Strain gradient membrane effects during cyclic Adenosine Monophosphate Pathway in human trophoblast cells

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ABSTRACT. Thickness changes in cell membranes, usually encountered in phase transition of lipid bilayers, may be initiated by conformational changes of some domains forming membrane receptors responding as a second messenger to external ligands. Unfortunately, thinning may indicate the possibility of fracture of the membrane, leading to loss of functionality of the cell aggregate. The mentioned response, whose manifestation is cAMP (cycling Adenosine MonophosPhate), may be directly linked to the coupling of conformational and mechanical effects, the former arising in some of the domains cited above. Stationarity of a new Helmholtz free energy, accounting for receptor density and conformation field and strain gradients in membrane thinning or thickening, is investigated. It turns out that the density of active receptors is directly related to the conformation field above and it enters as a source term in the resulting balance equation for the membrane stress. Henceforth, balance laws for the cAMP transporters and for the flux of active receptors, coupled with the former, must be supplied together with a balance between the diffusive powers to yield "sink" due to the outgoing flux provided by the transporters.

SOMMARIO. Variazioni di spessore nella membrana cellulare, che accompagnano la transizione di fase nei bi-strati lipidici puri, ovvero in assenza di proteine o altre inclusioni di carattere biologico, possono essere causate da variazioni conformazionali, provocate dalla risposta a ligandi chimici esterni, di domini recettoriali presenti sulla membrana stessa. Sfortunatamente, l'assottigliamento indica la possibilità di nucleazione di fratture nella biomembrana e la sua possibile perdita di funzionalità. La risposta suddetta, la cui manifestazione è il cAMP (adenosin-monofosfato ciclico), può essere correlata all'accoppiamento fra le variazioni conformazionali dei domini recettoriali e la variazione di spessore, che è un effetto meccanico. La stazionarietà dell'energia libera di Helmholtz, la cui espressione risulta nuova in letteratura, conduce ad un legame fra densità recettoriale variazione conformazionale, nonché al bilancio fra sforzi di membrana in cui compare la suddetta come termine sorgente. Le equazione di diffusione dei trasportatori e quella dei recettori, accoppiata con la precedente, devono essere tenute in conto per la soluzione del problema e così pure il bilancio fra le potenze spese per la diffusione per la determinazione della percentuale di cAMP extracellulare.

KEYWORDS. Strain gradients; Cell membrane; Receptors; Conformational changes; cAMP; cAMP transporters

INTRODUCTION

Thickening or thinning and-or shearing through thickness of the lipid membrane surrounding human trophoblast cells may arise during cyclic Adenosine Mono Phosphate (cAMP) pathway [11]. This may be caused by conformational changes of transmembrane domains of beta-adrenergic receptors populating such cells [6]. The underlying kinematics may be interpreted through the *Theory of Structured Deformations* (see e.g. [5, 7, 8]).
The pathway mentioned above may be detected through measurements of cAMP. This is an intracellular second messenger that transduces inside the cell the effects of extracellular ligands, which cannot get through the cell membrane. Such ligands bind to a specific receptor, following the so called "lock and key mechanism". Beta-adrenergic receptors are essentially integral membrane proteins embedded in the lipid bilayer and they belong to the so called G protein-coupled receptor (GPCR) family, which is characterized by seven transmembrane helices, denoted either bi I-VII or by *TM*1-*TM*7 (see Fig. 1 below and e.g. [4]).

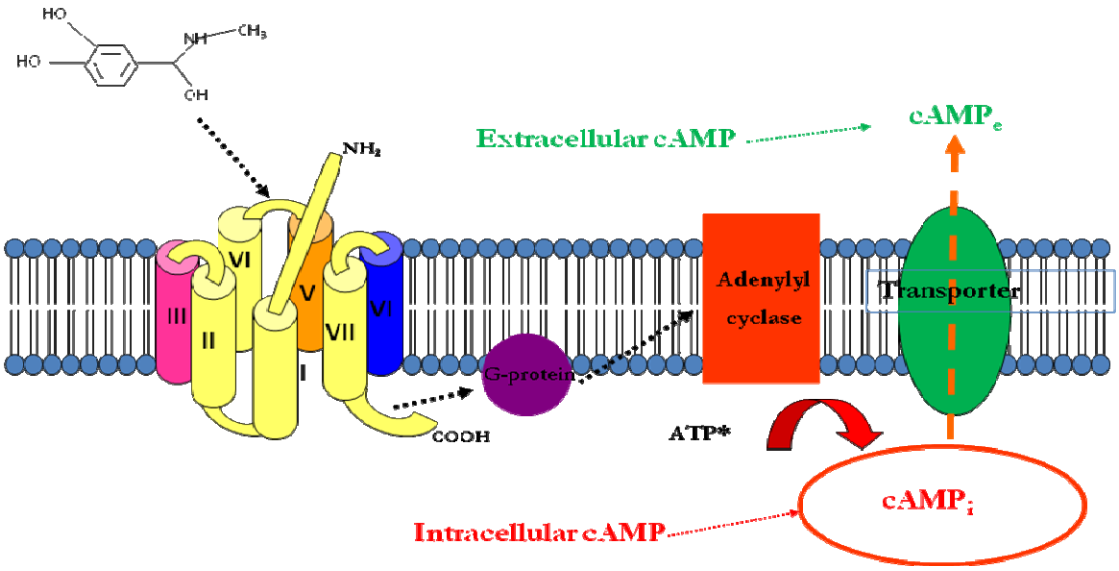

Figure 1: Description of the adenylyl cyclase transduction pathway

The conformational changes cited above may involve both a rotation about the axis of *TM*6 and a shear of *TM*6 towards *TM*5. Once the adrenergic ligand (i.e. epinephrine) is recognized, the receptor changes conformation and, thus, activates the G protein. This detaches from the receptor and, in turn, activates another membrane protein, called adenylyl cyclase. This enzyme transforms adenosine triphosphate (ATP) in cAMP.

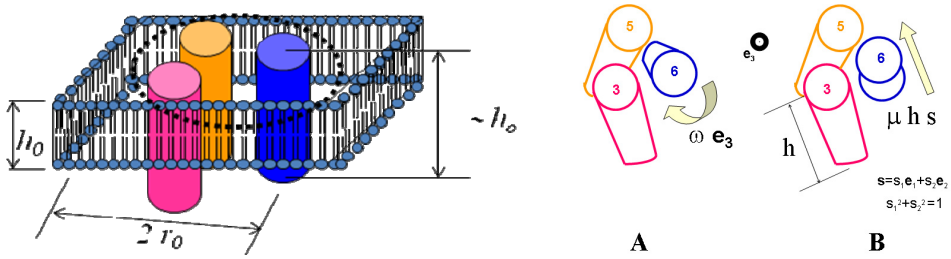

Figure 2: Reference configuration (Fig. 2a) and conformational changes involving the transmembrane domains (Fig. 2b and 2c)

This may be observed in human trophoblast cells (see e.g. [12, 1, 2]) forming the fetal site of the placenta. The response of aggregates of such cells to epinephrine is detected through enhancement of intracellular cyclic adenosine monophosphate,

denoted by $cAMP_i$ in Fig. 1. During the process, part of it, the $cAMP_e$, is transported to the extracellular medium through membrane proteins called MRP1 (see e.g. [3]).
During the conformational changes described above, the surrounding lipid bilayer may either exhibit neutrally out of plane shear or undergo shearing together with thickening or thinning. Predictions about such mechanical events coupled with conformational changes are sought through the evaluation of stationary points of a new Helmholtz free energy [11]. Thinning of the membrane is clearly precursor of possible fracture and yet loss of functionality of the membrane and eventually of the cell aggregate [15, 16, 17].

MATERIAL AND METHODS

The HTR-8/SVneo trophoblast cell line, obtained from human first-trimester placenta explant cultures and immortalized using SV40 large T antigen (kindly provided by Dr. CH Graham, Queen's University,Kingston, On, Canada). Cells were cultured at 37°C in an atmosphere of 5% CO2/95% air in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown to confluence (2-3 days) in twenty four well plate (see e.g. Fig.3), 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 measuring $cAMP_i$, i.e. intracellular cAMP.

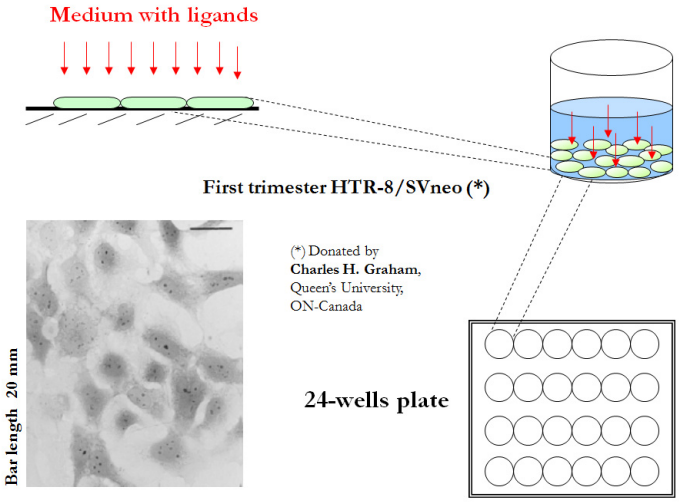

Figure 3: Reference A schematic of the performed experiment.

The effect of the receptor ligand epinephrine was examined on cAMP production in HRT-8/SVneo cells. Epinephrine enhanced cAMP concentration in a dose-related fashion, reaching a plateau at around 10-5 M. The calculated EC50 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/106 cells, not shown). In the presence of epinephrine, intracellular cAMP production, i.e. $cAMP_i$, increased as a function of incubation time up to 15 min (14-fold), thereafter a reduction of the nucleotide level was observed. At the same time, extracellular cAMP, denoted by $cAMP_e$ in the sequel, gradually increases in time, at least during the 60 min of observation.

THE MODEL

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 diffuse across the membrane to lower the (Helmholtz) free energy of the system in a purely entropic way (see e.g. [13]); The following term, evaluated in [13],

$$\xi\left(-e_{RL}+\ln\left(\frac{\xi}{\xi_0}\right)\right) \tag{1}$$

accounts for such effect, 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_o is a reference value of such density. An analog reasoning holds for the density of transporters, typically MRP1, for which the diffusion term of the free energy reads as follows

$$\zeta\left(-e_{RT}+\ln\left(\frac{\zeta}{\zeta_0}\right)\right) \tag{2}$$

where ζ represents the density of activated receptors in the membranes, e_{RT} is the specific activation energy for the complex transporter-cAMP$_i$, and ζ_o is a reference value.

A conformational term φ_{CR} must be accounted for in the energetic: this has an entropic nature and it penalizes both ω, the rotation of the sixth transmembrane domain about its axis (which basically coincide with the membrane one) and μ, the amount of shear of the sixth domain towards the fifth. Although a statistical mechanics derivation of the conformational term may be found in [11], neither the membrane thickness h in the current configuration nor the related membrane stretch J were accounted for in this treatment. Actually, quasi-incompressibility of the surrounding watery environment allows for quasi-incompressibility of the cell membrane (see e.g. [12]), yielding $h/h_0 \sim J$, where h_0 is a reference value for the undeformed thickness. It is worth noting that the thickness ratio, owing either thinning or thickening, is a measure of lipids order. If N_A denotes the Avogadro's number, it is possible to show that the change in entropy per unit receptor associated with rotations and shear is assumed to be additive and it may be written as follows

$$\phi_{CR}=3N_A\ln\left(K\frac{\eta}{J}\right), \qquad K:=\left(\frac{h_0}{2\pi r_0}\right)^3, \tag{3}$$

where $\eta=\mu\ \omega$ denotes the *conformational field,* [14], h_0 and r_0 are referential values of the membrane thickness and of the radius of the zone involved in the conformational changes. Somehow relation (3) explicitly accounts for a conformational-mechanical coupling, since it has been pointed out above that J measures both the in plane stretch of the membrane and, roughly, the inverse of the thickness ratio h_0/h. Although transporters do also exhibit conformational changes they do not cause the efflux of cAMP.

Henceforth, the part of the free energy storable by the lipid membrane, denoted by φ_{DPZ} and derived in [12], may be written as follows

$$\phi_{DPZ}=h_0\phi^{loc}(J)+\frac{h_0}{24}^3\frac{\phi^{loc}_{,J}(J)}{J^3}\|\nabla J\|^2 \tag{4}$$

where φ^{loc}, a double well potential represented in Fig. 4 and derived in [12] starting from a Landau expansion and motivated by statistical mechanics, is capable to account for different lipid constituents.

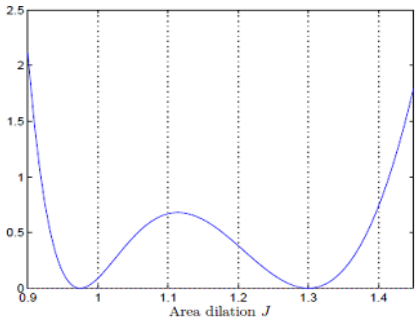

Figure 4: Behaviour of (normalized) φ^{loc} at the lipid ordered-disordered transition temperature for a given composition of constituents

It is worth noting that the gradient term in the energy the procedure performed in [12] shows that micromechanics (or statistical mechanics) suffices to determine the free energy directly stored through membrane elasticity. Indeed the elastic modulus of such higher gradient term, besides scaling like a bending constant, is proportional to $\varphi^{loc}_{,J}(J)$, the partial derivative of φ^{loc} with respect to its argument evaluated at the current value of the areal stretch J.
Henceforth, because receptors activate on the basis of conformational changes, the density ξ may turn out to depend upon η; without loss of generality, an unknown (invertible) constitutive relation between such variables is assumed to hold ξ and η (keeping J fixed). Stationarity of the total Helmholtz free energy is required at any time and it reads as follows:

$$\delta \int_{\Gamma} \left\{ \xi \left(-e_{RL} + \ln\left(\frac{\xi}{\xi_0} \right) + \phi_{CR}(\eta, J) \right) + \zeta \left(-e_{RT} + \ln\left(\frac{\zeta}{\zeta_0} \right) \right) + \phi_{DPZ}(J) \right\} dA = 0 \tag{5}$$

This is evaluated against variations $\delta\xi$ and δJ, because conformational changes of the transporters are not accounted for and hence variations against $\delta\zeta$ simply yields (2) to be constant, leads to the two equations below

$$\left(K \frac{\eta}{J} \right)^{3N_A} = \frac{\xi}{\xi_0} e^{-(1+e_{RL})+\xi_0^{-1}} \tag{6}$$

$$\Delta J + \left(\frac{\phi^{loc}_{,J}(J)}{J^3} \right)_{,J} \left(\frac{\phi^{loc}_{,J}(J)}{J^3} \right)^{-1} \|\nabla J\|^2 - \frac{24}{h_0^3} \left(\frac{\phi^{loc}_{,J}(J)}{J^3} \right)^{-1} \left(h_0 \phi^{loc}(J) - (3N_A)^2 \xi_0 \xi \right)_{,J} = 0 \tag{7}$$

where the former is the expected constitutive relation among ξ, η and J, and the latter represents a nonlinear PDE owing the balance of membrane stresses. Boundary conditions also arise from stationarity, although here they are not explicitly worked out in details. Strain gradients do explicitly enter in the balance Eq. (7) giving rise to both the linear term in the laplacian ΔJ and in the nonlinear one, penalizing the magnitude squared of the areal stretch gradient.
The time-space evolution of the density of active receptors entering in (6) and (7) can be specified once their diffusion is accounted for. Of course the cAMP transporters influence such a balance and, in turn they also must have a balanced flux. To this end, if D_i and D_e denotes the diffusivities of receptors and transporters respectively (assumed to be independent of the lipid ordering, namely on J), the following equations may be deduced:

$$-D_e \Delta\zeta + \zeta_{,t} = 0 \tag{8}$$

$$-D_i \Delta\xi + \xi_{,t} = \alpha\zeta_{,t} \tag{9}$$

where, roughly speaking, $\alpha\zeta_{,t}$ represents a sink term in the balance of fluxes involving the active receptors due to the fraction $cAMP_e$ swept away from the cell aggregate. It is worth to note that (8) is an eigenvalue problem which gives rise to suitable eigenfuntions, depending on the geometry of the domain and, eventually, on the boundary conditions. Furthermore, an expansion of (9) in terms of eigenfunctions may also be considered.
Initial conditions on the evolution of both ξ and ζ may be provided in terms of their referential values, to get

$$\int_{\Gamma} \zeta(X,0^+)^2 dA = \zeta_0{}^2 area(\Gamma) \qquad \int_{\Gamma} \xi(X,0^+)^2 dA = \xi_0{}^2 area(\Gamma) \tag{10}$$

where

$$\xi_0 = \frac{cC_w}{area(\Gamma)} \tag{11}$$

is proportional to the concentration c of ligands in the medium (C_w denotes the capacity of a well, mentioned above). The expression of may be determined by equation the powers expended to diffuse both receptors and transporters across the cell during the process.

CONCLUSIONS

The proposed approach highlights the presence of strain gradients due to at the level of the cell membrane. In particular, a linearized version of (7), together with its resulting boundary conditions, and the diffusion Eq. (8) and (9) are under investigations by looking at values of J in the spinoidal region of φ^{loc}, i.e. roughly speaking ner the local maximum (see Fig. 4 for its location). This linearization allows for predicting the expected (strain) gradients between thick and thin zones of the cell membrane and yet to relate it to the $cAMP_i$ measured in the experiments.

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