Cyclic adenosine monophosphate pathway and human trophoblast cells: is a predictive mechanical model needed?

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SUMMARY. The main features of a predictive mathematical model for the prediction the cell aggregate response to external ligands, quantified through the cAMP release, are outlined in this paper. The arising second messenger response is directly linked to the coupling of conformational and mechanical effects. A new Helmholtz free energy is constructed for such a system. The evaluation of the stationary points of this functional together with the balance between the influx of active receptors and the rate of change of their density yield the desired result.

INTRODUCTION.

Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger that transduces inside the cell the effects of extracellular ligands, which cannot get through the cell membrane.

The ligand binds to a specific receptor which is an integral membrane protein embedded in the lipid bilayer belonging to the G protein-coupled receptor (GPCR) family and characterized by seven transmembrane helices. Once the ligand is recognized, the receptor shifts conformation and thus mechanically activates the G protein, which detaches from the receptor and, in turn, activates another membrane protein, called adenylyl cyclase. This enzyme transforms adenosine triphosphate (ATP) in cAMP. The cyclic nucleotide works by activating protein kinase A that transfers a phosphate group into other proteins involved in several cell functions, including proliferation and migration (Figure 1).

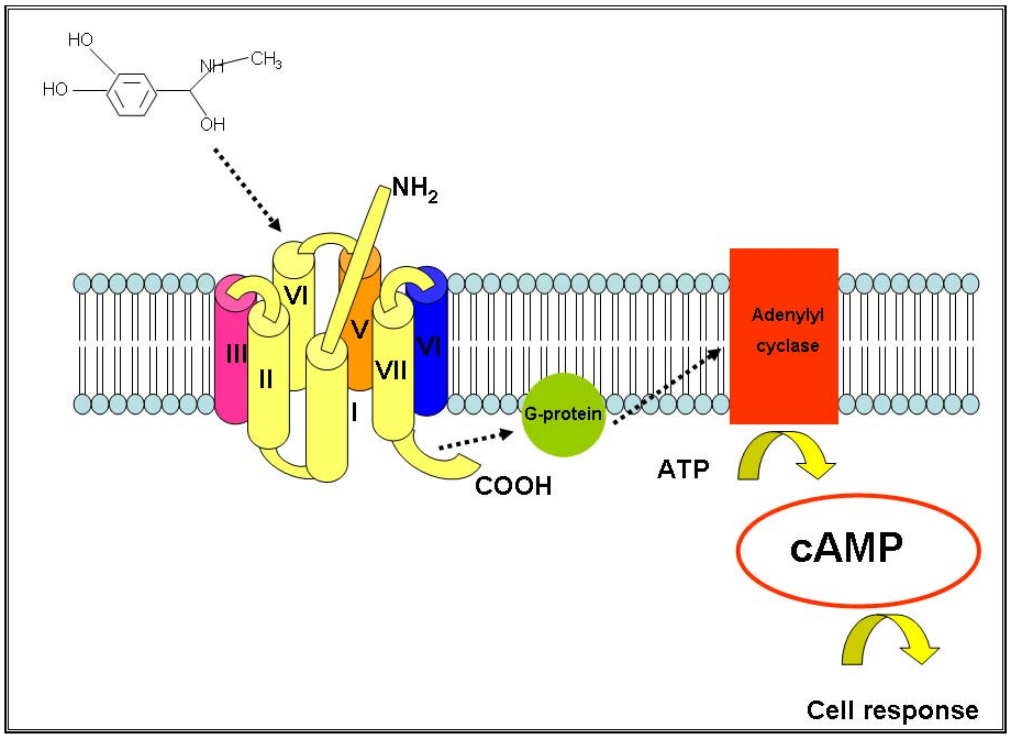


Figure 1: description of the adenylyl cyclase transduction pathway

Among this class of receptors, β-adrenergic receptor has been the first human GPCR whose structure has been solved [1]. It has been reported that three of the seven transmembrane (TM) helices, in particular TM3, 5 and 6, are involved in the conformational changes undergone by this receptor after interaction with its ligand [2] (Figure 2). Such changes may involve both a rotation (ω) about the axis of TM6, whose unit vector is denoted by $\boldsymbol{e}_3$, and a shear μ of TM6 towards TM5. The unit vector of the direction TM5-TM6 is denoted by $\boldsymbol{s}$ in Fig.2. There h denotes the average height of such domains.

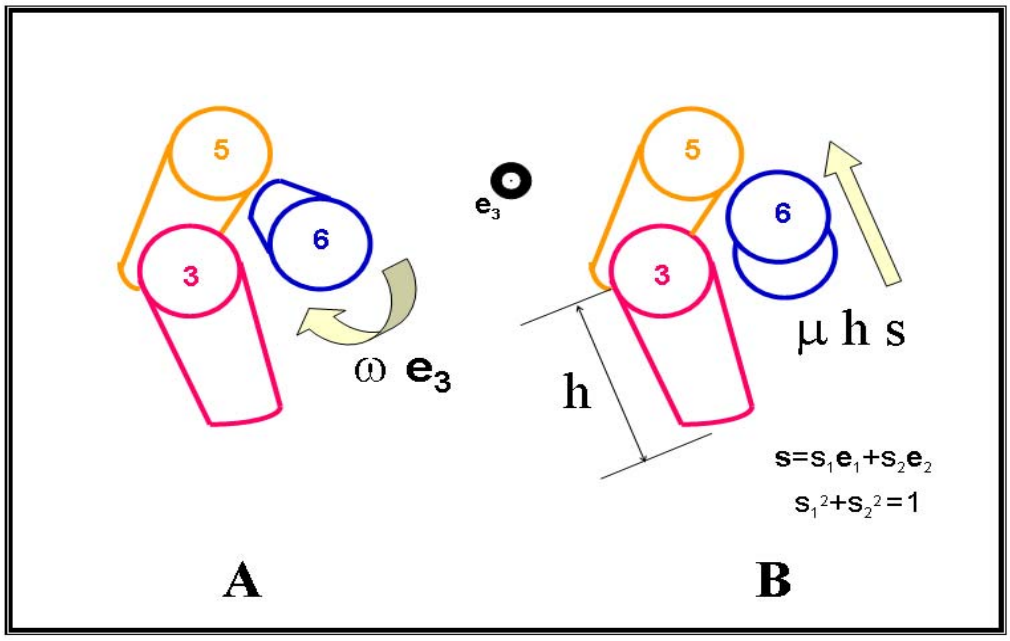


Figure 2: β-adrenergic receptor conformational changes induced by ligand binding

It has been known that β-adrenergic receptors are expressed in trophoblast cells [3], that form the fetal side of the placenta. These cells are implicated in nutrient and gas exchanges between the mother and the fetus as well as in maternal spiral arterioles remodelling, a process that is fundamental for a successful pregnancy outcome. In order to obtain a physiological vessel remodelling, proliferation, migration and invasiveness of a subpopulation of trophobalst cells, called extravillous trophoblast cells, must be finely controlled [4]. We have recently demonstrated a cAMP involvement in the control of human extravillous trophoblast cell proliferation and migration. Indeed prostaglandin E_2 (PGE_2) and forskolin (FSK), two compounds that highly enhance cAMP concentration, inhibit cell proliferation and migration, whereas somatostatin, which reduces PGE_2- and FSK-enhanced cAMP levels, stimulates these responses [5, 6].

In addition to β-adrenergic receptors, human trophoblast cells produce the adrenoceptor natural ligands cathecolamines [7], whose involvement in the regulation of hormone secretion from this tissue has been demonstrated [8-10].

MATERIALS AND METHODS

1.1 Cell Culture

The HTR-8/SVneo trophoblast cell line, obtained from human first-trimester placenta explant cultures and immortalized using SV40 large T antigen, was kindly provided by Dr. CH Graham, Queen's University, Kingston, Ontario (Canada). Cells were cultured at 37°C in an atmosphere of 5% CO_2/95% air in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

1.2 cAMP level determination

Cells were grown to confluence (2-3 days) in twenty four well plate (see e.g. fig. ….), then the medium was removed and replaced by serum-free RPMI. The incubation was carried out in the absence or in the presence of the

phosphodiesterase inhibitor IBMX and of epinephrine, for the indicated time. Media were then collected and immediately frozen at -70°C until cAMP levels were measured. Ice-cold 0.1 N HCl (0.25 ml) was added to the cells and, after centrifugation at 12,500 x g for 10 min, supernatants were neutralized adding 0.5 M Trizma base (0.05 ml) and utilized for intracellular cAMP determination.

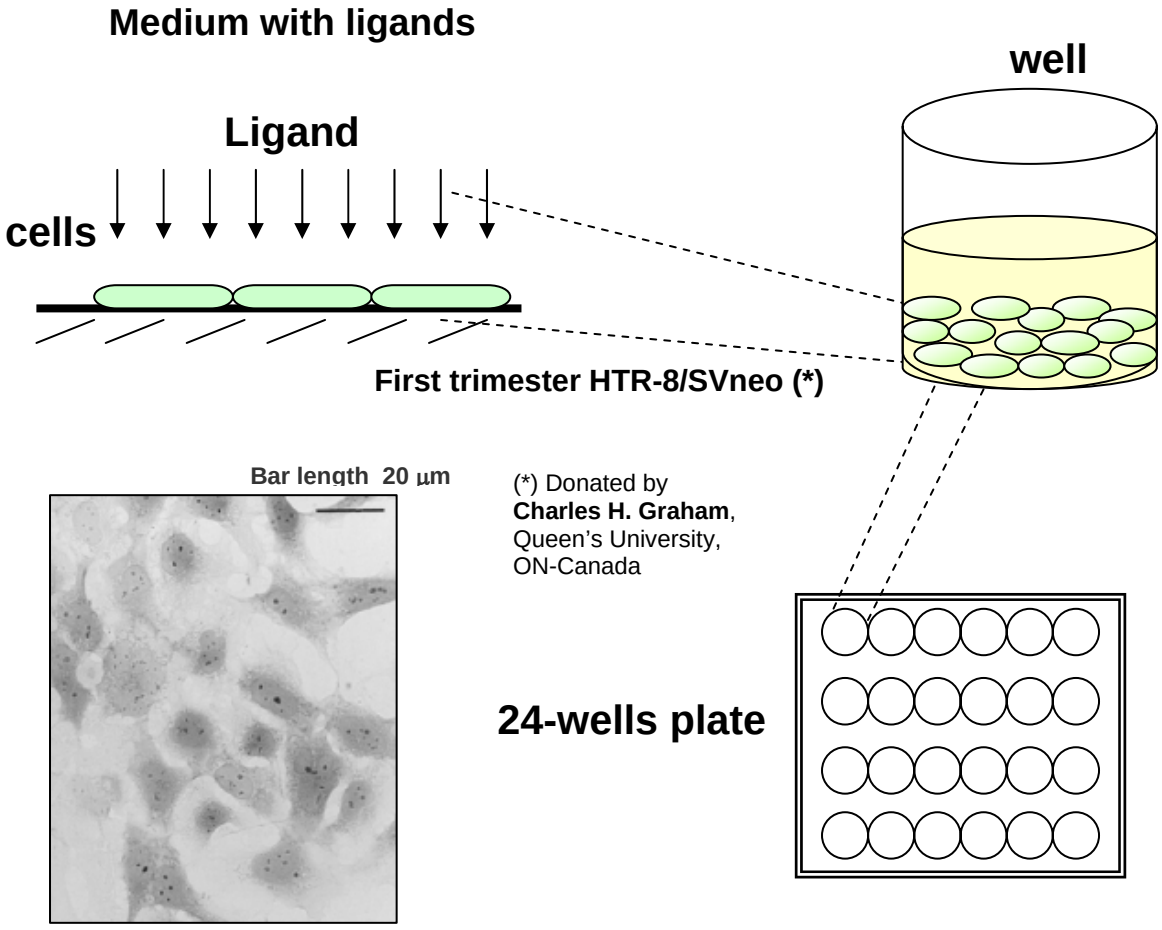


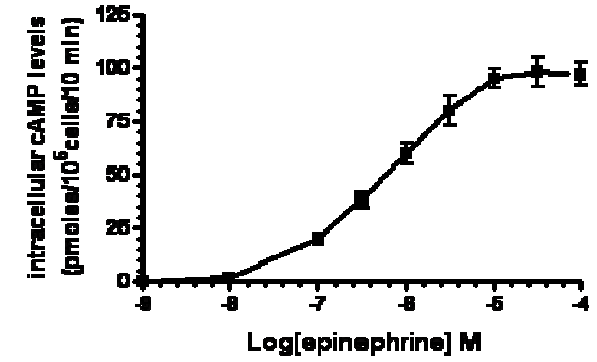
Figure 3: A schematic of the performed experiment.

2 RESULTS

2.1 cAMP evaluation

The effect of the receptor ligand epinephrine was examined on cAMP production in HRT-8/SVneo cells. As reported in Figure 4 a, epinephrine enhanced cAMP concentration in a dose-related fashion, reaching a plateau at around 10^{-5} M. The calculated EC_{50} value was 690 nM and the extent of stimulation was 15-fold [5].

We then measured intracellular cAMP levels in cells incubated up to 60 min in the absence and presence of 10^{-6} M epinephrine. In basal conditions, cAMP concentrations remained almost constant at all tested times (around 6.0 pmoles/10^6 cells, not shown). In the presence of epinephrine, intracellular cAMP production increased as a function of incubation time up to 15 min (14-fold), thereafter a reduction of the nucleotide level was observed (Figure 4 b).



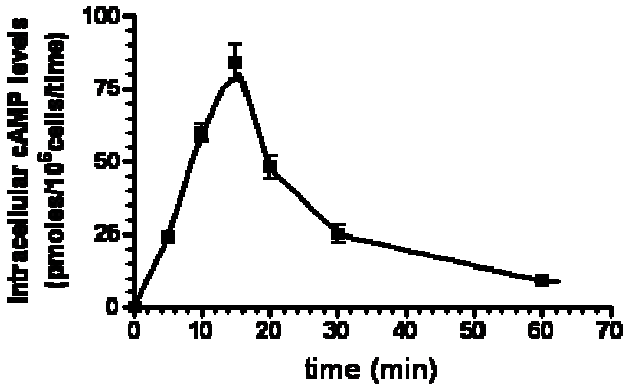


Figure 4: In A: effect of various concentrations of epinephrine on intracellular cAMP levels in HTR-8/SVneo cells. In B: Time-courses of intracellular cAMP levels in HTR-8/SVneo cells, incubated with 10^{-6} M epinephrine. Data are the mean ± SEM of four experiments, performed in duplicate on different cell cultures.

INTERPRETATION AND A CONSTRUCTION OF A PREDICTIVE MODEL

The fact that the release of cAMP of a given cell population is related to the number of active receptors that binds to affine ligands is supported by experiments and it is the leading idea for constructing a predictive model [11].

In the previous section it has been reported that three of the seven transmembrane (TM) helices, in particular TM3, 5 and 6, are involved through conformational changes during binding of ligands to receptors. During such movements, the surrounding membrane may comply by changing its thickness, leading to area changes in the cell membrane due to quasi-incompressibility [12].

The interpretation of the observed cAMP release due to the introduction of a ligand in a cell aggregate relies upon the occurrence of the conformational and mechanical changes discussed above, as well as the activation and diffusion of active receptors through the cell membrane. Such receptors are interpreted to diffuse, i.e. to "move around", to lower the (Helmholtz) free energy of the system in a purely entropic way (see e.g. [13]); The term in the energy that would account for such effects may be borrowed by [13] and written as follows:

$$\xi(-e_{RL} + \ln[\frac{\xi}{\xi_0}]), \tag{1}$$

where ξ represents the density of activated receptors in the membranes, e_{RL} is the specific activation energy for the complex ligand-receptor, where ξ_0 is a reference value of such density.

The same entropic nature (see e.g. [14]) is attributed to the energetics governing the accompanying conformational changes listed above (although a specific derivation of it may be found in [11]). If N_A denotes the Avogadro's number, following [14], it is possible to show that the change in entropy associated with rotations may be written as follows:

$$3N_A \ln(\frac{\omega}{2\pi^{2/3}}), \tag{2}$$

whereas the one due to translational changes may be written by calculating the (natural) logarithm of the translation μh (see Fig. 2) relative to the available free volume, calculated by multiplying the thickness by the available area $r^2{}_0 J$,

where r_0 represents the referential radius of the involved domains. Henceforth, the resulting translational change in entropy may be written as follows:

$$3N_A \ln\left(\frac{\mu h}{(J r_0^2 h)^{1/3}}\right). \tag{3}$$

Since $J=h/h_0$, where h_0 is the referential value of the membranal thickness and them, by assumption, the thickness h of the transmembranal domains has been assumed to be equal to the membrane thickness in the current configuration, the total change of conformational entropy turns out to be the sum of (2) and (3), i.e.:

$$\varphi_{CR} := 3N_A \ln\left(K\frac{\eta}{J}\right), \tag{4}$$

where

$$K := \left(\frac{h_0}{2\pi r_0}\right)^{2/3}, \tag{5}$$

$$\eta := \omega\mu \tag{6}$$

is the *conformational field*. The latter describes the combination of shear and rotation as a whole that may occur at the level of transmembranal domains.

Finally, the expression of the total Helmholtz free energy writes as follows

$$\int_\Gamma \left\{\xi(-e_{RL} + \ln[\frac{\xi}{\xi_0}] - \varphi_{CR}) + \varphi_{DPZ}\right\} dA, \tag{7}$$

where

$$\varphi_{DPZ} := h_0\,\varphi^{loc}(J) + \frac{h_0^3}{24J^3}\,\varphi^{loc}_{,J}(J)\,\|\nabla J\|^2, \tag{8}$$

represents the elastic membrane energy, obtained from (29) in [12] by neglecting the curvatures.

An unknown constitutive dependence of the conformational field on the density of active receptors and J is assumed, i.e.:

$$\eta = \tilde{\eta}(\xi, J), \tag{9}$$

this is because η definitely depends on the number of the active receptors (through their density) and, possibly, it may be affected by the local change in thickness of the membrane. Another way to state the same concept is to assume the invertibility of the constitutive relation that could be assumed between ξ and η holding J fixed (in this case the consequences of stationatity of (7) either with respect to ξ or η are indeed the same). Assuming that (9) holds, the first variation of the Helmholtz free energy (7) must vanish, so that the following condition holds true

$$0 = \int_\Gamma \left\{\left[(\xi\,\varphi_{CR})_{,\xi} - \xi_0^{-1} + e_{RL} - \ln\left(\frac{\xi}{\xi_0}\right)\right]\delta\xi - 3N_a\xi\left(\frac{\eta_{,J}}{\eta} - \frac{1}{J}\right)\delta J + \delta\varphi_{DPZ}\right\} dA. \tag{10}$$

The Euler-Lagrange equation coming from (10) which renders stationary (7) with respect to ξ may be converted in the following form:

$$(\xi\,\varphi_{CR})_{,\xi} = \xi_0^{-1} - e_{RL} + \ln\left(\frac{\xi}{\xi_0}\right), \tag{11}$$

which may be integrated to obtain the following relationship between the conformational Energy density and the items on the right-hand side:

$$\varphi_{CR} = \xi_0^{-1} - e_{RL} - 1 + \ln\left(\frac{\xi}{\xi_0}\right), \tag{12}$$

where, without loss of generality, the constant of integration has been set equal to zero (this would only fix a value of the conformational energy at the reference state). The latter expression yields a relationship between the ratio of the field monitoring the conformational changes and the areal stretch of the membrane versus the density of active receptors:

$$\left(K\frac{\eta}{J}\right)^{3N_A} = e^{-(1+e_{RL})+\xi_0^{-1}} \ \frac{\xi}{\xi_0}. \tag{13}$$

Hence, whatever relations will enable us to find both the fields J and ξ,, expression (13) allows for calculating the conformational field η in terms of known quantities. Thanks to the quasi incompressibility of the membrane [12], such a relationship allows for stating that stationary points of the Helmholtz free energy are characterized by the circumstance that the density of active receptors is proportional to the $3N_A$-th power of the product of the conformation variable and the change in thickness.

After making use of (8), (11) and (13) into (10), after some trivial manipulation, we are left with the following stationarity condition:

$$0 \ = \int_\Gamma \left\{ \left(h_0\, \varphi^{loc} \ - \ (3N_A)^2 \xi_0 \ \xi \right)_{,J} \delta J \ + \ \delta\left(\frac{h_0^3}{24 J^3}\, \varphi^{loc}_{,J} \, \|\nabla J\|^2 \right) \right\} dA, \tag{14}$$

which may be further expanded by evaluating the variation of the second term in the sum and, after making use of the divergence theorem,

$$0 \ = \int_\Gamma \left[\frac{h_0^3}{24} \left(\frac{\varphi^{loc}_{,J}}{J^3}\, \Delta J \ + \ (\frac{\varphi^{loc}_{,J}}{J^3})_{,J} \, \|\nabla J\|^2 \right) \ - \left(h_0\, \varphi^{loc} \ - \ (3N_A)^2 \xi_0 \ \xi \right)_{,J} \right] \delta J \, dA \ + \ boundary\, terms. \tag{15}$$

After recognizing the (trivial) relation yielding the variation of J in terms of variations of the underlying kinematics, i.e. the motion allowed for the points of the cell membrane under exam, localization of the bulk term yields:

$$\Delta J \ + \ (\frac{\varphi^{loc}_{,J}}{J^3})_{,J} \ \frac{J^3}{\varphi^{loc}_{,J}} \, \|\nabla J\|^2 \ = \ \frac{24}{h_0^3} \frac{J^3}{\varphi^{loc}_{,J}} \left(h_0\, \varphi^{loc} \ - \ (3N_A)^2 \xi_0 \ \xi \right)_{,J}, \tag{16}$$

whenever, without loss of generality, a constant of integration is set to be zero the first derivative of the membranal energy does not vanish. Besides the boundary terms, in this highly non-linear PDE it appears evident he coupling between the mechanical field J and the density ξ of active receptor.

Obviously, the latter must obey the usual balance condition, i.e.

$$div\mathbf{j} + \xi_{,t} = 0 \tag{17}$$

where **j** is the diffusive flux of active receptors across the cell membrane and t is the time; such a flux is assumed to follow the usual constitutive law, namely:

$$\mathbf{j} = D\,\nabla\xi \tag{18}$$

where D is the diffusivity of the receptors through the membrane. The resulting field relation (in the hypothesis that D would not depend upon J) is the usual

$$D\nabla\xi + \xi_{,t} = 0 \tag{19}$$

supplemented by the initial condition:

$$\xi(X,0) = 0 \tag{20}$$

because there are no active receptor at the beginning of the process.

Henceforth, because one of the experiments (see Fig. 4 a) deals with introducing a certain given mole concentration c

of ligands in the medium above the cell aggregate, and leave it there for a prescribed time duration t_f , a further condition must be imposed on the overall rate of change of active receptors, namely:

$$\int_\Gamma \int_0^{t_f} \xi_{,t}\, dt\, dA = c C_w \tag{21}$$

where C_w represents the capacity of the well. Of course, here and in the actual experiments c represents a value of concentration lower (or at most equal to) than the one leading to saturation. By substituting equation (20) in the latter relationship yields:

$$\int_\Gamma \xi(X, t_f)\, dA = c C_w \tag{22}$$

which, together with (20), supplements the linear PDE (19). Once the diffusion coefficient D is inferred, (19), (20) and (22) allow for a solvable linear problem.

The high nonlinearity of (16) on the one hand and the impossibility of finding an explicit dependence of either ξ or η in terms of the areal stretch J suggest that the problem must be tackled by appealing more closely to the actual experiment.

Indeed, the response of the system to the introduction of ligands may be monitored starting from the underlying "fundamental" state of the cell aggregate, which is the one observable right before the introduction of the ligands themselves in the medium. This state is indeed very well identifiable through measurements .

First of all, a binding experiment may be easily performed. This leads to the determination of the (homogeneous) density ξ_* (per unit area) of active receptors, i.e. the moles of active receptors present in the cell membrane of the whole aggregate. Here, the (averaged) relative thickening (or thinning) with respect to an assumed reference configuration may also be detected, leading to the corresponding value of in plane stretch J_*. It is about this state that the changes in the cells aggregate may be studied. The details of the analysis are not pursued in this paper, although they may be found in [11].

In particular, it is reasonable to study "small" changes in J with respect to such configuration to predict the corresponding cAMP release. The order of magnitude of the latter may finally be expressed in the following form:

$$cAMP \simeq 10^{-4} \int_0^{t_f} \int_\Gamma \xi(X, t) dA\, dt. \tag{23}$$

CONCLUSIONS

A model for the prediction of the response of cell aggregates undergoing ligand binding is presented. This arises from conformational changes of the potentially active receptors located in the cell membrane. It has been reported above that three of the seven transmembrane (TM) helices forming β-adrenergic receptors (TM3, 5 and 6) are involved through conformational changes during binding of ligands to receptors. During such movements, the surrounding membrane may comply by changing its thickness, leading to area changes in the cell membrane due to quasi-incompressibility [12].

The cAMP release is a very well detectable measure of cells response and it is related to the number of active receptors that binds to affine ligands. The leading ideas for constructing a predictive model capable to predict the cAMP release and account for the coupled conformational and mechanical effects is based on the construction of the Helmholtz free energy of the system, on the determination of its stationary points and on the balance between the influx of active receptors and the rate of change of their density. Details may be found in [11].

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