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High-Throughput Screening Strategies for Cardiac Ion Channels

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In this article we discuss new technologies for high-throughput screening (HTS) of cardiac ion channels. We review the current state of cell based HTS and discuss new technologies and approaches using voltage-sensing Fluorescence Resonance Energy Transfer (FRET) dyes and "native" cell lines. The advantages and disadvantages of a gene-centric approach in which a target protein is overexpressed in a non-native cell line are addressed and the role of such primary screens in the drug discovery process outlined. Primary and secondary screens using "native" type cell lines either endogenously expressing the ion channel of interest or overexpressing it are discussed with respect to HTS capacity and sensitivity for FRET voltage sensing dyes and other technologies. Finally the advantages and approaches of screening against multiple targets in an endogenous cardiac cell line are discussed. (Trends Cardiovasc Med 2001;11:54–59). © *2001 Elsevier Science Inc.*

Advances in the fields of genomics, molecular and cell biology, and chemistry have profoundly impacted the process of drug discovery in recent years. It is now standard to identify and clone a disease-associated gene, express it in a suitable, heterologous cell line, and determine its functional properties. Often, a cell-based, high-throughput assay is then employed to screen a chemical library (often >100,000 compounds) for modulators of the target (Persidis 1998, Sittampalam et al. 1997). The active compounds are subsequently tested in vivo to validate the therapeutic relevance of the gene and/or to serve as the basis for further chemical optimization toward drug development.

Genes that encode ion channel targets are particularly well suited to cell-based functional screens for several reasons. First, ion channels are membrane proteins and cannot be easily studied or screened as purified proteins. Second, ligand-displacement assays are unable to probe various functional states of channels and are relatively insensitive to allosteric modulators. Finally, new technologies have made functional screens for ion channel targets more convenient, informative, and robust. Because the cardiac action potential reflects the activity of over 20 ion channels (Carmeliet 1999, Kirsch 1999, Nattel 1999, Roden and Kupershmidt 1999, Snyder 1999, Walker and Spinale 1999), these technologies promise to accelerate the discovery of cardiac drugs. At the same time, the cardiac action potential involves the coordinated activity of many genes and therefore tests some of the assumptions regarding a gene-centric approach to drug discovery. A summary of some of the ion channel genes involved in the cardiac action potential is shown in Figure 1.

This review focuses on the cardiac action potential and the search for anti-arrhythmic drugs as a case study that highlights a dilemma in drug discovery: how to exploit advances in genomics and high-throughput drug screening while maintaining therapeutic relevance for complex cellular processes. We review the current state of high-throughput screening for ion channels and comment on the opportunities for improvements that could facilitate the search for new heart medicines.

• The Drug Discovery Challenge for Cardiac Drugs

More than 40 drugs are currently available to treat various heart conditions, with cardiac arrhythmia representing a significant segment of the drug market. Drugs available to treat arrhythmia act on a variety of ion channels, including Na^+, K^+, and Ca^{2+} channels (see Table 1). All anti-arrhythmic drugs exert their effects either directly (e.g., Ca^{2+} channel blockers) or indirectly (e.g., β-blockers) on ion channels, thus validating ion channels as drug targets. Although many of the current cardiac drugs are selective for cardiac targets, others that are just as useful have complex modes of actions on multiple targets. The challenge for the next generation of drugs will be to

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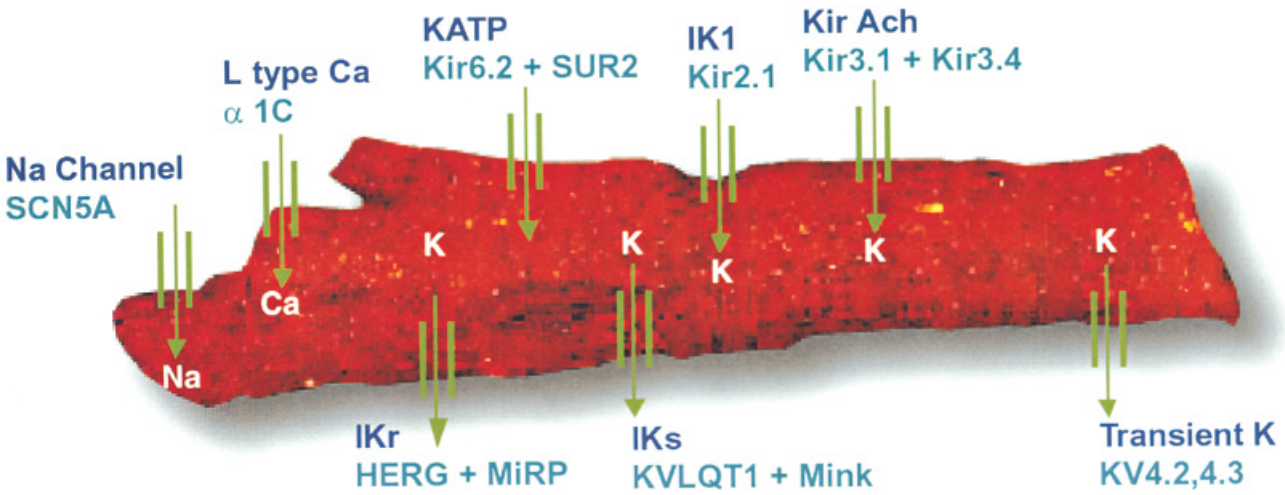

Figure 1. Representation of a cardiac ventricular myocyte with the following ionic conductances: *Inward:* Cardiac Na^+ channel (SCN5A); L type Ca^{2+} channel (α1C); KATP channel (Kir6.2 + SUR2); inward rectifier (Kir2.1); acetylcholine activated inward rectifier (Kir3.1 + Kir 3.4) *Outward:* Transient K^+ channel (Kv4.2 + Kv4.3); IKr (HERG + MiRP); IKs (KVLQT1 + MinK).

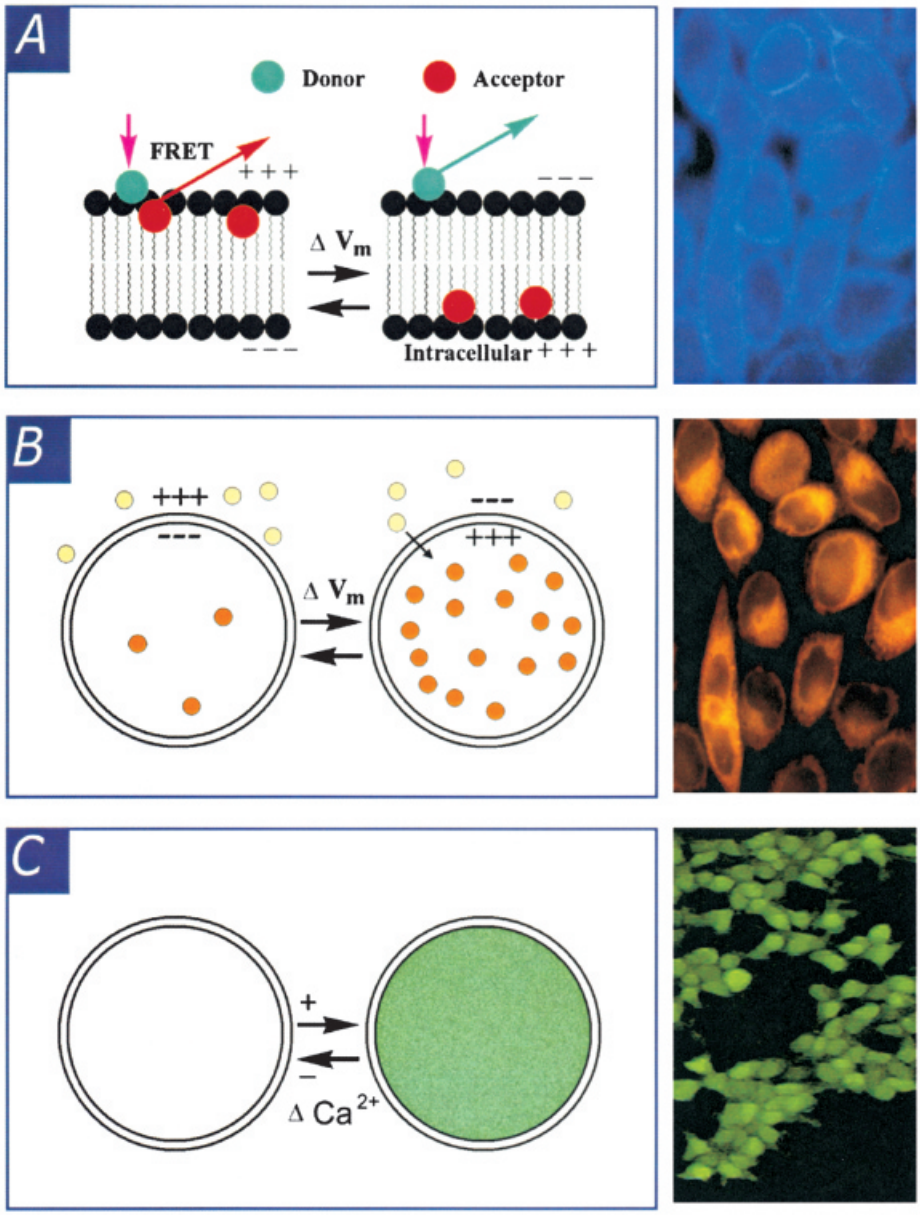

Figure 2. Voltage and Ca^{2+} dye sensor approaches. **(A)** Voltage-sensitive FRET mechanism employed by a voltage sensor. FRET interaction between the acceptor (oxonol) and donor (coumarin) is disrupted, with depolarization resulting in an increase in blue fluorescence of the CHO-K1 cells shown in the right panel. **(B)** DiBAC voltage-sensing dye showing increased loading in depolarized CHO-K1 cells with a resulting increase in staining. **(C)** Ca^{2+} sensing with the fluorescent dye Flu3. The dye is loaded into the cell, where it reversibly binds intracellular Ca^{2+} and fluoresces.

Table 1. Antiarrhythmic agents

	Target
Sodium channel blockers (class I)	
Class IA	Cardiac Na^+ channel
Quinidine (Quinidex)	
Procainamide (Pronestyl)	
Disopyramide (Norpace)	
Class IB	Cardiac Na^+ channel
Lidocaine (Xylocaine)	
Tocainide (Tonocard)	
Mexiletine (Mexitil)	
Class IC	Cardiac Na^+ channel
Encainide (Enkaid)	
Flecainide (Tambocor)	
Beta-adrenergic blockers (class II)	Beta receptor
Propranolol (Inderal)	
Acebutolol (Sectral)	
Esmolol (Brevibloc)	
Sotalol (Betapace)	
Drugs that prolong repolarization	
K^+ channel blockers (class III)	Cardiac IK (IKr, IKs) channels
Sotalol (Betapace)	
Amiodarone (Cordarone)	
Dofetilide (Tikosyn)	
Ibutilide (Corvert)	
Calcium channel blockers (class IV)	L type Ca^{2+} channel
Verapamil (Calan, Isoptin)	
Diltiazem (Cardizem)	
Mibefradil (Posicor)	T type Ca^{2+} channel
Miscellaneous	
Adenosine (Adenocard)	
Digoxin (Lanoxin)	

address the complicated nature of cardiac disease states while retaining target selectivity and specific modes of action to minimize side effects.

- **Functional Screens for Ion Channel Targets in Recombinant Cell Lines**

The pharmaceutical industry is currently taking a methodical, gene-centric approach to cardiac drug discovery by developing high-throughput screens using recombinant cell lines that functionally express cloned ion channel genes. This approach has been driven by both the availability of genes cloned from the heart and by improved cell-based assay technologies (González et al. 1999). There are several good reasons for screening ion channels in a non-native cell background. First, a transfected cell line containing a null background allows one to be certain of the molecular identity of the gene responsible for the observed signal. Second, the protein of interest can be over-expressed, thus improving the signal-to-noise of the screening readout (especially for flux assays). Third, host cells with low background conductances can be chosen to allow very sensitive assays of certain channels. Finally, heterologous cell lines such as HEK-293, CHO-K1, and COS-7 are relatively easy to culture and are robust enough to be handled by automated screening systems.

- **Assay and Screening Technologies**

Historically, most cell-based assays were carried out using flux assays such as guanidinium for Na channels and ^{86}Rb for K channels. The ^{86}Rb flux assay has been used in screening against KATP and KV1.3 K channels (Daniel et al. 1991, Hanson et al. 1999). The limitations of flux assays include: (1) detection of the target channel often requires the use of agonists, toxins, or other non-physiological methods; (2) the cells must undergo a lengthy loading procedure in the tracer followed by a thorough wash; (3) non-specific background fluxes may be large and must be subtracted from the data; and (4) the assays use radioactive materials.

Recently, a variety of optical technologies have become available for detecting ion channel activity in living cells with improved sensitivity and convenience (Figure 2). The advent of fluorescent Ca^{2+} indicator dyes such as fluo-3 (Minta et al. 1989) and fura-2 (Grynkiewicz et al. 1985) has allowed for the screening of Ca^{2+} channels and compounds that trigger intracellular Ca^{2+} release through G-protein-mediated signals. These non-toxic dyes can be loaded into most cell types and the cells can be assayed in multi-well microtiter plates using specialized kinetic plate readers such as the Fluorimetric Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA; Sullivan et al. 1999). These readers are equipped with integrated liquid handlers to introduce compounds or other reagents to the assay wells in order to initiate and observe effects on intracellular Ca^{2+}. In an assay for blockers of voltage-gated Ca^{2+} channels, for example, cells are seeded into multi-well plates, incubated with compounds and then the cells are depolarized by addition of high potassium to the wells. Blockers would appear as wells which showed no Ca^{2+} response to the depolarizing response. The most common formats utilize 96- or 384-well plates although higher densities are technically feasible. When integrated with other automation, these systems are capable of high-throughput screening of over 10,000 compounds per day.

Ca^{2+} indicator dyes work well for measuring Ca^{2+} channels because there is a large concentration gradient for Ca^{2+} influx into cells and because Ca^{2+} acts as a messenger inside cells. Therefore, the cell has evolved mechanisms to change Ca^{2+} concentration rapidly and dramatically. Other ions such as Na^+, K^+, and Cl^- do not change intracellular concentration as extensively and therefore those channels are not easily measured with indicators of ion concentration. However, these channels do alter membrane potential and can be assayed with indicators of cell membrane potential. Measurement of membrane potential can be extremely sensitive because relatively small currents can cause large voltage changes if the resistance of the cell is high. A commonly used optical method to measure membrane potential is based

on the distribution of a negatively charged, lipophilic, fluorescent oxonol dye between the extracellular medium and the cells (Zochowski et al. 2000). The oxonol preferentially accumulates and fluoresces in the membranes of depolarized cells, increasing their fluorescence. This assay is reasonably sensitive and gives a fairly large signal, but the dye redistribution mechanism is too slow to track rapid voltage changes. Typically these assays are configured to detect a steady-state voltage change occurring over many minutes. In addition, oxonol fluorescence is very sensitive to the environment and is perturbed by small changes in temperature and the presence of many compounds. These factors limit the utility of oxonol dyes for rapid or transient voltage-sensing applications and high-throughput screening.

A significant improvement to the oxonol redistribution method utilizes fluorescence resonance energy transfer (FRET) between the voltage-sensing oxonol dye and a second, fixed donor fluorophore (González and Tsien 1997). In the version shown in Figure 2, the donor fluorophore is a coumarin-linked phospholipid that integrates into the outer leaflet of the plasma membrane. When the coumarin is excited with violet light (409 nm) it will either fluoresce blue (460 nm) or transfer energy via FRET to the longer wavelength oxonol which will then emit light at its characteristic orange wavelength (e.g., 580 nm). FRET is extremely sensitive to the distance between the two fluorophores, so the location of the mobile, voltage-sensitive oxonol determines the ratios of the blue vs. orange emissions. In a cell with a negative resting membrane potential, for example, the negatively charged oxonol is repelled from the cytoplasmic side of the membrane and preferentially distributes to the outer leaflet, allowing FRET between the coumarin and the oxonol. As the cell is depolarized, the oxonol partner shifts to the inside surface of the membrane and this disrupts FRET, shifting the color emission from orange to blue.

The FRET voltage-sensing method has two major advantages over other techniques. First, the assay reflects real-time changes in membrane potential and is suitable for tracking action potentials, which presents the possibility to screen for modulators of the cardiac action potential. Second, the ratiometric aspect of the readout minimizes many artifacts typically seen in cell-based fluorescent assays. Namely, the readout is independent of the number of cells being assayed, variations in the degree of dye loading, and minimizes the effects of compound interaction with the dyes. A high-throughput screening instrument designed to measure fast ratiometric responses in a screening mode has been described (González et al. 1999).

The recombinant approach coupled with fluorescent Ca^{2+} indicator dyes and appropriate plate readers has proven successful in screening for blockers of Ca^{2+} channels. This may be due to the fact that a single α subunit confers most of the function to Ca^{2+} channels and it is therefore a relatively simple molecular target to reconstitute in a heterologous system. This is also true of monomeric Na^{+} channel α subunits, which are amenable to a recombinant high-throughput screening (HTS) approach using voltage-sensing fluorescent dyes (Catterall 1996).

• Limitations of the Recombinant Approach

The recombinant, functional approach to ion channel screens represents an important step in combining our knowledge of gene function with assay tools for drug discovery. As our appreciation of the complexity of certain biological processes grows, it is useful to consider the limitations of such an approach. First, a discovery process based on single genes can be inefficient when addressing a complex physiological process such as the cardiac action potential, because cloning, expressing and individually screening numerous genes is labor and time intensive. Second, the recombinant gene screening method assumes that individual genes can recapitulate an important attribute of a disease and that a cloned target will retain relevant functional characteristics in a non-native cell line. Ion channels can be particularly challenging in this regard owing to their heteromultimeric nature. Potassium channels, for example are heteromultimers composed of four individual α subunits and associated β subunits that all play a role in the function of the channel. Various alpha subunit combinations can occur among K^{+} channels so the molecular identity of the native channel may be very difficult to establish and reconstitute in a heterologous system. In addition, some of the most important cardiac K^{+} channels are now known to be comprised of two genes. For example IKs is composed of both KvLQT1 and MinK (Barhanin et al. 1996, Sanguinetti et al. 1996) while IKr is probably composed of HERG and MiRP1 (Abbott et al. 1999). The cardiac inward rectifier may be a complex of IK1 clone(s) and associated polyamines (Ishihara 1997). Thus, the standard HTS approach of heterologous expression in standard cell lines can become limiting as one attempts to build increasingly complex molecular physiology into a cell. This is particularly true of new Class III anti-arrhythmics, which rely on K^{+} channel blockade.

• Is Specificity of Drug Action Required at the Molecular Level?

A third reason to consider alternatives to the recombinant approach is that it implicitly assumes that specificity of action on the molecular target is desirable. However, many useful therapeutic compounds have multiple actions on several molecular targets. The stereotypic example of this is aspirin, but some of the better cardiac drugs to date also modulate multiple targets. One such example is Carvedilol, which is a α_1- and β-adrenergic receptor antagonist, calcium channel blocker and antioxidant especially useful in the treatment of hypertension. One of the most effective anti-arrhythmics is amiodarone (Cordarone™), which blocks a variety of cardiac ion channels, including INa, IKr, IKs, and ICa (Kodama et al. 1999). With the possible exception of dofetilide for IKr block, all of the approaches at controlling atrial and ventricular arrhythmias by modulating a single ion channel have failed. Recently, there has even been some concern about dofetilide owing to the occurrence of a serious irregular heart rhythm termed torsade de pointes in a small percentage of patients taking the anti-arrhythmic drug (Torp-Pedersen et al. 1999). Compounds that block two channels have also been found, but only ibutilide, which blocks IKr and activates INa, has been developed into a marketable drug (although azimolide which blocks both IKr and IKs is close to market). To mimic and improve upon drugs such as Corderone™ it will be necessary to screen for compounds that produce a pheno-

typic change in the action potential, possibly through effects on a number of ion channels.

• Future Opportunities for Cardiac HTS Technologies

Given the above discussion, improvements to cardiac HTS screens lie in improving and expanding the application of functional assays to drug discovery. For example, one possible goal could be to make the cardiac action potential the "target" and probe for functional changes such as spike amplitude, duration and frequency. For this to occur, advances are needed in several areas, including the use of more physiological cell backgrounds, more sophisticated assay methods and probes, improved data analysis for complex signals, and introducing new targets and modes of action.

• Cell Lines and Miniaturization

The potential of native cells for drug discovery is demonstrated by the fact that significant effort is currently expended on low-throughput electrophysiological screens using freshly isolated cardiac myocytes. As a more convenient alternative, the AT1 cell line from a mouse atrial cardiomyocyte tumor has been used as a model for cardiac myocytes (Maltsev et al. 1994, Yang et al. 1994). This cell line has recently been immortalized, and the resulting line HL-1 cell line appears to have many properties of native myocytes, including an ability to contract spontaneously and the presence of INa, ICa, and IKr (Claycomb et al. 1998). It remains to be seen if this cell line has a consistent enough phenotype to be useful for screening. This approach is also under way for other excitable tissue types, including neocortical (Hoshimaru et al. 1999) and dorsal root ganglion cells (Raymon et al. 1999) as well as immortalized progenitor cells that can be differentiated into neurons or astrocytes using oncogenic transformation (Sah et al. 1997). The role of telomerase in immortalized cells is likely to lead to other immortalized cell lines and improve our understanding of this process (Meyerson 1998, Yeager and Reddel 1999). Another goal for cardiac cell lines would be to create conditions where intercellular communication is preserved, which would further enhance the physiological significance of using these cells in an HTS approach.

Differentiated cell lines or primary cultures will likely be difficult to culture or obtain in large quantities. Therefore, to utilize them effectively in screens, the assay formats will need to be miniaturized as much as possible. Fluorescence-based assays are inherently amenable to miniaturization owing to the large signals, and it is expected that these assays will routinely be carried out in 384-well or higher density plates and employ fewer than 10,000 cells per compound tested. In principle, assays from fewer than 100 cells should be possible. Assays utilizing fewer than 100 cells may not be advantageous owing to the need to average the response from a number of cells. In addition to continued miniaturization, the optical technologies for detecting cell voltage will likely shift to longer wavelength fluorophores or time-resolved probes to reduce background signals from cells, plates and compounds.

• New Formats and Data Processing

To take advantage of these more physiological systems, there will need to be improvements in the generation and analysis of information-rich data from more complex cell lines and assays. For example, in the case of voltage signals, excitable cells may need to be stimulated electrically to either initiate the action potential, synchronize the response of a population, or to select for compounds with effects on particular firing patterns. This will open the door to HTS of compounds with more complex drug properties such as modulation of multiple channel types and use-dependent modulation of channels. As methods for manipulating membrane potential become more sophisticated, high-throughput screening methods will approach the data content afforded by low-throughput electrophysiologic recording for characterization of channel function and compound pharmacology. Sophisticated computational analysis will be needed to analyze the data and generate predictive analyses to interpret the results. This should help to direct the selection of hits to those with a more favorable activity profile, for example compounds that exhibit use-dependence. Companies such as Physiome (Princeton, NJ) are actively working on models to help guide such processes.

• New Targets and the Impact of Genomics

In addition to voltage, it is likely that assays for other physiological readouts could also prove useful, including second messengers such as cAMP (e.g., Mucignat-Caretta and Caretta 1997). Given the importance of apoptosis in heart failure (Sabbah et al. 1998) cardiac cells could be used to measure ionic readouts related to metabolic stress including pH, mitochondrial function, or hypoxia.

Gene expression patterns underlie most physiological processes. It is known that the pattern of expressed genes changes under pathological conditions related to cardiac disease (Yue et al. 1998). The identification of potential new targets using gene array technologies will undoubtedly produce new candidate targets. Many of these will likely be intracellular signaling molecules such as kinases, phosphatases or proteases. It will be interesting to see how ion channel expression is altered in disease and whether regulation of ion channel expression becomes a potential therapeutic strategy.

• Heart and Drug Safety

As our knowledge of drug metabolism and safety related to side effects increases, it is becoming clear that heart drugs must be shown to have minimal effects on other organ systems, especially the liver, which is where most drugs are metabolized. The withdrawal of the antihypertensive Ca^{2+} channel blocker mibefradil (Posicor) because of major metabolic drug interactions illustrates this problem (Clozel et al. 1999). Conversely, drugs intended for non-cardiac applications must be shown to leave cardiac function intact. As such, the cardiac action potential has also acquired considerable importance as a safety screen for non-cardiac drugs. For example, the human *ether-a-go-go* related K^+ channel gene, HERG, has been identified as the site of action of several antihistamine and antifungal drugs with cardiac side effects (Yap and Camm 1999). Functional assays for drug action on the cardiac action potential is becoming a standard filter for all drug candidates.

• Conclusions

Drugs modulating heart function are among the oldest and most established

drugs and were identified largely without the use of miniaturized high-throughput methods of screening drugs. Therefore, the success and impact of functional assays and high-throughput screening in identifying and developing drugs for cardiac ion channels remains to be seen. It is clear, however, that our ability to functionally probe the cell, or groups of cells, with the use of automated, high-throughput methods offers a new way to define novel chemical structures which may act in desirable ways to improve cell function. However, the complexity of the cardiac action potential challenges the current gene-centric standard usually employed for cell-based screens and forces consideration of a system-based assay that addresses the cardiac action potential directly and points to the need for better cell models of physiology. The lessons learned in comparing the two approaches may have profound implications as to how cell-based technologies are used to find drugs in the future.

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