction of HSA with the cell membrane and the resulting effects on signal transduction cannot be explained by small changes in the structure of HSA. Interaction of the whole structure with the membrane seems to be important. Recently, Laursen *et al.* [5] suggested the possibility that albumin binding to a human breast cancer cell line could induce structural changes in the membrane, thereby deregulating the binding of growth factors to their surface receptors. For these reasons the distortion of membrane lipids and the consequent perturbation of membrane fluidity could explain reasonably well the changes in the binding behavior of the embedded receptor proteins or the activity of adenylase cyclase in PBMC. Nevertheless, an allosteric process in adenylate cyclase itself cannot be excluded. If this is the case, HSA probably interacts with the outer membrane part of the enzyme, thereby indirectly controlling activity at a distance by means of conformational changes in adenylate cyclase.

It seems unlikely that structural aspects of albumin play a part in the overall transduction process, but the species dependence (Table 1) suggests this. From the similarity in amino acid sequence among the different albumins used, it may be expected that inhibitory actions on cAMP production will be identical. However, BSA and chicken albumin at the same concentration show quite remarkable activity differences. The 3-D structures of HSA and chicken albumin are not known, therefore explanations are speculative and further research is necessary. To see if inhibition of cAMP production is an albumin-specific process we performed some preliminary experiments with the unrelated protein casein showing comparable effects. Results are however unreliable due to solubility problems with this protein exerting side-effects on the cAMP assay.

The question arises as to whether the inhibitory effects of albumin on the adenylate system are part of a general phenomenon of different cell types. Van Oosterhout (unpublished results) found no effects of BSA on isoprenalin-stimulated cAMP production in tracheal and epithelial cells. Very recently, it was found that phosphoinositide turnover in human platelets was drastically stimulated in seconds by binding to cells of HSA and BSA [28]. This stimulation was not shared by ovalbumin and was abolished by albumin hydrolysis, thereby indicating the necessity for the intact polypeptide structure to exert its effects and eliminating the influence of albumin-bound fatty acids.

Inclusion or omission of serum albumin in the study of cellular processes without consideration of the consequences could eventually lead to artefacts. The fact that *in vivo* albumin is present in plasma at a considerably high concentration (5%) compared with that used in cell culture systems and the generally accepted influence of albumin in very diverse biological systems underlines the necessity for more detailed mechanistic studies.

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