J. Pharm. Pharmacol. 1994, 46: 731–734
Received August 26, 1993
Accepted January 27, 1994

Use of Cultured Human Neuroblastoma Cells in Rapid Discovery of the Voltage-gated Potassium-channel Blockers

JOYCE TORAL, WILLIAM HU, LYNDA YI, JAMES E. BARRETT, PATRICIA T. SOKOL AND M. REZA ZIAI

CV/CNS Research Section, Medical Research Division, American Cyanamid Company, Lederle Laboratories, Pearl River, New York 10965, USA

Abstract—Depolarization of human neuroblastoma cells by high concentrations of extracellular potassium ions, leads to the activation of the voltage-gated potassium channels. The activity of such potassium channels can be effectively and rapidly monitored by tracking the efflux of ^{86}Rb from pre-loaded target cells in response to the depolarizing stimulus. The inclusion of compounds with unknown activity in the assay medium, can result in the identification of novel blockers of the voltage-gated potassium channels. Since this functional assay is performed in 96-well microtitre plates, it represents a rapid and high-volume primary screening method for the detection and identification of the voltage-gated potassium-channel blockers, which may have therapeutic utility in several indications including memory degeneration and cardiac arrhythmias.

Potassium channels are integral membrane proteins of great molecular and functional diversity present in virtually all mammalian cells. In neurons they are primarily responsible for maintaining a negative resting membrane potential, as well as controlling membrane repolarization following an action potential. Depending on the sub-family to which a given potassium channel belongs, it can be activated by a change in the membrane potential, an increase in the intracellular concentration of Ca^{2+}, or binding of ligands to their receptors including acetylcholine, adrenaline, dopamine, galanin, calcitonin gene-related peptide, somatostatin, and ATP (Cook 1988; Rudy 1988; Halliwell 1990; Pfaffinger & Siegelbaum 1990; Daniel et al 1991). In addition, some potassium channels are activated by mechanical forces (e.g. the stretch-sensitive potassium channels), or by a change in intracellular pH (Davies 1990; Halliwell 1990). Some potassium channels are activated in response to a change in membrane potential. A shift to a more negative potential (hyperpolarization) activates a class of potassium channels known as the inward rectifier (Adams & Nonner 1990). A change in the membrane potential to a more positive value (depolarization), on the other hand, leads to the activation of the voltage-gated potassium channels including the fast transient currents (the A-type, I_A), delayed rectifiers (I_K), or the intermediate (IK_{Ca}) and large type calcium-activated potassium channels, BK_{Ca} (Rudy 1988; Adams & Nonner 1990).

The pharmacological identity of various voltage-gated potassium-channels is established by their sensitivity to standard compounds capable of blocking one or more types of potassium channels. These compounds, known as potassium-channel blockers, include tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP, as well as 2-AP and 3-AP), 3,4- and 2,3-diaminopyridine, $BaCl_2$, CsCl, strychnine, phencyclidine, pyridostigmine, 9-aminoacridine, DuP-996 (3,3-bis (4-pyridinylmethyl)-1-phenylindolin-2-one; linopiridine), clofilium, quinidine, aminoquinolines and quinine (Cook & Quast 1990; Atwal 1992; Lavretsky & Jarvik 1992; Harvey 1993).

This extensive molecular and functional diversity renders potassium channels a potential target of drug discovery for a variety of pathophysiological indications, including memory degeneration, stroke; epilepsy, multiple sclerosis, Huntington's chorea, anxiety, depression, psychosis, urinary incontinence, diabetes, asthma, premature labour, hypertension, cardiac ischaemia and arrhythmias (Cook 1988; Colatsky & Follmer 1989; Rosen 1991; Dunne & Petersoen 1991; Escande & Cavero 1992; Lavretsky & Jarvik 1992).

The availability of a functional assay with a considerable through-put is an indispensable part of any drug discovery program focusing on potassium-channel modulators. Such an assay should be able to screen hundreds of compounds on a routine basis.

In this report we describe a high-volume ^{86}Rb efflux assay in 96-well microtitre plates, and its application in detecting blockers of the voltage-gated potassium channels in cultured human neuroblastoma cells, TE671.

Materials and Methods

Buffers and reagents

The main buffer used was designated MOPS-PSS, pH 7·4 (NaCl 120 mM; KCl 7·0 mM; $CaCl_2$ 2·0 mM; $MgCl_2$ 1·0 mM; ouabain 10 μM; 4-morpholinepropanesulphonic acid, MOPS 20 mM).

Depolarizing solutions consisted of MOPS-PSS containing KCl (40, 80, or 100 mM) replacing the equivalent concentrations of NaCl.

Chemicals and peptide toxins were purchased from Sigma Chemical Co. (St Louis, MO) and Research Biochemicals Inc. (Natick, MA). Chemicals were dissolved at a stock concentration of 10–100 mM, either in MOPS-PSS or dimethylsulphoxide (DMSO), and were subsequently

Correspondence: M. R. Ziai, CV/CNS Research Section, Medical Research Division, American Cyanamid Company, Pearl River, New York 10965, USA.

diluted in the incubation buffer to the desired concentration. Peptide toxins were dissolved in MOPS-PSS containing bovine serum albumin (0·1% w/v) at 50–500 μM stock concentration.

Cell culture and ^{86}Rb loading

Human neuroblastoma cells TE671 were obtained from American Type Culture Collection (HTB 139) and were maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 4·5 g L^{-1} glucose and 2·0 mM L-glutamine. Cells were plated and loaded with ^{86}Rb in 96-well microtitre plates as described by Daniel et al (1991).

^{86}Rb efflux assay procedure

The growth medium in the microtitre plate was discarded by a sharp flicking of the plate. The adherent cell layer was washed three times with 200 μL MOPS-PSS using a 12-channel pipetter. The cells were incubated for 30 min at room temperature, either with 200 μL MOPS-PSS, or 20 μL of the depolarizing solutions, in the presence or absence of a potassium-channel blocker. Supernatant (150 μL) from each well was removed and counted. Cell layer was solubilized in 200 μL 0·1% Tween 20 in water and 150 μL was also counted in a Packard 2200 CA liquid scintillation counter. All supernatants were counted in 7·0 mL distilled water.

The percent efflux was calculated as follows:

$$\% \text{ total efflux} = \frac{(\text{counts min}^{-1} \text{ in supernatant})}{(\text{counts min}^{-1} \text{ in supernatant} + \text{counts min}^{-1} \text{ in cell extract})} \times 100 \quad (1)$$

and the value of percent net efflux was calculated as:

$$\% \text{ net efflux} = \% \text{ total efflux} - \% \text{ basal efflux} \quad (2)$$

where % total efflux is that induced by the depolarizing solution containing 100 mM KCl. The basal efflux is the efflux (leak) of ^{86}Rb observed in the physiological saline, MOPS-PSS.

Results and Discussion

We have previously characterized the ATP-dependent K^+-channels (K_{ATP}) in human neuroblastoma cells TE671 (Daniel et al 1991). We showed that this cell line has a typical glibenclamide-sensitive K_{ATP} which can be activated by a low intracellular ATP concentration, calcitonin gene-related peptide and some standard potassium-channel openers such as diazoxide.

Since there is no high-throughput functional assay currently available to discover modulators of the voltage-gated potassium channels, in this work, we extended our studies to the voltage-gated potassium channels present in TE671 cells.

Voltage-gated potassium channels are normally activated in response to a depolarizing stimulus such as a rise in the extracellular K^+ concentration, $[K^+]_{outside}$. Therefore, the initial step in the development of this functional assay was to determine the optimal KCl concentration capable of inducing a depolarization state and an efflux of ^{86}Rb.

Table 1. Blockade of ^{86}Rb efflux from TE671 cells by tetraethylammonium chloride (TEA).

	% total ^{86}Rb efflux	
Treatment	Without TEA	With 10 mM TEA
MOPS-PSS	20 ± 1·4 (0)*	21 ± 1·5 (0)
KCl 40 mM	25 ± 1·7 (5)	26 ± 1·8 (5)
80 mM	32 ± 2·3 (12)	33 ± 2·3 (12)
100 mM	34 ± 2·4 (14)	34 ± 2·4 (13)

* Percent net efflux shown in parentheses was calculated as described in the text.

Since each cell line appears to have a somewhat different $[K^+]_{outside}$ requirement for depolarization (Arner & Stallcup 1981), we studied the effect of three concentrations of $[K^+]_{outside}$ on ^{86}Rb efflux from TE671 cells. To this end, cells were loaded overnight with ^{86}Rb and the excess radioactivity was washed out. Cells were subsequently incubated for 30 min at room temperature (21°C) with the depolarizing solutions in the presence or absence of a series of standard potassium-channel blockers. The use of ouabain in the incubation buffers was intended to inhibit the Na^+-K^+ pump, which is responsible for the reuptake of ^{86}Rb by the target cells during subsequent incubation periods (Daniel et al 1991). Although ouabain itself can induce depolarization, leading to an increased ^{86}Rb efflux and contributing to the level of the basal efflux, it does not affect the value of the net efflux. In each assay, the latter value is derived by subtraction of the basal efflux from the total efflux. Following the incubation period, supernatants from the microtitre wells were collected and counted. The cell-bound radioactivity was also measured and percent efflux was calculated for each experiment.

The optimal time course for the efflux assay in TE671 cells was determined. The most suitable incubation time is the longest period during which ^{86}Rb continually exits the target cells through various potassium channels, while the background leak (basal efflux) of ^{86}Rb remains relatively small. For this assay, 20–30 min incubation at room temperature results in a suitable signal-to-noise ratio (results not shown).

Table 1 shows that raising $[K^+]_{outside}$ to 80 mM resulted in an increase in ^{86}Rb efflux from the basal level of 20% (in MOPS-PSS), to a depolarized state of 35%. No further increase in the net efflux was observed when KCl concentration was raised to 100 mM. TEA, a low-affinity blocker of the delayed rectifier and several other K^+-channels (Latorre &

Table 2. Blockade of ^{86}Rb efflux from TE671 cells by Ba^{2+} ion.

	% total ^{86}Rb efflux	
Treatment	Without $BaCl_2$	With 10 mM $BaCl_2$
MOPS-PSS	20 ± 1·2 (0)*	20 ± 1·2 (0)
KCl 40 mM	25 ± 1·5 (5)	20 ± 1·2 (1)
80 mM	32 ± 1·9 (12)	23 ± 1·3 (3)
100 mM	34 ± 2·1 (14)	25 ± 1·5 (5)

* Present net efflux shown in parentheses was calculated as described in the text.

Table 3. Blockade of ^{86}Rb efflux from TE671 cells by 4-aminopyridine (4-AP) and mast cell degranulating peptide (MCDP).

	% total ^{86}Rb efflux		
Treatment	Control	With 5 mM 4-AP	With 100 nM MCDP
MOPS-PSS	20 ± 1·0 (10)*	18 ± 1·0 (0)	20 ± 1·2 (0)
KCl 40 mM	25 ± 1·2 (5)	17 ± 1·0 (0)	20 ± 1·2 (0)
80 mM	32 ± 1·6 (12)	16 ± 0·9 (0)	19 ± 1·1 (0)
100 mM	34 ± 1·7 (14)	17 ± 1·0 (0)	18 ± 1·0 (0)

* Percent net efflux shown in parentheses was calculated as described in the text.

Miller 1983; Cook & Quast 1990; Dunne & Petersoen 1991), did not affect the efflux. Higher concentrations of TEA resulted in an anomalous increase in ^{86}Rb efflux, raising the background count (not shown). These results indicate that TE671 cells probably do not contain a typical TEA-sensitive delayed rectifier channel, similar to those in the frog node of Ranvier or the squid axon (Latorre & Miller 1983; Cook & Quast 1990).

In common with several voltage-gated potassium channels, including the I_K and I_A channels (Cook & Quast 1990), the channels activated by depolarizing solution (80 or 100 mM KCl) in TE671 cells appeared to be largely (approx. 75%) sensitive to a blockage by 10 mM $BaCl_2$ (Table 2). Ba^{2+} has been reported to block potassium channels, presumably because it has approximately the same crystal radius as K^+ (Latorre & Miller 1983; Adams & Nonner 1990). Although Ba^{2+} is a non-specific potassium-channel blocker, it can be effectively used to verify that the efflux of ^{86}Rb is indeed through the potassium channels and not other ion channels and pumps. In the present assay, the inclusion of larger concentrations of $BaCl_2$ in the buffers led to an anomalous increase in the background ^{86}Rb leak (not shown).

The ^{86}Rb efflux induced by the three depolarizing solutions was totally blocked (Table 3) by pre-incubation of TE671 cells with 5·0 mM 4-aminopyridine, or with 100 nM mast cell degranulating peptide (MCDP). Bee venom-derived peptide MCDP, is a highly specific and an avid blocker of the fast transient K-current, the A type potassium channel, or I_A (reviewed by Ziai et al 1990). A dose-response study of the inhibitory action of 4-aminopyridine has shown that the maximum blockage of ^{86}Rb efflux is achieved by using 2–5 mM 4-aminopyridine with IC50 1·0 mM (unpublished results). MCDP at 10 nM also appeared unable to inhibit the ^{86}Rb efflux induced by DS-100 in TE671 cells (unpublished results).

Aminopyridines inhibit K^+ current through delayed rectifiers, transient (I_A), and a few other voltage-dependent K^+ currents (Halliwell 1990; Adams & Nonner 1990; Cook & Quast 1990). 4-Aminopyridine, however, has little effect on the large conductance (charybdotoxin-sensitive), and small conductance (apamin-sensitive) Ca^{2+}-activated potassium channels (Cook & Quast 1990). A complete blockage of ^{86}Rb efflux from TE671 cells by 4-aminopyridine may indicate that the channels activated by high $[K^+]_{outside}$ are primarily of the I_A and 4-aminopyridine-sensitive I_K types. A total inhibition of ^{86}Rb efflux by MCDP may imply that the observed efflux is predominantly through the I_A channels with little, if any, contribution from the I_K channels. At present, this conclusion is speculative and should be verified by electrophysiological techniques.

Next, we tested the effect of apamin (a peptide blocker of the small conductance Ca^{2+}-activated potassium channel, SK_{Ca}) and charybdotoxin which blocks the BK_{Ca} channel. In the depolarized state induced by the three depolarizing solutions, apamin (100 nM) did not significantly affect the rate of efflux of ^{86}Rb from TE671 cells (Table 4). However, the efflux of ^{86}Rb in the resting state, in MOPS-PSS (basal efflux), was significantly reduced by apamin. Larger concentrations of apamin did not lead to a further decrease in the basal efflux (not shown). This indicates that, at the resting membrane potential, SK_{Ca} channels in TE671 cells are probably active and can be blocked by apamin. When depolarized by 80 or 100 mM KCl, the ^{86}Rb efflux through the voltage-gated potassium channels appears to mask the activity of SK_{Ca} (Table 4). Charybdotoxin (100 nM), blocked the efflux induced by 80 or 100 mM KCl by approximately 70%. This is probably due to the activation of BK_{Ca} by membrane depolarization. In contrast to the small conductance Ca^{2+}-activated potassium channel, the BK_{Ca}-channel can be activated by a change in the membrane potential or an increase in the intracellular calcium concentrations (Haylett & Jenkinson 1990). However, in view of the cross-reactivity of charybdotoxin with a number of different potassium

Table 4. Blockade of ^{86}Rb efflux from TE671 cells by charybdotoxin and apamin.

	% total ^{86}Rb efflux		
Treatment	Control	With 100 nM charybdotoxin	With 100 nM apamin
MOPS-PSS	20 ± 1·2 (0)*	21 ± 1·2 (0)	12 ± 0·8 (0)
KCl 40 mM	25 ± 1·5 (5)	25 ± 1·5 (4)	22 ± 1·3 (10)
80 mM	32 ± 1·9 (12)	27 ± 1·6 (6)	32 ± 1·9 (20)
100 mM	34 ± 2·0 (14)	27 ± 1·6 (6)	32 ± 1·9 (20)

* Percent net efflux shown in parentheses was calculated as described in the text.

channels, it is likely that voltage-gated and charybdotoxin-sensitive potassium channels, other than BK_{Ca} are also activated in this system. This possibility could explain the substantial blockade of the KCl-induced ^{86}Rb efflux by 4-aminopyridine (Table 3), since BK_{Ca} is largely insensitive to the 4-aminopyridine block (Cook & Quast 1990).

The results described above clearly show that the human neuroblastoma cells TE671 possess a repertoire of voltage-gated potassium channels sensitive to MCDP, 4-aminopyridine, Ba^{2+}, and charybdotoxin. The voltage-gated potassium channels, individually or in concert, are activated following depolarization of the cell membrane by 80–100 mM KCl. The extent by which ^{86}Rb exits the cell at any time probably reflects the sum of activities of a number of voltage-gated potassium channels. Blocking of individual K^+-channels by a reagent can result in a significant decrease in ^{86}Rb efflux which can be readily detected by this assay.

The TE671 cell line has been maintained and passaged, by us and others, for several years and has shown no inconsistencies in the expression and the pharmacological behaviour of its various ligand-gated and voltage-gated ion channels and other drug receptors, as ascertained by sensitive electrophysiological and binding assay criteria.

The present high-throughput functional assay is likely to be sufficient for detecting the majority of compounds capable of blocking the various voltage-dependent (depolarization-activated) potassium-channel sub-families in neurons, cardiocytes, vascular smooth muscle cells, or potentially any other adherent cultured cell lines targeted for drug discovery. This assay, by itself, is incapable of a precise identification of the potassium channel isoforms with which a given compound interacts. Its sole purpose is to screen rapidly a very large number of chemical entities and select putative potassium-channel blockers with potential for further studies.

Since in a typical potassium-channel-related drug discovery program, more than one potassium channel is usually targeted, a precise identification of the potassium channel mediating the depolarization-induced efflux of ^{86}Rb from TE671 cells is not of an immediate concern.

Detailed studies by electrophysiological techniques could subsequently identify the potassium channels targeted by compounds discovered by the high-throughput screen. Electrophysiological techniques such as voltage and patch clamping, however, are of extremely low through-put and cannot be used as primary screens in routine drug discovery programs.

We have successfully used this assay to discover a number of novel chemical structures capable of blocking the voltage-gated potassium channels in neurons and cardiocytes. The potassium-channel blocking activity of these compounds has been verified by electrophysiological techniques, as well as by ^{86}Rb efflux from cultured mammalian cells transfected with cloned neuronal voltage-gated potassium channels.

References

Adams, D. J., Nonner, W. (1990) Voltage-dependent potassium channels: gating, ion permeation and block. In: Cook, N. S. (ed.) Potassium Channels: Structure, Classification, Function and Therapeutic Potential. Ellis Horwood, London, pp 40–60

Arner, L. S., Stallcup, W. B. (1981) Rubidium efflux from neural cell lines through voltage-dependent potassium channels. Dev. Biol. 83: 138–145

Atwal, K. S. (1992) Modulation of potassium channels by organic molecules. Med. Res. Rev. 12: 569–591

Colatsky, T. J., Follmer, C. H. (1989) K-Channels blockers and activators in cardiac arrhythmias. Cardiovasc. Drug Rev. 7: 199–209

Cook, N. S. (1988) The pharmacology of potassium channels and their therapeutic potential. Trends Pharmacol. Sci. 9: 21–28

Cook, N. S., Quast, U. (1990) Potassium channel pharmacology. In: Cook, N. S. (ed.) Potassium Channels: Structure, Classification, Function and Therapeutic Potential. Ellis Horwood, London, pp 181–231

Daniel, S., Malkowitz, L., Wang, H.-C., Beer, B., Blume, A. J., Ziai, M. R. (1991) Screening for potassium channel modulators by a high through-put 86-rubidium efflux assay in a 96-well microtiter plate. J. Pharmacol. Methods 25: 185–193

Davies, N. W. (1990) Modulation of ATP-sensitive K-channels in skeletal muscle by intracellular protons. Nature 343: 375–377

Dunne, M. J., Petersoen, O. H. (1991) Potassium selective ion channels in insulin-secreting cells: physiology, pharmacology and their role in stimulus-secretion coupling. Biochim. Biophys. Acta 1071: 67–82

Escande, D., Cavero, I. (1992) K-Channel openers and natural cardioprotection. Trends Pharmacol. Sci. 13: 269–272

Halliwell, J. V. (1990) K Channels in the central nervous system. In: Cook, N. S. (ed.) Potassium Channels: Structure, Classification, Function and Therapeutic Potential. Ellis Horwood, London, pp 348–372

Harvey, A. L. (1993) Neuropharmacology of potassium ion channels. Med. Res. Rev. 13: 81–104

Haylett, B. G., Jenkinson, D. H. (1990) Calcium-activated potassium channels. In: Cook, N. S. (ed.) Potassium Channels: Structure, Classification, Function, and Therapeutic Potential. Ellis Horwood, London, pp 70–95

Latorre, R., Miller, C. (1983) Conduction and selectivity in potassium channels. J. Membrane Biol. 71: 11–30

Lavretsky, E. P., Jarvik, L. F. (1992) A group of potassium-channel blockers—acetylcholine releasers: new potentials for Alzheimer's disease? A Review. J. Clin. Psychopharmacol. 12: 110–118

Pfaffinger, P. J., Siegelbaum, S. A. (1990) K-Channel modulation by G proteins and second messengers. In: Cook, N. S. (ed.) Potassium Channels: Structure, Classification, Function and Therapeutic Potential. Ellis Horwood, London, pp 117–153

Rosen, M. P. (1991) The 'Sicilian Gambit': a new approach to the classification of antiarrhythmic drugs based on their action on arrhythmogenic mechanisms. Eur. Heart J. 12: 1112–1131

Rudy, B. (1988) Diversity and ubiquity of K channels. Neuroscience 25: 729–749

Ziai, M. R., Russek, S., Wang, H.-C., Beer, B., Blume, A. J. (1990) Mast cell degranulating peptide: a multi-functional neurotoxin. J. Pharm. Pharmacol. 42:457–461