TURN ON AND TURN OFF REACTIONS OF β-ADRENERGIC-SENSITIVE ADENYLATE CYCLASE IN CONTROL AND DESENSITIZED C_6 GLIOMA CELLS

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1. Introduction

In a study of β-adrenergic-sensitive adenylate cyclase in C_6 glioma cells [1] we described the coupling function, i.e., the function relating receptor occupancy by an agonist and adenylate cyclase activation. We showed that this function is of Michaelis-Menten type. A coupling constant K was defined representing the apparent affinity of the hormone–receptor complex (HR) for the adenylate cyclase (E). Different models have been proposed to explain the adenylate cyclase activation by an agonist: the properties of the collision coupling model in [2] agree with our data. Thus the adenylate cyclase activation model we proposed for C_6 glioma cells is the following:

$$H + R \underset{K_d}{\rightleftharpoons} HR + E \underset{k_{off}}{\overset{k_a}{\rightleftharpoons}} (HRE) \rightarrow HR + E^*$$

where H is the β-adrenergic agonist, R the β-adrenergic receptor, E and E* are the inactive and the active forms of adenylate cyclase, respectively. These forms had been proposed as corresponding to an association with a nucleotide regulatory protein binding either GDP (E) or GTP (E*) [3]. (HRE) is a very transient complex, k_a the rate activation constant of adenylate cyclase and k_{off} the rate constant of enzyme deactivation.

There is now considerable evidence that the deactivation is due to hydrolysis of GTP to GDP by a GTPase associated with the adenylate cyclase [3].

From this model, it can be seen that the coupling function is given by:

$$E^* = E_T \frac{[HR]}{(k_{off}/k_a) + [HR]} \quad (1)$$

where E_T is the maximal theoretical adenylate cyclase activity obtained when all the enzyme is in active state. The apparent affinity of the hormone–receptor complex (HR) for the adenylate cyclase was defined in this model as the coupling constant K, equal to k_{off}/k_a.

This was designed to verify the validity of the above collision coupling model by comparing the K-values of the coupling constant obtained at equilibrium and those obtained by measuring k_{off} and k_a.

We have shown that desensitization of C_6 glioma cells by a β-adrenergic agonist altered the coupling constant [1]. Such alteration might be due to a change in either the adenylate cyclase rate activation constant (k_a) or its rate deactivation constant (k_{off}) or in both constants. That means modification of either the turn-on or the turn-off reaction of adenylate cyclase. The second aim of this study was therefore to measure the value of k_a and k_{off} in a desensitized system. A change in either parameter would imply different molecular mechanisms.

2. Materials and methods

Culture of C_6 glioma cells, preparation of particulate fractions and measurement of [^{3}H]DHA binding and adenylate cyclase activity were all performed as in [4]. [^{3}H]DHA binding and adenylate cyclase activities were determined under strictly identical conditions.

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2.1. *Determination of the rate constant of adenylate cyclase deactivation (k_{off})*

The rate constant of adenylate cyclase deactivation was measured by the decline of β-adrenergic-stimulated adenylate cyclase activity after adding an excess of the antagonist propranolol according to [3]. The extrapolation of the cAMP decay curve plateau obtained with propranolol intersects the line obtained for cAMP production by the uninhibited enzyme at a time point equivalent to the reciprocal of k_{off}.

2.2. *Determination of the rate constant for adenylate cyclase activation*

The cAMP accumulation by agonist-stimulated adenylate cyclase as a function of time has been described in [5]. The intercept of the linear portion of the obtained curve with the abscissa gives the value:

$$\tau = \frac{1}{k_a[R_T] + k_{off}} = \frac{1}{k_{obs}}$$

When experiments were performed in the presence of Gpp(NH)p, propranolol did not inhibit the activated enzyme [6,7], thus, the deactivation constant, k_{off}, was equal to zero and $1/k_{obs} = 1/k_a[R_T]$. To determine τ, we performed linear regression analysis of the linear portion of the cAMP production curve. The standard error for τ was calculated by dividing the standard error for y ($S\,y.x/\sqrt{n}$) by the value of the regression line slope [8]. τ-Values were compared by Student's t-test.

3. Results

3.1. *Coupling function characteristics of 2 full agonists in C_6 glioma cells*

Isoproterenol and epinephrine gave the same maximal adenylate cyclase stimulations (E^*_{max} was identical) but displayed different app. K_a-values (4×10^{-8} M and 5.6×10^{-7} M, respectively) (fig.1A). Both dose–activation curves were Michaelis-Menten functions of the free agonist concentration since the Hofstee plots of these curves were linear (not shown). The curves for displacement of [^{3}H]DHA binding by isoproterenol and epinephrine, respectively, indicated that both these agonists interacted with all β-adrenergic receptors [4] and that agonist binding exhibited no cooperativity (Hill coefficient close to 1, fig.1B). The respective K_d of isoproterenol and epinephrine for the β-adrenergic receptors were 1.7×10^{-7} M and 2.2×10^{-6} M.

In fig.1C an Eadie-Scatchard plot of the coupling function was obtained with isoproterenol and epinephrine, where [RH] is normalized to $[R_T]$ and E*, to

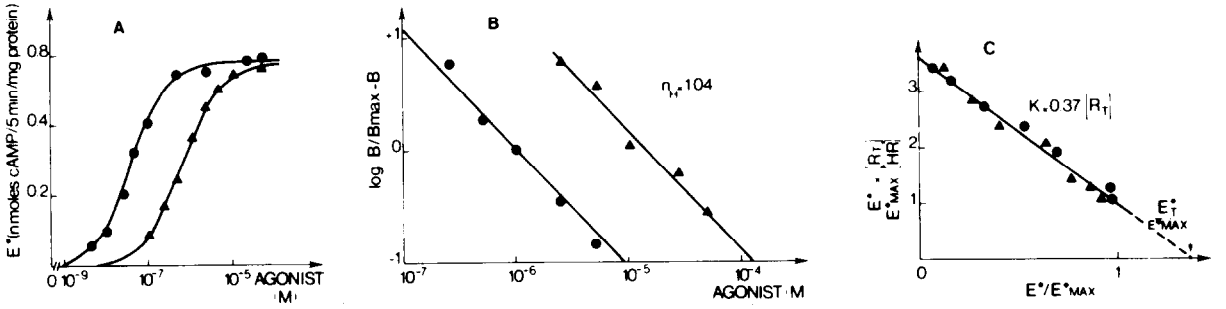

Fig.1. Characteristics of the coupling function of 2 full agonists in C_6 glioma cells: (●) (–)isoproterenol; (▲) (–)epinephrine. (A) Dose–response curves for adenylate cyclase activation by isoproterenol and epinephrine: E* = adenylate cyclase activity minus basal activity (0.07 nmol cAMP . 5 min^{-1} . mg $protein^{-1}$); app. K_a isoproterenol = 4.0×10^{-8} M; app. K_a epinephrine = 5.6×10^{-7} M; cAMP accumulation was measured 8–13 min after starting incubation. (B) Hill plots for displacement of [^{3}H]DHA by agonists in C_6 glioma cells. [^{3}H]DHA concentration 15.3 nM, K_d [^{3}H]DHA = 3.1×10^{-9} M; B = specific [^{3}H]DHA binding, B_{max} = 0.5 pmol/mg protein, K_d was 1.7×10^{-7} M and $2.2. \times 10^{-6}$ M for isoproterenol and epinephrine, respectively. Measurements were performed 10 min after the beginning of incubation. (C) Eadie-Scatchard plot of the function relating receptor occupancy to adenylate cyclase activation. Data are taken from the experiments (A,B): E^* = adenylate cyclase activity minus basal activity (percent of E^*_{max}); E^*_{max} = maximal minus basal adenylate cyclase activity (fig.1A); HR/R_T = fractional receptor occupancy by isoproterenol (●——●) calculated taking $K_d = 1.7 \times 10^{-7}$ M, or by epinephrine (▲——▲), calculated taking $K_d = 2.2 \times 10^{-6}$ M; K = apparent affinity of the agonist–receptor complex for the enzyme; E^*_T = theoretical maximal adenylate cyclase activity.

E^*_{max}. The slope of the line obtained allows calculation of the coupling constant K, expressed in relation to $[R_T]$. Note that, since these reactions occurred within membranes of unknown volume, it was impossible to ascertain the absolute values of K and R_T.

Despite their differences in app. K_a and K_d, both isoproterenol and epinephrine exhibited the same coupling function (fig.1C). The maximal theoretical adenylate cyclase activation (E_T) and the coupling constant (K) were identical. The K-value in this experiment was equal to 0.37 $[R_T]$ (fig.1C). (Mean value for epinephrine 0.35 ± 0.04 $[R_T]$, $n = 3$ and mean value for isoproterenol $K = 0.26 \pm 0.06$ $[R_T]$, $n = 3$; not statistically different.)

3.2. *Determination of the rate constant for enzyme deactivation (k_{off}) in normal and desensitized system*

Fig.2 shows that in the control system k_{off} was 5.2 min^{-1} (mean value 4.8 ± 0.1 min^{-1}, $n = 6$). The k_{off} value obtained in the isoproterenol-desensitized system (fig.2) was significantly higher 7.5 min^{-1} (mean value 7.3 ± 0.2 min^{-1}, $n = 3$).

The method proposed in [3] for k_{off} determination implies that dissociation of (HR) complexes was not a limiting factor of the decline observed in curve 2 and that the excess of propranolol added to the reaction medium immediately stopped further adenylate cyclase activation. Two arguments indicate that these conditions were fulfilled in C_6 glioma cells:

(1) The k_{off}-values measured either with isoproterenol (4.3 ± 0.3 min^{-1}, $n = 3$) or epinephrine (4.8 ± 0.1 min^{-1}, $n = 6$) were not statistically different despite the 10-fold difference in the respective K_d of these agonists and the fact that they therefore displayed a large difference in the rate constants for dissociation of their binding to the receptors.

(2) When Gpp(NH)p and propranolol were added together and with [α-^{32}P]ATP the cAMP production measured for 5 min was 151 and 99 pmol/mg protein for the enzyme preincubated with and without epinephrine, respectively. The difference (52 pmol cAMP) almost equalled the amount of cAMP produced during the deactivation process, turn-off (42 pmol cAMP/mg protein). If propranolol had not immediately stopped the activation reaction Gpp(NH)p would have caused persistent activation of the adenylate cyclase and therefore the amount of cAMP obtained would have been much larger than the amount of cAMP formed during the turn-off.

3.3. *Determination of the rate constant for adenylate cyclase activation (k_a)*

With 10^{-4} M isoproterenol, k_{obs}-values were equal to 14.3 min^{-1} and 9.2 min^{-1} in the absence and presence of Gpp(NH)p, respectively. This difference was expected since Gpp(NH)p suppressed the k_{off} reaction [3,9,10]. Therefore k_{obs} was equal to $k_a[R_T] + k_{off}$ and to $k_a[R_T]$ in the absence and presence of

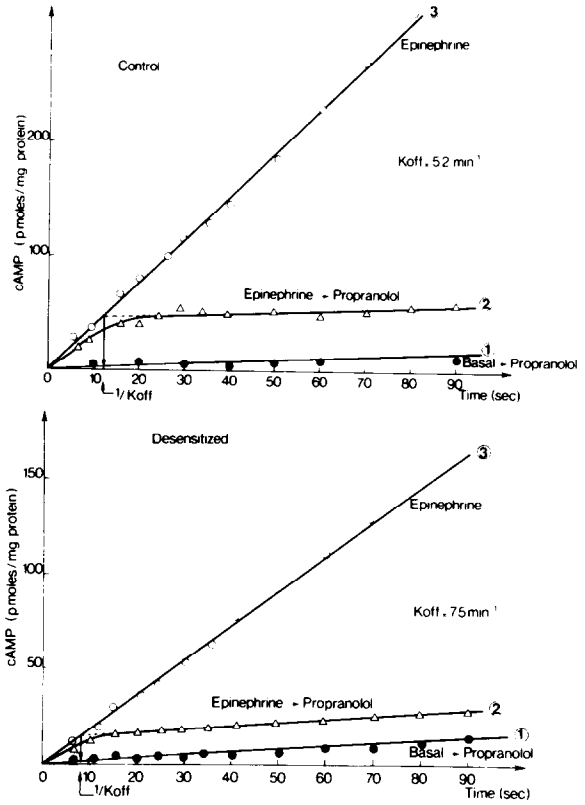

Fig.2. Adenylate cyclase deactivation in particulate fractions of control and desensitized C_6 glioma cells: determination of the rate constant k_{off}. Particulate fractions at a final concentration of 0.38 and 0.36 mg protein/ml for control and desensitized systems, respectively, were incubated in the standard medium for 5 min at 30°C with or without 20 μM of (−)epinephrine. At zero time tracer amounts of [α-^{32}P]ATP were added with (−)propranolol to a final concentration of 0.5 mM (curves 1 and 2) and without (curve 3). At the time indicated, 100 μl aliquots were removed and added to a stopping solution and the amount of c[^{32}P]AMP formed was determined: (●——●) basal activity of adenylate cyclase; (△——△) decline in epinephrine-stimulated adenylate cyclase activity on addition of (−)propranolol; (○——○) epinephrine-stimulated adenylate cyclase activity.

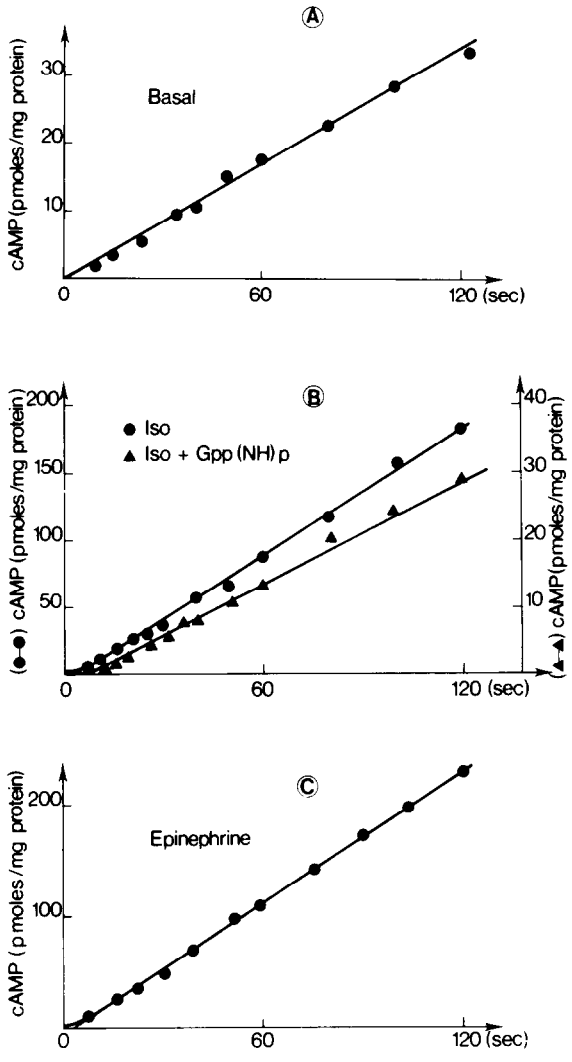

Fig.3. Determination of the rate constant of adenylate cyclase activation. (A) cAMP accumulation in the absence of an agonist in control C_6 glioma cell membranes; protein was 0.57 mg/ml. The line was determined by linear regression analysis ($r = 0.999$). It intercepts with the abscissa at $t = 0.8 \pm 0.6$ s. (B) cAMP accumulation in the presence of 10^{-4} M isoproterenol (●——●) and 10^{-4} M isoproterenol plus 10^{-5} M Gpp(NH)p (▲——▲). Membranes were preincubated for 20 min with or without Gpp(NH)p. The reaction was started by adding tracer amounts of [α-^{32}P]ATP and isoproterenol. Characteristics of the lines were determined by linear regression analysis of the linear portion of the curve. (●——●) $r = 0.999$, $\tau = 1/(k_a [R_T] + k_{off}) = 1/k_{obs} = 4.2 \pm 0.4$ s, $k_{obs} = 14.3$ min^{-1} (▲——▲) $r = 0.995$, $\tau = 1/k_a [R_T] = 1/k_{obs} = 6.5 \pm 1.0$ s, $k_{obs} = 9.2$ min^{-1} basal activities without or with Gpp(NH)p were both linear and were subtracted from the respective curves. (C) cAMP accumulation in the presence of 10^{-4} M (−)epinephrine in C_6 glioma cell control membranes. The protein concentration was 0.30 mg/ml. Characteristics of the line were determined from linear regression analysis of the linear portion of the curve from which basal activity was subtracted. $r = 0.999$ and $\tau = 1/(k_a [R_T] + k_{off}) = 1/k_{obs} = 3.6 \pm 0.3$ s, $k_{obs} = 16.7$ min^{-1}. τ-Values were determined ± SEM. The following sets of τ-values were compared by Student's t-test: basal (1A) and $t = 0$, not significant; basal (1A) and epinephrine (1C) $p < 0.001$; Iso (1B) and Iso + Gpp(NH)p (1B) $p < 0.05$; Epi (1C) and Iso (1B), not significant.

Gpp(NH)p, respectively. Note that the difference (14.3 min^{-1}–9.2 min^{-1}) gave an estimation of k_{off} (5.1 min^{-1}) close to the k_{off} determined directly in deactivation experiments (4.3 ± 0.3 min^{-1}, $n = 3$).

The k_{obs} values obtained with epinephrine were 16.7 min^{-1} (fig.3C) in the absence of Gpp(NH)p and 11.1 ± 0.8 min^{-1} ($n = 4$) in its presence (table 1).

Table 1

Comparison in control and desensitized C_6 glioma cell membranes of the coupling constant K, the activation rate constant k_a and the deactivation rate constant k_{off} for adenylate cyclase

	k_{off} (min^{-1})	k_{obs} (min^{-1}) in the presence of 10^{-5} M Gpp(NH)p		k_a ([R_T]$^{-1}$. min^{-1})	$\frac{k_{off}}{k_a}$	K (equilibrium)
		$k_a [R_T]$	$k_a [R_T^d]$			
Control system	4.8 ± 0.1 (6)	11.1 ± 0.8 (4)		11.1 ± 0.8 (4)	0.43 [R_T]	0.35 ± 0.04 [R_T] (3)
Desensitized system	7.3 ± 0.2 (3)		3.8 ± 0.5 (3)	7.6 ± 1.0 (3)	0.96 [R_T]	1.0 ± 0.2 [R_T] (3)

All these values were determined with (−)epinephrine. Values are means ± SEM. Number of experiments is indicated in brackets. Under our experimental conditions of desensitization: $[R_T^d] = [R_T]/2$; $[R_T^d]$ and $[R_T]$ being the total number of β-adrenergic receptors in the desensitized and control systems, respectively; k_a is expressed as a function of $[R_T]$ in both systems. Comparison of $k_a [R_T]$, k_{off} and K values in control and desensitized systems by Student's t-test gave $p < 0.05$

Determination of activation rate constant is only valid if hormone–receptor complex formation is not a limiting step in adenylate cyclase activation. This is not the case since, with two agonists, isoproterenol and epinephrine, displaying a 10-fold difference in their respective K_d, the activation rate constants were identical (fig.3).

In a desensitized system, the k_{obs}-values obtained in the presence of 10^{-5} M Gpp(NH)p plus 10^{-4} M epinephrine were lower than in the control system (3.1 min^{-1} in fig.4, mean value 3.8 ± 0.5 min^{-1}, $n = 3$). Since in the desensitized system the total number of receptors (R_T^d) was 50% of the control (R_T), the value of k_{obs} in the desensitized system was equal to $k_a[R_T]/2$. Thus, k_a in the desensitized system was equal to 7.6 ± 1.0 $[R_T]^{-1} . min^{-1}$, $n = 3$ (table 1). For direct comparison of k_a-values in normal and desensitized systems, we performed an experiment in which concentration of hormone–receptor complexes was the same in both systems. This was achieved by using 10^{-4} M epinephrine for the desensitized system (a concentration leading to maximal receptor occupancy) and 2×10^{-6} M epinephrine in the control system (a concentration giving half receptor occupancy). This experiment confirmed that k_a diminished in the desensitized system (fig.4). Table 1 gives the different values of k_{off}, k_a and K determined with epinephrine. It indicates that the values of the coupling constant K determined at equilibrium or by the ratio k_{off}/k_a are very close in both control and desensitized systems.

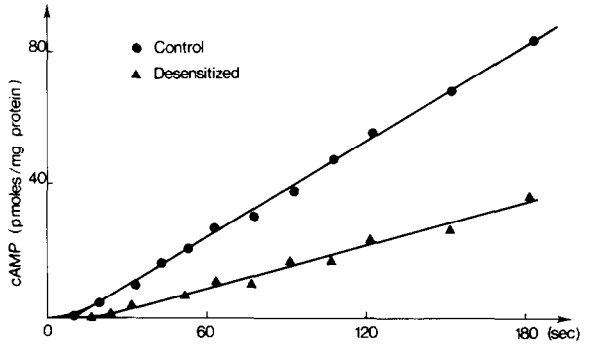

Fig.4. Determination of the rate constants of adenylate cyclase activation in control and desensitized C_6 glioma cell membranes. Cells were treated for 3 h with 10^{-5} M isoproterenol. Protein concentrations in control and desensitized systems were 0.34 mg protein/ml and 0.38 mg protein/ml, respectively. Membranes were preincubated for 20 min in the presence of 10^{-5} M Gpp(NH)p. cAMP accumulation was measured in the presence of 2×10^{-6} M epinephrine plus 10^{-5} M Gpp(NH)p in the control system (●——●) and in the presence of 10^{-4} M epinephrine plus 10^{-5} M Gpp(NH)p in the isoproterenol desensitized system (▲——▲). In the desensitized system, the total number of β-adrenergic receptors [R_T^d] was equal to 0.25 pmol/mg protein, which is half the concentration found in the control system, [R_T] = 0.48 pmol/mg protein. In the control system, we therefore used a concentration of epinephrine giving half receptor occupancy to study the activation at the same concentration of (*HR*) complexes in both control and desensitized systems. Basal adenylate cyclase activities in the presence of 10^{-5} M Gpp(NH)p were linear and were subtracted from each curve. Characteristics of the lines were determined by linear regression analysis of the linear portion of the curve. Control system: $r = 0.997$, $\tau = 1/(k_a [R_T]/2) = 10.4 \pm 0.7$ s, $k_a [R_T]/2 = 5.8$ min^{-1}. Desensitized system: $r = 0.981$, $\tau = 1/k_a [R_T^d] = 19.3 \pm 3$ s, $k_a [R_T^d] = 3.1$ min^{-1}. Values were determined ± SEM; the τ-values were compared by Student's t-test. Comparison of control and desensitized systems gave $p < 0.01$.

4. Discussion

These results suggest that the collision coupling model adequately describes the molecular events occurring during adenylate cyclase activation in C_6 glioma cells. We also showed that the increase in the coupling constant occurring during desensitization results from a decrease in the rate constant of activation and a rise in the rate constant of deactivation.

The change in the activation rate constant observed here might be due to an alteration at or near the receptor sites, thus impairing the receptor–nucleotide-binding protein interaction necessary for coupling. Such a hypothesis agrees with recently reported data. Fusion experiments have shown that desensitization of frog erythrocytes appears to originate from changes in the receptor component of the system [11]. Reconstitution experiments with a solubilized β-adrenergic receptor obtained after desensitization of wild-type S49 lymphoma cells and S49 mutant *cyc*$^-$ indicated that during desensitization the GTP-binding protein does not alter but that the locus of change is on or near the β-adrenergic receptor [12,13]. Such a change accounts for the homologous nature of the desensitization observed.

The increase found in k_{off} in the present work might indicate that GTPase activity is enhanced during agonist desensitization. This effect might explain why, in some systems in which 2 hormones act through different receptors but on the same enzyme, cell exposure to one of these hormones makes both of them refractory to the hormonal response, a process defined as heterologous desensitization [14].

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References

[1] Homburger, V., Lucas, M., Cantau, B., Barabe, J., Penit, J. and Bockaert, J. (1980) J. Biol. Chem. 255, 10436–10444.
[2] Levitzki, A. and Helmreich, E. J. M. (1979) FEBS Lett. 101, 213–219.
[3] Cassel, D., Levkovitz, H. and Selinger, Z. (1977) J. Cyclic Nucl. Res. 3, 393–406.
[4] Lucas, M. and Bockaert, J. (1977) Mol. Pharmacol. 13, 314–329.
[5] Tolkovsky, A. M. and Levitzki, A. (1978) Biochemistry 17, 3795–3810.
[6] Schramm, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 2232–2237.
[7] Sevilla, N., Steer, M. L. and Levitzki, A. (1976) Biochemistry 15, 3493–3499.
[8] Snedecor, G. W. (1962) in: Statistical methods applied to experiments in agriculture and biology, 5th edn, p. 125, Iowa State University Press, Ames IA.
[9] Arad, M. and Levitzki, A. (1979) Mol. Pharmacol. 16, 749–756.
[10] Cassel, D. and Selinger, Z. (1977) Biochem. Biophys. Res. Commun. 77, 868–873.
[11] Pike, L. J. and Lefkowitz, R. J. (1980) Biochim. Biophys. Acta 632, 354–365.
[12] Green, D. A. and Clark, R. B. (1981) J. Biol. Chem. 256, 2105–2108.
[13] Iyengar, R., Krishna-Bath, M. and Riser, M. E. (1981) J. Biol. Chem. 256, 4810–4815.
[14] Su, Y. F., Cubeddu, L. X. and Perkins, J. P. (1976) J. Cyclic Nucl. Res. 2, 257–270.