FOUNDATIONS OF THE ION FLUX METHOD

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Introduction

The control of passive transmembrane ion transport by special gating systems plays a fundamental role in many cellular signal-transfer processes. Examples for such control systems, that regulate the opening and closing of ion channels, are divers neuroreceptors, and the sodium and potassium channel gating molecules connected with the generation of action potentials in nerves and muscles. For several decades the investigation of the molecular foundations of gating processes was dependent on crude pharmacological methods of measurement. The analysis of the resulting data often led to the determination of empirical parameters whose significance in relation to the fundamental phenomena usually remained obscure. Through progressive improvement of experimental techniques it has recently become possible to carry out much more detailed investigations.

A promising new method for studying ion gating processes is the ion flux technique originally introduced by Kasai and Changeux (1971). The measurement of the gating process dependent exchange of ions between a large bath and suspended

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microscopic closed membrane structures (CMS) permits determination of detailed information about gating reactions. The technique entails measurement of the total amount of a certain ionic species contained in the CMS. Any parameter that directly reflects the ion content can be used as a variable of measurement (e.g., CPM of a radioactive ion species). A change of the total ion content can be determined under two separate experimental conditions: (a) when there is a net efflux of ions from the CMS into the bath, (b) when there is a net influx of ions from the bath into the CMS. The resulting data will reflect either the time course of the respective increase or decrease of ion content, or when flux is very rapid on the time scale of measurement, the time independent overall change of ion content (i.e. the so-called flux amplitude). Examples for CMS that have been used in ion flux studies are ion channel containing membrane fragments, isolated cells and reconstituted lipid vesicles.

A number of investigations have verified that the ion flux method constitutes a potentially broadly applicable procedure for investigating the elementary reaction events underlying channel gating processes. Here, unlike with more indirect measurement techniques, that rely on the detection of a fluorescence signal connected with a specific ligand or label, the ion flux process itself serves as a signal source. The system to be investigated is therefore practically unperturbed by the process of measurement. In comparison with techniques based on measuring electrical signals, that also fulfil this criterion (e.g., voltage clamp and current fluctuation methods), the ion flux method offers several advantages. For example the amounts of the chemical species that participate in gating processes (e.g., neuroactivator ligands, and gating molecules) are more accessible to accurate experimental control, when suspensions of small uniform membrane structures are used. Further, with such suspensions, it is possible to employ many experimental

manipulations conventionally used in the study of chemical reactions in homogeneous solution (e.g., effective mixing through stirring).

Three fundamental aspects that had to be delt with in the progressive refinement of quantitatively accurate procedures for studying ion flux were: (a) the development of reliable experimental of techniques for rapidly initiating gating processes, and for measuring suitable signals reflecting the subsequent ion transport events (b) the development of procedures for preparing suspensions of CMS particularly suited for flux studies (c) the development of analytic schemes for extracting information about channel gating processes from flux data.

Techniques of Flux Measurement

In excitable biomembranes, ion transport phenomena controlled by a channel gating system are activated by special physical processes (e.g., release of a transmitter substance, or change of the membrane potential). In ion flux experiments, corresponding transport phenomena must be initiated by experimental simulation of these activation events. Up to now only experimental procedures for the investigation of ligand induced gating processes have been developed. These entail initiation of ion fluxes by rapid mixing of a suspension of CMS with a solution of a suitable neuroactivator substance. The course of the flux process is most commonly measured by rapid filtration using synthetic membrane filters. The individual filtrations are carried out at set intervals after the initial mixing step. Corresponding to the experimental conditions chosen, this allows determination of the increase or decrease of the ion content of the CMS retained on the filters. Information about the gating processes that control flux can then be obtained through an analysis of the flux time course and flux amplitudes.

The channel gating system that has been most extensively investigated is the nicotinic acetylcholine receptor - an intrinsic membrane protein that regulates nerve-nerve and nerve-muscle signal transmission processes. At neuromuscular and electromotor synapses the action of acetylcholine initiates receptor processes, that lead to a change in the postsynaptic membrane potential. This involves two types of reaction events. Short pulses (millisecond) of the presynaptically released transmitter at submillimolar concentrations, lead to receptor activation connected with a net opening of ion channels in the postsynaptic membrane. If acetylcholine is present for longer periods (fractions of seconds to minutes) at sufficiently high concentrations (micromolar), the initial channel opening phase is followed by a complex reduction of the number of open channels - the so-called desensitization of the acetylcholine receptor.

Most fundamental ion flux studies have been performed using acetylcholine receptor containing membrane fragments derived from the electric organs of Torpedo and Electrophorus electricus. In early investigations attempts were made to measure the ligand-induced efflux of $^{22}Na^{+}$ on the time scale of minutes (Kasai & Changeux, 1971; Hess et al., 1975; Popot et al., 1976) using a filter assay technique. Bernhardt & Neumann (1978) found that such methods permit the determination of ligand concentration dependent flux amplitudes, but not the actual time course of receptor controlled ion flux. With the introduction of more complex measurement techniques, it is now possible to measure flux processes on the millisecond to minute time range. These refined methods require the use of rapid mixing instruments, that were originally designed for the kinetics of fast chemical reactions. It is thereby possible to measure the time course of the change in ion content of a suspension of CMS using quench-flow (Hess et al., 1979; Neubig & Cohen, 1980) or stopped-flow (Moore & Raftery, 1980) techniques.

Investigations of ion flux regulated by the acetylcholine receptor permit several conclusions about the gating events connected with receptor processes. The time course of the ion flux initiated by neuroactivator ligand binding to the receptor consists of at least two separate, apparently exponential phases that occur on a progressively more rapid time scale, with increasing neuroactivator concentration (Neubig & Cohen, 1980; Hess et al., 1982; Heidmann et al., 1983). This flux behavior is compatible with the assumption that, following a rapid initial channel opening step (receptor activation), the receptor process induced by neuroactivators leads to at least two successively slower channel closing steps (receptor inactivation). Such a finding can also be deduced from measurements with other techniques (Heidmann & Changeux, 1979).

Further it could be shown that at low neuroactivator concentrations the slow receptor inactivation step, also known as desensitization, leads to the appearance of concentration dependent flux amplitudes (Bernhardt & Neumann, 1978; Walker et al., 1982). The amplitude was found to increase steadily with increasing neuroactivator concentration, until a limiting maximum amplitude is reached. A reverse dependence was found in inhibition studies, where acetylcholine receptor containing membrane fragments were inhibited by preincubation with an irreversibly binding snake toxin. Inhibition by sucessively greater amounts of toxin leads to a stepwise reduction of the flux amplitudes induced by neuroactivator ligands (Moore et al., 1979; Neubig & Cohen, 1980; Bernhardt & Neumann, 1982b).

Preparative Techniques

Not all CMS suspensions are equally suitable for flux studies. It is to be expected that sizeable admixtures of open and nonfunctional membrane structures may, on the one hand, complicate data analysis, and, on the other hand, lead to artifacts.

Special preparative techniques are therefore required to separate a suspension of highly homogeneous vesicular structures from crude homogenates of diverse membrane fragments and other cell components.

Generally applicable methods for producing such suspensions were first developed for acetylcholine receptor containing membrane fragments. Homogenization of whole electric organs from Torpedo or Electrophorus electricus can be used to generate a crude cell fraction. In initial studies (Kasai & Changeux, 1971; Duguid et al., 1973) sedimentation on sucrose gradients was used to separate fractions from such homogenates, that are rich in acetylcholine receptor content, but poor in acetylcholinesterase content. Due to progressive refinement of such techniques, it is now possible to produce uniform suspensions of membrane fragments with a high surface density of receptors (Sobel et al., 1977; Elliot et al., 1980). Unfortunately, such suspensions are not optimally suited for flux studies (a) because using gradient fractionation under these conditions does not necessarily lead to a separation of open and closed membrane structures, and (b) because non-functional vesicles, which contain proteolytically cleaved receptors, are not separated.

To minimize the contribution of open and non-functional membrane fragments in flux measurements, special methods were developed. Thus, through addition of the neuroactivator carbamoylcholine in the presence of $^{22}Na^{+}$ to a suspension obtained through gradient separation, a selective filling with tracer of closed, functionally intact vesicles was achieved (Hess et al., 1975). In tracer flux measurements with such preincubated suspensions, only the selectively filled vesicles contribute to the flux signal. Unfortunately, the open and non-functional membrane fragments still present as a background, nevertheless constitute an undesirable admixture. Due

to the receptor binding sites they contain, they lead to a reduction of the neuroactivator concentration which is difficult to estimate. A more efficient gradient separation was achieved by preincubating the crude homogenate with CsCl. In the subsequent sucrose gradient sedimentation there is an enrichment of receptor containing closed membrane vesicles (Hess & Andrews, 1977).

A consideration of the detailed factors that control gated ion flux Bernhardt & Neumann, 1980 a,b) led to the proposal of more stringent criteria CMS suspensions optimally suited for quantitative flux experiments must fulfill: (a) there should be as little variation as possible in the ion channel content and volumes of the CMS (b) these parameters should lie in a range for which the flux time course is slow enough to be measured, and is on the same time scale as the gating process to be investigated. Bernhardt et al. (1981) developed a procedure for generating suspensions of acetylcholine receptor containing membrane fragments, that comply with these criteria. A crude homogenate of electric organs is first incubated in 0.2M CsCl. A selective exchange of Li^+ for Cs^+ in solely the functionally intact closed membrane structures is then effected by the stepwise addition of acetylcholine in the presence of 0.2M LiCl. These can then be separated from the heavier non-functional fragments, filled with CsCl, and other cell components, by sedimentation on a percoll gradient. Uniform suspensions of vesicular fragments having roughly the same volume and receptor content, can be obtained from fractions of the gradient derived from different density regions. With minor modifications this procedure should be generally applicable for arbitrary CMS suspensions.

Methods of Analysis

The aim of the analysis of flux data is to derive quantitative information about channel gating events from the time course and amplitude of the flux process. In initial studies, empirically defined parameters were employed to analyse curves (Kasai & Changeux, 1971). By explicitely considering the physical foundations of flux and channel gating processes, it was possible to develop theoretically well-founded, generally applicable methods of analysis (Bernhardt & Neumann, 1978, 1980 a,b, 1981). A main postulate in the derivation was, that the overall flux process, measured on a collection of CMS, is composed of a superposition of independent contributions of the individual CMS. The quantity actually measured in ion flux experiments - the total amount X(t) of a certain ion species present in the CMS at time t - is thus given as the sum of the ion content of the separate CMS. To derive practically applicable expressions for X(t) it was first necessary to examine the time course of ion flux from or into a CMS. It was possible to formulate a kinetic equation that explicitly encompasses all factors that determine flux. The rate of flux was found to be directly proportional to the rate constant for flux through a channel k', to the number of channels n_i, the fraction of these $\alpha_i(t)$ that are open at time t, and inversely proportional to the CMS volume v_i

$$\frac{dx_i}{dt} = - (n_i/v_i) \cdot k' \cdot \alpha_i(t) \cdot x_i(t) \qquad (1)$$

where $x_i(t)$ is the CMS ion content at time t, and the index i implies that the corresponding quantities may vary from CMS to CMS.

In principle the explicit evaluation of this equation should lead to an expression for X(t). Unfortunately such a direct mode of solution is complicated by the following circumstances (Bernhardt & Neumann, 1978, 1980 a,b): (a) In every actual suspension of CMS a variation of the values n_i and v_i is to be expected. Since the flux rate depends on these magnitudes, this entails that the time course of X(t) will reflect a complex superposition of the time course of ion flux from or into the individual CMS. (b) Even in the ideal case of a suspension of perfectly uniform CMS a complex mathematical espression for X(t) is expected to result, due to an always present variation of the fractions $\alpha_i(t)$. This follows from the fact that the fraction of open channels $\alpha_i(t)$ on a given CMS is not necessarily identical to the total fraction of open channels on all CMS $\alpha(t)$. Rather, on the basis of probability laws, a statistical variation of the $\alpha_i(t)$ values is to be expected.

A quantitatively exact evaluation of X(t) is only possible, if the variations of n_i, v_i and $\alpha_i(t)$ are explicitly incorporated into the framework of the mathematical treatment. Despite these difficulties it was nevertheless possible to derive simple expressions for X(t) by introducing several approximations (Bernhardt & Neumann, 1978, 1980 a,b). If in equation (1) one substitutes the mean values $\bar{n}$ and $\bar{v}$ for the corresponding parameters n_i and v_i, and $\alpha(t)$ for $\alpha_i(t)$, one obtains an easily solvable equation, the solution of which is a simple exponential expression for X(t) - the approximate flux equation, which, for efflux conditions, is given by

$$X(t) = X(0) \cdot \exp\left[-\bar{n} \cdot \bar{k}' \cdot \int_0^t \alpha(\tau) d\tau \right]. \qquad (2)$$

This expression, or variants thereof having the same functional form, have been frequently employed to analyze flux data. However, due to the implicit approximations, the range of applicability remained uncertain. Heidmann et al. (1983) recently compared the results of an ion flux analysis based on equation (2), with corresponding results of a separate stopped flow kinetic investigation carried out under identical conditions. They were able to verify that this approximate expression leads to quantitatively correct rate constants and dissociation constants for a ligand induced gating process.

That the applicability of equation (2) is limited, follows from a number of inhibition studies. Thus, Moore et al. (1979), and Neubig & Cohen (1980) were forced to postulate the presence of "spare" or "excess" receptors, in order to explain the stepwise reduction of flux amplitude accompanying inhibition of receptors by snake toxin. Bernhardt & Neumann (1982b) were able to show that such ad hoc assumptions are superfluous, and result from an incorrect modelling of the underlying flux phenomena by expression (2).

Recently Bernhardt & Neumann (1981) were able to derive generally applicable flux equations, that constitute exact solutions of the kinetic equation (1):

$$X(t) = \begin{cases} X(0) \sum_n P_n \left[\langle e^{-kt} \rangle^n \right]_v & \text{(efflux)} \quad (3) \\ X(\infty) \sum_n P_n \left\{ 1 - \left[\langle e^{-kt} \rangle^n \right]_v \right\} & \text{(influx)} \quad (4) \end{cases}$$

where the summation is over subpopulations of CMS having n gated channels per CMS. The quantity actually measured in ion flux experiments - the time dependent overall ion content of

a collection of CMS - is thus seen to depend on: (a) the mean single channel flux contribution $\langle e^{-kt} \rangle$, which is a function solely of the kinetic parameters for ion flux and channel gating, and (b) the weight factors P_n and an average over a volume distribution function (expressed by the square brakets $[\ldots]_v$), which explicitly incorporate the variations in CMS ion channel content and volume. Employing equation (3) Bernhardt & Neumann (1982 a,b) were able to analyze snake toxin inhibition of acetylcholine receptor controlled ion flux, without recourse to additional assumptions.

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