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Nonradioactive Rubidium Efflux Assay Technology for Screening of Ion Channels

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8.1 INTRODUCTION

Ion channels are pore-forming integral membrane proteins which enable the fast passage of ions across cell membranes. Their ion conductivity is typically highly specific and has been used for general classification

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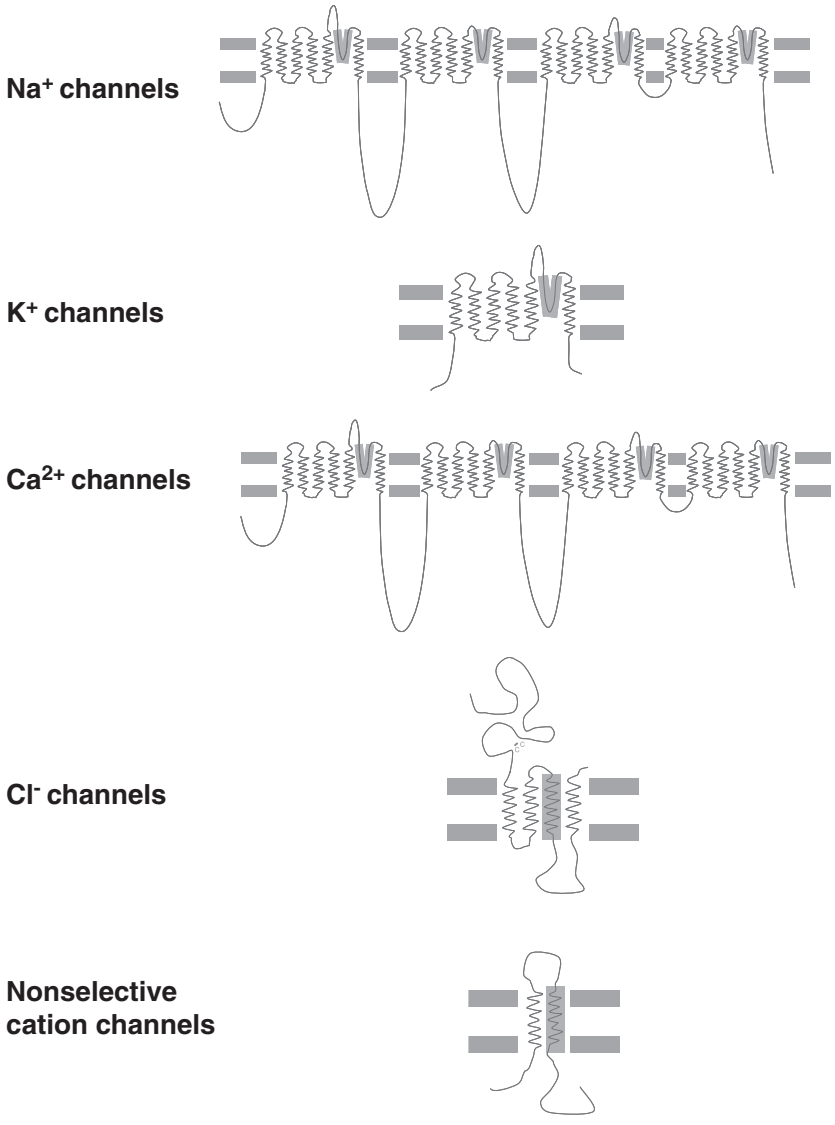

Figure 8.1 Schematic representation of different ion channel classes and their membrane topology. Adapted with permission from Elsevier. Copyright 2005.

into sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), chloride (Cl^-) and nonselective cation channels (Figure 8.1). As opposed to active transport by membrane pumps such as the Na^+/K^+ ATPase, ion channel proteins allow only passive transport of ions along a concentration gradient. The opening and closing ("gating") of ion channels is regulated

by a number of different stimuli, such as transmembrane voltage, ligand binding, mechanical stress and temperature. As the first two stimuli are the most common, these membrane proteins are broadly grouped into voltage-gated and ligand-gated ion channels. Ion channels are involved in many biological and disease processes and are particularly important for the regulation of electrical properties of excitable cells such as neurons and myocytes. In many other cell types they contribute to important physiological processes, such as hormonal secretion and blood pressure regulation, to name only two. Although ion channels represent a complex gene class, they are all characterized by a pore-forming region that determines ion selectivity and mediates ion flux across cell membranes.

The sequencing of the human genome has identified about 400 pore-forming ion channel genes, which corresponds to about 1.3% of the human genome (1). The pore-forming ion channel subunits comprise a minimum of two transmembrane domains (e.g., the inward rectifying K^+ channel Kir) and up to 24 transmembrane domains (e.g., the voltage-gated Na^+ and Ca^{2+} channels). Some of the K^+ channels even comprise two pore-forming regions in tandem. Functional ion channels often are homo- or heteromeric protein complexes that can co-assemble with accessory subunits, thus generating a vast number of physiological ion channel complexes with different functions and pharmacology.

8.2 ION CHANNELS AS DRUG TARGETS

Although a large number of disease relevant ion channels have been identified, drugs targeting ion channels constitute only approximately 7% of drugs currently on the market. The majority of these drugs block Ca^{2+} channels and have been registered for treatment of hypertension, angina pectoris and arrhythmia as main disease indications. Blockers of Na^+ channels are mainly prescribed for treatment of epilepsy, arrhythmia and depression. Activators of K^+ channels have major disease indications for cardiac failure and hypertension, and Cl^- channel activators for treatment of cystic fibrosis. Blockers of the nonselective cation channel 5-HT3 are mainly in use for the treatment of emesis and nausea, and K^+ channel blockers are prescribed for indications such as arrhythmia and noninsulin dependent diabetes. In 2000, ion channel drugs generated over $18 billion of world-wide revenues and constituted 10% of all prescription drug sales. Today, around 15% of the top 100 best selling drugs target ion channels. Examples are the Ca^{2+} channel blockers Norvasc and Cardizem, the 5-HT3 blocker Zofran and the Na^+ channel

blocker Lamotrigene. Since only a fraction of the ion channels have been explored as drug targets, there is great potential for novel ion channel therapeutics. In conjunction with novel assay technologies and structural and mechanistic insights into channel function, the development of selective and state dependent drugs is on the horizon.

8.3 ION CHANNEL ASSAYS AND SCREENING

With the rational design of ion channel modulators still in its infancy, the emphasis for ion channel drug discovery programs remains random or focused screening (2, 3). Whereas high throughput screening (HTS) assays are well established for target classes such as G-protein coupled receptors (GPCRs) and enzymes, ion channel drug discovery is less developed due to the technical difficulties in developing such assays. The "gold standard" for functional analysis of ion channels, patch clamp electrophysiology (4), traditionally required hand-drawn glass capillary electrodes and highly skilled micromanipulation, allowing acquisition of only tens of data points per day. Recent advances in the development of functional ion channel assays, however, are currently enabling a more 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 (Figure 8.2). Both of these consequences of ion channel activation are being employed for the development of functional ion channel screening assays (a review has been given elsewhere, 5).

8.4 NONRADIOACTIVE RUBIDIUM EFFLUX ASSAY BASED ON ATOMIC ABSORPTION SPECTROMETRY

To avoid problems caused by 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, in consequence, safety and environmental hazards (6), the author developed a nonradioactive Rb^+ efflux assay for the functional analysis of native and recombinant ion channels in the early 1990s when working at the Pharma Research Center of Bayer AG, Wuppertal, Germany (7). Rubidium is an alkali metal with atomic number 37 and an ionic radius of 1.61 Å which is not present in eukaryotic cells and tissues. Its close similarity to K^+ results in a high permeability in K^+ channels and

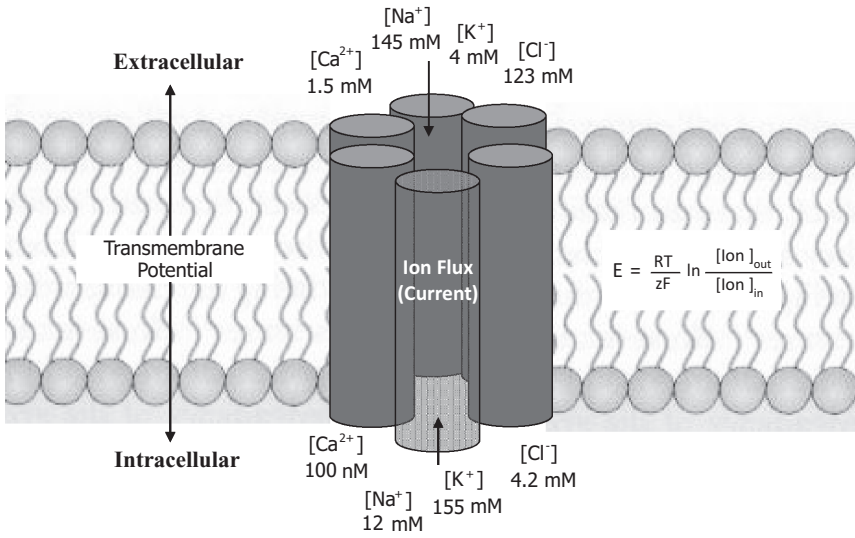

Figure 8.2 Schematic representation of an ion channel embedded in the cell membrane. Activation of the ion channels leads to flux of ions along their concentration gradients and concomitant changes in membrane potential, which can be quantified by the Nernst equation. Both of these aspects can be exploited for development of functional ion channel assays. Adapted with permission from Elsevier. Copyright 2005.

also nonselective cation channels (8). Importantly, it can easily be detected by using atomic absorption spectroscopy ("flame photometry") with a sensitivity (so-called "characteristic concentration") of 0.11 mg/l by measuring its light absorption at 780 nm. Interestingly, its name derives from the Latin word *rubidus*, which means deep red. This is the color its' salts impart to flames and which actually has led to its discovery in 1860/1861 by the German scientists Robert Bunsen and Gustav Kirchhoff.

Atomic absorption spectroscopy (AAS) is a well established technology traditionally employed for detection of trace elements in environmental, biological and medical samples. It uses thermal energy to generate free ground state atoms in a vapor phase that absorb light of a specific wavelength, which is 780 nm in the case of rubidium. In practice, atomization is usually achieved by spraying a sample into the flame of an atomic absorption spectrometer and measuring absorption of light – typically emitted by a hollow cathode lamp – with a photomultiplier (Figure 8.3). Thus, an atomic absorption spectrometer can also be imagined as a photometer where the *cuvette* is replaced by a burner generating the flame, hence the name "flame photometry". Although the law of

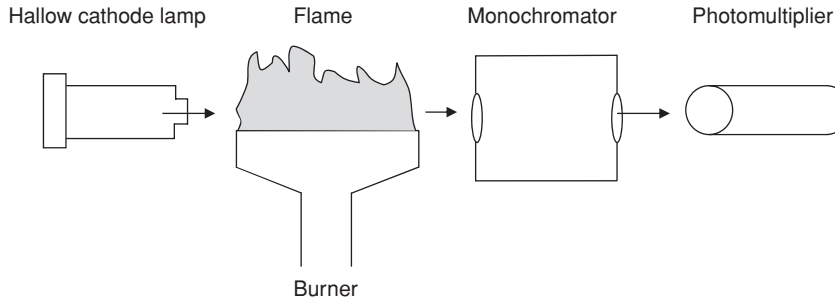

Figure 8.3 Schematic diagram of an atomic absorption spectrometer (AAS). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Lambert–Beer–Bouger applies and can be employed to determine the concentration of an element by measuring its absorption, in practice this is typically accomplished by comparing the light absorption of the sample with a standard curve obtained under identical experimental conditions.

The experimental protocol for a nonradioactive Rb^+ efflux assay comprises two parts, the first concerning cell culture and manipulation and the second concerning determination of the tracer rubidium by AAS (Figure 8.4). Thus, cells expressing the ion channel under investigation, either natively or recombinantly, are cultured in cell compatible microplates and loaded with the tracer rubidium by simply exchanging potassium in a cell compatible buffer solution with the same concentration

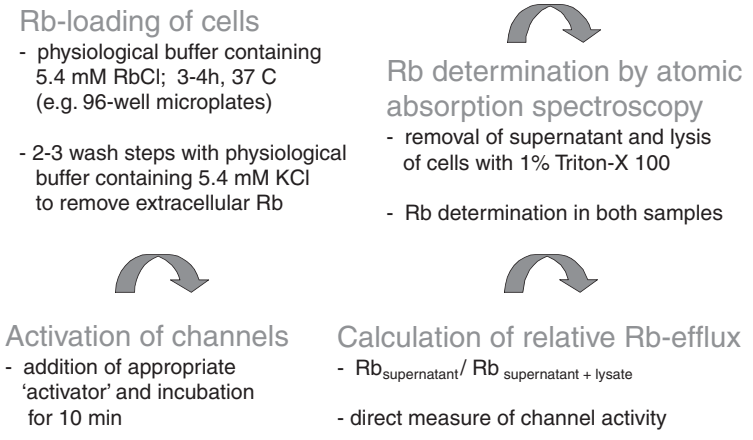

Figure 8.4 Schematic representation of the experimental workflow for the nonradioactive rubidium efflux assay.

of rubidium. This loading phase, which typically takes 2–4 hours, can be blocked by cardiac glycosides like oubain, as cellular Na^+/K^+-ATPases are mainly responsible for transporting Rb^+ into the cells. Prior to starting efflux experiments, excessive RbCl needs to be removed by a series of quick wash steps with isotonic buffer The frequency and buffer volumes necessary for these wash steps mainly depend on the cell type, cell density, microplate formats and washing devices used. They need to be optimized on a case-by-case basis, which is very important, as appropriate removal of excessive Rb^+ is essential for obtaining good signal-to-background ratios, and thus the specific signal window. Activation of the ion channel under investigation leads to Rb^+ efflux into the cell supernatant as a consequence of the established concentration gradient over the cell membrane for this tracer ion (Figure 8.2). Voltage-gated potassium channels can be activated by addition of a depolarizing concentration of KCl (typically $\geq$ 50 mM) to the cells, whereas ligand-gated channels (e.g., nicotinic acetylcholine receptors) are activated by addition of an appropriate concentration of the respective activating ligand (e.g., acetylcholine). Although it is highly recommendable to optimize the incubation time empirically on a case-by-case basis in order to achieve optimal efflux results, in most cases a period of 5–10 minutes was found to be sufficient. Compounds to be screened for channel blocking effects should be added prior to channel activation for at least 10 minutes because of kinetic considerations, but also this parameter should be optimized for the very channel under investigation. Cell supernatants which contain the "effluxed" Rb^+ are removed and collected and the remaining cells are lysed and collected as well. Both of these Rb^+ containing matrices can either be used directly for AAS analysis or stored at room temperature prior to rubidium determination, which is not disturbed by cell debris.

Although, in principle, rubidium determinations can be carried out with any high quality flame atomic absorption spectrometer, the development of innovative AAS instrumentation specifically for ion channel analysis (the Ion Channel Reader, ICR, series of instruments, Aurora Biomed Inc., Vancouver, Canada; http://www.aurorabiomed.com) has largely facilitated application of this assay technology over the last few years; this is also demonstrated by many published examples (Table 8.1) after the first description of this technology was published 10 years ago (7). In fact, this assay technology has been made compatible with the throughput requirements of HTS in drug discovery with the development of the ICR 12 000, which features a sophisticated microsampling process utilizing 96- or 384-well microplates and simultaneous

Table 8.1 Published examples of ion channels that were analysed employing nonradioactive Rb^+ efflux assay technology.

Voltage-gated K^+ channels	Ca^{2+}-activated K^+ channels	Ligand-gated nonselective cation channels
Kv1.1 (7)	SK (7)	nAChR (7)
Kv1.3 (9)	BK (7, 10, 11)	P2X (79)
Kv1.4 (7)		
Kv1.5 (12)		
KCNQ1/KCNE1 (mink) (13)		
Kv7.2 (KCNQ2) (14)		
Kv7.2/3 (KCNQ 2/3) (15)		
Kv11 (hERG) (16, 17)		
Kir2.1 (18)		
Kir6.2/SUR2A (19)		

measurements of 12 samples at a time (Figure 8.5). It is claimed that this system allows measurements of up to 60 000 samples per day (http://www.aurorabiomed.com).

To determine the Rb^+ efflux, the relative amount of rubidium in the supernatant is calculated as a fraction of the total rubidium [Rb in

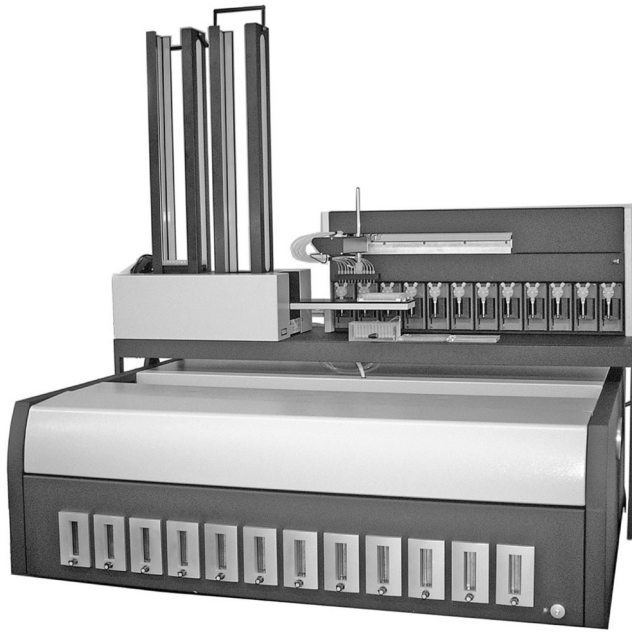

Figure 8.5 Photograph of the Ion Channel Reader 12000 for parallel rubidium efflux AAS measurements of ion channels.

supernatant / Rb in supernatant + Rb in cell lysate]. In this way, potential well-to-well differences in cell densities, cell loss during assay process and Rb^+ loading can be eliminated. This relative rubidium efflux is a robust and direct measure of ion channel activity, both of which are important features. In particular, the latter differentiates this assay technology from several other fluorescence-based HTS methods for ion channel drug discovery that measure indirect consequences of channel modulation based on membrane potential changes (5) and are thus much more prone to disturbances, resulting in a comparatively high "false–positive" rate. Typically, a more than twofold increase of Rb^+ efflux upon channel activation over basal efflux levels is sufficient for the development of good quality HTS assays (20), as standard deviations for rubidium measurements by AAS are low (7). If sample throughput needs to be further increased, under highly standardized experimental conditions it might be possible to measure rubidium in the supernatant only. However, since this might compromise the quality of the screening assay it is very important to test the reliability under these conditions very carefully on a case-by-case basis for the ion channel under investigation.

8.5 A TYPICAL ASSAY PROTOCOL

The following assay protocol for the analysis of calcium-activated SK channels was successfully used for screening of modulators of SK channels and can be employed using either recombinant HEK-293 cells stably expressing SK channels or PC-12 cells, which natively express three different SK channel members (SK1, SK2 and SK3). This protocol can also serve as a basis for the development of such efflux assays for other potassium and nonselective cation channels.

Cells are grown at 37 °C in cell culture-compatible microplates for 48 hours to a final cell density of about 1×10^4 cells per well of a 96-well plate for recombinant HEK-293 cells or 2×10^5 cells per well for PC-12 cells in a 24-well plate in standard cell culture medium. After aspirating the medium, 0.2 ml (or 0.5 ml for 24-well plates) cell buffer containing RbCl is added (5.4 mM RbCl, 150 mM NaCl, 2 mM $CaCl_2$, 0.8 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, 25 mM HEPES, pH 7.4) and cells are incubated for four hours at 37 °C. Cells are then quickly washed three times with buffer (the same as above, but containing 5.4 mM KCl instead of RbCl) to remove extracellular Rb^+. Subsequently, 0.2 ml buffer containing a saturating concentration of 10 μM thapsigargin (Figure 8.6) is added to the recombinant HEK-293 cells in order to

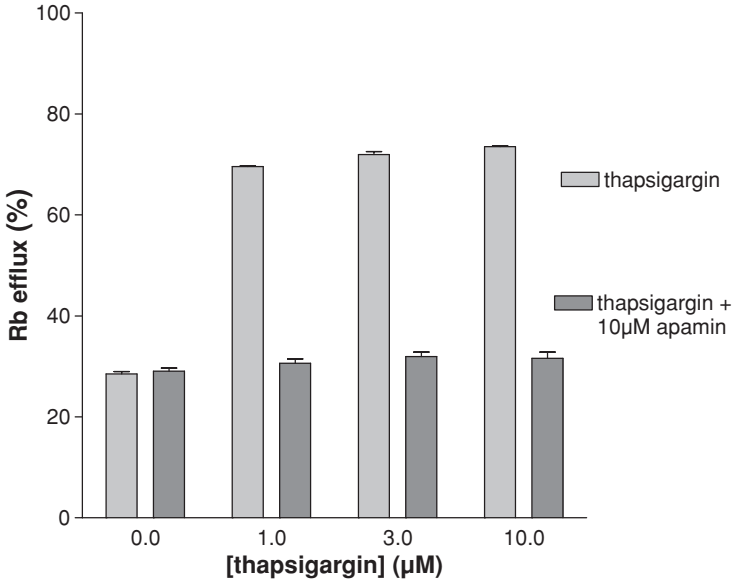

Figure 8.6 Activation of SK channels stably expressed in recombinant HEK293 cells using thapsigargin and its inhibition by the specific SK channel blocker apamin. (For details refer to the text.) Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

activate SK3 channels through release of intracellular Ca^{2+}, whereas 0.5 ml buffer containing 50 mM KCl is added to activate natively expressed SK channels in PC-12 cells (since SK channels also express voltage-sensitive L-type calcium channels, depolarization with KCl leads to calcium influx through these channels which in turn activates SK channels) (Figure 8.7). After incubation for 10 minutes the supernatant is carefully removed and collected for rubidium measurements. Cells are lysed by the addition of 0.2 ml (in the case of HEK-293 cells) or 0.5 ml (for PC-12 cells) 1% Triton X-100 and cell lysates are also collected for rubidium determinations. AAS measurements are carried out with a flame atomic absorption spectrometer. The stimulated relative Rb^+ efflux [Rb in supernatant / Rb in supernatant + Rb in cell lysate] with recombinant HEK-293 cells amounts to about 75% (Figure 8.6), while about 65% was obtained in the case of PC-12 cells (Figure 8.7). The specificity of the induced Rb^+ efflux is further demonstrated by the use of the specific SK channel blocking peptide apamin isolated from bee venom toxin (Figures 8.6 and 8.7) which blocks the channels in a concentration dependent manner with an IC_{50} of 10 nM (Figure 8.8).

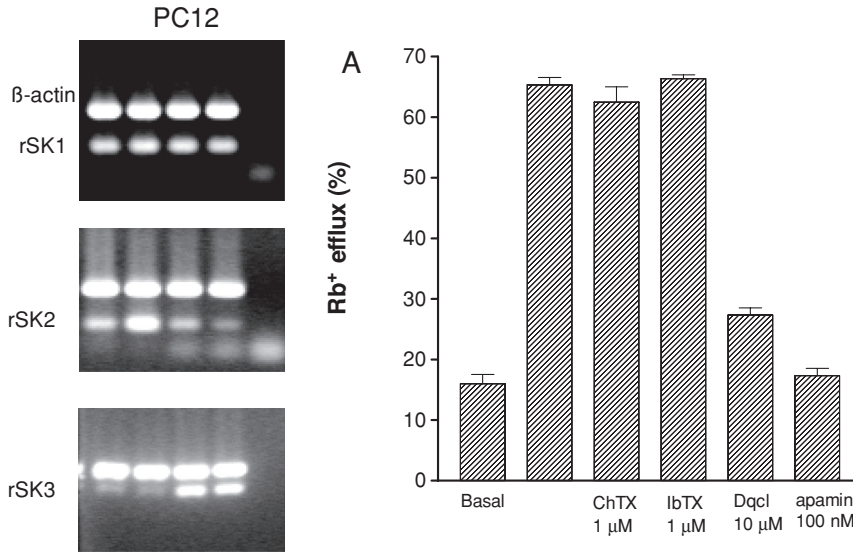

Figure 8.7 Activation of SK channels endogenously expressed in PC-12 cells. In the left-hand panel, RT-PCR with subtype-selective primers demonstrates presence of all three different SK channel members (SK1–3). In the right-hand panel, 50 mM KCl was used to activate SK channels in the presence of various blockers. Whereas the SK-specific blocker apamin completely abolishes channel activity, the BK blockers charybdotoxin (ChTX) and iberiotoxin (IbTX) have no effect. Dequalinium chloride (Dqcl), a nonspecific blocker of potassium channels, has only a weak effect. Adapted with permission from Elsevier. Copyright 1999.

If this protocol is used for the analysis of other potassium or nonselective cation channels, channel activation and specificity analysis have to be adapted on a case-by-case basis and the very ion channel under investigation.

8.6 CONCLUSIONS

After the original publication of the nonradioactive rubidium efflux assay in 1999, many pharmaceutical companies have implemented and used this assay technology in their screening cascades for ion channel drug discovery and/or general preclinical safety pharmacology tests, which include the hERG potassium channel (21). Published examples in the literature of such applications of the nonradioactive rubidium efflux assay technology comprise many different potassium and nonselective cation channels (Table 8.1). Moreover, this assay technology has also inspired others to investigate the Na^+/K^+-ATPase using rubidium and its uptake

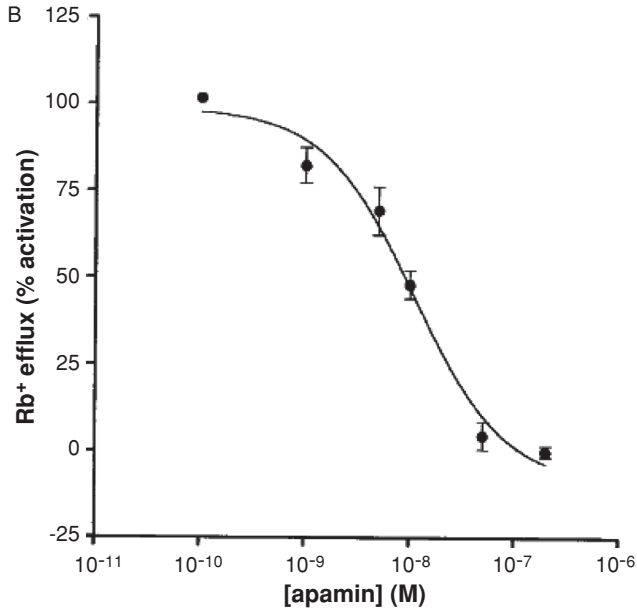

Figure 8.8 Application of the bee venom toxin peptide apamin leads to a concentration dependent block of SK channels endogenously expressed in PC-12 cells ($IC_{50} = 10\,nM$). Adapted with permission from Elsevier. Copyright 1999.

measured by AAS (22), chloride channels using silver and the formation of an AgCl complex measured by AAS (23), and sodium channels using lithium and its influx measured by AAS (24). Since functional ion flux assays with AAS as "readout" represent a direct measure of channel activity, they are robust and insensitive to disturbances. Compared to electrophysiological methods, which can be considered the "gold standard" for functional analysis of ion channels, their temporal resolution is limited to the seconds/minutes range and the membrane potential cannot be controlled precisely. Thus, these assays cannot be employed for screening of bona fide state dependent ion channel modulators. Nevertheless, owing to its ease, throughput and robustness the nonradioactive rubidium efflux assay technology has established itself as part of the standard repertoire of contemporary ion channel drug discovery.

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