# Inhibition of human platelet adenylate cyclase activity by adrenaline, thrombin and collagen: analysis and reinterpretation of experimental data

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Mathematical models based on the current understanding of stimulation and inhibition of adenylate cyclase (AC) activity have been developed and used to analyse experimental data [Farndale, Winkler, Martin and Barnes (1992) Biochem. J. **282**, 25–32] describing the inhibition of human platelet AC by collagen, thrombin and adrenaline. Here it has been demonstrated that neither affinities of receptors specific for adrenaline or thrombin nor the activity of cAMP phosphodiesterase are affected by collagen. Both collagen and thrombin at high doses act as effective inhibitors of AC activity. Inhibition of AC activity by collagen proceeds via two parallel pathways; the same is true for thrombin at moderate concentrations, and the two ligands act independently. The G-protein-dependence of these pathways is distinct from that mediating inhibition of AC activity by adrenaline, i.e. $G_{i2}$. Convergence of the inhibitory pathways takes place at the catalytic subunit of AC.

Keywords: enzyme kinetics, G-proteins, mathematical models, receptors, signal transduction.

## INTRODUCTION

The mammalian adenylate cyclases (ACs), including human platelet AC, comprise several enzymes of similar structure, some of which are dually controlled via heterotrimeric G-proteins [1–4]. Various experimental approaches have been adopted to investigate the inhibitory G-proteins which mediate control of AC activity.

For example, $\alpha_2$-adrenergic agonists are considered to inhibit AC [5] via the inhibitory G-protein $G_{i2}$ [6], based on the observations that an antibody specific for the common C-terminus of $G_{i1}$ and $G_{i2}$ blocked the inhibition of AC activity by adrenaline, and that $G_{i2}$ is more abundant in platelets than $G_{i1}$. AC is also inhibited by the platelet agonists thrombin [7] and collagen [8,9], but whether $G_{i2}$ mediates the action of these agonists is not known.

Using a kinetic approach, distinct GTPase activities were stimulated in platelets by thrombin, suggesting that more than one G-protein may mediate its action [10]. Similarly, the GDP analogue guanosine 5′-[$\beta$-thio]diphosphate (GDP[$\beta$S]) was used to show that thrombin inhibits AC via a G-protein with a lower affinity for GTP than those mediating the action of either collagen or adrenaline, which could not be resolved kinetically [9]. The inhibition of AC by collagen is pertussis-toxin-sensitive [9,11,12], but the actions of adrenaline and thrombin are similarly sensitive, so that this technique did not resolve the species involved [12].

The present work arose from the presumption that the results presented in a previous publication [9] contain more information on the mechanisms of AC inhibition than could be revealed by conventional analysis. The construction of working hypotheses and operational models, expressed in rigorous mathematical form, may allow reinterpretation of experimental data. In this

respect, the work of Farndale et al. [9] is of special interest, because of its weight of detailed experimental data.

The aim of the present study, therefore, was to relate quantitatively time-course and dose–response curves with known or putative properties of the AC system and the agents affecting its activity. We formulate basic postulates for a minimal model (presented in detail elsewhere [18]), which we apply to approximate the experimental data of the original work [9]. When that proves impossible, we extend the model (formally) to account for the properties of the system which are not known or have not been postulated initially. Next, we discuss the implications of the formal model-based analysis. Finally, we arrive at a generalized model for two inhibitors of AC acting in combination.

Part of this work was published in a preliminary form [13] and was presented at the 6th Erfurt Conference on Platelets [14].

## METHODS AND MODELS

Materials and methods used are described in [9]. Four series of experiments were carried out on either washed (intact) platelets (experiment A of Series 1) or four preparations of platelet membranes (Series 1–4) which were colour-coded for convenience; this colour code will be used for reference in Figures and Tables and in the Discussion. Two batches of platelet membranes were prepared from four different donations of blood on each of two occasions: Red and Green together, and Black and Blue together.

### AC assays

Platelet membranes were incubated with 0.1 $\mu$M prostaglandin $E_1$ (PGE$_1$), [$\alpha$-$^{32}$P]ATP and other additions described fully in [9],
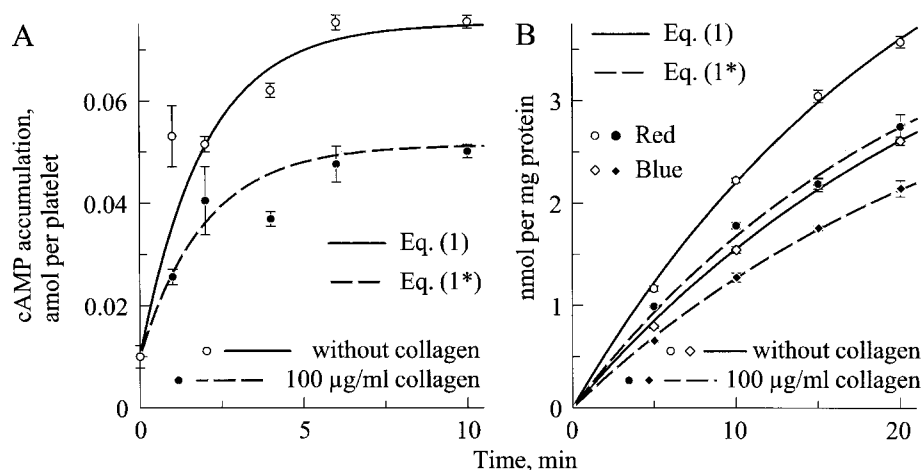
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**Figure 1   Time-course of 0.1 $\mu$M PGE$_1$-stimulated cAMP accumulation in incubation medium containing $4 \times 10^8$ washed (intact) human platelets (A) or platelet membranes (typically 25 $\mu$g of platelet membrane protein per assay) (B)**

AC assays were performed as described in the Methods and models section, except that the assays were terminated at the indicated times. Data represent means $\pm$ S.E.M. from triplicate determinations for control (open symbols) and collagen-treated platelets or membranes (closed symbols). Red and Blue are colour codes for different preparations (see the Methods and models section).

**Table 1   Parameters of the model (eqns. 1 and 1*) estimated by its fitting to experimental data obtained on washed (intact) platelets and platelet membranes (Series 1)**

| | | Value | | |
| | | | Membranes | |
| Parameter | Notation | Intact | Red* | Blue* |
|---|---|---|---|---|
| Initial quantity of cAMP (amol/platelet) ('Intact') | $q_0$ | 0.010 | 0† | 0 |
| (Initial) rate of cAMP synthesis by 0.1 $\mu$M-PGE$_1$-stimulated AC [amol/min per platelet ('Intact') or pmol/min per mg of protein ('Membranes')] | $a$ | 0.0376 | 274 | 187 |
| Average rate of cAMP accumulation [pmol/min per mg of protein ('Membranes')] | $\alpha$ | –‡ | 199 | 142 |
| Time constant of cAMP degradation or thermal inactivation of AC (min) | $\tau$ | 2.0 | 22.0 | 26.0 |
| Average efficacy of 100 $\mu$g/ml-collagen-inhibited AC complex | $\epsilon_c$ | 0.684 | 0.762 | 0.818 |
| $F$-ratio | $F$§ | – | 1.864 | 0.731 |
| Fisher's critical value | $F\,(0.05;\ n_1, n_2)$¶ | – | 2.621 | 2.621 |
| Degrees of freedom | $n_1,\ n_2$‡ | – | 5, 24 | 5, 24 |

* Colour codes for different preparations.
† $q_0 = 0$ for platelet membranes by assumption.
‡ Not determined.
§ For data of A and B variants of the experiments and eqns. (1) and (1*).
¶ For 0.05 confidence level and $n_1$ and $n_2$ degrees of freedom [19]; the model has been considered acceptable when $F <\ F\,(0.05;\ n_1, n_2)$.

with inhibitors (combinations of collagen, adrenaline and thrombin) as indicated for up to 20 min (Series 1) or for 15 min (Series 2–4) at 30 °C. The method is based on that of Saloman et al. [15] and is elaborated elsewhere [16].

## Modelling

cAMP accumulates in the course of time, $t$, with the rate $a$:

$$Q(t) = q_0 \exp(-t/\tau) + a\tau[1 - \exp(-t/\tau)] \qquad (1)$$

other notations being defined in Table 1 (and see the Appendix). The following equation:

$$Q(t) = q_0 \exp(-t/\tau) + \epsilon_c a\tau[1 - \exp(-t/\tau)] \qquad (1^*)$$

takes into account the effect of collagen on cAMP accumulation.

The main assumptions for modelling dose–response curves are as follows.

(1) Adrenaline and collagen, as well as thrombin and collagen, act as non-competitive incomplete inhibitors of AC.

(2) Binding of these ligands to their respective receptors is the main determinant of functional state and efficacy of AC (or, rather, the complex consisting of the catalytic and G-protein subunits, activated or not by ligand-specific receptors).

(3) The total observable AC activity comprises the activities of all its functional complexes, the contribution of the complexes in each state being proportional to their number and efficacy, depending on the inhibitor concentrations.

Several models based on similar assumptions have been developed for specific stimulators of AC [17,18]; eqn. (9) in [19] was used here directly, and eqn. (9) in [17] had to be extended.

Models were fitted to the experimental data by the method of least squares. Analysis of variance (Fisher's $F$ criterion) [19] was used to test the goodness-of-fit of the models.

## FORMAL DATA ANALYSIS (MODEL FITTING AND MODIFICATION)

Here we present approximations of experimental data using the simplest possible models described briefly above; the models were extended when necessary. Data from four series of experiments were analysed, each (except for dose–response curves for collagen) carried out in two variants (A, without collagen; B, with 100 $\mu$g/ml collagen fibres) with two to four membrane preparations (see the Figure legends). The results are presented, in Figures 1–4 and Tables 1–4 below. In Tables 1, 2 and 4 the
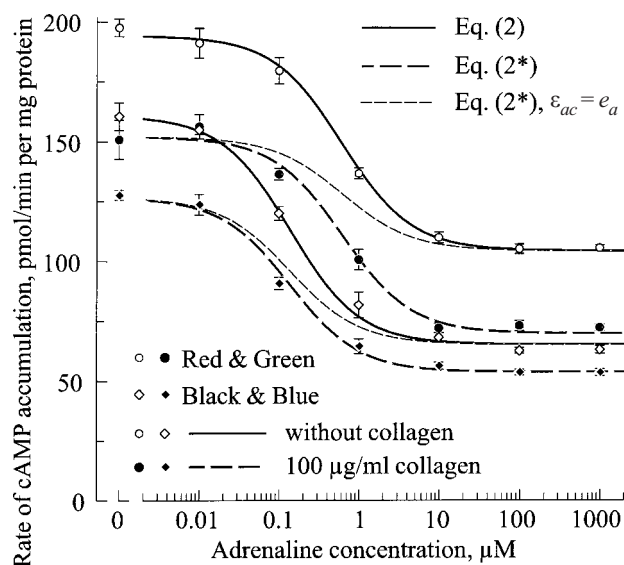
results of verification of goodness-of-fit of the models are also presented. Equations, Figures and Tables carry the number of the experiment from which they were derived. In the Figures, bold continuous curves and open symbols correspond to one inhibitor; bold broken curves and closed symbols to adrenaline or thrombin in combination with collagen; thin broken curves correspond to alternative models. The numbers of equations corresponding to the above combinations of either inhibitor with collagen carry an asterisk (*), and numbers of alternative equations carry primes (′ or ″). Greek characters denote those parameters of the models which are not directly related to the properties of the AC system or its elements. Subscripts in the equations refer to the functional states of AC complex (see previous section and Figure 5). When estimates of a parameter are exactly the same for all or some preparations, this is by assumption.

### Series 1 experiments: time-course of cAMP accumulation

Data obtained from two preparations (Red and Blue) were plotted and analysed separately (Figure 1 and Table 1) because of non-homogeneity of the two pairs of data sets (variants A and B). To analyse data obtained on both washed (intact) platelets and platelet membranes, eqns. (1) and (1*) were used. For intact platelets, the model deviates considerably from the data. No other reasonable model, however, might fit better: the deviations are best explained by the large errors of experiment. The average rate of cAMP accumulation in a 15 min interval, $\alpha$, was determined as $Q$(15 min)/15 min.

### Series 2 experiments: inhibition of AC activity by adrenaline (without and with collagen)

Data obtained from four membrane preparations were taken in two pairs of sets (Red and Green together, and Black and Blue together), which were plotted and analysed separately because of non-homogeneity between the two pairs (Figure 2 and Table 2), the data sets within the pairs being homogeneous. For the analysis, the following equations (see eqn. 9 in [18]):

$$A(x) = \alpha\left(e_0\frac{x_a}{x+x_a}+e_a\frac{x}{x+x_a}\right) \qquad (2)$$



**Figure 2   Inhibition of 0.1 $\mu$M PGE$_1$-stimulated cAMP accumulation by adrenaline alone and in combination with collagen fibres**

AC assays were performed in a medium containing 31 $\mu$g of platelet membrane protein per assay as described in the Methods and models section. Data represent means $\pm$ S.E.M. from four replicates of each experiment. Red and Green, and Black and Blue, are colour codes for different preparations (see the Methods and models section).

**Table 2   Parameters of the model (eqns. 2 and 2*) estimated by its fitting to experimental data of Series 2**

| Parameter | Notation | Value Red* and Green* | Black* and Blue* |
|---|---|---|---|
| Average rate of cAMP accumulation by 0.1 $\mu$M-PGE$_1$- stimulated AC (pmol/min per mg of protein) | $\alpha$ | 194 | 161 |
| Apparent equilibrium dissociation constant characterizing the affinity of adrenaline for its receptor ($\mu$M) | $x_a$ | 0.620 | 0.138 |
| Efficacy of AC complex in state† $a$ | $e_a$ | 0.538 | 0.406 |
| Average efficacy | $\epsilon_c$ | 0.784 | 0.787 |
| Average efficacy | $\epsilon_{ac}$ | 0.360 | 0.334 |
| $F$-ratio | $F$‡ | 0.634 | 0.963 |
| Fisher's critical value | $F$ (0.05; $n_1,n_2$) | 1.978 | 1.980 |
| Degrees of freedom | $n_1, n_2$ | 9, 97 | 9, 95 |

\* See footnote to Table 1.
† See Figure 5(A).
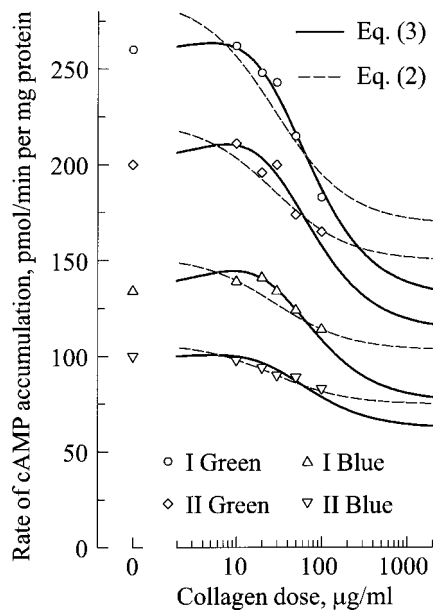‡ For data of A and B variants of the experiments and eqns. (2) and (2*).

**Figure 3    Inhibition of 0.1 $\mu$M PGE$_1$-stimulated cAMP accumulation by collagen fibres**

AC assays were performed in a medium containing typically 25 $\mu$g of platelet membrane protein per assay as described in the Methods and models section. Data represent means $\pm$ S.E.M. from four replicates of each experiment. Green and Blue are colour codes for different preparations (see the Methods and models section).

and

$$A_c(x) = \alpha \left( \epsilon_c \frac{x_a}{x+x_a} + \epsilon_{ac} \frac{x}{x+x_a} \right) \tag{2*}$$

where $x$ is concentration of adrenaline, other notations being defined in Table 2, were used.

### Series 3 experiments: inhibition of AC activity by collagen

Results from this series, each experiment being performed twice on two preparations, were plotted and analysed separately. Attempts were made to approximate these results using eqn. (2).

The model deviates considerably from the data points of all the experiments, which suggest a slight increase in AC activity at 10 $\mu$g/ml collagen followed by a steep decrease in the activity at higher collagen levels (see Figure 3). For this reason, the following equation (see eqn. 9 in [17]):

$$A(y) = \alpha \left( e_0 \frac{y_c^2}{(y+y_c)^2} + e_c \frac{2y_c y}{(y+y_c)^2} + e_{cc} \frac{y^2}{(y+y_c)^2} \right) \tag{3}$$

where $y$ is collagen dose, was used. (For interpretation of this model, see the subsubsection 'Pathways of AC inhibition by adrenaline, collagen and thrombin' below.) The results are presented in Figure 3 and Table 3. This model fits better; but its goodness-of-fit cannot be verified without replicate measurements. Average efficacy of the AC complex inhibited by 100 $\mu$g/ml collagen ($\epsilon_c$) was determined as $A(100\ \mu\text{g/ml})/\alpha$.

### Series 4 experiments: inhibition of AC activity by thrombin (without and with collagen)

Data of this series (from four membrane preparations) were analysed separately because of non-homogeneity of all the data sets. Neither of the above models could be applied directly, since these data suggest further decline in AC activity at high concentrations of thrombin. Therefore, the following multiplyer:

$$\zeta_h/(z+\zeta_h)$$

where $z$ is concentration of thrombin, $\zeta_h$ being defined in Table 4, was introduced. As a result, from eqns. (2) and (2*) the following equations were obtained:

$$A(z) = \alpha \left( e_0 \frac{z_t}{z+z_t} + e_t \frac{z}{z+z_t} \right) \frac{\zeta_h}{z+\zeta_h} \tag{4'}$$

and

$$A_c(z) = \alpha \left( \epsilon_c \frac{z_t}{z+z_t} + \epsilon_{tc} \frac{z}{z+z_t} \right) \frac{\zeta_h}{z+\zeta_h} \tag{4''}$$

Similarly, modification of eqn. (3) resulted in:

$$A(z) = \alpha \left( e_0 \frac{z_t^2}{(z+z_t)^2} + e_t \frac{2z_t z}{(z+z_t)^2} + e_{tt} \frac{z^2}{(z+z_t)^2} \right) \frac{\zeta_h}{z+\zeta_h} \tag{4}$$

**Table 3    Parameters of the model (eqn. 3) estimated by its fitting to experimental data of Series 3**

| Parameter | Notation | Green*† I | Green*† II | Blue* I | Blue* II |
|---|---|---|---|---|---|
| Average rate of cAMP accumulation (pmol/min per mg of protein) | $\alpha$ | 258 | 201 | 135 | 99 |
| Equilibrium dissociation constant characterizing the affinity of collagen for its receptor ($\mu$g/ml) | $y_c$ | 25.0 | 25.0 | 25.0 | 25.0 |
| Efficacy of AC complex: | | | | | |
| In state‡ $c$ | $e_c$ | 1.13 | 1.19 | 1.26 | 1.10 |
| In state $cc$ | $e_{cc}$ | 0.509 | 0.564 | 0.563 | 0.628 |
| Average efficacy of 100 $\mu$g/ml-collagen-inhibited AC complex | $\epsilon_c$ | 0.726 | 0.783 | 0.803 | 0.794 |

\* See footnote to Table 1.
† The experiments on each preparation were performed twice.
‡ See Figure 5.

**Table 4    Parameters of the model (eqns. 4 and 4* or 3/4) estimated by its fitting to experimental data of Series 4**

| Parameter | Notation | Value Red* | Green* | Black* | Blue* |
|---|---|---|---|---|---|
| Average rate of cAMP accumulation by 0.1 $\mu$M-PGE$_1$- stimulated AC (pmol/min per mg of protein) | $\alpha$ | 262 | 202 | 186 | 178 |
| Equilibrium dissociation constant characterizing the affinity of thrombin for its receptor (units/ml) | $z_t$ | 0.0259 | 0.0259 | 0.0259 | 0.0259 |
| Efficacy of AC complex | | | | | |
| In state† $t$ | $e_t$ | 1.24 | 1.23 | 0.634 | 0.586 |
| In state $tt$ | $e_{tt}$ | 0.334 | 0.622 | 0.516 | 0.475 |
| In state $c$ | $e_c$ | 1.13 | 1.13 | 1.13 | 1.13 |
| In state $cc$ | $e_{cc}$ | 0.689 | 0.647 | 0.588 | 0.527 |
| In states $ct$ or $cct$ | $\eta_{ct}$ | 0.591 | 0.718 | 0.407 | 0.359 |
| In states $ctt$ or $cctt$ | $\eta_{ctt}$ | 0.298 | 0.438 | 0.504 | 0.439 |
| Average efficacy of 100 $\mu$g/ml-collagen-inhibited AC complex | $\epsilon_c$ | 0.842 | 0.816 | 0.775 | 0.734 |
| Average efficacy | $\epsilon_{tc}$ | 0.624 | 0.726 | 0.409 | 0.371 |
| Average efficacy | $\epsilon_{ttc}$ | 0.299 | 0.452 | 0.502 | 0.442 |
| Factor characterizing thrombin's potency of inhibition at high concentrations (units/ml) | $\zeta_h$ | 39.0 | 39.0 | 39.0 | 39.0 |
| $F$-ratio | $F$‡ | 1.603 | 5.526 | 1.911 | 1.296 |
| Fisher's critical value | $F(0.05; n_1, n_2)$ | 2.295 | 2.295 | 2.005 | 2.109 |
| Degrees of freedom | $n_1, n_2$ | 6, 48 | 6, 48 | 10, 56 | 8, 52 |
| $F$-ratio | $F$ | | 1.275§ | | |
| Fisher's critical value | $F(0.05; n_1, n_2)$ | | 3.009§ | | |
| Degrees of freedom | $n_1, n_2$ | | 3, 24§ | | |

*See footnotes to Table 1.
†See Figure 5(B).
‡For data of A and B variants of the experiments and eqn. (3/4).
§For data of experiment 4 B Green and eqn. (4*).
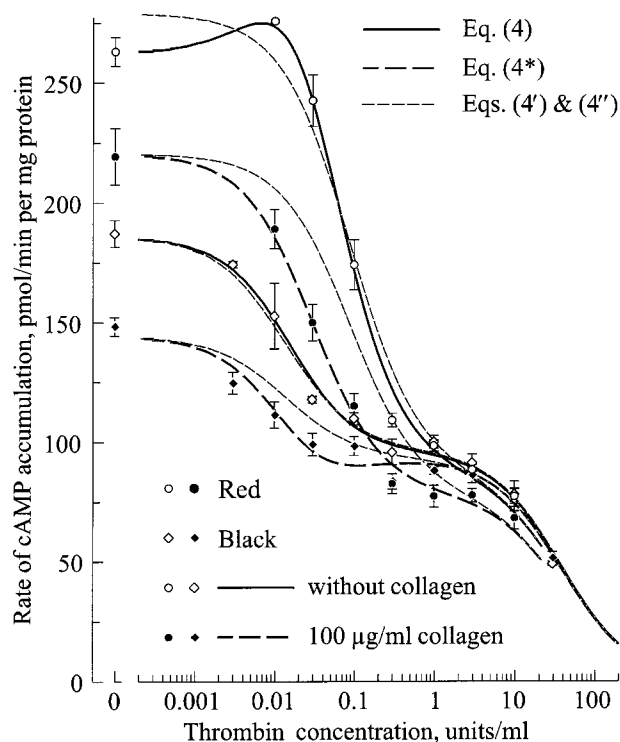


**Figure 4. Inhibition of PGE$_1$- (0.1 $\mu$M) stimulated cAMP accumulation by thrombin alone and in combination with collagen fibres**

AC assays were performed in a medium containing 29 $\mu$g of platelet membrane protein per assay as described in the Methods and models section. Data represent means ± S.E.M. from four replicates of each experiment. Red and Black are colour codes for different preparations (see the Methods and models section).

and

$$A_c(z) = \alpha \left( \epsilon_c \frac{z_t^2}{(z+z_t)^2} + \epsilon_{tc} \frac{2z_t z}{(z+z_t)^2} + \epsilon_{ttc} \frac{z^2}{(z+z_t)^2} \right) \frac{\zeta_h}{z+\zeta_h} \qquad (4^*)$$

all the notations being defined in Table 4. (For interpretation, see the subsection 'Pathways of AC inhibition by adrenaline, collagen and thrombin' below.)

Eqns. (4′) and (4″) fit well to some experimental results (Red with collagen and Black without collagen), but deviate considerably from the other sets (Red without collagen and Black with collagen, see Figure 4), which suggest, as in the previous case, non-monotony of the dose–response curves. Eqns. (4) and (4*) were fitted to all the data (Figure 4 and Table 4). In one case of eight (presented only in Table 4), however, the fit is not satisfactory.

When comparing estimated parameters, the assessment of their identity or difference was based on the goodness-of-fit of corresponding models. Thus if a pair of different estimates of a parameter corresponds to a pair of model curves, each of which can be fitted to different sets of data (or only one can be fitted while the other cannot), the estimates defining different curves are considered to differ from each other.

## DISCUSSION

The parameters of the models used to analyse the experimental data were assumed to reflect intrinsic properties of the (macro)molecular components of the AC system. Ideally, the properties of these components and, therefore, the estimates of the corresponding parameters, would be expected to be identical in all the experiments. Three parameters ($\alpha$, $e_0$ and $\epsilon_c$) are shared by all the models. Parameter $e_0$ is assumed to be 1
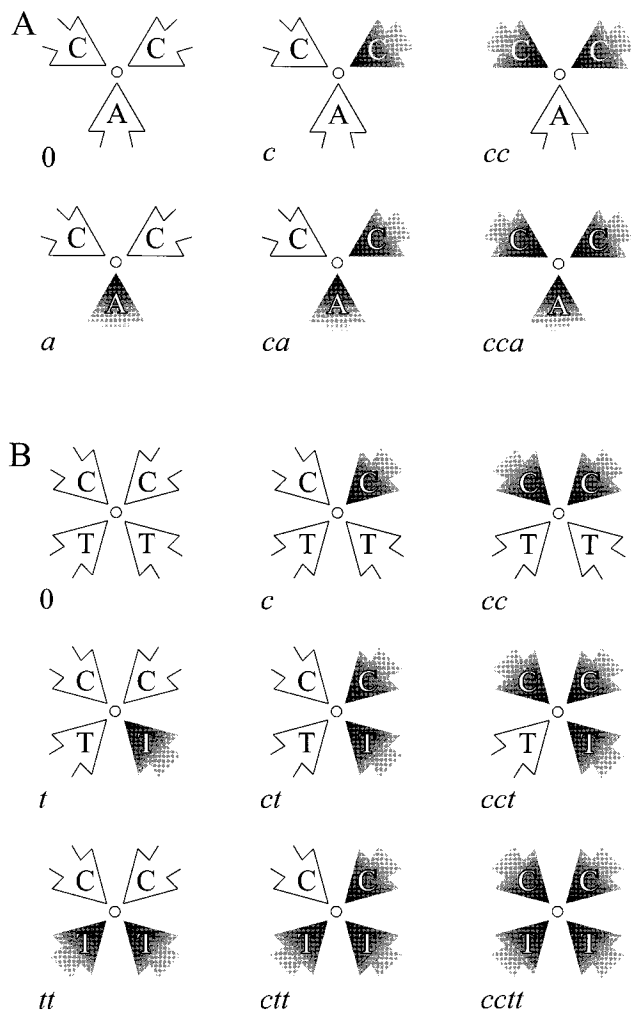
**Figure 5    Schematic representation of all the possible combinations of the pathways of inhibition of AC activity by collagen and adrenaline (A) and by collagen and thrombin (B)**

Activated pathways are symbolized by shading. The states of the AC complex determined by the combinations of activated and non-activated pathways are denoted in *lower-case italics* below the cartoons depicting each combination of the pathways; these notations are used as subscripts in the equations (see the Methods and models and Formal data analysis (model fitting and modification) sections and the Discussion.

throughout. The estimates of parameter $\epsilon_c$ are close for all experiments (Tables 1–4).

## Rate of cAMP accumulation

We have used the rising phase of the cAMP accumulation curve (Figure 1), previously analysed by others [20], to derive parameters $a$ and $\alpha$, which are directly related (but not equivalent) to the intrinsic activity of AC or molecular catalytic-centre activity ('turnover number'). They characterize the initial and average rate of cAMP accumulation respectively, in a given assay medium where the exact concentration of AC is not known. The estimates of $a$ and $\alpha$, therefore, can be assigned only to each preparation

separately and should be expected to be approximately the same in all experiments using the same preparation.

Although the estimates of $\alpha$ vary considerably (see Tables 1–4), the range, as expected, is much narrower for data from the same membrane preparation. Strikingly, the data from Series 2 experiments obtained from Red and Green membranes (prepared on the same day) proved to be homogeneous; therefore, the corresponding parameters of eqns. (2) and (2*) (including $\alpha$) for both Red and Green preparations are identical. The same holds true for Black and Blue membranes, prepared together on a different day. It is noteworthy that $\alpha$ estimated from the data of the time-course experiment of Series 1 carried out on preparation Red is very close to that estimated from the dose–response experiment of Series 2 carried out on the same preparation (see Tables 1 and 2); the estimates of this parameter from experiments of Series 3 and 4 carried out on preparation Green are close again to that obtained from the experiment of Series 2 carried out on the same preparation (see Tables 2–4). On the differences, see the subsection 'Essential differences between the two groups of membrane preparations' below.

## Time constant ($\tau$) of cAMP breakdown or thermal inactivation of AC

Non-linearity of time course of cAMP accumulation implies either (in intact platelets) its breakdown by cAMP phosphodiesterase (PDE) where inhibition by isobutylmethylxanthine proves to be incomplete, or (in membrane preparations) a modest decline in AC activity, since cAMP PDE activity is negligible; the latter effect, termed thermal inactivation, is believed to reside in loss of function of the catalytic subunit [21], rather than of either $G_s$ or the hormone receptor. So in both cases parameter $\tau$ has different meanings in intact platelets and membranes; the difference in its estimates (Table 1), therefore, is not surprising.

Parameter $\tau$ is shared by eqns. (1) and (1*). This implies the assumption that neither the activity of cAMP PDE nor thermal inactivation of AC is affected by collagen; this proves to agree with experimental data (see Figure 1A and Table 1).

The estimates of $\tau$ obtained on preparations Red and Blue are quite close (Table 1).

## Equilibrium dissociation constants and efficacies of inhibition of AC activity

Parameter $x_a$ is shared by eqns. (2) and (2*). That implies that the affinity of adrenaline for its receptor is not affected by collagen, which is in agreement with experimental data (Figure 2 and Table 2). The same holds true for $z_t$ as well as for $\zeta_h$ [see eqns. (4) and (4*), Figure 4 and Table 4].

As follows from eqn. (2):

$$A(\infty)/A(0) = e_a$$

Parameter $e_a$ ($e_a < 1$; see Table 2), therefore, is the determinant of relative asymptotic decrease in AC activity, or relative inhibition. Similar interpretations apply to $e_{cc}$ (eqn. 3) and $e_{tt}$ (eqn. 4) if it is assumed that:

$$\zeta_h/(z + \zeta_h) \equiv 1$$

(which means that inhibition at infinitely high thrombin concentration caused by different mechanisms is ignored).

Similarly, the absolute asymptotic inhibition by adrenaline (see eqn. 2) is:

$$I(\infty) = \alpha(1 - e_a)$$

And, as follows from eqn. (2), for $x = x_a$:

$$I(x_a) = \alpha(1 - e_a)/2 = I_a(\infty)/2$$

Parameter $x_a$, therefore, being equal to the concentration of inhibitor corresponding to half-asymptotic inhibition of AC activity, is equivalent to $IC_{50}$. Unlike $IC_{50}$, a formal parameter conventionally used to characterize an inhibitor's potency, $x_a$ has a clearly defined physical meaning (see Table 2); besides, being a parameter of a mathematical model, it can be estimated more accurately than $IC_{50}$.

## Inhibition of AC activity at high concentrations of thrombin

The need for the formal multiplier $\zeta_h/(z + \zeta_h)$ in eqns. (4')–(4*) to account for further inhibition of AC at high concentrations of thrombin implies a separate mechanism. This high-concentration inhibition, as it will soon become clear (see the subsection 'Essential differences between the two groups of membrane preparations' below) cannot be mediated by the G-proteins which mediate the lower-concentration inhibition The existence of a G-protein specialized for this high-concentration inhibition seems unlikely. We conclude that it occurs at a level different from inhibitory G-proteins. It has been proposed that the proteolytic activity of thrombin may degrade components of the system [7]. These could include the $PGE_1$ receptor, the AC catalytic subunit or, indeed, $G_s$.

## Pathways of AC inhibition by adrenaline, collagen and thrombin

Eqn. (3) (as well as eqns. 4 and 4*) and the shape of the corresponding curves suggests that inhibition of AC activity by collagen (or thrombin) proceeds via two independent parallel pathways which determine the states of the AC complex corresponding to neither, either or both pathways activated by collagen (or thrombin) (see Figure 5). The two pathways of action of either collagen or thrombin were assumed to be identical. The first, second and third terms in eqns. (3) and (4) determine the contribution to the inhibition of AC activity of the AC complexes inhibited via neither, either or both pathways (see Figure 5).

No single simpler model fits all the experimental data satisfactorily. It can be concluded that separate and independent pathways are involved in the actions of adrenaline and collagen. Indeed, whereas to approximate the experimental data of Series 2 (for adrenaline), a single-pathway model is sufficient, a two-pathways model is needed to approximate the data of Series 3 (for collagen).

In contrast, two parallel pathways are involved in the action of both thrombin and collagen. In addition, seeming convergence of dose–response curves for thrombin alone and thrombin with collagen (Figure 4) suggests possible convergence of the pathways of action of these inhibitors at a level other than the catalytic subunit of AC.

Certainly, collagen and thrombin do not share a common receptor. In platelets, the main receptors for collagen are the integrin $\alpha 2\beta 1$, CD36 and glycoprotein VI [24], with glycoprotein VI considered as the major signalling receptor [25,26]. However, a collagen-like ligand [27] specific for glycoprotein VI [28] does not inhibit AC [29], and neither $\alpha 2\beta 1$ nor CD36 has so far been

associated with G-protein activation. The nature of the collagen receptor mediating inhibition of AC remains, therefore, to be established. Thrombin is recognized by glycoprotein Ib–IX complex, reviewed in [30], as well as the tethered-ligand G-protein-linked receptor [31,32].

Collagen and thrombin do not seem to share a common G-protein [9] either, a statement supported by analysis of the experimental data of Series 4. Indeed, let us make the opposite assumption: these inhibitors share a common G-protein, on two molecules of which all inhibitory pathways (Figure 5B) converge. Under this assumption, the action of collagen and that of thrombin would produce the same result. It should be kept in mind that G-proteins transducing stimulation or inhibition from receptors to the effector (the catalytic subunit) are considered to act as switches triggered by receptors [22]. The switch having been triggered 'on' is neither controlled any longer by the receptor nor can it be affected by any other receptor (and, consequently, by the same or other inhibitor). In terms of the model, the states of the AC complex determined by the pathways activated by collagen, by thrombin, or both, would exhibit identical efficacies. The model based on this assumption could not be fitted to the data (results not shown), which means that the above assumption is false. It is necessary, therefore, to take into account all the possible combinations of activated and non-activated pathways of inhibition depicted in Figure 5(B). Available experimental data, however, are not sufficient to estimate the efficacies of all the possible states of the AC complex determined by all those combinations of the pathways (see the Appendix). For this reason, the following equation:

$$A(y, z) =$$

$$\alpha\left(e_0\frac{y_c^2}{(y+y_c)^2} + e_c\frac{2y_c y}{(y+y_c)^2} + e_{cc}\frac{y^2}{(y+y_c)^2}\right)\frac{z_t^2}{(z+z_t)^2}\frac{\zeta_h}{z+\zeta_h}$$

$$+ \alpha\left(e_t\frac{y_c^2}{(y+y_c)^2} + \eta_{ct}\frac{2y_c y}{(y+y_c)^2} + \eta_{ct}\frac{y^2}{(y+y_c)^2}\right)\frac{2z_t z}{(z+z_t)^2}\frac{\zeta_h}{z+\zeta_h}$$

$$+ \alpha\left(e_{tt}\frac{y_c^2}{(y+y_c)^2} + \eta_{ctt}\frac{2y_c y}{(y+y_c)^2} + \eta_{ctt}\frac{y^2}{(y+y_c)^2}\right)\frac{z^2}{(z+z_t)^2}\frac{\zeta_h}{z+\zeta_h}$$

$$(3/4)$$

with two formal parameters ($\eta_{ct}$ and $\eta_{ctt}$) was used to model inhibition of AC activity by collagen and thrombin in combination. Eqn. (3/4) accommodates eqns. (3) and (4) and was applied to the data of both variants of experiments of Series 4. Since experimental data available are not sufficient to estimate both $e_c$ and $e_{cc}$ independently (see the Appendix), one of these parameters ($e_c$) was chosen to be fixed and close to estimates of $e_c$ obtained from experimental data of Series 3 (Table 3), and the other ($e_{cc}$) was estimated as a result of fitting model 3/4 to the data of Series 4. Note that the estimates of $e_{cc}$ from Series 4 are close to those from Series 3 (Tables 3 and 4). What is more important, $e_{tt} \neq e_{cc}$ for each experiment of Series 4, performed on four different preparations. The same applies to another pair of parameters, $e_t \neq e_c$. Note that $\eta_{ctt} < \eta_{tt}$ for each preparation, which means that collagen further inhibits AC upon reaching asymptotic inhibition by thrombin.

These findings strongly suggest that collagen and thrombin do not share a common G-protein, i.e. inhibition of AC activity by thrombin and collagen is mediated by distinct G-proteins. This conclusion agrees with the functional resolution (using GDP[$\beta$S]) of the actions of thrombin and collagen which were described previously [9]. That means that the inhibitory pathways can be

identified, in each case, with a specific receptor and a (specific) G-protein; convergence of these pathways, therefore, is possible only on the catalytic subunit of AC, as shown in Figure 5.

### Essential differences between the two groups of membrane preparations

As pointed out above, two groups of data sets of Series 2 had to be analysed separately because of non-homogeneity between the groups. All data sets of Series 4 proved to be non-homogeneous. All estimates of parameter $\alpha$ produced using preparations Red or Green are higher than those obtained on Black or Blue (see Tables 1–4).

Minor differences in the estimates of other parameters (e.g. $e_a$, $\epsilon_{ac}$) produce clear differences in model curves. In Figure 2, bold continuous and bold broken curves for Red and Green are vertically shifted from each other, whereas corresponding curves for Black and Blue tend to converge with increasing adrenaline concentration. In the first case one might be tempted to speculate on apparent 'additivity' of the collagen effect, whereas, in the second, the effect seems to be 'non-additive'. Two incompatible conclusions on the inhibitory mechanisms of AC activity in the same system would follow from such speculations. As pointed out elsewhere [18], the notion of 'additivity' is useless and misleading here. Nonetheless, regardless of the criteria used to compare the two sets of model curves, these sets are different. Here, however, both sets of data are modelled adequately by the same equations (eqns. 2 and 2*). The differences are quantitative (see Table 2) rather than qualitative. One might suppose these differences to reflect possible differences in the properties of the AC system's components involved. This is not likely to be the case, either, as will be shown below.

Another difference between the Red and Green and Black and Blue groups is that the two pairs of curves are shifted horizontally with respect to each other. This is reflected in the different estimates of the corresponding parameter, $x_a$ (see Table 2). It should be noted that the difference is again quantitative, considerable ($\approx 4.5$-fold) and highly significant. This finding has to be explained.

Mixtures of platelet membranes from several donors might be expected to differ (which could account for the above differences in the estimates of $x_a$) if one assumes considerable heterogeneity in (or bimodal distribution of) certain properties of blood from different donors. But then one would have the same heterogeneity within each mixture, which would result in an extension of the dose–response curves over a wider range of concentrations. This is not the case: experimental data do not suggest any deviation from the model applied. It should be concluded, therefore, that in both groups of preparations adrenaline receptors were homogeneous. The different estimates of $x_a$ should be considered to reflect different experimental conditions most likely during preparation of batches of membranes. The source(s) of the difference may be connected with the storage medium in which the membranes were suspended, e.g. trace levels of detergent, or something more akin to that than any variation in the performance of the AC assay. Formally, the shift of the curves along the concentration axis of one group with respect to the other could be explained as a result of action of some unidentified antagonist of adrenaline, the antagonist being present in one group of preparations and absent in the other. Similar reasoning could be applied to explain other differences.

Pronounced differences can be seen also in Figure 4. These differences are also reflected in the different estimates of corresponding parameters (see Table 4).

### CONCLUSIONS

(1) In intact platelets, the time constant of cAMP degradation, $\tau$, is not affected by collagen, which means that collagen does not affect the activity of cAMP PDE.

(2) The affinities of receptors specific for adrenaline or thrombin are not affected by collagen.

(3) In contrast with the action of adrenaline, inhibition of AC by collagen proceeds via two parallel pathways; the same is true for thrombin at moderate concentrations. These pathways each involve distinct G-proteins, different from that mediating inhibition of AC by adrenaline, i.e. $Gi_2$. Convergence of the inhibitory pathways takes place on the catalytic subunit of AC.

(4) All differences found in the estimates of model parameters can be explained in terms of corresponding models used for data analysis. The sources of these differences have to be attributed to possible differences in experimental procedures, including preparation and storage of platelet membranes.

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# APPENDIX

## Time-course of cAMP accumulation

Accumulation of cAMP can be considered as a result of two enzymic processes: its synthesis and degradation. According to classical Michaelis–Menten kinetics:

$$V_{\text{synthesis}} = k_{\text{cat AC}} [\text{activated AC}] \frac{[\text{ATP}]}{K_{m\ \text{ATP}} + [\text{ATP}]}$$

and

$$V_{\text{degradation}} = k_{\text{cat, cAMP-degrading enzyme}} [\text{cAMP-degrading enzyme}]$$
$$\times \frac{[\text{cAMP}]}{K_{\text{m, cAMP}} + [\text{cAMP}]}$$

Under the assumption that, in intact platelets, $[\text{ATP}] \gg K_{\text{m,ATP}}$:

$$V_{\text{synthesis}} = k_{\text{cat, AC}} [\text{activated AC}] = V_{\text{max}}$$

and for $[\text{cAMP}] \ll K_{\text{m cAMP}}$:

$$V_{\text{degradation}} = \frac{k_{\text{cat, cAMP-degrading enzyme}}}{K_{\text{m, cAMP}}} [\text{cAMP-degrading}$$

enzyme].[cAMP]

or

$$V_{\text{degradation}} = \frac{c}{\tau}$$

where

$c = [\text{cAMP}]$,

$$\frac{1}{\tau} = \frac{k_{\text{cat, cAMP-degrading enzyme}}}{K_{\text{m, cAMP}}} [\text{cAMP-degrading enzyme}]$$

As a result

$$V_{\text{accumulation}} = V_{\text{max}} - \frac{c}{\tau}$$

or

$$\frac{dc}{dt} = V_{\text{max}} - \frac{c}{\tau}$$

and ($c_0$ denoting initial concentration)

$$c = c_0 \exp(-t/\tau) + V_{\text{max}} \tau [1 - \exp(-t/\tau)] \tag{A 1}$$

A similar equation (eqn. 1 in the Methods and models section of the main paper) holds true for the mass of cAMP accumulated in the course of time.

## Activity of AC affected by collagen and thrombin in combination

In accordance with assumption (3) in the Methods and models section of the main paper, the activity of AC depends on collagen and thrombin doses as follows:

$$A(y;z) = \alpha \left( e_0 \frac{y_c^2}{(y+y_c)^2} + e_c \frac{2y_c y}{(y+y_c)^2} + e_{cc} \frac{y^2}{(y+y_c)^2} \right) \frac{z_t^2}{(z+z_t)^2} \frac{\zeta_h}{z+\zeta_h}$$
$$+ \alpha \left( e_t \frac{y_c^2}{(y+y_c)^2} + e_{ct} \frac{2y_c y}{(y+y_c)^2} + e_{cct} \frac{y^2}{(y+y_c)^2} \right) \frac{2z_t z}{(z+z_t)^2} \frac{\zeta_h}{z+\zeta_h}$$
$$+ \alpha \left( e_{tt} \frac{y_c^2}{(y+y_c)^2} + e_{ctt} \frac{2y_c y}{(y+y_c)^2} + e_{cctt} \frac{y^2}{(y+y_c)^2} \right) \frac{z^2}{(z+z_t)^2} \frac{\zeta_h}{z+\zeta_h}$$
$$\tag{A 2}$$

with all the parameters remaining the same as in the main paper, $e_{ct}$, $e_{cct}$, $e_{ctt}$ and $e_{cctt}$ corresponding to the states of the AC complex determined by the inhibitory pathways activated by both collagen and thrombin (see Figure 5B of the main paper). This equation can be easily obtained by following Appendix 1 in [19]. It is clear (see eqn. 4* of the main paper) that, at collagen dose used ($y = 100\ \mu\text{g/ml}$):

$$e_0 \frac{y_c^2}{(y+y_c)^2} + e_c \frac{2y_c y}{(y+y_c)^2} + e_{cc} \frac{y^2}{(y+y_c)^2} = \epsilon_c \tag{A 3}$$

$$e_t \frac{y_c^2}{(y+y_c)^2} + e_{ct} \frac{2y_c y}{(y+y_c)^2} + e_{cct} \frac{y^2}{(y+y_c)^2} = \epsilon_{tc} \tag{A 4}$$

$$e_{tt} \frac{y_c^2}{(y+y_c)^2} + e_{ctt} \frac{2y_c y}{(y+y_c)^2} + e_{cctt} \frac{y^2}{(y+y_c)^2} = \epsilon_{ttc} \tag{A 5}$$

Six parameters ($e_c$, $e_{cc}$, $e_{ct}$, $e_{cct}$, $e_{ctt}$ and $e_{cctt}$) in eqns. (A3)–(A5) are unknown, and the number of equations available is not sufficient to solve them with respect to these parameters. For this reason (keeping in mind that the estimates of either $e_c$ or $e_{cc}$ obtained from Series 3 can be considered as known parameters) $e_{ct}$ and $e_{cct}$ were substituted by a formal parameter, $\eta_{ct}$, and $e_{ctt}$ and $e_{cctt}$, by $\eta_{ctt}$, (this is equivalent to considering both parameters in each pair (or corresponding pathways) being identical), making the system solvable with respect to all parameters. The above substitution in eqn. (A2) yields eqn. (3/4) in the Discussion section of the main paper.