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(54) Title: SYSTEM AND METHOD FOR PLANAR VOLTAGE-CLAMP ELECTROPHYSIOLOGICAL MEASUREMENTS ON MULTIPLE CELLS AND THEIR USE IN IDENTIFYING AGENTS THAT MODULATE ION CHANNELS

(57) Abstract: The present invention relates to an assay method for identifying an agent that modulates the electrical property of a membranous sample comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture; (c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration seal phase internal solution; (d) exchanging the low ion concentration seal phase internal solution for an access phase internal solution; (e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and (f) taking electrophysiological recordings before and after incubation of the cell with one or more agents; wherein a difference between: (i) the electrophysiological recording in the presence of the agent; and (ii) the electrophysiological recording in the absence of the agent is indicative of an agent that modulates the electrical property of a membranous sample.

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System and method for planar voltage-clamp electrophysiological measurements on multiple cells and their use in identifying agents that modulate ion channels.

FIELD OF INVENTION

The present invention relates to improved electrophysiological methods.

In particular, the present invention relates to, *inter alia*, improved assay methods involving planar voltage-clamp electrophysiological measurements on multiple cells in parallel and their use in identifying, testing and/or screening of agents that modulate ion channels.

BACKGROUND TO THE INVENTION

Over the past decade, compounds both on the market and in development, have been withdrawn due to undesirable effects on cardiac repolarisation in man, observed as a prolongation of the QT interval on electrocardiograms (ECG). In extreme circumstances, this drug-induced prolongation of the QT interval can lead to a type of cardiac arrhythmia called Torsades de Pointes (TdP; Vandenberg et al. hERG K+ channels: friend and foe. Trends Pharmacol Sci 2001; 22: 240-246) leading ultimately to ventricular fibrillation and sudden death. The primary event in this syndrome is inhibition of the rapid component of the delayed rectifying potassium current (IKr) by these compounds. The compounds bind to the apeture-forming alpha sub-units of the channel protein carrying this current – sub-units that are encoded by the human ether-a-go-go-related gene (hERG). Since IKr plays a key role in repolarisation of the cardiac action potential, its inhibition slows repolarisation and this is manifested as a prolongation of the QT interval. Whilst QT interval prolongation is not a safety concern per se, it carries a high risk of cardiovascular adverse effects and in a small percentage of people it can lead to TdP and degeneration into ventricular fibrillation.

The hERG channel contains a binding site for the class III antiarrhythmic methanesulphonanilides (dofetilide, E-4031 and MK-449) and many drugs that show prolonged QT in the clinic share this site. Consequently they have either warning labels (e.g. pimozide) or have been withdrawn from the market (e.g. terfenadine). The lack of an appropriate therapeutic margin between activity at the hERG channel and the desired target will prevent a potential candidate drug from progressing further. It is therefore necessary to evaluate the hERG activity of candidate drugs as early as possible to allow reduction in this activity for novel compound classes.

From a drug safety perspective, the ideal position is for compounds not to interact with ion channels – such as hERG, but in practice this has been extremely difficult to achieve.

The most widely used and accepted test for the ability of a compound to affect ion channel function is the use of voltage clamp electrophysiology (Hamill et al. Pflugers Arch. 1981; 391:85-100). This technique allows the voltage of a cell expressing the channel of interest to be accurately controlled with millisecond precision. Thus, the channel gating can be controlled, subsequent current flow measured and the ability of compounds to modulate this current assessed. However, this technique requires a high degree of expertise and, because only one cell at a time can be measured, the throughput is low. A typical experimental day will allow testing of compounds on approximately 10 cells.

There is therefore a need for improved assay systems that enable a higher throughput.

Recent advances in technology have opened up the possibility of simultaneous and parallel voltage-clamp electrophysiology measurements on multiple cells using planar substrates rather than the conventional borosilicate glass micropipettes ('parallel planar voltage clamp electrophysiology'). These systems, exemplified by Molecular Devices IonWorks™HT and Axon Instruments PatchXpress, consist of multiwell plates made of various materials (plastic, silicon, glass, Sylgard™) that contain a single, micrometer-scale apeture or pore in each well. Cells in suspension are manoeuvred over the apeture or pore by suction or by electric field potential where they form a high resistance seal over said apeture or pore. The cells can subsequently be permeablised locally at the apeture or pore by suction, electroporation or by applying a solution containing a permeablising compound - such as amphotericin. Voltage-clamp electrophysiological recordings can then be made on all or a subset of the attached cells simultaneously.

The Molecular Devices IonWorks™HT system is described in Kiss *et al.* (2003) *Assay and Drug Development Technologies* 1, 1-2, 127-135 and also by Schroeder *et al.* (2003) *Journal of Biochemical Screening* 8(1), 50-64.

In view of the importance of overcoming the throughput restrictions of classical patch clamp electrophysiology and the need for safer drugs that do not, or substantially do not, interact with ions channels – such as hERG ion channels - there is a need in the art for improved high throughput assay methods for identifying agents that modulate ion channel activity.

SUMMARY OF THE INVENTION

The present invention is based, in part, upon the surprising finding that exchanging a low ion concentration internal solution that is used in an electrophysiological measurement apparatus for another internal solution – such as a low or a high ion concentration internal solution – leads to improved assay methods.

In particular, the inventor's have found that exchanging a low ion concentration internal solution for another internal solution – such as a low or a high ion concentration internal solution – leads to optimised seal quality in the electrophysiological measurement apparatus and/or an optimised potency estimate for an agent being assayed.

Moreover, the inventor's have found that exchanging a low ion concentration internal solution for another internal solution – such as a low or a high ion concentration internal solution – and reducing the number of cells contained in each compartment of the electrophysiological measurement apparatus, surprisingly results in an optimal potency estimate.

The invention described herein may be used, *inter alia*, in the development of assays, which utilise parallel planar voltage clamp electrophysiology to determine the potency at which compounds block ion channels.

SUMMARY ASPECTS OF THE INVENTION

Aspects of the present invention are presented in the accompanying claims.

In a first aspect, the present invention relates to an assay method for identifying an agent that modulates the electrical property of a membranous sample comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture; (c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration seal phase internal solution; (d) exchanging the low ion concentration seal phase internal solution for an access phase internal solution; (e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and (f) taking electrophysiological recordings before and after incubation of the cell with one or more agents; wherein a difference between: (i) the electrophysiological recording in the presence of the agent; and (ii) the electrophysiological recording in the absence of the agent is indicative of an agent that modulates the electrical property of a membranous sample.

In a second aspect, the present invention relates to a method for maintaining the optimum seal quality of a compartment in an electrophysiological measurement apparatus comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture; (c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration seal phase internal solution; and (d) exchanging the low ion concentration seal phase internal solution for an access phase internal solution.

In a third aspect, the present invention relates to a method for maintaining optimum seal quality and determining the potency of a non-lipophillic agent that modulates the electrical

property of a membranous sample comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture; (c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration seal phase internal solution; (d) exchanging the low ion concentration seal phase internal solution for a high ion concentration access phase internal solution; (e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and (f) taking electrophysiological recordings before and after incubation of the cell with one or more agents; wherein a difference between: (i) the electrophysiological recording in the presence of the agent; and (ii) the electrophysiological recordings in the absence of the agent is indicative of the potency estimate for the non-lipophillic agent.

In a fourth aspect, the present invention relates to a method for determining the potency of an agent comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture using a reduced number of cells per compartment; (c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration internal solution; (d) exchanging the low ion concentration internal solution for an access phase internal solution; (e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and (f) taking electrophysiological recordings before and after incubation of the cell with one or more agents.

In a fifth aspect, the present invention relates to an assay method for determining the potency of an agent comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture using a reduced number of cells per compartment; (c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration internal solution; (d) exchanging the low ion concentration internal solution for an access phase internal solution; (e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and (f) taking electrophysiological recordings before and after incubation of the cell with one or more agents; wherein a difference between: (i) the electrophysiological recording in the presence of the agent; and (ii) the electrophysiological recordings in the absence of the agent is indicative of the potency estimate for the agent.

In a sixth aspect, the present invention relates to a low ion concentration seal phase internal solution.

In a seventh aspect, the present invention relates to a process comprising the steps of: (i) performing the assay method according to the first aspect of the present invention; (ii) identifying an agent capable of modulating the electrical property of a membranous sample; and (iii) preparing a quantity of that agent.

In an eighth aspect, the present invention relates to a process comprising the steps of: (i) performing the assay according to the first aspect of the present invention; (ii) identifying an

agent capable of modulating the electrical property of a membranous sample; (iii) preparing a quantity of that agent; and (iv) preparing a pharmaceutical composition comprising that agent.

In a ninth aspect, the present invention relates to a process comprising the steps of: (i) performing the assay according to the first aspect of the present invention; (ii) identifying an agent capable of modulating the electrical property of a membranous sample; (iii) modifying said agent; and (iv) preparing a pharmaceutical composition comprising said modified agent.

In a tenth aspect, the present invention relates to a pharmaceutical composition comprising an agent identified by the assay method according to the first aspect of the present invention or the process according to the sixth, seventh and eighth aspects of the present invention admixed with a pharmaceutically acceptable carrier, diluent, excipient or adjuvant and/or combinations thereof.

In an eleventh aspect, the present invention relates to a process of preparing a pharmaceutical composition comprising admixing an agent identified by the assay method according to the first aspect of the present invention or the process according to the sixth, seventh and eighth aspects with a pharmaceutically acceptable diluent, carrier, excipient or adjuvant and/or combinations thereof.

In a twelfth aspect, the present invention relates to a method of treating a disease in a human or animal which method comprises administering to an individual an effective amount of a pharmaceutical composition comprising an agent identified by the assay method according to the first aspect of the present invention or the process according to the sixth, seventh and eighth

aspects, wherein the agent is capable of modulating the disease and wherein said composition is optionally admixed with a pharmaceutically acceptable carrier, diluent excipient or adjuvant and/or combinations thereof.

In a thirteenth aspect, the present invention relates to an agent identifiable, preferably identified by the assay method according to the first aspect of the present invention.

In a fourteenth aspect, the present invention relates to an agent identifiable preferably identified by the assay method according to the first aspect of the present invention for use in the treatment and/or prevention of disease.

In a fifteenth aspect, the present invention relates to a kit for performing the method according to the first, second, third of fourth aspects of the present invention comprising: (a) a first vessel comprising a seal phase internal solution according to the sixth aspect of the present invention; and (b) a second vessel comprising an access phase internal solution.

In a sixteenth aspect, the present invention relates to the use of a seal phase internal solution according to the sixth aspect of the present invention for measuring an electrical property of a membranous sample.

In a seventeenth aspect, the present invention relates to the use of a seal phase internal solution according to the sixth aspect of the present invention for identifying an agent that modulates the electrical property of a membranous sample.

In an eightteenth aspect, the present invention relates to the use of a seal phase internal solution according to the sixth aspect of the present invention for maintaining the optimum seal quality of a compartment in an electrophysiological measurement apparatus.

In a nineteenth aspect, the present invention relates to the use of a seal phase internal solution according to the sixth aspect of the present invention for determining the potency of a non-lipophillic agent that modulates the electrical property of a membranous sample.

In a twentieth aspect, the present invention relates to the use of a seal phase internal solution according to the sixth aspect of the present invention for determining the potency of an agent.

Other aspects of the present invention are presented in the accompanying claims and in the following description and discussion. These aspects are presented under separate section headings. However, it is to be understood that the teachings under each section heading are not necessarily limited to that particular section heading.

PREFERRED EMBODIMENTS

Preferably, the assay method according to the first aspect of the present invention comprises the step of exchanging the low ion concentration seal phase internal solution for a low or a high ion concentration access phase internal solution.

Preferably, the assay method according to the first aspect of the present invention comprises the step of exchanging the low ion concentration seal phase internal solution for a high ion concentration access phase internal solution.

Preferably, the agent is a lipophillic agent.

Preferably, the lipophillic agent is astemizole or pimozide.

Preferably, each compartment of the electrophysiological measurement apparatus containing an aperture contains from 1-900 cells in a volume of 10.5 µl buffer solution.

Preferably, the membranous samples comprises an ion channel.

Preferably, the ion channel is a myocardial I_{Kr} ion channel.

Preferably, the myocardial I_{Kr} ion channel is encoded by ERG, including hERG.

Preferably, the electrophysiological measurement apparatus is a Molecular Devices IonWorks™HT or an Axon Instruments PatchXpress.

Preferably, the access phase internal solution is a low or high ion concentration solution.

Preferably, the number of membranous samples in each compartment is modulated such that the pIC_{50} value is at a level comparable to that obtained in conventional voltage clamp electrophysiology apparatus.

Preferably, the low ion concentration internal solution is a seal phase internal solution.

Preferably, the low ion concentration internal solution is exchanged for a low or a high ion concentration access phase internal solution.

Preferably, the ion in the low ion concentration seal phase internal solution is a chloride ion.

Preferably, the ion is present at a concentration of about 40 mM.

Preferably, the low ion concentration seal phase internal solution comprises about 40mM KCl, about 100mM K gluconate, about 3.2 mM $MgCl_2$, about 3.0mM EGTA, and about 5.0mM HEPES.

Preferably, one or more agents are formulated into one or more compositions for use in medicine.

Preferably, the access phase internal solution has a chloride ion concentration of about 40 mM or about 140 mM.

Preferably, the low ion concentration seal phase internal solution comprises about 40mM KCl, about 100mM K gluconate, about 3.2 mM $MgCl_2$, about 3.0mM EGTA, and about 5.0mM HEPES and a permeabilising agent.

Preferably, the high ion concentration access phase internal solution comprises about 140mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent.

ADVANTAGES

The present invention has a number of advantages. These advantages will be apparent in the following description.

By way of example, the present invention is advantageous since it provides commercially useful methods and assay methods.

By way of further example, the present invention is advantageous since it provides a commercially useful method for assaying lipophillic and non-lipophillic agents.

By way of further example, the present invention is advantageous since it provides a commercially useful method for obtaining optimum seal quality in an electrophysiological measurement apparatus.

By way of further example, the present invention is advantageous since it provides a commercially useful method for obtaining an optimum potency estimate of an agent that is assayed in an electrophysiological measurement apparatus.

By way of further example, the present invention is advantageous since it provides a commercially useful method for obtaining both an optimum seal quality and also an optimum

potency estimate for an agent that is assayed in an electrophysiological measurement apparatus.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Effect of cell number titration on potency of pimozide (1-[1-[4,4-Bis(4-fluorophenyl)butyl]-4-piperidinyl]-1,3-dihydro-2*H*-benzimidazol-2-one), astemizole (1-[(4-Fluorophenyl)methyl]-*N*-[1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl]-1*H*-benzimidazol-2-amine), loratadine (4-(8-Chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene)-1-piperidinecarboxylic acid ethyl ester), terfenadine (α-[4-(1,1-dimethylethyl)phenyl]-4-(hydroxydiphenylmethyl)-1-piperidinebutanol) and cisapride (*cis*-4-amino-5-chloro-N-[1-[3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide).

Potency (pIC_{50}) was assessed using an 8-point dose-response curve. Devation from conventional pIC_{50} (y axis) was assessed by comparing the pIC_{50} value obtained using conventional, pipette-based electrophysiology recordings against the pIC_{50} value obtained using the IonWorks™HT system (conventional pIC_{50} minus IonWorks™HT pIC_{50}). The x-axis represents cells/ml in the stock cell suspension (x1000). 3.5ul of this suspension is added to each well on the IonWorks™HT system. For all compounds, there is higher deviation from conventional pIC_{50} at higher cell concentrations than at lower cell concentrations (see Table 1).

Figure 2

Effect of cell number titration on useable cells obtained on the IonWorks™HT system. The y-axis represents the percentage of cells on a PatchPlate which gave a seal resistance of greater than 50Mohms as measured during Scan #2 on the IonWorks™HT system. The x-axis represents cells/ml in the stock cell suspension (x1000). 3.5ul of this suspension is added to each well on the IonWorks™HT system.

The graph shows that the number of usable cells falls off significantly if the stock cell suspension contains less than $0.25x10^6$ cells/ml.

Figure 3

Effects of different assay conditions on the potency correlation of 51 different hERG channels blockers, estimated using the IonWorks™HT system, compared to the potency estimated using conventional whole-cell voltage clamp electrophysiology.

Potency (pIC_{50}) was assessed using an 8-point dose-response curve on the IonWorks™HT system or a 3-6 point dose response curve using conventional whole-cell voltage clamp electrophysiology. The graphs show the correlation between the two different methods under the following conditions:

a. Low ion concentration seal-phase internal solution and low ion concentration access-phase internal solution (40mM KCl, 100mM K gluconate, 3.2 mM $MgCl_2$, 3.0mM EGTA, and 5.0mM HEPES). Cell number was greater than $1x10^6$ cells/ml in the stock cell suspension. Correlation co-efficient, $R^2 = 0.58$.

b. Low ion concentration seal-phase internal solution (40mM KCl, 100mM K gluconate, 3.2 mM $MgCl_2$, 3.0mM EGTA, and 5.0mM HEPES) and high ion concentration access-phase internal solution (140mM KCl, 1.0mM $MgCl_2$, 1.0mM EGTA, 20mM HEPES). Cell number was greater than $1x10^6$ cells/ml in the stock cell suspension. Correlation co-efficient, $R^2 = 0.68$.

c. Low ion concentration seal phase internal solution (40mM KCl, 100mM K gluconate, 3.2 mM $MgCl_2$, 3.0mM EGTA, and 5.0mM HEPES) and high ion concentration access-phase internal solution (140mM KCl, 1.0mM $MgCl_2$, 1.0mM EGTA, 20mM HEPES). Cell number was $0.25x10^6$ cells/ml in the stock cell suspension. Correlation co-efficient, $R^2 = 0.76$.

The graph shows that using the preferred assay conditions on the IonWorks™HT system (fig c) gives the best correlation between potency estimated using the IonWorks™HT system and that estimated using conventional whole-cell voltage clamp techniques.

Figure 4

Potency correlation of 140 different hERG channels blockers, estimated using the IonWorks™HT system (y-axis), compared to the potency estimated using rubidium (Rb^+) efflux and atomic absorption spectroscopy (x-axis). Potency (pIC_{50}) was assessed using an 8-point dose-response curve on the IonWorks™HT system and the following assay conditions; low ion concentration seal phase internal solution (40mM KCl, 100mM K gluconate, 3.2 mM $MgCl_2$, 3.0mM EGTA, and 5.0mM HEPES) and high ion concentration access-phase internal solution (140mM KCl, 1.0mM $MgCl_2$, 1.0mM EGTA, 20mM HEPES). Cell number was $0.25x10^6$ cells/ml in the stock cell suspension.

The graph shows that using the preferred assay conditions on the IonWorks™HT system gives approximately 10-fold higher estimate for potency than Rb^+ efflux / atomic absorption spectroscopy.

DETAILED DESCRIPTION OF THE INVENTION

ION

As used herein, the term "ion" refers to any atom or other molecular entity that has acquired an electric charge by loss or gain of one or more electrons.

The ion may be a cation or an anion.

Preferably, the ion is an anion.

In a highly preferred embodiment, the ion is a chloride ion.

LOW ION CONCENTRATION

As used herein, the term "low ion concentration", preferably low chloride ion concentration, means that the ion concentration is from 0 to about 60 mM.

In a preferred embodiment, the low ion concentration is from 0 to about 50 mM – such as about 5-50mM, about 10-50mM, about 15-50mM, about 20-50mM, about 25-50mM, about 30-50mM,about 35-50mM, about 40-50mM, about 45-50mM, about 45-49mM, about 45-

48mM, or about 45-47mM or any suitable combination of start or end points, for example, from about 37-48mM.

In a highly preferred embodiment, the low ion concentration is about 46 mM, preferably, 46.4 mM.

Suitably, the low ion concentration will be such that the osmolarity of the final solution will be within normal physiological parameters (about 250 to 310 mOsm/liter).

The low ion concentration may be from 0 to about 40 mM – such as about 5-40mM, about 10-40mM, about 15-40mM, about 20-40mM, about 25-40mM, about 30-40mM or about 35-40mM or any suitable combination of start or end points, for example, from about 37-39mM.

Advnatgaeosuly, ion substitutes – such as non-chloride ion substitutes - may be used in the low ion concentration solutions, which allow the ion (preferably, chloride ion) concentration to be lowered without changing the overall ionic strength of the solution.

Suitably, the ion substitute may be in the form of potassium chloride or magnesium chloride and the like.

Suitably, the following may also be used as ion substitutes to maintain osmolarity: gluconate, aspartate, methanesulfonate, isethionate, cyclamate, glutamate, sulphate, methanesulphonate, formate, acetate, iodide, bromide, SCN^-, $C(CN)_3^-$, $N(CN)_2^-$, ClO_4^-, NO_3^-, ClO_3^-, BrO_3^- or HCO_3^- or combinations thereof.

Preferably, the substitute is a non-chloride ion potassium salt – such as potassium gluconate, potassium aspartate, potassium methanesulfonate, potassium isethionate, potassium cyclamate, potassium glutamate, potassium sulphate, potassium methanesulphonate, potassium formate, potassium acetate, potassium iodide, potassium bromide, K^+SCN^-, $K^+C(CN)_3^-$, $K^+N(CN)_2^-$, $K^+ClO_4^-$, $K^+NO_3^-$, $K^+ClO_3^-$, $K^+BrO_3^-$ or $K^+HCO_3^-$ or combinations thereof

Suitably, the concentration of these salts – such as potassium gluconate - will be in the range of from 70-180mM, preferably from 80-170mM, more preferably from 80-160mM, more preferably from 80-150mM, more preferably from 80-140mM, more preferably from 80-130mM, more preferably from 80-120mM, more preferably from 80-110mM, or more preferably from 90-110mM potassium gluconate or any suitable start or end point – such as from 90-160mM.

HIGH ION CONCENTRATION

As used herein, the term "high ion concentration", preferably high chloride ion concentration, means that the ion concentration is from about 120 to about 180 mM, preferably, from about 130 to about 170 mM.

In a highly preferred embodiment of the present invention, the high ion concentration is from about 130 to about 160 mM – such as from about 130-160mM , about 135-150 mM, about 135-145 mM, about 138-142mM or any suitable combination of start or end points, for example, from about 136-149mM.

In a highly preferred embodiment, the high ion concentration is about 140 mM, preferably, about 142mM.

Suitably, the high ion concentration will be such that the osmolarity of the final solution will be within normal physiological parameters (about 250 to 310 mOsm/liter).

The high ion concentration may be from about 140 to about 160 mM – such as from about 145-160mM , about 150-160 mM, or about 155-159 mM or any suitable combination of start or end points, for example, from about 156-160mM. .

ACCESS PHASE INTERNAL SOLUTION

As used herein, the term "access-phase" relates to the period during which whole-cell access is being obtained and maintained and where the portion of cell membrane sealed to the apeture is exposed to an internal solution containing a permeablisation compound. This includes the time when the electrophysiological recordings are made and compounds are applied to the cells.

Accordingly, an "access phase internal solution" in the context of the present invention refers to a solution containing a permeablisation compound that is used during the period when whole-cell access is being obtained and maintained, including the time when the electrophysiological recordings are made and compounds are applied to the cells.

The access phase internal solution may have either a high or a low ion concentration.

By way of example, the access phase internal solution may be a low chloride ion access phase internal solution containing from 0 to about 40mM chloride ions, preferably, 40mM chloride ions.

By way of further example, the access phase internal solution may be a low chloride ion access phase internal solution containing from 0 to about 60mM chloride ions.

By way of further example, the access phase internal solution may be a high chloride ion seal phase internal solution containing from about 120 to about 180 mM chloride ions, preferably, 140mM chloride ions.

Typically, the access phase internal solution will comprise additional reagents – such as potassium gluconate and/or magnesium chloride or the like, and/or EGTA and/or a buffer.

The access phase internal solution may comprise 70-180mM potassium gluconate, preferably from 80-170mM, more preferably from 80-160mM, more preferably from 80-150mM, more preferably from 80-140mM, more preferably from 80-130mM, more preferably from 80-120mM, more preferably from 80-110mM, or more preferably from 90-110mM potassium gluconate or any suitable start or end point – such as from 90-160mM.

The access phase internal solution may comprise 0-20mM $MgCl_2$, preferably from 0-15mM, more preferably from 0-10mM, more preferably from 0-5mM, more preferably from 0-4mM, more preferably from 1-4mM, more preferably from 2-4mM or more preferably 3mM $MgCl_2$ or any suitable start or end point – such as from 2-13mM.

The access phase internal solution may comprise 0-40mM EGTA, preferably from 0-30mM, more preferably from 0-20mM, more preferably from 0-10mM, more preferably from 0-5mM, more preferably from 1-5mM or more preferably from 2-4mM or any suitable start or end point – such as from 1-9mM.

The access phase internal solution may comprise 0-40mM HEPES, preferably from 0-30mM, more preferably from 0-20mM, more preferably from 0-10mM, more preferably from 2-10mM, more preferably from 4-8mM or more preferably from 4-6mM or any suitable start or end point – such as from 2-18mM.

The access phase internal solution may comprise range 0.01 to 1mg/ml permeabilising agent, preferably from 0.05- 1 mg/ml, more preferably from 0.1-1 mg/ml, more preferably from 0.5-1 mg/ml or more preferably from 0.8-1 mg/ml or any suitable start or end point – such as from 0.06–0.8 mg/ml.

Preferably, the low ion concentration access phase internal solution comprises or consists of about 40mM KCl , about 100mM K gluconate, about 3.2mM $MgCl_2$, about 3.0mM EGTA about 5.0mM HEPES, and a permeabilising agent – such as Amphotericin B at about 0.1mg/ml.

Preferably, the high ion concentration access phase internal solution comprises or consists of about 140mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent – such as Amphotericin B at about 0.1mg/ml.

For some embodiments of the present invention, the composition of the access phase internal solution, aside from the presence of the permeablisation compound, may be the same or substantially the same as the seal phase internal solution.

PERMEABILISING AGENT

An important part of the electrophysiological measurement apparatus used in accordance with the present invention is the ability to form an electrical seal between the surface of a substrate and a biological membrane. To achieve this, the membranous sample is placed in suspension in a compartment, and drawn to an aperture – such as pore or a hole - through the use of differential pressure applied between two compartments, thus creating an electrical seal.

The formation of high-resistance electrical seals enables the measurement system to detect very small physiological membrane currents (eg., 10-12 amp). In addition, by perforating a portion of the cell membrane, it is possible to control the voltage (voltage clamp) or current (current clamp) across the remaining intact portion of the cell membrane. This greatly enhances the utility of the technique for making physiological measurements of ion channel/transporter activity, since quite often this activity is dependent on transmembrane voltage. By being able to control the trans-membrane voltage (or current), it is possible to stimulate or deactivate ion channels or transporters with great precision and as such greatly enhance the ability to study complex drug interactions.

To achieve the voltage clamp of the membrane, an electrode must be placed in electrical contact with the inside of the membranous sample. This requires electrically permeabilising the part of the membranous sample which separates the two compartments.

This can be achieved by chemical means.

The advantage of the chemical technique is that the membrane patch remains intact so that larger intracellular molecules remain inside the cell and are not flushed out as with other techniques – such as the zapping technique. This approach is well known in the field and is commonly referred to as a "perforated patch".

Any chemical that is capable of permeabilising a membranous sample such that it is permeable to monovalent ions - such as chloride ions - may be used.

Preferably, permeabilisation is achieved by using certain antibiotics - such as Nystatin and Amphotericin B. These antibiotics work by forming chemical pores in the cell membrane that are permeable to monovalent ions - such as chloride.

A person skilled in the art will appreciate that other permeabilising agents – such as gramicidin or ß-escin - may also be used.

SEAL PHASE INTERNAL SOLUTION

As used herein, the term "seal-phase" relates to the period within which one or more cells are sealed to the apeture of the electrophysiological measurement apparatus, between addition of

the cell suspension to the wells and up to the point where the internal solution is exchanged for one containing a permeablisation compound.

Accordingly, a "seal phase internal solution" in the context of the present invention refers to a solution that does not contain a permeablisation compound and is used during the period in which one or more cells are sealed to the apeture.

In a highly preferred embodiment of the present invention, the seal phase internal solution has a low ion concentration.

Accordingly, in this embodiment of the present invention, the seal phase internal solution may be a low chloride ion seal phase internal solution containing, for example, from 0 to about 50mM chloride ions, preferably, 46mM chloride ions

Typically, the seal phase internal solution will comprise additional reagents – such as sodium gluconate and/or magnesium chloride or the like, and/or EGTA and/or a buffer.

The access phase internal solution may comprise 0-60mM potassium chloride, preferably from 10-60mM, more preferably from 20-60mM, more preferably from 30-60mM, more preferably from 30-50mM or any suitable start or end point – such as from 11-59mM.

The access phase internal solution may comprise 70-180mM potassium gluconate, preferably from 80-170mM, more preferably from 80-160mM, more preferably from 80-150mM, more preferably from 80-140mM, more preferably from 80-130mM, more preferably from 80-

120mM, more preferably from 80-110mM, or more preferably from 90-110mM potassium gluconate or any suitable start or end point – such as from 90-160mM.

The access phase internal solution may comprise 0-20mM $MgCl_2$, preferably from 0-15mM, more preferably from 0-10mM, more preferably from 0-5mM, more preferably from 0-4mM, more preferably from 1-4mM, more preferably from 2-4mM or more preferably from 3mM $MgCl_2$ or any suitable start or end point – such as from 2-13mM.

The access phase internal solution may comprise 0-40mM EGTA, preferably from 0-30mM, more preferably from 0-20mM, more preferably from 0-10mM, more preferably from 0-5mM, more preferably from 1-5mM or more preferably from 2-4mM or any suitable start or end point – such as from 1-9mM.

The access phase internal solution may comprise 0-40mM HEPES, preferably from 0-30mM, more preferably from 0-20mM, more preferably from 0-10mM, more preferably from 2-10mM, more preferably from 4-8mM or more preferably from 4-6mM or any suitable start or end point – such as from 2-18mM.

Preferably, the low ion concentration access phase internal solution comprises or consists of about 40mM KCl, about 100mM K gluconate, about 3.2mM $MgCl_2$, about 3.0mM EGTA and about 5.0mM HEPES.

ELECTROPHYSIOLOGICAL MEASUREMENT APPARATUS

In accordance with the present invention, any electrophysiological measurement apparatus may be used that utilises a single, small (eg., several micron diameter) hole, aperture or pore in an at least substantially planar substrate to provide the sealing function resulting in a high-resistance electrical seal.

The substrate may include, for example, thin plastic films in which small holes have been machined or silicon substrates, where standard photolithographic/wet etching techniques are used to make the holes.

Preferably, the electrophysiological measurement apparatus is a Molecular Devices IonWorks™HT or an Axon Instruments PatchXpress.

Another planar patch systems that is commercially available is the Nanion Port-a-Patch System.

Those planar patch systems in development include: Sophion Q Patch and Cytocentrics Cytopatch, for example.

MEMBRANOUS SAMPLE

The term "membranous sample" as used herein, has its natural meaning.

The membranous sample may be or may be derived from a mammal.

Preferably, the membranous sample is or is derived from an animal or a human. Most preferably, the membranous sample is or is derived from a human.

The membranous sample may be or may be derived from biological material.

The membranous sample may include living cells, isolated cell fragments - such as organelles, and/or artificial membranes - such as vesicles

In a highly preferred embodiment, the membranous sample comprises one or more ion channels.

ION CHANNEL

The present invention may be used to study membrane components that are associated with or capable of bringing about measurable voltage changes and/or current flows across biological membranes.

Suitable membrane components may include ion channels and ion transporters, ligand-gated channels and transporters.

Ion channels are membrane proteins that allow ions to flow across biological membranes, including the plasma membrane and organelle membranes. Ion channels are believed to create water-filled pores through which ions and some small hydrophilic molecules can pass by diffusion.

Ligand-gated channels open or close in response to a binding, reaction, and/or other association of signalling molecules, known as "ligands". These channels may be gated by the binding of extracellular or intracellular ligands. In either case, the ligand is different than the substance that is transported when the channel opens.

External ligands gate a variety of ion channels, including ATP gated- channels, glutamate-activated cationic channels, and cys-loop superfamily channels, for example. External ligands are commonly neurotransmitters – such as acetylcholine, amino acids, catecholamines, miscellaneous monoamines, and peptides.

Internal ligands also gate a variety of ion channels – such as G-protein coupled receptors, chloride channels, and calcium-gated potassium channels. These channels generally are controlled by second messengers, which are small signalling molecules - such as cyclic AMP (cAMP), cyclic GMP (cGMP), and calcium ions.

Ion transporters are membrane proteins that use energy - such as that derived from ATP to force ions or small molecules though the membrane via an electrochemical gradient. The transporters may be direct active transporters, binding ATP directly and using the energy of its hydrolysis to drive active transport, or indirect active transporters, using ATP indirectly by using the downhill flow of a different type of ion to drive active transport, where the gradient of the different type of ion is created by a direct active transporter. allowing another transporter to create a gradient of a different type of ion, and then using.

Indirect transporters may be further subdivided into symporters and antiporters depending on whether the driving ion and the pumped ion (or other molecule) pass through the membrane in the same or opposite directions, respectively. Exemplary direct active transporters include the Na+/K+ ATPase and the H+ ATPase. Exemplary indirect active transporters include (1) symporters such as the Na+/glucose transporter, the various amino acid/Na+ transporters, and the Na+/iodide transporter, and (2) antiporters such as the Na+/K+ ATPase.

Preferably, the ion channel is a cardiac ion channel – such as SCN5A, KvLQT1, Kv1.5, Kv4.2, Kv4.3, Kir2.1, Kir2.2, Kir2.3, Kir2.4, CaV1.2, CaV3.1, CaV3.2, HCN1, HCN4 or CFTR.

Preferably, the ion channel is a myocardial I_{Kr} channel – such as a myocardial I_{Kr} ion channel encoded by the ERG channel, including hERG.

hERG

Background teachings on hERG have been presented by Victor A. McKusick *et al* on http://www.ncbi.nlm.nih.gov/Omim. The following information concerning hERG has been extracted from that source:

> A novel human cDNA from a hippocampal cDNA library was isolated by homology to the *Drosophila* 'ether-a-go-go' (eag) gene, which encodes a Ca(2+)-modulated potassium channel. The authors called the cDNA HERG (human ether-a-go-go-related gene). By PCR analysis of a somatic cell hybrid panel, the HERG gene was localised to human chromosome 7.

The HERG gene contains 15 exons spanning approximately 19 kb on chromosome 7q35. Oligonucleotide primers have been synthesised to cover the entire coding region and mutations in 36 Japanese families with the familial long QT syndrome have been identified. The HERG gene contains 16 exons (which includes an alternative exon 1b), ranging from 100 bp to 553 bp.

The HERG gene has been expressed in *Xenopus laevis* oocytes and the potassium channel's biophysical properties and its sensitivity to various pharmacological agents studied. Data have indicated that HERG proteins form I(Kr) channels, but that another subunit may be required for certain drug sensitivities. Since blockage of I(Kr) is a known mechanism for drug-induced cardiac arrhythmias, a mechanistic link between certain types of inherited and acquired LQT have been identified. Acquired long QT syndrome occurs following treatment with certain medications and in association with reduced serum potassium levels (hypokalemia). Both acquired and inherited LQTs are associated with torsade de pointes and polymorphic ventricular tachycardia resulting from abnormal cardiac depolarization (as detected by QT prolongation on the electrocardiogram). LQT is also characterized by sinusoidal twisting of the QRS axis around the isoelectric line. Torsade de pointes can degenerate into ventricular fibrillation, which can lead to sudden death.

hERG has also been demonstrated to encode an inwardly rectifying potassium channel. Inward rectifiers are a large class of potassium channels that preferentially conduct inward potassium currents at voltages negative to the potassium equilibrium potential. In the heart, these channels also have small outward conductances that regulate the resting potential and contribute to the terminal phase of repolarization. At positive voltages, these

channels close and thus help maintain the level of the resting potential. HERG channels show gating properties consistent with many of the outwardly rectifying potassium channels, but they also have an inactivation mechanism that attenuates efflux during depolarization.

The HERG potassium channel is unusual in that it seems to have the architectural plan of the depolarization-activated K+ channel family (6 putative transmembrane segments), yet it exhibits rectification like that of the inward-rectifying K+ channels, a family with different molecular structure (2 transmembrane segments). HERG channels expressed in mammalian cells have been studied and it has been found that this inward rectification arises from a rapid and voltage-dependent inactivation process that reduces conductance at positive voltages. The inactivation gating mechanism resembles that of C-type inactivation, often considered to be the 'slow inactivation' mechanism of other K+ channels. The characteristics of this gating suggested a specific role for this channel in the normal suppression of arrhythmias. The role for HERG in suppressing extra beats might help explain the increased incidence of cardiac sudden death in patients that lack HERG currents, either because they carry a genetic defect (familial long QT syndrome type 2) or because they are being treated with class III antiarrhythmics that block HERG channels.

A subunit interaction domain, termed the NAB domain, in the hydrophilic cytoplasmic N terminus of HERG has also been identified. This domain is responsible for the oligomerization of the protein into functional tetramers. Truncated HERG proteins, including the deletion mutant at position 1261, contain the NAB domain but lack the rest of the channel and thus inhibit the expression of functional tetrameric HERG channels in

transfected cells. The authors suggested that LQT may be the result of decreased expression of a functional HERG potassium channel in the heart.

Single-strand conformation polymorphism and DNA sequence analyses has been performed and HERG mutations detected in 6 LQT families, including 2 intragenic deletions, 1 splice-donor mutation, and 3 missense mutations. In 1 kindred, the mutation arose *de novo*. Northern blot analyses showed that HERG is highly expressed in the heart. The data were interpreted as indicating that HERG is LQT2. Two hypotheses for LQT had previously been proposed. One suggested that a predominance of left autonomic innervation caused abnormal cardiac repolarization and arrhythmias. This hypothesis was supported by the finding that arrhythmias can be induced in dogs by removal of the right stellate ganglion. In addition, anecdotal evidence suggested that some LQT patients are effectively treated by beta-adrenergic blocking agents and by left stellate ganglionectomy. The second hypothesis for LQT-related arrhythmias suggested that mutations in cardiac-specific ion channel genes (or genes that modulate cardiac ion channels) cause delayed myocellular repolarization. Delayed myocellular repolarization could promote reactivation of L-type Ca(2+) channels, resulting in secondary depolarizations. These secondary depolarizations are the likely cellular mechanism of torsade de pointes arrhythmias. This hypothesis is supported by the observation that pharmacologic block of potassium channels can induce QT prolongation and repolarization-related arrhythmias in human and animal models. The discovery that one form of LQT results from mutations in a cardiac potassium channel gene supported the myocellular hypothesis.

Electrophysiologic, biochemical, and immunohistochemical methods to study the molecular mechanisms of HERG channel dysfunction caused by LQT2 mutations have been studied. Some mutations, e.g., tyr611 to his and val822 to met, caused defects in

biosynthetic processing of HERG channels with the protein retained in the endoplasmic reticulum (ER). Other mutations, e.g., ile593 to arg and gly628 to ser, were processed similarly to wildtype HERG protein, but these mutations did not produce functional channels. In contrast, the thr474-to-ile mutation expressed HERG current but with altered gating properties. These findings suggested that the loss of HERG channel function in LQT2 mutations is caused by multiple mechanisms including abnormal channel processing, the generation of nonfunctional channels, and altered channel gating.

The clinical features and prognostic implications of mutations involving the pore and nonpore regions of the HERG channel in LQT2 have been studied. Forty-four different mutations in this gene were identified in 201 subjects, with 14 localized to the pore region (amino acid residues 550 through 650). A total of 35 individuals had mutations in the pore region and 166 in nonpore regions. Those with pore mutations had a markedly increased risk for arrhythmia-associated cardiac events (syncope, cardiac arrest, or sudden death) compared with those with nonpore mutations.

Failure of cell surface expression because of defective HERG trafficking is known to cause LQT2 in some cases. Defective trafficking of membrane proteins results in a variety of other human diseases.

Recent reviews on hERG and its role in disease can be found in *Cardiovasc Res.* (2003) 1;60(2):235-41 and *Curr Opin Drug Discov Devel.* (2003) 6(5):667-74.

ASSAY METHOD

The assay methods described herein may be used with single samples, for example, using pipette-based or planar-substrate-based measurement devices.

Preferably, the methods are used with planar-substrate-based measurement devices. Preferably, the methods are used with multiple samples, sequentially and/or simultaneously, thereby enabling screening, including high-throughput screening.

If the methods of the present invention are implemented on a Molecular Devices IonWorks™HT system, then the assay may be performed as follows.

Cells – such as CHOk1 cells - heterologously expressing an ion channel – such as the hERG K^+ channel - may be prepared in accordance with known methods.

Using the IonWorks™HT system which uses the parallel planar voltage-clamp technique, the cells are sucked down onto a small pore or aperture (approx. 2 μm diameter) which is engineered into a flat substrate. Essentially, the conventional glass micropipette is replaced with a planar pore in a multiwell microplate-type insert (the PatchPlate™). The PatchPlate™ may be a 384 well plate in an 8 x 48 well configuration. The well format may be that of a 1536 well (32 x 48 well) plate and the PatchPlate™ resembles a 1536 well plate cut into four along the wider aspect. The volume of each well is approximately 15 μl. The bottom surface of the plate consists of a proprietary membrane into which a single hole per well (approx. 2 μm diameter) is machined.

A PatchPlate™ is manually placed into the system and sealed into place with a manifold. Negative pressure is applied to the bottom surface of the plate and about 3.5 µl 'external solution' is added automatically via the fluidics head to each well of the plate.

Suitable external solutions are described in Kiss *et al.* (2003) *Assay and Drug Development Technologies* 1, 1-2, 127-135 and also by Schroeder *et al.* (2003) *Journal of Biochemical Screening* 8(1), 50-64, and may comprise, for example, 0.9 mM $CaCl_2$, 2.67 mM KCl, 1.47 mM K_3PO_4, 0.5 mM $MgCl_2$, 138 mM NaCl, 8.10 mM Na_3PO_4, pH 7.4

The bottom surface of the plate is primed by filling the plenum beneath the PatchPlate™ with a low ion concentration internal solution – such as low chloride ion seal phase internal solution. Since each well is electrically isolated and only a single 2 µm apeture separates the well from the intracellular reservoir, each well represents an individual electrophysiologically-enabled chamber.

The quality of the PatchPlate™ apetures may be tested using, for example, a 10mV test pulse applied sequentially to 48 wells at a time using the electronic measurement head. Each apeture is required to reach a threshold of 2.8 MΩ ± 0.5 MΩ. Wells that fall outside this are marked as blocked. This equates to the initial in-bath pipette test in conventional patching. Cells in suspension (approx. 3.5 µl volume in extracellular solution) are added to each well using the fluidics head. The sub-plate negative pressure is maintained at a level more negative than atmospheric pressure to suck the cells onto the apetures.

Seal formation is monitored sequentially across the plate in batches of 48 wells while holding at about –70mV by applying a 10mV voltage pulse. This equates to the cell-attached mode in conventional voltage-clamp electrophysiology. Once a seal has been established, the low chloride ion internal solution – such as the low chloride ion seal-phase internal solution - in the bottom reservoir solution is exchanged for an internal solution containing a permeabilising agent – such as an access-phase internal solution - in order to produce a permeabilised patch configuration. Cells are then tested by the application of a voltage protocol that activates the hERG channel. This process equates to the whole-cell mode in conventional patching. By applying the voltage protocol before and after incubation with a test compound, the inhibitory effects of that compound on the membranous sample current magnitude can be measured.

Agents may be added via, for example, the automated pipette system from a 96 or 384 well compound plate.

Advantageously, the methods described herein may be used to identify agents that do not, or substantially do not interact with certain ion channels – such as hERG ion channels. This has been extremely difficult to achieve in a modern drug discovery environment since to do so by design requires testing large numbers of compounds to check for activity at these ion channels and, where appropriate, to screen out the activity. The inability to do this reliably relates to conventional methodologies for ion channel screening: the assays either have a throughput that is far too low to make testing large numbers of compounds for activity tenable, or they lack the ability to reliably test for effects on channel function.

In the context of the present invention, the term "modulating" may refer to preventing, suppressing, blocking, alleviating, restorating, elevating, increasing or otherwise affecting an electrical property of a membranous sample.

Preferably, the term refers to preventing, suppressing or blocking an electrical property of a membranous sample.

In particular, the term refers to preventing, suppressing or blocking an electrical property of an ion channel – such as hERG ion channel - such that an agent does not, or substantially does not interact with an ion channel.

Advantageously, the methods described herein may also be used to identify agents that block ion channels – such as hERG ion channels – in, for example, toxicological screening.

POTENCY ESTIMATION

Advantageously, the methods of the present invention may also be used to obtain optimal potency estimates, for example, for determining the potency at which the agents block ion channels.

Thus, in one aspect, there is provided a method for determining the potency of an agent comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture using a reduced number of cells per compartment; (c) forming a seal between the membranous samples and the

aperture in the presence of a low ion concentration internal solution; (d) exchanging the low ion concentration internal solution for an access phase internal solution; (e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and taking electrophysiological recordings before and after incubation of the cell with one or more agents.

Preferably, optimal potency estimates are obtained for lipophilic and/or non-lipophilic agents.

In one embodiment, the methods are used to assay non-lipophillic agents – such as cisapride (ClogP = 3.65) - to obtain optimal potency estimates, and for determining the potency at which the agents block ion channels

In a further aspect, there is provided an assay method for determining the potency of an agent comprising the steps of: (a) providing an electrophysiological measurement apparatus; (b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture using a reduced number of cells per compartment; (c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration internal solution; (d) exchanging the low ion concentration internal solution for an access phase internal solution; (e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and (f) taking electrophysiological recordings before and after incubation of the cell with one or more agents; wherein a difference between: (i) the electrophysiological recording in the presence of the agent; and (ii) the electrophysiological recordings in the absence of the agent is indicative of the potency estimate for the agent.

In one embodiment, in order to obtain an optimal potency estimate for an agent – such as a non-lipophillic agent - the low ion concentration internal solution – such as the low ion concentration seal phase internal solution – is exchanged for a high ion concentration internal solution – such as a high ion concentration access phase internal solution.

Preferably, the low ion concentration internal solution is a low ion concentration seal phase internal solution comprising about 40 mM chloride ions.

More preferably, the low ion concentration seal phase internal solution may comprise about 40mM KCl, about 100mM K gluconate, about 3.2 mM $MgCl_2$, about 3.0mM EGTA, and about 5.0mM HEPES.

Preferably, the high ion concentration internal solution is a high ion concentration access phase internal solution comprising about 140 mM chloride ions.

More preferably, the access phase internal solution may comprise about 140mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent.

Advantageously, an optimum seal quality is also obtained using these assay conditions.

In a further embodiment of the present invention, in order to obtain an optimal potency estimate for the agent – such as the non-lipophillic agent - a low or a high ion concentration internal solution – such as a low or a high ion concentration seal phase internal solution – is

exchanged for a high ion concentration internal solution – such as a high ion concentration access phase internal solution.

The methods of the present invention may also be applied to lipophillic agents – such as terfenadine (ClogP = 6.09), astemizole (1-[(4-fluorophenyl)methyl]-N-[1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl]-1*H*-benzimidazol-2-amine; ClogP = 5.89) or pimozide (1-[1-[4,4-Bis(4-fluorophenyl)butyl]-4-piperidinyl]-1,3-dihydro-2*H*-benzimidazol-2-one). In particular, the methods of the present invention may be used for determining the potency at which lipophillic agents block ion channels.

When using either low ion concentration access-phase internal solution or high ion concentration access-phase internal solution during compound potency estimation, it has been found that the pIC_{50} estimates for compounds – such as lipophillic compounds (eg. the standard hERG channel blocking agents astemizole and pimozide)are up to 2 log units less than the pIC_{50} estimates obtained using conventional voltage-clamp electrophysiological experiments. The drop-off in potency is positively correlated to the measures of compound lipophillicity, ACDlogP, ClogP and % non-polar surface area.

The use of a cell suspension at 1 x 10^6 cells per ml for addition to the individual compartments of the PatchPlate has been recommended (IonWorks HT Users Guide, Molecular Devices Corp. 2002, Chapter 5, page 5-4). 3.5 µl of this cell suspension is added to each compartment and the total final assay volume in the compartments is 10.5 µl. Hence, each compartment will contain approximately 3500 cells in a volume of 10.5 µl buffer solution.

Surprisingly, the present inventors have found that by reducing or adjusting the cell number in each compartment, the pIC_{50} estimates for the compounds – such as lipophillic compounds - can be increased to a level comparable to those obtained in conventional voltage-clamp electrophysiology experiments.

Without wishing to be bound by theory, this indicates that the lipid of the cell membranes is acting as a 'sink'. As the number of cells in the well increases, the concentration of lipid (cell membrane) in the well will also increase linearly giving a greater volume of lipid for compound to partition into and reducing the effective concentration in the well. This causes the potency of these compounds to be underestimated. Reducing the cell number also reduces the lipid:buffer ratio and hence the amount of compound that partitions into the membrane. The potency estimate is thus closer to that obtained with conventional voltage-clamp electrophysiology experiments.

As the number of cells added to each well is reduced, the chances of obtaining a useable recording from a cell in that well is also reduced due to the statistically random nature of the sealing process.

An optimum range for cell number has been determined where the number of usable seals obtained across the plate is high enough for meaningful analysis while maximising the pIC_{50} values of the compounds.

In a volume of 10.5 µl buffer per well the number of cells is reduced to about 1-900 cells per well - such as about 1-875 cells per well, about 50–875 cells per well, about 100-875 cells per well, about 150-875 cells per well, or about 175-875 cells per well, or any combination of start or end points – such as about 173-854 cells per well.

A person skilled in the art will understand that the optimal cell number will vary depending on the volume of buffer added, since this will alter the cell number:buffer ratio.

The optimal cell number:buffer ratio is in the range about 1-90 cells per 1 µl buffer – such as about 1-85 cells per 1 µl buffer, about 5-85 cells per 1 µl buffer, about 10-85 cells per 1 µl buffer, about 15-85 cells per 1 µl buffer, or about 17-83 cells per 1 µl buffer, or any combination of start or end points- such as 20-82 cells per 1 µl buffer.

Accordingly, in a further embodiment of the present invention, when using an access phase internal solution that has a low or a high chloride ion concentration, reducing or adjusting the number of membranous samples in each compartment advantageously provides for an optimal potency estimate for the agent.

In this embodiment, the access phase internal solution may typically comprise about 40mM KCl, about 100mM K gluconate, about 3.2 mM $MgCl_2$, about 3.0mM EGTA, about 5.0 mM HEPES and a permeabilising agent or about 140mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent.

Preferably, the number of membranous samples – such as cells - in each compartment is between about 100 and 1000 in a volume of 10.5 µl buffer solution, such as about 100-950, about 100-925, about 100-900, about 100-875, about 125-1000, about 125-975, about 125-950, about 125-925, about 125-900, about 125-875, about 150-1000, about 150-950, about 150-925, about 150-900, about 150-875, about 175-1000, about 175-950, about 175-925, or about 175-900 or any suitable combination of start or end points.

In a highly preferred embodiment, the number of membranous samples in each compartment is between about 175 and 875 in a volume of 10.5 µl buffer solution.

In another highly preferred embodiment, the number of membranous samples in each compartment is between about 17 and 83 in a volume of 1 µl buffer solution.

Advantageously, improved pIC_{50} estimates for agents can also be obtained using an access phase internal solution that does not contain a permeabilising agent. Therefore, adjusting the number of cells in each compartment can be used to improve pIC_{50} estimates when used in conjunction with permeabilisation methods other than those that include a permeablisation agent. As a person skilled in the art will understand, 'whole cell access' or 'electrical access' can be induced in many ways. By way of example, access may be achieved using membrane permeabilisation with voltage pulses of sufficient strength and duration that the membrane inside the pipette physically breaks down. This approach is well known in the field and is commonly referred to as "zapping". The membrane may also be 'permeabilised' by adding negative pressure (suction) to the back of the pipette, or to the side of the planar substrate containing the internal solution.

OPTIMUM SEAL QUALITY

The maintenance of an electrical seal between the surface of a substrate and a biological membrane is particularly important since this results in a high-resistance electrical seal. Maintaining the optimum seal quality throughout the assay period is therefore important to obtain an optimum assay result.

As described herein, the inventors have surprisingly found that the optimum seal quality can be obtained by using a low ion concentration internal solution - such as a low ion concentration seal phase internal solution – and can be maintained by exchanging the low ion concentration internal solution for a high or a low ion concentration internal solution - such as a high or a low ion concentration access phase internal solution.

Accordingly, in this aspect of the present invention, the seal phase internal solution may typically comprise about 40mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, and about 20mM HEPES

The access phase internal solution may typically comprise about 40mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent, or about 140mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent.

The seal quality of the aperture, hole or pore may be tested using a 10mV test pulse applied sequentially to the chambers or wells using the electronic measurement head of an electrophysiological measurement apparatus.

Preferably, each apeture is required to reach a threshold of 2.8 MΩ ± 0.5 MΩ.

Wells that fall outside this threshold are marked as blocked.

This equates to the initial in-bath pipette test in conventional patching.

Cells in suspension (eg. 3.5 µl volume in extracellular solution) are added to each well using the fluidics head. The sub-plate negative pressure is maintained at a level more negative than atmospheric pressure to suck the cells onto the apetures. Seal formation is monitored sequentially across the plate in batches – such as in batches of 48 wells - while holding at, for example, -70mV by applying a 10mV voltage pulse. This equates to the cell-attached mode in conventional voltage-clamp electrophysiology. Once a seal has been established, the seal-phase internal solution in the bottom reservoir solution is exchanged for an access-phase internal solution in order to produce a permeabilised patch configuration. Cells are then tested by the application of a voltage protocol. This process equates to the whole-cell mode in conventional patching.

DISEASE

Many disease states are related to dysfunctional ion channels.

Among prominent groups of diseases are cardiac arrhythmias, diabetes, hypertension, angina pectoris, cystic fibrosis and neurological disorders - such as epilepsy and myasthenia gravis.

Dysfunctional hERG ion channels may lead to the development of LQT. Dysfunctional hERG ion channels causing LQT may occur due to mutations in specific genes and/or treatment with any of a variety of drugs. These drugs include, but are not limited to those being taken to treat cardiac arrhythmias and also other drugs including antihistamines and some antibiotics - such as erythromycin.

Regardless of whether the LQT is a result of mutations or drug induced, it is due to an effect on an ion channel.

The drugs interact with the major subunit of the hERG ion channel, thereby affecting the flow of potassium ions in cells – such as cardiac cells.

Mutations in hERG also can affect potassium ion flow through this channel. This can result in LQT syndrome and may lead to torsade de pointes.

OTHER THERAPIES

It is also to be understood that the agents of the present invention may have other important medical implications.

For example, the agents of the present invention may be useful in the treatment of the disorders listed in WO-A-99/52890.

In addition, or in the alternative, the agents of the present invention may be useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now provided: diabetes including Type II diabetes, obesity, cancer, inflammation or inflammatory disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion; cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease, atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis or endosclerosis.

In addition, or in the alternative, the agents of the present invention may be useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds,

treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity (for treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

In addition, or in the alternative, the agents of the present invention may be useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal

diseases or other dental diseases, orchitis or epididimo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis,.encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillaim-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic

inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

AGENT

As used herein, the term "agent" may be a single entity or it may be a combination of entities. The agent may be an organic compound or other chemical. The agent may be a compound, which is obtainable from or produced by any suitable source, whether natural or artificial. The agent may be an amino acid molecule, a polypeptide, or a chemical derivative thereof, or a combination thereof. The agent may even be a polynucleotide molecule - which may be a sense or an anti-sense molecule, or an antibody, for example, a polyclonal antibody, a monoclonal antibody or a monoclonal humanised antibody.

Various strategies have been developed to produce monoclonal antibodies with human character, which bypasses the need for an antibody-producing human cell line. For example, useful mouse monoclonal antibodies have been "humanised" by linking rodent variable regions and human constant regions (Winter, G. and Milstein, C. (1991) *Nature* 349, 293-299). This reduces the human anti-mouse immunogenicity of the antibody but residual

immunogenicity is retained by virtue of the foreign V-region framework. Moreover, the antigen-binding specificity is essentially that of the murine donor. CDR-grafting and framework manipulation (EP 0239400) has improved and refined antibody manipulation to the point where it is possible to produce humanised murine antibodies which are acceptable for therapeutic use in humans. Humanised antibodies may be obtained using other methods well known in the art (for example as described in US-A-239400).

The agent may be attached to an entity (e.g. an organic molecule) by a linker.

The entity may be designed or obtained from a library of compounds, which may comprise peptides, as well as other compounds, such as small organic molecules.

By way of example, the entity may be a natural substance, a biological macromolecule, or an extract made from biological materials such as bacteria, fungi, or animal (particularly mammalian) cells or tissues, an organic or an inorganic molecule, a synthetic agent, a semi-synthetic agent, a structural or functional mimetic, a peptide, a peptidomimetics, a peptide cleaved from a whole protein, or a peptides synthesised synthetically (such as, by way of example, either using a peptide synthesizer or by recombinant techniques or combinations thereof), a recombinant agent, an antibody, a natural or a non-natural agent, a fusion protein or equivalent thereof and mutants, derivatives or combinations thereof.

Typically, the entity will be an organic compound. For some instances, the organic compounds will comprise two or more hydrocarbyl groups. Here, the term "hydrocarbyl group" means a group comprising at least C and H and may optionally comprise one or more

other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen. For some applications, preferably the entity comprises at least one cyclic group. The cyclic group may be a polycyclic group, such as a non-fused polycyclic group. For some applications, the entity comprises at least the one of said cyclic groups linked to another hydrocarbyl group.

The entity may contain halo groups - such as fluoro, chloro, bromo or iodo groups.
The entity may contain one or more of alkyl, alkoxy, alkenyl, alkylene and alkenylene groups – which may be unbranched- or branched-chain.

The agent of the present invention may have known therapeutic properties and a reduced, diminished or decreased activity towards an electrical property of a membranous sample – such as an ion channel *eg.* a hERG ion channel.

From a drug safety perspective, the ideal position is for compounds not to interact with ion channels – such as hERG.

Accordingly, the agent of the present invention may have known therapeutic properties and have no or substantially no activity towards an electrical property of a membranous sample – such as an ion channel *eg.* hERG.

As will be appreciated by a skilled person, all drugs require reduced hERG activity. In this regard, there is extreme regulatory pressure to reduce the likelihood of drug-induced long QT syndrome. Part of this will be showing potency of candidate drugs at hERG channels before administration to humans (for draft ICH guidelines see http://www.fda.gov/cber/gdlns/ichqt.pdf sections 3.2.1 and 3.3).

Drugs that have been withdrawn/suspended from market due to an unacceptable risk of Torsade de Pointes (TdP) for the condition being treated include astemizole, cisapride, droperidol, grepafloxacin, levomethadyl, prenylamine, terfenadine, terodiline.

Measurable incidence of TdP in humans has been reported with include aprindine, bepridil, chlorpromazine, flecainide, halofantrine, haloperidol, lidoflazine, maprotiline, pentamidine, pimozide and thioridazine.

TdP in humans has been reported in isolated cases with amantadine, amitriptyline, chloral hydrate, chloroquine, ciprofloxacin, clarithromycin, cocaine, desipramine, diphenhydramine, domperidone, doxepin, erythromycin i.v., fexofenadine, fluoxetine, furosemide, imipramine, ketanserin, mexiletine, mibefradil, nifedipine, papaverine, perhexiline, probucol, propafenone, sparfloxacin, spiramycin, sultopride, tacrolimus and zimeldine.

Thus, by way of example, the agent may be a modified form of astemizole, cisapride, droperidol, grepafloxacin, levomethadyl, prenylamine, terfenadine, terodiline, aprindine, bepridil, chlorpromazine, flecainide, halofantrine, haloperidol, lidoflazine, maprotiline, pentamidine, pimozide and thioridazine, amantadine, amitriptyline, chloral hydrate, chloroquine, ciprofloxacin, clarithromycin, cocaine, desipramine, diphenhydramine, domperidone, doxepin, erythromycin i.v., fexofenadine, fluoxetine, furosemide, imipramine, ketanserin, mexiletine, mibefradil, nifedipine, papaverine, perhexiline, probucol, propafenone, sparfloxacin, spiramycin, sultopride, tacrolimus or zimeldine with a reduced hERG ion channel activity – such as no or substantially no hERG ion channel activity.

PHARMACEUTICAL SALT

The agent may be administered in the form of a pharmaceutically acceptable salt.

Pharmaceutically acceptable salts are well known to those skilled in the art, and for example include those mentioned by Berge *et al*, in J. Pharm. Sci., 66, 1-19 (1977). Suitable acid addition salts are formed from acids which form non-toxic salts and include the hydrochloride, hydrobromide, hydroiodide, nitrate, sulphate, bisulphate, phosphate, hydrogenphosphate, acetate, trifluoroacetate, gluconate, lactate, salicylate, citrate, tartrate, ascorbate, succinate, maleate, fumarate, gluconate, formate, benzoate, methanesulphonate, ethanesulphonate, benzenesulphonate and p-toluenesulphonate salts.

When one or more acidic moieties are present, suitable pharmaceutically acceptable base addition salts can be formed from bases, which form non-toxic salts and include the

aluminium, calcium, lithium, magnesium, potassium, sodium, zinc, and ammonium salts such as diethanolammonium, salts.

A pharmaceutically acceptable salt of an agent may be readily prepared by mixing together solutions of an agent and the desired acid or base, as appropriate. The salt may precipitate from solution and be collected by filtration or may be recovered by evaporation of the solvent.

An agent may contain one or more asymmetric carbon atoms and therefore exist in two or more stereoisomeric forms. Where an agent contains an alkenyl or alkenylene group, cis (E) and trans (Z) isomerism may also occur. The present invention includes the individual stereoisomers of an agent and, where appropriate, the individual tautomeric forms thereof, together with mixtures thereof.

Separation of diastereoisomers or *cis*- and *trans*-isomers may be achieved by conventional techniques, e.g., by fractional crystallisation, chromatography or H.P.L.C. of a stereoisomeric mixture of an agent or a suitable salt or derivative thereof. An individual enantiomer of an agent may also be prepared from a corresponding optically pure intermediate or by resolution, such as by H.P.L.C. of the corresponding racemate using a suitable chiral support or by fractional crystallisation of the diastereoisomeric salts formed by reaction of the corresponding racemate with a suitable optically active acid or base, as appropriate.

The present invention also encompasses all suitable isotopic variations of an agent or a pharmaceutically acceptable salt thereof. An isotopic variation of an agent or a pharmaceutically acceptable salt thereof is defined as one in which at least one atom is

replaced by an atom having the same atomic number but an atomic mass different from the atomic mass usually found in nature. Examples of isotopes that may be incorporated into an agent and pharmaceutically acceptable salts thereof include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorus, sulphur, fluorine and chlorine such as ^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{15}N, ^{17}O, ^{18}O, ^{31}P, ^{32}P, ^{35}S, ^{18}F and ^{36}Cl, respectively. Certain isotopic variations of an agent and pharmaceutically acceptable salts thereof, for example, those in which a radioactive isotope such as ^{3}H or ^{14}C is incorporated are useful in drug and/or substrate tissue distribution studies. Tritiated, i.e., ^{3}H, and carbon-14, i.e., ^{14}C, isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with isotopes such as deuterium, i.e., ^{2}H, may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements and hence may be preferred in some circumstances. Isotopic variations of an agent of the present invention and pharmaceutically acceptable salts thereof of this invention can generally be prepared by conventional procedures using appropriate isotopic variations of suitable reagents.

It will be appreciated by those skilled in the art that an agent may be derived from a prodrug. Examples of prodrugs include entities that have certain protected group(s) and which may not possess pharmacological activity as such, but may, in certain instances, be administered (such as orally or parenterally) and thereafter metabolised in the body to form an agent of the present invention which are pharmacologically active.

It will be further appreciated that certain moieties known as "pro-moieties", for example as described in "Design of Prodrugs" by H. Bundgaard, Elsevier, 1985 (the disclosured of which

is hereby incorporated by reference), may be placed on appropriate functionalities of agents. Such prodrugs are also included within the scope of the invention.

The present invention also includes the use of zwitterionic forms of an agent of the present invention. The terms used in the claims encompass one or more of the forms just mentioned.

SOLVATES

The present invention also includes the use of solvate forms of an agent.

PHARMACEUTICALLY ACTIVE SALT

An agent may be administered as a pharmaceutically acceptable salt.

Typically, a pharmaceutically acceptable salt may be readily prepared by using a desired acid or base, as appropriate. The salt may precipitate from solution and be collected by filtration or may be recovered by evaporation of the solvent.

CHEMICAL SYNTHESIS METHODS

An agent may be prepared by chemical synthesis techniques.

It will be apparent to those skilled in the art that sensitive functional groups may need to be protected and deprotected during synthesis of a compound of the invention. This may be achieved by conventional techniques, for example as described in "Protective Groups in Organic

Synthesis" by T W Greene and P G M Wuts, John Wiley and Sons Inc. (1999), and by P.J.Kocienski, in "Protecting Groups", Georg Thieme Verlag (1994).

It is possible during some of the reactions that any stereocentres present could, under certain conditions, be racemised, for example if a base is used in a reaction with a substrate having an optical centre comprising a base-sensitive group. This is possible during e.g., a guanylation step. It should be possible to circumvent potential problems such as this by choice of reaction sequence, conditions, reagents, protection/deprotection regimes, etc. as is well-known in the art.

The compounds and salts of the invention may be separated and purified by conventional methods.

Separation of diastereomers may be achieved by conventional techniques, e.g. by fractional crystallisation, chromatography or H.P.L.C. of a stereoisomeric mixture of a compound of formula (I) or a suitable salt or derivative thereof. An individual enantiomer of a compound of formula (I) may also be prepared from a corresponding optically pure intermediate or by resolution, such as by H.P.L.C. of the corresponding racemate using a suitable chiral support or by fractional crystallisation of the diastereomeric salts formed by reaction of the corresponding racemate with a suitably optically active acid or base.

An agent or variants, homologues, derivatives, fragments or mimetics thereof may be produced using chemical methods to synthesize an agent in whole or in part. For example, if they are peptides, then peptides may be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g.,

Creighton (1983) Proteins Structures And Molecular Principles, WH Freeman and Co, New York NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, *supra*).

Synthesis of peptide agents may be performed using various solid-phase techniques (Roberge JY *et al.* (1995) Science 269: 202-204) and automated synthesis may be achieved, for example, using the ABI 43 1 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer. Additionally, the amino acid sequences comprising an agent or any part thereof may be altered during direct synthesis and/or combined using chemical methods with a sequence from other subunits, or any part thereof, to produce a variant agent.

In an alternative embodiment of the invention, the coding sequence of a peptide agent (or variants, homologues, derivatives, fragments or mimetics thereof) may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al.* (1980) Nuc Acids Res Symp Ser 215-23, Horn T *et al.* (1980) Nuc Acids Res Symp Ser 225-232).

MIMETIC

As used herein, the term "mimetic" relates to any chemical, which includes, but is not limited to, a peptide, polypeptide, antibody or other organic chemical, which has the same qualitative activity or effect as a reference agent.

CHEMICAL DERIVATIVE

The term "derivative" or "derivatised" as used herein includes chemical modification of an agent. Illustrative of such chemical modifications would be replacement of hydrogen by a halo group, an alkyl group, an acyl group or an amino group.

CHEMICAL MODIFICATION

The chemical modification of an agent may either enhance or reduce hydrogen bonding interaction, charge interaction, hydrophobic interaction, Van Der Waals interaction or dipole interaction between the agent and the target.

In one aspect, the identified agent may act as a model (for example, a template) for the development of other compounds.

RECOMBINANT METHODS

An agent may be prepared by recombinant DNA techniques.

OTHER ACTIVE COMPONENTS

A composition may comprise other therapeutic substances in addition to the agent.

THERAPY

Agents identified by the method of the present invention may be used as therapeutic agents – i.e., in therapy applications.

As with the term "treatment", the term "therapy" includes curative effects, alleviation effects, and prophylactic effects.

The therapy may be on mammals such as humans or animals.

The therapy may be for treating disorders associated with dysfunctional ion channels – such as the development of LQT and/or torsade de pointes.

PHARMACEUTICAL COMPOSITIONS

Pharmaceutical compositions useful in the present invention may comprise a therapeutically effective amount of agent(s) and pharmaceutically acceptable carrier, diluent or excipient (including combinations thereof).

Pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent may be selected with regard to the intended route of administration and standard pharmaceutical practice. Pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s) or solubilising agent(s).

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, pharmaceutical compositions useful in the present invention may be formulated to be administered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be administered by a number of routes.

If an agent is a protein, then said protein may be prepared *in situ* in the subject being treated. In this respect, nucleotide sequences encoding said protein may be delivered by use of non-viral techniques (e.g., by use of liposomes) and/or viral techniques (e.g., by use of retroviral vectors) such that the said protein is expressed from said nucleotide sequence.

ADMINISTRATION

The components useful in the present invention may be administered alone but will generally be administered as a pharmaceutical composition – e.g., when the components are in admixture with a suitable pharmaceutical excipient, diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

DOSE LEVELS

Typically, a physician will determine the actual dosage, which will be most suitable for an individual subject. The specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age,

body weight, general health, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the individual undergoing therapy.

FORMULATION

The component(s) may be formulated into a pharmaceutical composition, such as by mixing with one or more of a suitable carrier, diluent or excipient, by using techniques that are known in the art.

HOST CELLS

As used herein, the term "host cell" refers to any cell that is of use in the present invention – such as cell comprising a recombinant ion channel.

Host cells may be transformed or transfected with a nucleotide sequence contained in a vector e.g. a cloning vector. Preferably said nucleotide sequence is carried in a vector for the replication and/or expression of the nucleotide sequence. The cells will be chosen to be compatible with the said vector and may, for example, be prokaryotic (for example bacterial), fungal, yeast or plant cells.

The gram-negative bacterium *E. coli* is widely used as a host for cloning nucleotide sequences. This organism is also widely used for heterologous nucleotide sequence expression. However, large amounts of heterologous protein tend to accumulate inside the cell. Subsequent purification of the desired protein from the bulk of *E. coli* intracellular proteins can sometimes be difficult.

In contrast to *E. coli*, bacteria from the genus Bacillus are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera Streptomyces and Pseudomonas.

Depending on the nature of the polynucleotide and/or the desirability for further processing of the expressed protein, eukaryotic hosts including yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because yeast cells are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (*e.g.* hyperglycosylation in yeast). In these instances, a different fungal host organism should be selected.

Preferably, the host cells are mammalian cells – such as CHO cells.

TRANSFECTION

Introduction of a vector into a host cell can be effected by various methods. For example, calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction or infection may be used. Such methods are described in many standard laboratory manuals - such as Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Host cells containing the expression vector can be selected by using, for example, G418 for cells transfected with an expression vector carrying a neomycin resistance selectable marker.

TRANSFORMATION

Teachings on the transformation of cells are well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

A host cell may be transformed with a nucleotide sequence. Host cells transformed with the nucleotide sequence may be cultured under conditions suitable for the replication or expression of the nucleotide sequence.

CONSTRUCTS

Nucleotide sequences – such as nucleotide sequences encoding ion channels - may be present in a construct.

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron

sequence including the Sh1-intron or the ADH intron, intermediate to the promoter and the nucleotide sequence. The same is true for the term "fused" which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type nucleotide sequence promoter and when they are both in their natural environment.

The construct may even contain or express a marker, which allows for the selection of the nucleotide sequence construct in, for example, a bacterium, preferably of the genus Bacillus, such as *Bacillus subtilis*, or plants into which it has been transferred. Various markers exist which may be used, for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - *e.g.* resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

VECTORS

Nucleotide sequence - such as nucleotide sequences encoding ion channels - may be present in a vector.

The term "vector" includes expression vectors and transformation vectors and shuttle vectors.

The term "transformation vector" means a construct capable of being transferred from one entity to another entity - which may be of the species or may be of a different species. If the construct is capable of being transferred from one species to another e.g. from an *E. coli* plasmid to a bacterium, such as of the genus Bacillus, then the transformation vector is

sometimes called a "shuttle vector". It may even be a construct capable of being transferred from an *E. coli* plasmid to an Agrobacterium to a plant.

The vectors may be transformed into a suitable host cell as described below to provide for expression of a polypeptide.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter.

KIT

In a further aspect, the present invention relates to a kit for performing the methods of the present invention comprising: (a) a first vessel comprising a seal phase internal solution according to the present invention; and (b) a second vessel comprising an access phase internal solution.

Preferably, the seal phase internal solution has a chloride ion concentration of about 40 mM. More preferably, wherein the seal phase internal solution comprises about 40mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, and about 20mM HEPES.

Preferably, the access phase internal solution has a chloride ion concentration of about 40 mM or more. More preferably, wherein the access phase internal solution comprises about 40mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising

agent or about 140mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent.

GENERAL RECOMBINANT DNA METHODOLOGY TECHNIQUES

The present invention employs, unless otherwise indicated, conventional techniques of molecular biology, microbiology, and recombinant DNA, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press. Each of these general texts is herein incorporated by reference.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1

Materials and Methods

Cell culture

CHOK1-hERG cells are maintained at 37°C in a humidified environment (5% CO_2) in the following medium: HAM'S F-12 + L-glutamine (SIGMA N-6658) supplemented with 10% FBS (SIGMA CR0848) and 0.6mg/ml Hygromycin (SIGMA H-5527). 15ml medium per T75 flask is routinely used.

CHO-Kv1.5 cells are maintained at 37°C in a humidified environment (5% CO_2) in the following medium: HAM'S F-12 + L-glutamine (SIGMA N-6658) supplemented with 10% FBS (SIGMA CR0848) and 0.5mg/ml Hygromycin (SIGMA H-5527). 15 ml medium per T75 flask is routinely used.

Cells should be split every 2-3 days at a dilution of 1:10 to 1:20.
For the purposes of experimental runs, cells should be split at a range of dilutions between 1:6 and 1:10. These values are meant as guidelines. If cells are split early or late in the day the range of dilutions should be altered accordingly in order to get a plate density of approx. 70% after 2 days incubation.

Molecular Devices IonWorks™HT system

i. Passaging protocol

Pre-warm Cell dissociation solution (Sigma C5789), PBS (Gibco 14040-091) and media prior to use (37°C, 30mins)

1) Pour off media and rinse cells in 5ml PBS.
2) Pipette off PBS and add 3ml Cell dissociation solution down side of flask.
3) Gently tilt flask to ensure all cell layer is covered in solution.
4) Incubate cells at 37°C for 5 minutes.
5) Gently tap flask to dislodge cells.
6) Add 5ml media and swill around the bottom of flask.
7) Pipette into a 15ml centrifuge tube and centrifuge at 1000rpm for 5mins.
8) Pour off supernatant and gently resuspend cells in 10mls media.
9) Add 1-2ml to a fresh T75 flask containing 15ml media for a 1:10-1:5 split respectively.
10) Return flasks to incubator.

ii Daily Protocol

1) Empty both waste bottles.
2) Turn on vacuum pump.
3) Power up IonWorks™HT unit.
4) Power up host computer.
5) Start IonWorks™HT software.
6) Check around the plenum for fluid traces. Dry off with a clean tissue.

7) Replace old patch plate and clamp into place. Apply vacuum and press down on either end of the clamp until no hissing can be heard.

8) To perform flush and rinse empty all source bottles and rinse in distilled water

9) Fill bottles accordingly:

Fluidics wash = PBS

Electrode wash = PBS

Internal solution = Potassium Gluconate buffer

Antibiotic solution = KCl buffer (2 inches will suffice)

10) Clean buffer boat with distilled water, ethanol and then in distilled water for a second time and dry with compressed air.

11) Replace buffer boat into the IonWorks™HT system and fill with 5-6mls fresh PBS.

12) When the green light is visible on the screen, under the utilities tab select 'Flush and Rinse'.

13) A dialog box will enquire if buffer has been placed in the buffer boat, Select yes.

14) After the flush and rinse, when the green light is visible on the screen, remove buffer boat and clean with distilled water, ethanol and again with distilled water before drying with compressed air.

15) On the computer, under the File tab select load protocol. Select required protocol from the list.

16) Select edit protocol and amend the name of the run. Check all of the other parameters are correct i.e. Plate type, incubation time, pulse protocol, etc.

III Cell preparation

Pre-warm Versene (Gibco 15040-033) prior to use (37°C, 30mins). PBS should be kept at room temperature.

1) Cells should be grown to 50-70% confluence in a T75 flask.
2) Pour off media and wash cells with 3ml Versene, gently tilting flask several times.
3) Pipette off Versene.
4) Add 1.8ml fresh Versene and tilt flask repeatedly to ensure complete coverage of the cell layer.
5) Incubate cells (37°C, 5mins).
6) Detach cells by gently tapping the flask twice.
7) Add 10ml PBS to the cell suspension and gently tilt flask to mix.
8) Transfer to a 15ml centrifuge tube.
9) Centrifuge cells (50g, 4mins); pipette off supernatant.
10) Very gently resuspend cells in 3ml PBS using a 10ml pipette.
11) Triturate five times to fully dissociate cells using a 1000µl pipette set at 200µl at a rate of one full stroke (up and down) per second. Position end of pipette half way up the fluid height.
12) Place cells on rocker.
13) Count cells and check cell viability using the Cedex cell counter (see Counting the Cells).
14) Determine correct dilution to obtain 250,000 cells/ml. Dilute in PBS.
15) Add 3ml cell suspension to a clean cell suspension boat in the IonWorks™HT system.

IV Counting the cells

1) Turn on the Cedex machine.

2) Turn on the computer.

3) Check all source and waste bottles.

4) Click on Liquid Management tab on bottom right of screen.

5) Select Prime (This will clean system and prime tube with Trypan Blue).

The machine is now ready to count cells.

6) Add 0.1ml cell suspension to 0.9ml PBS.

7) Pour cell mixture into a Cedex cup and click into place on the Cedex cell counter.

8) Click onto Microscope button.

9) If ready to count, click on traffic light symbol (Turns green).

10) Check counting parameters (Dilution 1:10, number of images = 10).

11) Click on second traffic light symbol (Turns green).

12) Machine will start up and prepare for counting.

13) Machine will ask again if you are ready to count the cells, if so click on Yes, if not click No.

14) Machine will automatically count the cells and assess their viability.

15) Determine the dilution required to obtain 250,000 cells/ml.

16) Dilute cells in fresh PBS.

v. Perforation agent

The perforation agent used is Amphotericin B (Sigma A4888).

1) Weigh out 25-30mg Amphotericin B into a 1.5ml Eppendorf tube.

2) Add 830µl DMSO to the tube and vortex immediately.

3) Place the tube in the sonicator until every trace of powder has gone.

4) Measure out 250ml freshly made Potassium Chloride buffer into a clean bottle.

5) Add the tube of Amphotericin B and shake the bottle vigorously for approx. 1min.

6) Add the Amphotericin B solution to the Antibiotic bottle in the IonWorks™HT.

VI Running the machine

1) Clean the buffer and cell boats with ethanol then water and dry with compressed air.

2) Add 5-6mls of fresh PBS to the buffer boat.

3) Replace the old patch plate with a new one and set vacuum switch to hold.

4) Add a compound plate if necessary. Always position with A1 in the bottom left hand corner.

4) Add the cell suspension to the cell boat.

5) Close the IonWorks™HT lid.

6) Click on the green light.

7) Select start.

8) The Run will now start.

9) When the green light appears at the end of the run it is safe to open the lid.

10) Remove the buffer boat and clean with distilled water, ethanol and water again. Dry with compressed air.

11) Replace buffer boat into unit and add 5-6mls of ethanol.

12) Close lid and when green light shows, go to Utilities, Wash pipettor.

13) Wait for the green light to appear before removing buffer boat and repeating the wash procedure.

VII Observations

1) Directly after starting the run, the vacuum pressure should be checked. On the digital dial the pressure should increase rapidly and settle at around 9.2 in. water within a minute.

2) After around 30 secs, the F-head will aspirate PBS from the buffer station and dispense it into the patch plate. It will then move to the wash station for cleaning.

3) The fluidics system will then start to prime the lines feeding the plenum with internal solution. (You should look for fluid in the upper de-bubbler. Initially it will fill to the height of the black clamp and then drop down to half this height. It will then move up and down as the fluid is debubbled). This takes around 2 mins.

4) After approx. 4 mins the E-head will take Hole Test recordings and move to the wash station for cleaning.

5) After 5 mins the F-head will aspirate cells from the cell boat and dispense them into the patch plate. The cells are then given a few minutes to reach and then seal at holes in the bottom of the patch plate.

6) The E-head will then take Seal Test recordings (after approx. 10 mins) and then move to the wash station for cleaning.

7) The Amphotericin is then introduced to the lines and switched for the internal solution under the plenum (you should see this in the de-bubbler). It will be cycled through the plenum in order to gain access to the cells. The fluid height in the debubbler should rest just above the inlet pipe.

8) After approx. 22 mins the E-head will start to take pre-compound recordings from half of the plate (groups 1-4).

9) The F-head will apply compound to the first half of the plate, from rows A-D of the compound plate, with washes in between each addition.

10) After 25 mins. the E-head will take post-compound recordings from the first half of the plate with washes in between each recording. It will then take pre-compound records from the remainder of the plate (groups 5-8).

11) The F-head will dispense compound into the remaining half of the plate, from rows E-H of the compound plate, with washes in between each addition.

12) After approx. 40 mins, the E-head will take post-compound recordings from the remaining half of the plate, with washes in between each recording.

A clean up sequence is carried out to wash both the E and F-head and drain all pipes of fluid.

Example 2

Internal solutions

The results in Table 1 show the effect of four combinations of different seal- and access-phase solutions *ie.* 40mM and 140mM KCl, 0 and 100mM K Gluconate.

The data show the effects on 3 parameters (seal resistance, seal number and % seals) measured at two different points in the assay - at the start - (seal test) and at the end (scan#2).

Higher seal resistances and higher seal numbers (and therefore %) are preferable as described herein.

If a poor number of seals are obtained at the start of the experiment, this cannot be significantly improved during the course of the experiment. However, a poor seal resistance at the start of the experiment can be improved during the course of the experiment.

Example 3

Optimisation of seal quality

For optimal seal quality, a seal-phase internal solution that is low in chloride (Cl^-) ions (low chloride ion seal-phase internal solution) is used.

The seal phase internal solution has the following composition:

40mM KCl, 100mM K gluconate, 3.2mM $MgCl_2$, 3.0mM EGTA and 5.0mM HEPES

Using a seal-phase internal solution that contains higher concentrations of chloride ion ions (high chloride ion seal-phase internal solution) results in a poorer seal quality.

Such a solution may have the following composition:

140mM KCl, 1.0mM $MgCl_2$, 1.0mM EGTA and 20mM HEPES

The optimal seal quality obtained using low chloride ion seal-phase internal solution can be maintained through the access-phase by using either low chloride ion seal-phase internal solution containing amphotericin B (low chloride ion access-phase internal solution) or high chloride ion seal-phase internal solution containing amphotericin B (high chloride ion access-phase internal solution) during the access phase.

The poor seal quality obtained using high chloride ion seal-phase internal solution is maintained through the access-phase by using high chloride ion access-phase internal solution during the access phase and cannot be improved by using low chloride ion access-phase internal solution during the access-phase.

Thus for optimal seal quality throughout the assay period, a low chloride ion seal-phase internal solution must be used during the seal-phase followed by either low chloride ion access-phase internal solution or high chloride ion access-phase internal solution during the access phase.

Example 4

Optimal potency estimates

When using low chloride ion access-phase internal solution during compound potency estimation, the potency (log of the concentration of compound in moles/litre (M) which inhibit the current magnitude by 50%; pIC_{50}) of compounds – such as the standard hERG channel blocking agents cisapride - is around 0.5 log units less than the pIC_{50} obtained using conventional voltage-clamp electrophysiological experiments.

Use of high chloride ion access-phase internal solution during compound potency estimation results in compound pIC_{50} estimates that are closer to the pIC_{50} values obtained using conventional voltage-clamp electrophysiological experiments.

Example 5

Obtaining both optimal seal quality and optimal potency estimate for non-lipophillic compounds

For both optimal seal quality throughout the assay period and optimal potency estimate for non-lipophillic compounds, a low chloride ion seal-phase internal solution is used during the seal-phase followed by high chloride ion access-phase internal solution during the access phase.

Example 6

Optimal potency estimates for lipophillic compounds

When using either low chloride ion access-phase internal solution or high chloride ion access-phase internal solution during compound potency estimation, the pIC_{50} estimates for some lipophillic compounds, notably the standard hERG channel blocking agents astemizole and pimozide, are up to 2 log units less than the pIC_{50} estimates obtained using conventional voltage-clamp electrophysiological experiments. The drop-off in potency is positively correlated to the measures of compound lipophillicity, ACDlogP, ClogP and % non-polar surface area.

Molecular Devices recommends the use of a cell suspension at $1x10^6$ cells per ml for addition to the individual wells of the PatchPlate (IonWorks HT Users Guide, molecular Devices Corp. 2002, Chapter 5, page 5-4. 3.5ul of this cell suspension is added to each well and the total final assay volume in the wells is 10.5 µl. Hence, each well will contain approximately 3500 cells in a volume of 10.5 µl buffer solution.

By reducing the cell number in each well, the pIC_{50} estimates for the lipophillic compounds can be surprisingly increased to a level comparable to those obtained in conventional voltage-clamp electrophysiology experiments. This indicates that the lipid of the cell membranes is acting as a 'sink' for lipophillic compounds. As the number of cells in the well increases, the concentration of lipid (cell membrane) in the well will also increase linearly giving a greater volume of lipid for compound to partition into and reducing the effective concentration in the well This causes the potency of these compounds to be underestimated. Reducing the cell number reduces the lipid:buffer ratio and hence the amount of compound that partitions into

the membrane. The potency estimate is thus closer to that obtained with conventional voltage-clamp electrophysiology experiments.

As the number of cells added to each well is reduced, the chances of obtaining a useable recording from a cell in that well is also reduced due to the statistically random nature of the sealing process. We have determined an optimum range for cell number where the number of usable seals obtained across the plate is high enough for meaningful analysis while maximising the pIC_{50} values of the lipophillic compounds. In a volume of 10.5 µl buffer per well the optimal range is 175-875 cells per well. The optimal cell number will vary depending on the volume of buffer added, since this will alter the cell number:buffer ratio. The optimal cell number:buffer ratio is in the range 17-83 cells per 1ul buffer.

Example 7

Effect of cell number

The data presented in Table 2 and Figures 1 and 3 shows the effect of cell number titration on potency.

The numbers on the Y axis are deviation from the pIC50 measured by conventional electrophysiology versus IonWorksHT (*i.e.* conventional pIC50 – IonworksHT pIC50).

Compounds which correlate well with conventional estimates should give values close to 0.

Compounds that give poor correlations and good correlations are used. The poor correlators can be improved by reducing cell number (see steep trendline moving from high to low cell number). This has a less pronounced effect in the good correlators.

FURTHER ASPECTS

In a further aspect, there is provided a method for determining the potency of a lipophillic agent comprising the steps of:

(a) providing an electrophysiological measurement apparatus;

(b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture using a reduced number of cells per compartment;

(c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration internal solution;

(d) exchanging the low ion concentration internal solution for an access phase internal solution;

(e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and

(f) taking electrophysiological recordings before and after incubation of the cell with one or more agents;

wherein a difference between:

(i) the electrophysiological recording in the presence of the agent; and

(i) the electrophysiological recordings in the absence of the agent

is indicative of the potency estimate for lipophillic agent.

Table 1. Effects of various combinations of seal- and access-phase internal solutions on seal resistance and seal number at the start (Seal test) and end (Scan#2) of a typical experiment using CHO/hERG cells.

Seal-phase internal solution	K+ gluconate	KCl	K+ gluconate	KCl
Access-phase internal solution	K+ gluconate	KCl	KCl	K+ gluconate
Seal test seal resistance (Mohms)	102	88	114	78
Seal test number of seals	301	264	312	258
Seal test % seals	78	69	81	67
Scan#2 seal resistance (Mohms)	137	132	106	89
Scan#2 number of seals	301	266	310	251
Scan#2 % seals	78	69	81	65

Table 2. Effect of cell number on potency (pIC_{50}) against hERG for 5 different hERG channel blockers. Potency was assessed using an 8 point dose-response curve on an IonWorksHT system. For comparison, the pIC_{50} value obtained using conventional, pipette-based electrophysiology recordings is also shown.

		cells / ml (x1000)				
compound	Conventional pIC_{50} value	100	250	1000	1750	2500
pimozide	7.4	7.2	7.0	6.7	6.7	6.6
astemizole	8.4	7.2	7.2	6.9	7.1	6.9
loratidine	5.1	5.3	5.1	5.1	5.0	4.9
terfenadine	7.3	6.5	6.5	6.2	6.2	6.1
cisapride	7.1	7.1	7.0	6.8	6.8	6.7

All publications mentioned in the above specification, and references cited in said publications, are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biology, molecular biology and electrophysiology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. An assay method for identifying an agent that modulates the electrical property of a membranous sample comprising the steps of:

(a) providing an electrophysiological measurement apparatus;

(b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture;

(c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration seal phase internal solution;

(d) exchanging the low ion concentration seal phase internal solution for an access phase internal solution;

(e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and

(f) taking electrophysiological recordings before and after incubation of the cell with one or more agents;

wherein a difference between:

(ii) the electrophysiological recording in the presence of the agent; and

(iii) the electrophysiological recording in the absence of the agent

is indicative of an agent that modulates the electrical property of a membranous sample.

2. An assay method according to claim 1, comprising the step of exchanging the low ion concentration seal phase internal solution for a low or a high ion concentration access phase internal solution.

3. An assay method according to claim 1, comprising the step of exchanging the low ion concentration seal phase internal solution for a high ion concentration access phase internal solution.

4. An assay method according to claim 2, wherein the agent is a lipophillic agent.

5. The assay method according to claim 4, wherein the lipophillic agent is astemizole or pimozide.

6. The assay method according to any of the preceding claims, wherein the cell number in each compartment of the electrophysiological measurement apparatus containing an aperture contains from 1-900 cells in a volume of 10.5 µl buffer solution.

7. The assay method according to any of the preceding claims, wherein the membranous samples comprises an ion channel.

8. The assay method according to claim 7, wherein the ion channel is a myocardial I_{Kr} ion channel.

9. The assay method according to claim 8, wherein the myocardial I_{Kr} ion channel is encoded by ERG, including hERG.

10. The assay method according to any of the preceding claims, wherein the electrophysiological measurement apparatus is a Molecular Devices IonWorks™HT or an Axon Instruments PatchExpress.

11. A method for maintaining the optimum seal quality of a compartment in an electrophysiological measurement apparatus comprising the steps of:

(a) providing an electrophysiological measurement apparatus;

(b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture;

(c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration seal phase internal solution; and

(d) exchanging the low ion concentration seal phase internal solution for an access phase internal solution.

12. A method according to claim 11, wherein the access phase internal solution is a low or high ion concentration solution.

13. A method for maintaining optimum seal quality and determining the potency of a non-lipophillic agent that modulates the electrical property of a membranous sample comprising the steps of:

(a) providing an electrophysiological measurement apparatus;

(b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture;

(c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration seal phase internal solution;

(d) exchanging the low ion concentration seal phase internal solution for a high ion concentration access phase internal solution;

(e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and

(f) taking electrophysiological recordings before and after incubation of the cell with one or more agents;

wherein a difference between:

(i) the electrophysiological recording in the presence of the agent; and

(ii) the electrophysiological recordings in the absence of the agent

is indicative of the potency estimate for the non-lipophillic agent.

14. A method for determining the potency of an agent comprising the steps of:

(a) providing an electrophysiological measurement apparatus;

(b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture using a reduced number of cells per compartment;

(c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration internal solution;

(d) exchanging the low ion concentration internal solution for an access phase internal solution;

(e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and

(f) taking electrophysiological recordings before and after incubation of the cell with one or more agents.

15. An assay method for determining the potency of an agent comprising the steps of:

(a) providing an electrophysiological measurement apparatus;

(b) contacting one or more membranous samples with a compartment of the electrophysiological measurement apparatus containing an aperture using a reduced number of cells per compartment;

(c) forming a seal between the membranous samples and the aperture in the presence of a low ion concentration internal solution;

(d) exchanging the low ion concentration internal solution for an access phase internal solution;

(e) establishing electrical access, via the aperture, to the internal compartment of the membranous sample; and

(f) taking electrophysiological recordings before and after incubation of the cell with one or more agents;

wherein a difference between:

(i) the electrophysiological recording in the presence of the agent; and

(ii) the electrophysiological recordings in the absence of the agent

is indicative of the potency estimate for the agent.

16. The method according to claim 14 or claim 15, wherein the number of membranous samples in each compartment is modulated such that the pIC_{50} value is at a level comparable to that obtained in conventional voltage clamp electrophysiology apparatus.

17. The method according to any of claims 14-16, wherein the low ion concentration internal solution is a seal phase internal solution.

18. The method according to any of claims 14-17, wherein the low ion concentration internal solution is exchanged for a low or a high ion concentration access phase internal solution.

19. The method according to any of claims 14-18, wherein the cell number in each compartment of the electrophysiological measurement apparatus containing an aperture contains from 1-900 cells in a volume of 10.5 μl buffer solution.

20. A low ion concentration seal phase internal solution.

21. A low ion concentration seal phase internal solution according to claim 20, wherein the ion is a chloride ion.

22.A low ion concentration seal phase internal solution according to claim 20 or claim 21, wherein the ion is present at a concentration of about 40 mM.

23. A low ion concentration seal phase internal solution according to any of claims 20-22, wherein the solution comprises about 40mM KCl, about 100mM K gluconate, about 3.2 mM $MgCl_2$, about 3.0mM EGTA, and about 5.0mM HEPES.

24. A process comprising the steps of:

i) performing the assay method according to any one of claims 1 to 10;

ii) identifying an agent capable of modulating the electrical property of a membranous sample; and

iii) preparing a quantity of that agent.

25. A process comprising the steps of:

i) performing the assay according to any one of claims 1 to 10;

ii) identifying an agent capable of modulating the electrical property of a membranous sample;

iii) preparing a quantity of that agent; and

iv) preparing a pharmaceutical composition comprising that agent.

26. A process comprising the steps of:

i) performing the assay according to any one of claims 1 to 10;

ii) identifying an agent capable of modulating the electrical property of a membranous sample;

iii) modifying said agent; and

iv) preparing a pharmaceutical composition comprising said modified agent.

27. A pharmaceutical composition comprising an agent identified by the assay method of any one of claims 1 to 10 or the process of any one of claims 24 to 26 admixed with a pharmaceutically acceptable carrier, diluent, excipient or adjuvant and/or combinations thereof.

28. A process of preparing a pharmaceutical composition comprising admixing an agent identified by the assay method of any one of claims 1 to 10 or the process of any one of claims 24 to 26 with a pharmaceutically acceptable diluent, carrier, excipient or adjuvant and/or combinations thereof.

29. A method of treating a disease in a human or animal which method comprises administering to an individual an effective amount of a pharmaceutical composition comprising an agent identified by the assay method of any one of claims 1 to 10 or the process of any one of claims 24 to 26, wherein the agent is capable of modulating the disease and wherein said composition is optionally admixed with a pharmaceutically acceptable carrier, diluent excipient or adjuvant and/or combinations thereof.

30. A method according to claim 29 wherein said one or more agents are formulated into one or more compositions for use in medicine.

31. An agent identifiable, preferably identified by the assay method according to any one of claims 1 to 10.

32. An agent identifiable preferably identified by the assay method according to any one of claims 1 to 10 for use in the treatment and/or prevention of disease.

33. A kit for performing the assay method according to any of claims 1-10, or the method according to any of claims 11-19, comprising:

(a) a first vessel comprising a low ion concentration seal phase internal solution according to any of claims 20 to 23; and

(b) a second vessel comprising an access phase internal solution.

34. A kit according to claim 33, wherein the access phase internal solution has a chloride ion concentration of about 40 mM or about 140 mM.

35. A kit according to claim 33 or claim 34, wherein the access phase internal solution comprises about 40mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent.

36. A kit according to any of claims 33-35, wherein the access phase internal solution comprises about 140mM KCl, about 1.0mM $MgCl_2$, about 1.0mM EGTA, about 20mM HEPES and a permeabilising agent.

37. Use of a low concentration seal phase internal solution according to any of claims 20 to 23 for measuring an electrical property of a membranous sample.

38. Use of a seal phase internal solution according to any of claims 20 to 23 for identifying an agent that modulates the electrical property of a membranous sample.

39. Use of a seal phase internal solution according to any of claims 20 to 23 for maintaining the optimum seal quality of a compartment in an electrophysiological measurement apparatus.

40. Use of a seal phase internal solution according to any of claims 20 to 23 for determining the potency of an agent that modulates the electrical property of a membranous sample.

41. Use of a seal phase internal solution according to any of claims 20 to 23 for determining the potency of an agent.

Figure 1

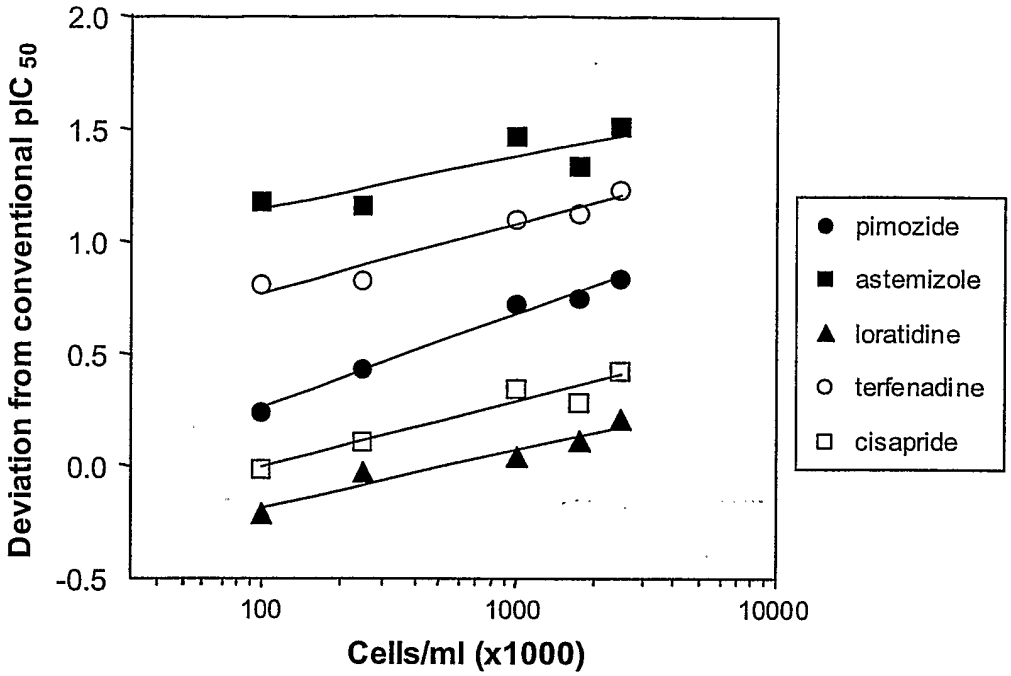

2.0
1.5
1.0
0.5
0.0
-0.5
Deviation from conventional pIC 50
100
1000
10000
Cells/ml (x1000)
pimozide
astemizole
loratidine
terfenadine
cisapride

Figure 2

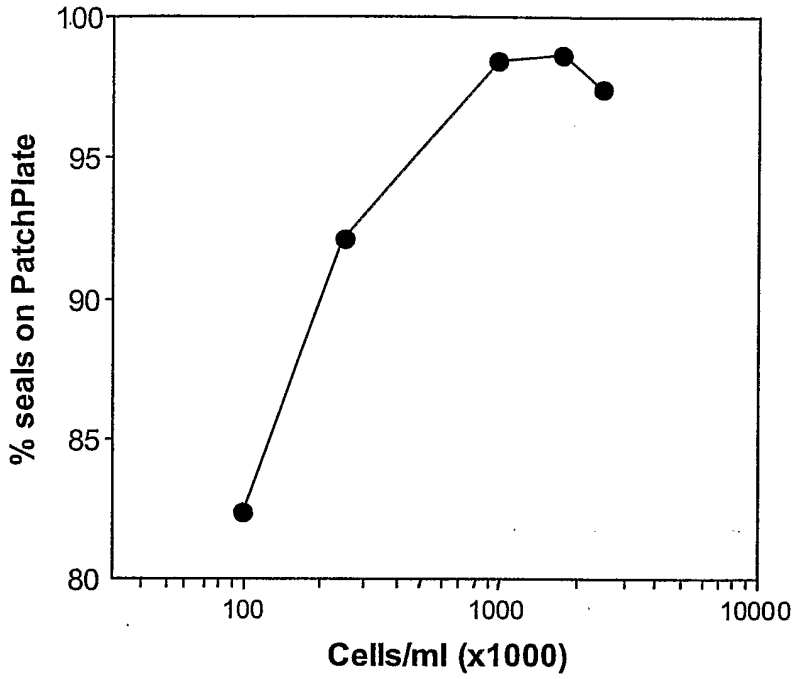
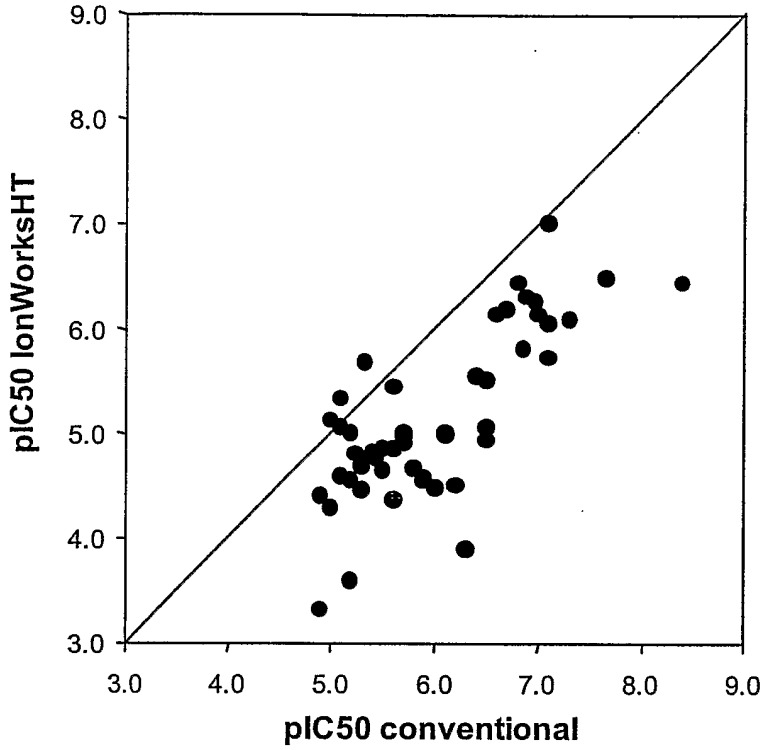
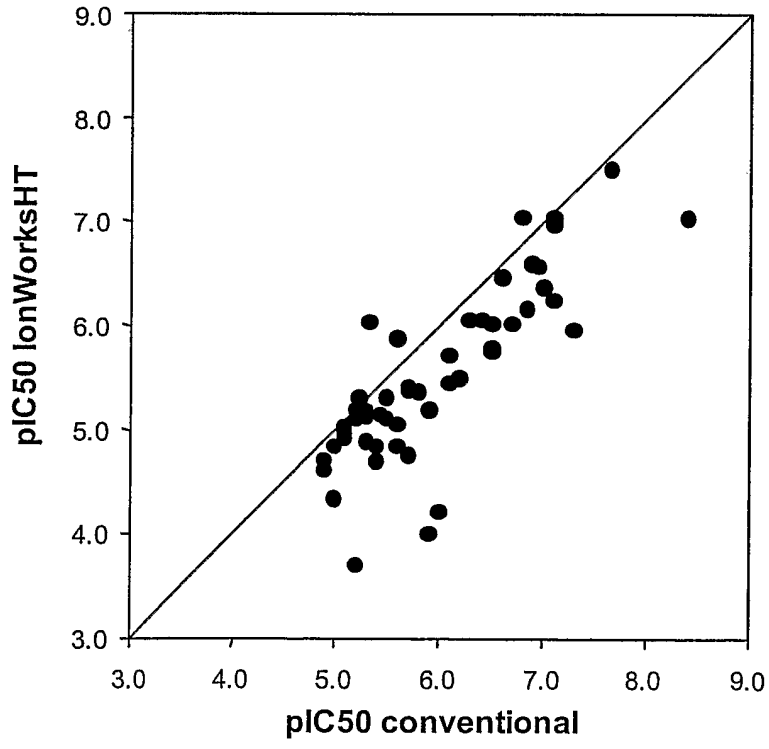

100
95
90
85
80
% seals on PatchPlate
100
1000
10000
Cells/ml (x1000)

Figure 3

a.


9.0
8.0
7.0
6.0
5.0
4.0
3.0
pIC50 IonWorksHT
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pIC50 conventional

b.


9.0
8.0
7.0
6.0
5.0
4.0
3.0
pIC50 IonWorksHT
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pIC50 conventional

c.

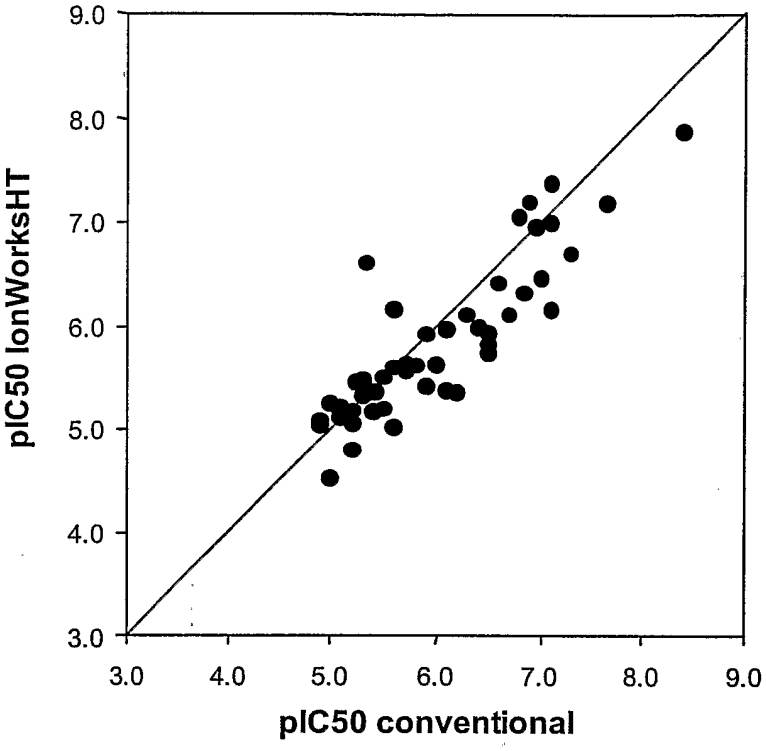
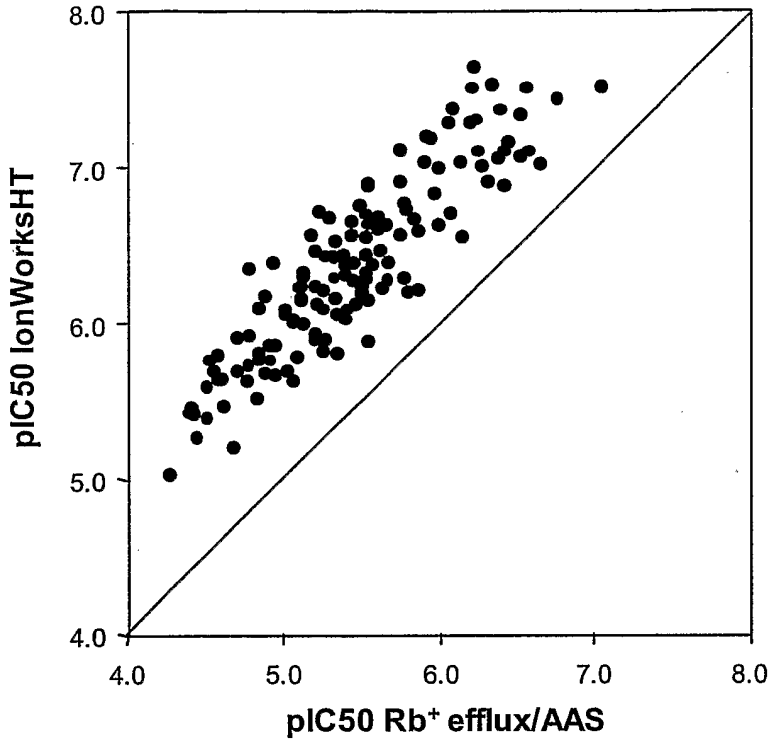

9.0
8.0
7.0
6.0
5.0
4.0
3.0
pIC50 IonWorksHT
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pIC50 conventional

Figure 4


8.0
7.0
6.0
5.0
4.0
pIC50 IonWorksHT
4.0
5.0
6.0
7.0
8.0
pIC50 Rb+ efflux/AAS

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2005/000239

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/487
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, se arch terms used)

EPO-INTERNAL, WPI DATA, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03021230 A2 (ESSEN INSTRUMENTS, INC), 13 March 2003 (13.03.2003), page 20 - page 21; page 38	1-2,11-12, 14-19,24-41
X	WO 0159447 A1 (YALE UNIVERSITY), 16 August 2001 (16.08.2001), page 8, line 9 - line 11	1-2,11-12, 14-19,24-41
A	US 20020053915 A1 (WEAVER, C D ET AL), 9 May 2002 (09.05.2002), paragraphs 0040, 0070-0073	1-2,11-12, 14-19,24-41
A	US 200300146091 A (VOGEL, H ET AL), 7 August 2003 (07.08.2003), paragraph [0212]	1-2,11-12, 14-19,24-41

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 July 2005	11 -07- 2005
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Sture Elnäs /OGU Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2005/000239

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02103354 A1 (SOPHION BIOSCIENCE A/S), 27 December 2002 (27.12.2002), page 9, line 15 - line 33	1-2,11-12, 14-19,24-41
A	WO 03104455 A1 (SOPHION BIOSCIENCE A/S), 18 December 2003 (18.12.2003), page 5, line 1 - line 16	1-2,11-12, 14-19,24-41
A	US 6063260 A (OLESEN, S ET AL), 16 May 2000 (16.05.2000), column 10; column 15; column 16	1-2,11-12, 14-19,24-41
A	US 20020164777 A1 (KELLY, J G ET AL), 7 November 2002 (07.11.2002), paragraph [0134]	1-2,11-12, 14-19,24-41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2005/000239

WO	03021230	A2	13/03/2003	EP	1434850	A	07/07/2004
				US	20030070923	A	17/04/2003
				EP	1405064	A	07/04/2004
WO	0159447	A1	16/08/2001	AU	3499601	A	20/08/2001
				EP	1257816	A	20/11/2002
				US	6699697	B	02/03/2004
				US	20020064841	A	30/05/2002
				US	20040168912	A	02/09/2004
US	20020053915	A1	09/05/2002	AU	7189801	A	21/01/2002
				CA	2413663	A	17/01/2002
				EP	1311848	A	21/05/2003
				IL	153545	D	00/00/0000
				JP	2004514417	T	20/05/2004
				MX	PA03000160	A	22/09/2003
				WO	0204943	A	17/01/2002
US	200300146091	A	07/08/2003	NONE			
WO	02103354	A1	27/12/2002	CN	1529814	A	15/09/2004
				EP	1397677	A	17/03/2004
				US	20040020773	A	05/02/2004
WO	03104455	A1	18/12/2003	AU	2003251124	A	00/00/0000
				CA	2488449	A	18/12/2003
				EP	1511843	A	09/03/2005
US	6063260	A	16/05/2000	AT	216080	T	15/04/2002
				AU	2788695	A	23/05/1996
				CA	2203886	A,C	09/05/1996
				DE	69526364	D,T	28/11/2002
				DK	788600	T	05/08/2002
				EP	0788600	A,B	13/08/1997
				SE	0788600	T3	
				ES	2171542	T	16/09/2002
				JP	10509794	T	22/09/1998
				PT	788600	T	31/07/2002
				SI	788600	T	30/06/2002
				US	6117291	A	12/09/2000
				WO	9613721	A	09/05/1996
US	20020164777	A1	07/11/2002	EP	1358474	A	05/11/2003
				JP	2004528539	T	16/09/2004
				WO	02065092	A	22/08/2002
				US	20020108869	A	15/08/2002

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/000239

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-41 (in part)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See extra sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-2,11-12,14-19,24-41

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/000239

Box II, 2.

The application does not fulfil the requirement of Article 5 PCT to clearly describe the invention. The description is extensive and contain for the inventive concept irrelevant information. The single general inventive concept of the 20 independent claims seems to solve the problem of an improved high throughput assay method for identifying agents that modulate ion channel activity. The solution is to isolate cells in the presence of a low ion concentration seal phase internal solution and subsequently exchanging the low concentration seal phase internal solution for an access internal solution (item c and d in claim 1). Remaining technical features are general knowledge or not common to all independent claims. The search is limited to the two specified technical features.

Box III

Á posteriori, the application will comprise six different groups of inventions.

The partial search covers the scope of the single general concept of all the independent claims, namely the possibility to exchange the seal phase internal solution for an access phase internal solution at electrophysiological recordings of an ion channel, where the seal phase solution is said to have low ion concentration, i.e. within the physiological range of a cell, and with this concept the unified first invention mentioned, claims 1-2. This group also covers claims 11-12, 14-19 and claims 24-41 (in part).

WO03021230 discloses a device and a method for performing measurements on ion channels. The document describes an exchange of different internal solutions for external solutions. (Page 20, page 21, especially lines 12-16. Page 38, lines 10-15) The concentration of the solution is the same as in a living cell.

In view of WO03021230, the technical feature and the concept mentioned are known. Hence, the application will comprise the following inventions that lack unity of invention:

II) Claims 3-6, directed to a high ion concentration of the access phase.
III) Claims 7-9, directed to a sample comprising an ion channel.
IV) Claim 10 directed to different instruments.
V) Claim 13, directed to measurement of nonlipophillic agent.
VI) Claims 20-23, directed to the seal phase internal solution.

The groups solve different problems. No features can be distinguished which can be considered as same or corresponding special technical features in the sense of Rule 13.2 PCT.

The inventions do not fulfil the requirement of PCT Rule 13.1 and 13.2 regarding stating a single general inventive concept or a special technical feature.

Accordingly, the application lacks unity of invention.