Interprotein interactions are responsible for the confined diffusion of a G-protein-coupled receptor at the cell surface

F. Daumas*, N. Destainville†, C. Millot*, A. Lopez*, D. Dean† and L. Salomé*†

*Institut de Pharmacologie et Biologie Structurale, UMR CNRS 5089, 205, route de Narbonne, 31077 Toulouse cedex, France, and †Laboratoire de Physique Théorique, IRSAMC, CNRS FRE 2603, 118, route de Narbonne, 31062 Toulouse cedex, France

Abstract

The monitoring of the movements of membrane proteins (or lipids) by single-particle tracking enables one to obtain reliable insights into the complex dynamic organization of the plasma membrane constituents. Using this technique, we investigated the diffusional behaviour of a G-protein-coupled receptor. The trajectories of the receptors revealed a diffusion mode combining a short-term rapid confined diffusion with a long-term slow diffusion. A detailed statistical analysis shows that the receptors have a diffusion confined to a domain which itself diffuses, the confinement being due to long-range attractive inter-protein interactions. The existing models of the dynamic organization of the cell membrane cannot explain our results. We propose a theoretical Brownian model of interacting proteins that is consistent with the experimental observations and accounts for the variations found as a function of the domain size of the short-term and long-term diffusion coefficients.

Introduction

G-protein-coupled receptors are seven-helix transmembrane proteins constituting the largest class of cell-membrane receptors. A tremendous amount of information is known about the multitude of different proteins involved in the G-protein-coupled signal-transduction pathway. However, an important question that remains to be elucidated is that of the mechanism of interaction between the membrane partners of the G-protein-coupled receptors leading to a cellular response after ligand binding by the receptor. It is now well accepted that the prevailing view of a random collision-coupling between receptors, G-protein and effectors needs to be revisited and alternative schemes proposed to account for the evidence, accumulated from pharmacological studies and lateral diffusion measurements, of a compartmentalization of the receptors and the G-proteins [1,2]. Here we address a part of this question using the single-particle tracking technique [3] to observe the movements of a G-protein-coupled receptor at the surface of living cells.

The μ opioid receptor chosen for these experiments, involved in pain phenomena, is the target of many analgesic drugs, including morphine. Its pharmacological properties have been the object of several studies in our laboratory [4,5]. Stably transfected in fibroblast cells and checked for its functionality, the receptor was tracked at video rate (25 frames/s) with a 15-nm spatial resolution at the cell plasma membrane by the observation of colloidal gold particles attached to the T7 tag at its N-terminal extracellular domain by specific antibodies. Some 90% of the trajectories revealed a diffusion mode combining a short-term rapid confined diffusion with a long-term slow free diffusion. A thorough statistical analysis showed that this mode consists of a diffusion confined to a domain which itself diffuses, we call it ‘walking confined diffusion’. Furthermore we could also deduce that an effective harmonic potential resulting from inter-protein long-range attractive interactions are responsible for the confinement of the receptors rather than extramembranous or membranous fences or barriers [6–8]. This paper is a shortened version of a previous detailed article [9].

Conducting single-particle tracking experiments

Conjugated gold colloids stabilized with BSA and bearing less than two anti-T7 antibody molecules were prepared as described in [10]. Cells plated on microscope coverslips were rinsed for the culture medium and incubated with 60 µl of the conjugated colloids suspended in PBS during 45 min at 4°C prior to microscope observations. Images were recorded at room temperature by video-enhanced differential interference microscopy with a microscope (Axioplan II; Zeiss) equipped with a ×63 objective, a ×4 projective lens and a CCD camera (C2400-75i; Hamamatsu) connected to a numerical videotape recorder (DSR30P; Sony).

After digitization, the particle position was determined on the successive images of the sequences with a precision, estimated from the standard deviation of the co-ordinates of fixed particles, of 15 nm for 2-min trajectories. The first

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Abbreviation used: MSD, mean square displacement

*To whom correspondence should be addressed (e-mail Laurence.Salome@ipbs.fr).
step of the analysis of the obtained trajectories proceeds by the calculation of the MSD (mean square displacement) as a function of the time interval according to the eqn (1), restricted to intervals smaller than 1/10 of the total number of images to eliminate long-range noise [11]:

\[
\text{MSD}(n\delta t) = \frac{1}{N-1-n} \sum_{j=1}^{N-1-n} \left[ (x(j\delta t + n\delta t) - x(j\delta t))^2 + (y(j\delta t + n\delta t) - y(j\delta t))^2 \right] \tag{1}
\]

where \(\delta t\) is the time interval between two successive frames (\(\delta t = 40 \text{ ms}\)), \(x(t)\) and \(y(t)\) are the colloid co-ordinates at time \(t\), \(N\) is the total number of frames and \(n\) is the number of time intervals.

In order to detect any variation with the duration of the observation, the same calculations were performed on consecutive subintervals of each trajectory.

**Statistical analysis of the receptor trajectories**

A simple examination of the overall trajectories (about 100) of 2 min duration each allows one to distinguish between two receptor populations with different types of movement. 10% of the receptors moved very slowly and the other 90% had a rapid diffusion apparently restricted to a region of about 1 \(\mu\text{m}\). Then, the fit of the MSD = \(f(t)\) plots with theoretical diffusion equations is used to characterize the mode of motion (Figure 1).

Let us first consider the smallest population of slowly diffusing receptors. The MSD versus time plots showed straight or slightly curved lines well fitted by the equation of either a simple diffusion MSD(t) = 4Dt or a directed diffusion MSD(t) = 4Dt + \(v^2t^2\). Taking into account that such behaviours with comparable small values of the diffusion coefficient \(D\) and drift velocity \(v\) were found for cytoskeleton-related proteins, we concluded that these
receptors are probably in the course of recycling through an internalization via clathrin-coated pits [12].

The second main population had MSD versus time plots that were well fitted by the sum of a short-time confined diffusion term and a long-time free diffusion:

\[
\text{MSD}(t) = 2L^2(1 - e^{-t/\tau}) + 4D_{\text{MACRO}}t
\]

(2)

where \( L \) is the characteristic size of the confining region and \( \tau \) is the equilibration time, i.e. the characteristic time taken by the receptor to visit the domain given by \( \tau = L^2/2D_{\text{micro}} \) for a diffusion with a diffusion coefficient \( D_{\text{micro}} \) inside the domain. \( D_{\text{MACRO}} \) is the diffusion coefficient associated with the long-time free diffusion.

The values of both diffusion coefficients were found to be spread over three decades with the most probable values of about \( 10^{-5} \) cm\(^2\)/s for \( D_{\text{micro}} \) and \( 1.3 \times 10^{-11} \) cm\(^2\)/s for \( D_{\text{MACRO}} \). The sizes \( L \) of the domains are distributed between 0.02 and 0.55 \( \mu \)m with a mean value of 0.15 \( \mu \)m. This gives a characteristic value of \( \tau \) of about 100 ms.

Before going further into the characterization of this mode of movement it was necessary to determine whether statistical fluctuations of simple random walk could be responsible for these trajectories. Such a control was performed according to the criterion of Kusumi et al. [6], showing that a random walk could be unambiguously excluded for all the trajectories.

Subsequently, in order to determine the origin of the confinement we calculated the histograms of the distribution of the distances travelled for two time intervals. The first, between 0 and 1 s, was chosen as the lowest interval ensuring that the distribution inside the domain had reached its equilibrium form, and the second, between 0 and 4 s, was determined as the longest time over which the distance diffused with \( D_{\text{MACRO}} \) remaining smaller than the domain size. Both normalized histograms are fitted with excellent agreement by a Gaussian probability distribution (Figure 2).

Let us note that the half height widths found for each distribution are indiscernible, confirming that the trajectories are not those of free random walks which would exhibit a significant broadening of the distribution curves. The existence of a locally isotropic harmonic potential to which the receptors are subjected and leading to their confinement is the simplest physical explanation for the observed Gaussian distributions. This potential most probably results from interactions of the receptors with proteins within the membrane and whatever the form of the potential, it can be expected that the receptor is localized about its minimum where it takes generically a quadratic form.

Averaging the particle positions over running intervals of 4 s revealed a free diffusive motion of the confining domain with a diffusion coefficient comparable with \( D_{\text{MACRO}} \) obtained from the MSD fit (eqn 2).

As the values of both diffusion coefficients and domain sizes were spread over broad ranges, we examined if there were any correlations between their variations. Both microscopic and macroscopic diffusion coefficients were found to increase with quadratic dependence on the domain size: \( D_{\text{micro}} \approx D_{\text{MACRO}} \approx L^2 \) (Figure 3).

**Modelling based on a system of interacting Brownian proteins**

Our analysis supported the idea of a walking confined diffusion of the receptors but the observed superimposition of a short-term confined diffusion with a long-term free diffusion could also be interpreted as the result of a hopping diffusion, that is a diffusion confined within compartments with jumps to adjacent compartments. Such behaviour identified for various proteins gave rise to the membrane skeleton fence model proposed by Kusumi et al. [6,7] and more recently to the anchored-membrane protein picket model [8] to account for similar behaviours observed for lipids. Several verifications were performed to determine whether the confinement observed in our experiments could be due to fences. We checked carefully for the existence of jumps in the trajectories by plotting each co-ordinate \( x \) and \( y \) as a function of time. No systematic jumps were apparent with only a few jumps detected over nearly 100 trajectories.

A Gaussian distribution of the travelled distances within a domain could result from the deformation of a square well distribution, expected in the case of barriers delineating the domain, due to the low time resolution (video rate) in our experiments. To check this possibility we have calculated the shrinking of a Gaussian distribution due to a decrease in the time resolution. It depends on the ratio of the equilibration time \( \tau \) to the time resolution. We then determined
Log–log plots are shown of the microscopic and macroscopic diffusion coefficients $D_{\text{micro}}$ and $D_{\text{MACRO}}$ as a function of the domain size $L$ determined for each trajectory from the MSD($t$) fit with eqn (2) and log–log plot of $D_{\text{micro}}$ (5 s) as a function of $L$ (5 s) extracted from a single trajectory over intervals of 5 s. The three sets of points are aligned on straight lines of slope 2 showing that $D_{\text{micro}} \approx D_{\text{MACRO}} \approx L^2$ and $D_{\text{micro}}$ (5 s) $\approx L^2$ (5 s).

First it was found to be in excellent agreement with the calculated corresponding values. Secondly we could verify for trajectories with large equilibration time that, as expected from the calculation, they do not show significant change with respect to the shape of the distribution or its width. This result demonstrates that we are effectively dealing with Gaussian distributions of the receptors within the domains. In addition, the dynamic corral models involving a stochastic gating mechanism to regulate the protein mobility also fail to explain the variation of the microscopic diffusion coefficient with the domain size [13,14]. Thus, the interpretation of our results requires the development of an alternative model of the dynamic organization of cell membranes.

The appropriate theoretical framework to deal with the question of the effect of protein interactions on the diffusion of a protein is the Brownian model. The motion of a protein can be described by the Langevin equation [15]:

$$\frac{dX_i}{dt} = -\mu \sum_{j \neq 1} \frac{\partial}{\partial X_i} V(X_i - X_j) + \eta_i$$

(3)

where $1 \leq i \leq N$ is the particle index and $\alpha$ the spatial index (here in two dimensions one may write $X_1^1 = X_1$ and $X_2^1 = Y_1$); $\mu$ is the mobility of the particle measuring the linear response of the particle to an applied external force. The particles interact via an effective pairwise potential $V$ for length scales above which the Langevin equation is valid. The term $\eta$ is Gaussian white noise such that $\langle \eta_i(t)\eta_j(t') \rangle = 2D_{\text{micro}} \delta(t-t')\delta_{ij}\delta_{\alpha\beta}$. The noise magnitude $D_{\text{micro}}$ and coupling of the long-range forces depend on the protein concentration. The Einstein or fluctuation dissipation relation [15] gives $D_{\text{micro}} = k_B T \mu$.

In the case of attractive interaction, an assembly of proteins will remain confined to a region about its centre of mass

$$X_c = \frac{1}{N} \sum_{i=1}^{N} X_i$$

(4)

For a pairwise interaction potential the equation of movement for $X_c$ is

$$\frac{dX_c}{dt} = \frac{1}{N} \sum_{i=1}^{N} \eta_i$$

(5)

Leading to a simple diffusion for $X_c$:

$$\langle X_c^2 \rangle = \frac{4D_{\text{micro}}}{N} t$$

(6)

with a diffusion coefficient $D_{\text{MACRO}} = D_{\text{micro}}/N$. 

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A simple argument [9] gives the following relationship for the dependence of $D_{\text{micro}}$ on the protein concentration $\rho = N/2L^2$:

$$D_{\text{micro}} \approx 1/\rho$$  \hspace{1cm} (7)$$

Assuming that the domain can vary in size with the number of proteins remaining constant gives:

$$D_{\text{micro}} \approx L^2$$  \hspace{1cm} (8)$$

The fact that we found a variation in the domain size from trajectory to trajectory suggested that the domain size itself may vary in time. In order to check this point, we established the histograms of $x(t + n\delta t) - x(t)$ and $y(t + n\delta t) - y(t)$ for $n\delta t$ between 0 and 1 s for consecutive segments of a 5-s duration over the total trajectory to determine the local values of $L$ within these time intervals (related to the half width at half maximum of the distribution) for each segment. Fifteen trajectories exhibited significant fluctuations (by a factor of at least 3) of the domain size with time. Interestingly, the associated variation of $D_{\text{micro}}$ (5 s) with $L$ (5 s) measured in each successive 5-s segment followed the prediction above, in eqn (8). One of the most explicit plots of $D_{\text{micro}}$ (5 s) = $f[L(5 \text{ s})]$ obtained for these trajectories is shown in Figure 3.

**Conclusions**

From the experimental point of view the most striking result observed is the absence of systematic jumps between confining regions but rather the slow free diffusion of these domains. The quadratic dependence between the domain size and $D_{\text{micro}}$ and $D_{\text{MACRO}}$ accounted for by our ‘walking confined diffusion’ model cannot be explained within a hopping model. It should be emphasized that there are differences between the system studied here and those where the hopping model satisfactorily describes the trajectories [6,7,16]: the membrane receptors and cells are different. Nevertheless, let us note that our model supports the view of Abney and Scalettar [17] of an organization of the membrane due to inter-protein interactions.

The confining potential responsible for the domain cohesion may have multiple origins such as purely electrostatic and van-der-Waals interactions, membrane-mediated interactions or interactions mediated by internal constituents close to the membrane. The nature of these interactions could be further elucidated by performing experiments on model membranes, whose composition can be controlled. Such investigations are in development in our laboratory.

**References**


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