ASSAY and Drug Development Technologies
Volume 2, Number 5, 2004

Technology Review

Nonradioactive Rubidium Ion Efflux Assay and Its Applications in Drug Discovery and Development

Georg C. Terstappen

Abstract: The recent sequencing of the human genome has created comprehensive information of all potential drug targets. Based on current estimations for the total number of genes, around 400 pore-forming ion channel genes can be expected corresponding to about 1.3% of the human genome. Since many ion channels are involved in diseases and the currently marketed drugs act only on a small fraction of these pore-forming membrane proteins, there is a big opportunity for innovative ion channel drug discovery. In fact, recent advances in the development of functional ion channel assays are currently enabling a more systematic exploitation of this important target class. In particular, fluorescence-based methods, automated electrophysiology, and ion flux assays are most important in this regard. This article will briefly describe these methods focusing on the nonradioactive Rb^+ efflux assay that I developed in the early 1990s since it has found widespread application in drug discovery and development and greatly displaced $^{86}Rb^+$ assays for the analysis of K^+ and nonselective cation channels in the pharmaceutical industry.

Ion Channel Assays and Screening

SINCE RATIONAL DRUG DESIGN is still in its infancy, the motor of innovative drug discovery programs is typically represented by screening activities with the aim of identifying chemical molecules that modulate the drug target under study.[1] The number of chemical entities to be tested is high, and usually random HTS of the whole corporate compound collection (hundreds of thousands of chemical molecules) is the strategy of choice in pharmaceutical companies in order to identify chemical entry points for subsequent lead optimization and drug development. In order to keep pace with the ever-increasing number of compounds to be tested, biological assay systems ("screens") need to be developed that are amenable to HTS. Whereas such assays exist for target classes such as G protein-coupled receptors and enzymes, ion channel drug discovery is less developed because of technical difficulties in configuring such assays. In fact, the "gold standard" for functional analysis of ion channels, patch clamp electrophysiology,[2] requires hand-drawn glass capillary electrodes and highly skilled micromanipulation, thus allowing acquisition of only tens of data points per day. Recent advances in the development of functional ion channel assays are currently enabling the systematic exploitation of this important target class. Since activation of ion channels leads to a movement (flux) of charged molecular species across the cell membrane, a concomitant transient change in membrane potential is evoked. Both of these consequences of ion channel activation are being employed for the development of functional ion channel screening assays (Fig. 1).

Assays Measuring Membrane Potential

Changes in membrane potential that are an indirect consequence of ion channel activation can be measured

Sienabiotech S.p.A., Discovery Research, Siena, Italy.

ABBREVIATIONS: AAS, atomic absorption spectrometry; CHO, Chinese hamster ovary; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; FLIPR, fluorometric imaging plate reader; FRET, fluorescence resonance energy transfer; hERG, human *ether-a-go-go*-related gene; HTS, high-throughput screening; SAR, structure–activity relationship.

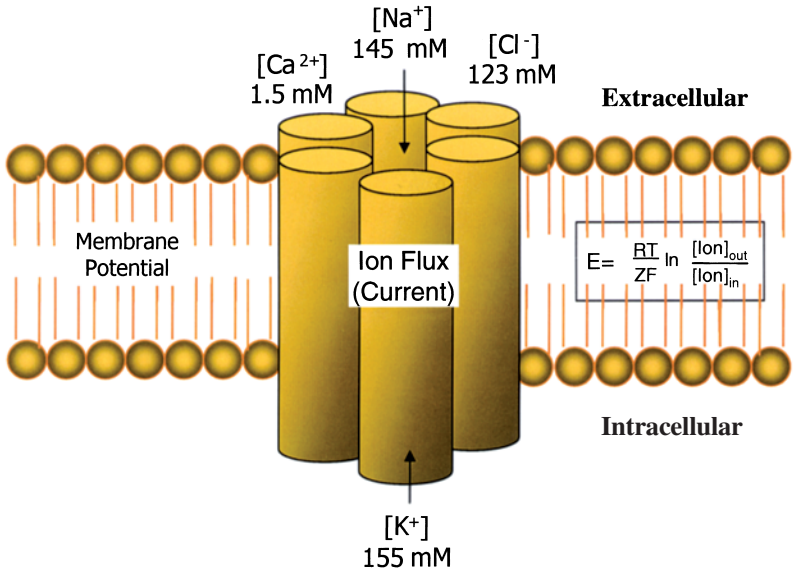

FIG. 1. Schematic representation of ion flux and its effect on membrane potential. Both properties, which are interconnected by the Nernst equation (shown in a simplified version), are exploited for ion channel assay development. For details refer to the text. Free intra- and extracellular ionic concentrations have been taken from Hille[3] and are only shown on the side of the membrane where the respective concentration is higher. Thus, the indicated arrows define the direction of ion flux upon channel activation. If K^+ is exchanged with Rb^+, channel activation will result in Rb^+ efflux.

using fluorescence probes such as the anionic oxonol $DiBAC_4(3)$, which partitions across the cell membrane according to the membrane potential.[4] Whereas cellular hyperpolarization leads to a net extrusion of the dye from the cells, depolarization results in an increase in cytoplasmic $DiBAC_4(3)$. Thus, determining changes in intracellular $DiBAC_4(3)$ fluorescence is a measure of ion channel activation that is independent from the channel's ion selectivity. In conjunction with appropriate instrumentation to measure fluorescence intensity (*e.g.*, FLIPR [Molecular Devices, Sunnyvale, CA])[5] this membrane-potential sensitive dye is widely used for ion channel HTS.[6,7] Since the slow response time of this dye, which is in the range of minutes, is a significant disadvantage, an FRET-based version[8] has been developed employing the modified oxonol $DiSBAC_2(3)$ as the FRET acceptor and the coumarin-linked phospholipid *N*-(6-chloro-7-hydroxycoumarin-3-carbonyl)-dimyristoylphosphatidylethanolamine, which attaches to the outer leaflet of the cell membrane and remains stationary, as the FRET donor.[9] In this case, hyperpolarization of cells leads to an increase in FRET within seconds, whereas depolarization has the opposite effect. In addition to the much faster response time, these FRET measurements are ratiometric since fluorescence intensities are measured at the emission maximum of donor and acceptor (460 and 570 nm, respectively) thus simultaneously eliminating some factors of variability (*e.g.*, differences in cell numbers and dye loading). For HTS purposes specialized instrumentation is necessary (e.g., voltage/ion probe reader VIPR™ [Aurora Biosciences, San Diego, CA]).[10] All of these cell-based assays represent indirect measures of ion channel activity and thus suffer from comparatively high numbers of "false-positive" compounds in HTS.

Assays Measuring Ion Flux

Classical patch clamp electrophysiology, which had revolutionized ion channel analysis in the past, enables a detailed real-time (milliseconds) characterization of any ion channel.[2] This "gold standard" technique, which is the most precise and information-rich method for measurement of ion flux (current), allows control of transcellular membrane potential by electronic negative-feedback circuits and thus the identification of state-dependent ion channel modulators. However, because of the very limited throughput of compounds that can be tested, this method is not amenable to HTS in drug discovery. Increasing the throughput of this technique is a highly active field of research (for overview, see special issue of *Receptors and Channels*,[11] 2003), and two main strategies are being pursued. In brief, the first develops automated robotic systems for voltage clamping of *Xenopus* oocytes or mammalian cells, and commercial systems are readily available. The second replaces traditional glass micropipettes (electrodes) by using planar micrometer-sized holes carrying arrays ("chips") as recording interfaces. The first such systems have been made available only recently. Whereas the automated patch clamping

systems increase throughput by a factor of about 10 compared to standard patch clamp technology, array-based recording interfaces were reported to increase throughput by 100–1,000-fold.[12] Although innovative electrophysiological patch clamp methods exhibit a greatly increased throughput, enabling the guidance of SAR studies for ion channel drug discovery, these methods are not (yet) compatible with HTS.

Radioactive isotopes of ions that pass through the channel under study can be used as tracers in cell-based assay systems. Such tracers can be radioactive isotopes of the naturally conducting ion species as in the case of $^{22}Na^{+}$[13] and $^{45}Ca^{2+}$[14] or other ion species that are conducted by the channel such as [^{14}C]guanidinium in the case of Na^+ channels[13] and $^{86}Rb^+$ in the case of K^+ and nonselective cation channels.[15] Depending on the channel, either influx or efflux is measured after channel activation. Since ion flux is a direct measure of channel activity, such assays are robust and less sensitive to disturbances. The main disadvantage is the use of radioisotopes and the associated safety and environmental hazards.

Other methods of measuring ion flux across membranes make use of ion-specific fluorescence indicators. Such probes exist for a variety of ions, including K^+, Na^+, and Cl^-,[16–18] but their use has generally been limited to measuring Ca^{2+} flux via Ca^{2+} channels or nonselective cation channels with conductivity for Ca^{2+} because of the availability of sensitive cell-permeable Ca^{2+} indicators with high binding affinities for this cation. In addition, the low intracellular Ca^{2+} concentration (~100 n*M*)[3] and the concomitant steep electrochemical gradient for this cation are favorable for assay development as well. Typically, cells are loaded with Ca^{2+}-specific fluorescence indicators such as Fura-2[19] and Fluo-3/4,[20] which change their emission characteristics upon Ca^{2+} binding following channel activation. In conjunction with specialized fluorescence plate reader instrumentation (*e.g.*, FLIPR) such assays have found widespread use in HTS.[21]

Nonradioactive Rb^+ Efflux Assay

In order to circumvent problems associated with the short-half life (18.65 days) and high-energy emission of radioactive ^{86}Rb (β_{max} 1.77 MeV; γ_{max} 1.08 MeV) and concomitant safety and environmental hazards I developed a nonradioactive Rb^+ efflux assay for the high-capacity analysis of native and recombinant ion channels in the early 1990s.[22] Rubidium is an alkali metal with atomic number 37 and an ionic radius of 1.61 Å that is not present in eukaryotic cells. Its similarity to K^+ leads to a high permeability in K^+ channels and also nonselective cation channels.[3] Moreover, it can easily be detected by using AAS ("flame photometry"). As a matter of fact the name derives from the Latin word *rubidus* (deep red), the color its salts impart to flames and led to its discovery in 1860/1861 by Bunsen and Kirchhoff. AAS is a well-established technology that uses thermal energy to generate free ground-state atoms in a vapor phase that absorb light of a specific wavelength (in the case of rubidium this is 780 nm). In practice, the atomization is achieved by spraying a sample into the flame of an atomic absorption spectrometer and measuring the absorption of light typically emitted by a hollow cathode lamp with a photomultiplier. Thus, an atomic absorption spectrometer can also be thought of as a photometer where the cuvette is replaced by a burner generating the flame, hence the name "flame photometry." In fact, the law of Lambert-Beer-Bouger applies and can be employed to determine the concentration of an element by measuring its absorption. In practice, however, this is usually accomplished by comparing the light absorption of a sample with a standard curve obtained under identical experimental conditions.

Experimental considerations

The original experimental protocol that I developed[22] was simple and is still used with or without minor modifications. It basically consists of two parts: cell biology and physical determination of the tracer rubidium by AAS. First, cells expressing the ion channel under study are cultured in microplates and loaded with rubidium by simply exchanging potassium in a cell-compatible buffer solution with the same concentration of rubidium (typically 5.4 m*M* RbCl). This loading phase, which is usually finished within 2–4 h, can be inhibited by the cardiac glycoside oubain, indicating the involvement of Na^+,K^+-ATPases in pumping Rb^+ into the cells. Prior to starting efflux experiments it is necessary to remove excessive RbCl, which is accomplished by a series of quick washing steps with buffer now containing 5.4 m*M* KCl. The frequency and buffer volumes of these washing steps mainly depend on the cell type, cell density, microplate formats, and washing devices used and should be optimized case by case since appropriate removal of excessive Rb^+ is essential for obtaining good signal-to-background ratios. Activation of the ion channel under study then leads to Rb^+ efflux into the cell supernatant due to the established concentration gradient for this tracer ion (see also Fig. 1). For voltage-gated potassium channels this can be accomplished by adding a depolarizing concentration of KCl (typically 50 m*M*) to the cells and for ligand-gated channels by adding the appropriate ligand (see the following section *Applications* in ion channel drug discovery). The incubation time with the channel opener has to be optimized empirically in order to achieve optimal efflux results, but in many cases a 10-

min period is sufficient. When compounds are tested for channel blocking effects they should be added prior to channel activation (*e.g.*, ≥10 min) because of kinetic considerations. Cell supernatants that contain the "effluxed" Rb^+ are then removed and collected as well as the lysed cells (by addition of 1% Triton X-100, for instance). Both of these Rb^+-containing matrices can be stored at room temperature prior to AAS analysis, which is not disturbed by cell debris. The relative amount of rubidium in the supernatant, calculated as [Rb in supernatant/(Rb in supernatant + Rb in cell lysate)], thus eliminating potential well-to-well differences in cell densities and Rb^+ loading, is a robust and direct measure of ion channel activity. For the configuration of good-quality HTS assays,[23] a twofold or greater increase of Rb^+ efflux upon channel activation over basal efflux levels is usually sufficient since the standard deviations for rubidium measurements by AAS are low.[22] It is worth mentioning that under highly standardized experimental conditions it might be possible to measure rubidium only in the supernatant in order to increase sample throughput. However, since this might compromise the quality of the screening assay it is very important to test its reliability under such conditions very carefully.

In principle, rubidium determination can be carried out with any type of flame AAS equipment. In the early 1990s I used an ATI Unicam 939 atomic absorption spectrometer equipped with an automatic sample diluting and autosampling device from Gilson carrying 10-ml test tubes, which allowed a throughput of about 200 samples/h. This throughput is clearly far from what is required for HTS. The recent development of an innovative AAS instrument for ion channel analysis (ICR 12000, Aurora Biomed, Inc., Vancouver, BC, Canada), featuring a sophisticated microsampling process utilizing 96- or 384-well microplates and simultaneous measurements of 12 samples at a time, is said to allow measurements of up to 60,000 samples per day (see: http://www.aurorabiomed.com/ICR12000.htm), thus making the nonradioactive Rb^+ efflux assay compatible with the throughput requirements of HTS.

Applications in ion channel drug discovery

Calcium-activated potassium channels. The initial ion channel targets used for the proof-of-concept experiments of this novel assay technology were small-conductance calcium-activated potassium (SK) channels, which belong to the 6TM/1P subclass of ion channels (Table 1). These channels were analyzed in PC-12 cells, which natively express all three SK channel members.[24] Channel activation in this pheochromocytoma cell line can either be achieved by direct Ca^{2+} "injection" with a calcium-specific ionophore such as A23187 or indirectly by activation of natively expressed L-type calcium channels with 50 m*M* K^+, which leads to an increase of the intracellular Ca^{2+} concentration. In either case a fourfold stimulation of Rb^+ efflux is evoked,[22] which can be blocked with the SK channel-specific ligand apamin, a peptide isolated from bee venom (IC_{50} = 10 n*M*).

Table 1. Published Examples of Ion Channels that Were Analyzed Employing the Nonradioactive Rb^+ Efflux Assay

Voltage-gated K^+ channels	*Ca^{2+}-activated K^+ channels*	*Ligand-gated nonselective cation channels*
Kv1.1	SK	nAChR
Kv1.3	BK	P2X
Kv1.4		
Kv1.5		
Kv7.2 (KCNQ2)		
Kv7.2/3 (KCNQ 2/3)		
Kv11 (hERG)		

For details refer to the text. nAChR = nicotinic acetylcholine receptor.

Another ion channel also belonging to the 6TM/1P subclass is the large-conductance calcium-activated (BK) channel (Table 1). Activation of this channel in recombinant CHO-K1 cells can easily be accomplished with the ionophore A23187, leading to a fivefold stimulation of Rb^+ efflux, which can be blocked with iberiotoxin (IC_{50} = 15 n*M*), a selective peptide ligand isolated from the scorpion *Buthus tamulus*.[22] More recently, scientists at Abbott Laboratories have developed a screening assay expressing BK channels in HEK293 cells.[25] The diphenylurea analogue NS1608, which in the mid-1990s was in preclinical development for cerebrovascular ischemia, was used to activate the channels, leading to a three- to fourfold increased Rb^+ efflux, which was completely blocked by iberiotoxin (IC_{50} = 12 n*M*). A pharmacological profile obtained with a set of known openers and blockers of BK channels compared very well with results from a conventional ^{86}Rb efflux assay,[25] demonstrating the utility of the nonradioactive Rb^+ efflux assay for HTS campaigns as well as SAR studies.

Voltage-gated potassium channels. Nonradioactive Rb^+ efflux assays were also developed for many voltage-gated potassium channels of the 6TM/1P subclass (Table 1). For Kv1.1 and Kv1.4 channels expressed in HEK293 cells, activation with 50 m*M* KCl led to a less than twofold increased Rb^+ efflux in 10 min, which was blocked by the nonspecific potassium channel blocker tetraethylammonium.[22] The comparatively low KCl-induced Rb^+ efflux of Kv1.1 and Kv1.4 channels, which was not sufficient to configure a robust screening assay, might be attributed to low expression levels and/or inactivation properties. More recently, researchers from Au-

rora Biomed configured a Kv1.3 assay using CHO-K1 cells where depolarization with 63 m*M* KCl led to a fourfold increased Rb^+ efflux in 15 min. This efflux could be blocked by agitoxin-2 (IC_{50} = 0.66 n*M*), which is a peptide isolated from the venom of the scorpion *Leiurus quinquestriatus* and a known blocker of this channel (see: http://www.aurorabiomed.com/New-Pro/CHO_Kv13_CellLine.pdf). Scientists from Merck Research Laboratories noted the generation of a Kv1.5-expressing cell line for the establishment of a Rb^+ efflux assay using 70 m*M* KCl for channel activation.[26] Researchers at AstraZeneca developed a screening assay for Kv7.2 (KCNQ2) leading to a fourfold increased Rb^+ efflux upon stimulation with 50 m*M* KCl.[27] These investigators also calculated Z' factors,[23] which were 0.73 for a 96-well plate format and 0.6 for a 384-well format, respectively, thus indicating the high suitability for screening and SAR studies.[27] Moreover, they also demonstrated that the pharmacological profile of Kv7.2 defined by electrophysiology was faithfully reflected by the Rb^+ efflux assay. Initially, this assay allowed measuring 1,000 data points/day in a 96-well plate format.[27] Subsequently, 15,360 data points/day were achieved in a 384-well plate format (40 384-well plates/8 h) using the ICR 12000.[28] For the identification of Kv7.2/3 (KCNQ2/3) channel modulators, scientists at Wyeth utilized a recombinant CHO-K1 cell line expressing these channels that they activated with 20 m*M* KCl. The calculated Z' value for the 96-well format was 0.8, and the throughput was about 250 compounds/week/full-time equivalent for obtaining EC_{50} values.[29]

Ligand-gated nonselective cation channels. Nonselective cation channels with conductivity for Rb^+ such as nicotinic acetylcholine receptors and purinergic P2X receptors (Table 1) led to three- and fourfold increased Rb^+ efflux when the ligand nicotine or ATP was used to activate these receptor channels in native PC-12 cells.[22] In the case of P2X receptors the specificity of channel activation was demonstrated by obtaining a limited pharmacological profile; the P2X receptor antagonist suramin (1 m*M*) blocked ATP-induced Rb^+ efflux completely, whereas the P2Y receptor agonist UTP (100 μM) did not elicit any Rb^+ efflux. Recently, scientists at Amgen described a Rb^+ efflux assay for a non-disclosed ligand-gated cation channel that was expressed in CHO-K1 cells.[30] Exposing the recombinant cells for 3 min with a non-disclosed agonist at 10 μM resulted in an about fourfold increased Rb^+ efflux. This efflux was blocked by a non-disclosed antagonist with an IC_{50} of 344 n*M*, which was in very good agreement with electrophysiological results. Initially, a large chemical library was screened using a luminescence assay format, and subsequently results were compared with a FLIPR and Rb^+ efflux assay. All three assay formats gave consistent results in terms of relative potencies for agonists and antagonists and thus can be used for screening and pharmacological characterization.[30]

Applications in drug development

The voltage-gated potassium channel Kv11, better known as hERG, has recently received much attention and gained additional importance since it seems to be particularly susceptible to inhibition by many xenobiotics and drugs, leading to potentially lethal arrhythmias.[31] In fact, several drugs have recently been withdrawn from the market because of hERG activity (the antihistamine terfenadine was the first such drug in 1998), and soon the safety of all new drugs will have to be tested accordingly. Thus, the pharmaceutical industry is extremely keen in identifying a potential "hERG liability" of novel compounds as early as possible in the research and development process, which needs functional assays that allow a certain throughput of compounds to be tested. In fact, at some pharmaceutical companies it was seriously discussed whether the whole corporate compound collection should be tested for hERG activity "upfront." Scientists at Aventis developed a nonradioactive Rb^+ efflux assay using hERG expressed in CHO-K1 cells (Table 1) and 50 m*M* KCl for channel activation.[32] After 10 min of activation an about twofold increased Rb^+ efflux was obtained. Although the signal-to-background ratio is comparatively low, a Z' value of 0.53 was calculated for a 96-well plate format, thus meeting HTS standards. A pharmacological characterization employing five known hERG blockers showed the same rank order as compared to results obtained with electrophysiology, although the absolute IC_{50} values were five- to 20-fold higher, indicating the suitability of the Rb^+ efflux assay for compound profiling. This hERG assay was also compared to fluorescence-based assays using $DiBAC_4(3)$ and FLIPR membrane potential, which did not allow ranking of compounds by their potency, generated higher numbers of "false-positive" compounds, and were less sensitive.[32] A similar recombinant cell line was also employed by researchers at AstraZeneca who measured a fourfold increased Rb^+ efflux after a 30-min incubation with 50 m*M* KCl.[28] These scientists configured the hERG assay in a 384-well plate format utilizing the ICR 12000 (Aurora Biomed) and calculated a Z' value of $\geq$0.5.

Conclusions

All of the described functional assays have individual advantages and disadvantages,[33–35] and their application largely depends on the ion channel under study and the number of compounds to be tested. They are widely applied in drug discovery and have already allowed the

screening of a broad range of ion channel targets.[36] The nonradioactive Rb^+ efflux assay has greatly replaced radioactive $^{86}Rb^+$ assays in the pharmaceutical industry, and the described examples represent only the published fraction (in the form of either articles in scientific journals or presentations at conferences) of its applications to date. Talking to representatives from the pharmaceutical industry at conferences and on various other occasions indicates that many other ion channels are currently being "screened" with this functional assay, thus contributing to innovative ion channel drug discovery that ultimately will lead to efficacious novel therapeutics.

References

1. Terstappen GC, Reggiani A: In silico research in drug discovery. *Trends Pharmacol Sci* 2001;22:23–26.
2. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch Eur J Physiol* 1981;391:85–100.
3. Hille B: *Ionic Channels of Excitable Membranes*, Sinauer Associates, Sunderland, MA, 1992.
4. Epps DE, Wolfe ML, Groppi V: Characterization of the steady-state and dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($Dibac_4(3)$) in model systems and cells. *Chem Phys Lipids* 1994;69:137–150.
5. Schroeder KS, Neagle BD: FLIPR: a new instrument for accurate high-throughput optical screening. *J Biomol Screen* 1996;1:75–80.
6. Terstappen GC, Pula G, Carignani C, Chen MX, Roncarati R: Pharmacological characterization of the human small conductance calcium-activated potassium channel hSK3 reveals sensitivity to tricyclic antidepressants and antipsychotic phenothiazines. *Neuropharmacology* 2001;40:772–783.
7. Whiteaker KL, Gopalakrishnan SM, Groebe D, Shieh CC, Warrior U, Burns DJ, Coghlan MJ, Scott VE, Gopalakrishnan M: Validation of FLIPR membrane potential dye for high throughput screening of potassium channel modulators. *J Biomol Screen* 2001;6:305–312.
8. Gonzalez JE, Tsien RY: Voltage sensing by fluorescence resonance energy transfer in single cells. *Biophys J* 1995;69:1272–1280.
9. Gonzalez JE, Tsien RY: Improved indicators of cell membrane potential that use fluorescence resonance energy. *Chem Biol* 1997;4:269–277.
10. Falconer M, Smith F, Surah-Narwal S, Congrave G, Liu Z, Hayter P, Ciaramella G, Keighley W, Haddock P, Waldron G, Sewing A: High-throughput screening for ion channel modulators. *J Biomol Screen* 2002;7:460–465.
11. Worley J III, ed.: *Receptors Channels* 2003;9:1–66.
12. Schroeder K, Neagle B, Trezise JD, Worley J: Ionworks HT: a new high-throughput electrophysiology measurement platform. *J Biomol Screen* 2003;8:50–64.
13. Willow M, Kuenzel EA, Catterall WA: Inhibition of voltage-sensitive sodium channels in neuroblastoma cells and synaptosomes by the anticonvulsant drugs diphenylhydantoin and carbamazepine. *Mol Pharmol* 1984;25:228–234.
14. Rasmussen CA, Sutko JL, Barry WH: Effects of ryanodine and caffeine on contractility, membrane voltage, and calcium exchange in cultured heart cells. *Circ Res* 1987;60:495–504.
15. Weir SW, Weston AH: The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. *Br J Pharmacol* 1986;88:121–128.
16. Kasner SE, Ganz MB: Regulation of intracellular potassium in mesangial cells: a fluorescence analysis using the dye PBFI. *Am J Physiol* 1992;262:462–467.
17. Minta A, Tsien RY: Fluorescent indicators for cytosolic sodium. *J Biol Chem* 1989;264:19449–19457.
18. Verkman AS, Sellers MC, Chao AC, Leung T, Ketcham R: Synthesis and characterization of improved chloride-sensitive fluorescent indicators for biological applications. *Anal Biochem* 1989;178:355–361.
19. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–3450.
20. Minta A, Kao JP, Tsien RY: Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem* 1989;264:8171–8178.
21. Mattheakis LC, Oehler LD: Seeing the light: calcium imaging in cells for drug discovery. *Drug Discov Today* 2000;1(HTS Suppl):15–19.
22. Terstappen GC: Functional analysis of native and recombinant ion channels using a high-capacity nonradioactive rubidium efflux assay. *Anal Biochem* 1999;272:149–155.
23. Zhang J-H, Chung TDY, Oldenburg KR: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67–73.
24. Terstappen GC: Nonradioactive rubidium efflux assay and emerging technologies in ion channel drug discovery. Presented at Ion Channel Retreat, Whistler, BC, Canada, 2003.
25. Parihar AS, Groebe DR, Scott VE, Feng J, Zhang X-F, Warrior U, Gopalakrishnan M, Shieh C-C: Functional analysis of large conductance Ca^{2+} activated K^+ channels: ion flux studies by atomic absorption spectrometry. *Assay Drug Dev Technol* 2003;1:647–654.
26. Karczewski J: Mapping the binding site for small molecule blockers of the Kv1.5 K^+ channel using high throughput technologies. Presented at Ion Channel Retreat, Vancouver, BC, Canada, 2004.
27. Scott CW, Wilkins DE, Trivedi S, Crankshaw DJ: A medium-throughput functional assay of KCNQ2 potassium channels using rubidium efflux and atomic absorption spectroscopy. *Anal Biochem* 2003;319:251–257.
28. Trivedi S: Nonradioactive ion flux assay using atomic absorption spectroscopy for identifying ion channel modulators. Presented at Ion Channel Retreat, Whistler, BC, Canada, 2003.
29. Wang K: Validation of an atomic absorption Rb^+ efflux assay for KCNQ2/3 channels (M-current) using the Ion Channel Reader (ICR) 8000. Presented at Ion Channel Retreat, Vancouver, BC, Canada, 2004.
30. Taylor B: Pharmacology of a ligand-gated cation channel: Rb^+ efflux versus fluorescence and luminescence. Presented at Ion Channel Retreat, Whistler, BC, Canada, 2003.
31. Vandenberg JI, Walker BW, Campbell TJ: HERK K^+ channels: friend and foe. *Trends Pharmacol Sci* 2001;22:240–246.
32. Tang W, Kang J, Wu X, Rampe D, Wang L, Shen H, Li Z, Dunnington D, Garyantes T: Development and evaluation of high throughput functional assay methods for hERG potassium channel. *J Biomol Screen* 2001;6:325–331.

33. Mattheakis LC, Savchenko A: Assay technologies for screening ion channel targets. *Curr Opin Drug Discov Dev* 2001;4:124–134.
34. Bennett PB, Guthrie HRE: Trends in ion channel drug discovery: advances in screening technologies. *Trends Biotechnol* 2003;21:563–569.
35. Gill S, Lee SS, Hesketh JC, Fedida D, Rezazadeh S, Stankovich L, Liang D: Flux assays in high throughput screening of ion channels in drug discovery. *Assay Drug Dev Technol* 2003;1:709–717.
36. Worley JF, Main MJ: An industrial perspective on utilizing functional ion channel assays for high throughput screening. *Recept Channels* 2002;8:269–282.

Address reprint requests to:
Dr. Georg C. Terstappen
Discovery Research
Sienabiotech S.p.A.
Via Fiorentina 1
53100 Siena
Italy

E-mail: gterstappen@sienabiotech.com