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Ion-Selective Optodes Measure Extracellular Potassium Flux in Excitable Cells

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Abstract

Optodes have been used for detection of ionic concentrations and fluxes for several years. However, their uses in biomedical applications have not yet been fully explored. This study investigates optodes as a potential sensor platform for monitoring cellular ion flux with attendant implications in the field of drug screening and toxicology. A prototype system was developed to quantitatively measure extracellular potassium flux from a monolayer of cardiomyocytes. Optodes were created and immobilized on a glass coverslip for fluorescent imaging. The system detected potassium (K^+) ion flux during the repolarization phase of the cardiac action potential and further detected a decrease in the magnitude of the flux in the presence of a known K^+ channel inhibitor by optically monitoring local K^+ ion concentrations during field stimulation of the cardiomyocyte monolayer.

Keywords

biological applications for polymers; fluorescence; microstructure; sensors

Introduction

The cardiac action potential is a stereotypical sequence of ion channel openings and closings that lead to depolarization of the cell (driven largely by the influx of Na^+ ions), followed by a “plateau phase” where the inward and outward currents are balanced, and finally repolarization of the membrane (driven primarily by efflux of K^+ ions). Inherited mutations in the ion channels and related proteins can therefore alter the shape and duration of the action potential leading to arrhythmias and cardiac dysfunction.^[1] Similarly, acquired alterations in ion channel functions may alter the excitability of cardiomyocytes and hence be proarrhythmic. Interestingly, multiple classes of drugs have been shown to inhibit the voltage-gated potassium channel HERG (human ether-a-go-go related gene) that is the molecular basis for the repolarizing current I_{KR} as an unintended side effect leading to proarrhythmia.^[2] This has led to the mandated withdrawal of several such drugs (including ternafidine and moxifloxacin) while others carry “blackbox” advisories about their potential side-effects.^[3] Hence screening new drugs for their ability to block the HERG channel is now a standard step in the drug development pipeline. Classically this is done with whole cell patch clamping to assess the effect of the drug on the I_{KR} current. However, these experiments can be work intensive and time consuming.^[4] Development of novel techniques to screen drugs for HERG blockade that are adaptable to high throughput technology would likely be a significant advance in the field of drug development.

Optodes are the optical equivalent of ion-selective electrodes and have previously been characterized and used for functions such as environmental monitoring.^[5] Optodes can be developed to provide exquisite specificity for the ion of interest and can be tuned to the range of ion concentrations that is biologically relevant by changing the ratio of optode components.^[6] These properties make optodes ideal for use in biological systems; however the use of optodes in biology remains limited.^[7,8]

Recently we have described optode nanosensors for intracellular measurements of sodium. Intracellular potassium measurements, however pose a different challenge, since the high intracellular K^+ concentration make any relative changes in K^+ concentration during the repolarization phase of the action potential very small and hence hard to measure.^[9] On the other hand, since extracellular K^+ concentrations are typically low ($3\text{--}6 \times 10^{-3}$ M), measuring local increases in K^+ ion concentration due to K^+ efflux during the repolarization phase of the action potential is more feasible by reducing background potassium concentration and optimizing the response of the sensors to this range of concentrations. However, since diffusion in the extracellular space limits potassium flux detection to regions

within microns of the cellular membrane, the optodes would have to be in close proximity to the cell membrane.

In this paper, we describe a method to detect extracellular K^+ ion fluxes. This method uses potassium optodes with ideal concentration detection, optical properties and response time. A device was designed to locate immobilized optodes close to cardiomyocyte monolayers to limit diffusion effects as described above. Our device is able to detect K^+ flux during the cardiomyocyte action potential, and further detects alteration in this flux in the presence of a known potassium channel inhibitor.

Experimental Part

Materials

All materials were obtained from Sigma in the highest purity possible unless otherwise noted. Haloperidol was purchased from Sigma, stock solutions were created in dimethyl sulfoxide (DMSO) and stored at 4 °C for up to 3 months.

Optode Composition/Performance

The optode consisted of the following, in wt.-%: 0.6 9-(Diethylamino)-5-[(2-octyldecyl)imino]benzo[a]phenoxazine (chromoionophore III or ETH 5350), 0.9 potassium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (KTFPB), 1.9 potassium ionophore III (BME-44), 32.1 poly(vinyl chloride) (PVC), and 64.5 bis(2-ethylhexyl) sebacate (DOS). These components were dissolved in tetrahydrofuran (THF) (32 mg PVC/1.0 mL THF).

Bulk optodes were plated onto glass discs and the THF was allowed to evaporate leaving a plasticized polymer macrosensor. These were loaded into 96 well plates to perform calibration experiments. Calibrations were conducted with solutions of varying potassium concentrations buffered with 1×10^{-4} M Tris at pH 7.4. The sensors were allowed to equilibrate and then excited at 485 nm with emission measured at 570 nm and 670 nm (Spectramax, Molecular Devices). Measurements were performed at each potassium concentration to build a calibration response curve.

Potassium microspheres were created by rapidly injecting through a pipette tip 200 μ L of optode in THF solution into 1.5 mL deionized water. Injection was performed into a microcentrifuge tube that was suspended in a sonicating water bath (Branson). The agitation caused the optode to disperse while the THF was dissolved out of the optode and eventually evaporated. This left micro-sized particles of optode suspended in water. The resulting mixture was then dispensed onto round glass coverslips and allowed to dry overnight (250 μ L per slide).

Cardiomyocyte Isolation

Cardiomyocytes were prepared as previously discussed.^[10] Briefly, ventricular tissue harvested from post-natal day 1 rat pups was minced and subjected to enzymatic digestion with $0.4 \text{ mg} \cdot \text{mL}^{-1}$ of collagenase/ $0.6 \text{ mg} \cdot \text{mL}^{-1}$ pancreatin in ADS buffer, followed by cell isolation on a Percoll gradient. Pure isolated cardiomyocytes were counted and plated at a density of 1×10^5 cells $\cdot \text{mL}^{-1}$ on laminin coated 25 mm glass coverslips (VWR). For ion flux measurements, the DMEM growth solution was replaced with a non-fluorescent cell buffer consisting of 1×10^{-4} M HEPES at pH 7.4 supplemented with 137×10^{-3} M NaCl, 2.7×10^{-3} M KCl, 0.9×10^{-3} M CaCl_2 and 10×10^{-3} M glucose.

Cell Mounting Device

The mounting device was modeled in SolidWorks 2006. All components (excluding hardware) were fabricated on a 3D Systems SLA 250 solid imaging system (SL 5220 resin). The device was designed to locate a monolayer of cardiomyocytes to within 5–30 μm of a potassium optode membrane. The system is also capable of providing a uniform an electric field via two silver chloride electrode rails.

Imaging

Microscope images during cardiomyocyte experiments were taken on a Zeiss LSM Meta 510 confocal microscope. Images were recorded using a $20\times$ lens. A HE/NE laser at 514 nm was used for excitation and emission was recorded with a center wavelength of 670 nm using the meta software. Myocytes were paced with an ECM 830 (Harvard Apparatus) at 1 Hz with a 30 V potential applied for 5 ms. Images were binned and recorded at 50 Hz. Images of optode dispersion on glass coverslips were taken on a Zeiss Axiovert 200 with a FITC (485/514 nm) filter cube.

Data Processing

Time series images were initially processed using Zeiss LSM Meta 510 imaging software. High intensity regions of interest (ROI) containing visible harmonic components were selected and the mean pixel intensity (within each ROI) was calculated for each frame. The resulting time series data was then exported and postprocessed in Origin 7.0.

Results and Discussion

The weight percentages of each of the components of the optode were chosen to produce a fluorescent response to potassium with a dissociation constant (K_d) around 3×10^{-3} M. This would make the optode ideally tuned to measure changes in the extracellular K^+ ion concentration, which is typically $3\text{--}5 \times 10^{-3}$ M in most systems, since the properties of the optode make it most responsive around its K_d .

Calibrations were performed to determine the K_d of the optode, Figure 1 (A) and (B). Optodes were excited at 485 nm in a plate reader in the presence of varying amounts of potassium and an emission spectrum was recorded. Two emission peaks in the emission spectrum were observed, centered near 570 and 670 nm, with an isosbestic point near 630 nm (Figure 1(A)); thus we chose to use the optode as a ratiometric indicator. The ratio of 570 over 670 nm was determined for each concentration of potassium. We chose to define the value α as:

$$\alpha = (\text{ratio}_{\text{maximum value}} - \text{ratio}_{[\text{potassium}]}) / (\text{ratio}_{\text{maximum value}} - \text{ratio}_{\text{minimum value}}) \quad (1)$$

This derived variable normalizes the response of the optode. This was calculated for each potassium concentration and plotted against the log of the potassium concentration (Figure 1(B)). A sigmoidal curve was fitted and the K_d was determined from this fit to be 5×10^{-3} M.

The optode was deposited onto a glass coverslip so that it could be placed in close proximity to the cardiomyocytes to determine extracellular K^+ concentration changes. The optode response is an equilibrium response throughout the entire plasticized polymer. Because of this, the optode needs to be small enough to have a fast response time capable of measuring K^+ concentration changes that occur on the order of tens of ms. Two methods were explored to achieve an even coating of optode on a glass coverslip with a minimal volume. First, the optode was spun-coated onto the glass coverslip using a custom built spinner. This provided

fairly even distribution of the optode over the entire surface of the coverslip (Figure 1(C)). However, because the plasticized polymer matrix is one continuous optode the total volume of the optode is large, leading to an increase in the response time which would make it difficult to measure small changes in K^+ ion concentrations in the time range of the action potential.

Our second technique was to create a deposit a thin film of optode on the surface of the coverslip. This was accomplished by injecting dissolved optode into deionized water in a sonicating bath. The resulting emulsion was then pipetted onto a coverslip and the water was evaporated to leave a uniform polymer layer (Figure 1(D)). While the average thickness of the layer is constant, the microspheres form a rough lattice with large surface area. Each microsphere is an individual optode with a small total volume. This significantly reduces the response time of the optode, as the equilibrium response can be achieved much faster in the small volume, in the order of ms.

The optode coated coverslips were mounted to a custom built device using vacuum grease (Figure 2 (A)). This device was created to minimize the volume between the optode and the cardiomyocytes and hence minimize the diffusion gradients of potassium. The mounting device consisted of a base plate which fits in to the microscope, two electrode rails which allow for pacing of the cardiomyocytes, a well which is the liquid bath area in which both the optode and the cardiomyocytes are immersed, and a positioning arm which clamps onto the cell-coated coverslip to lower the cardiomyocytes to a consistent height in the bath well.

Once the optode-coated coverslip was adhered to the bottom of the mounting device the bath area was filled with extracellular solution and the cardiomyocytes were brought down into the bath. The entire apparatus was then mounted onto the microscope for imaging. A side view of the device on the microscope is shown in Figure 2(B). The design of the device allows for both bright-field imaging of the cells and fluorescent imaging of the optode. Additionally, compounds can easily be added to the bath solution. The device also allows for fluorescent measurements without motion artifacts from the cardiomyocytes. In our experiments, we repetitively field stimulate the cardiomyocytes to initiate a train action potentials at a set frequency, which also initiates contraction of the cardiomyocytes at the same frequency. Hence measurement of the fluorescent intensity through the cardiomyocytes could be confounded by changes in the optical properties of the cells as they contract and relax. The design of our device allows for the optode to be in close proximity to the cardiomyocytes, but with the ability to make measurements without having to image through the cardiomyocytes and hence removing the possibility of motion artifacts from the cells.

Both the cells and the optode-coated coverslip were mounted into the mounting device and the well was filled with extracellular solution. A region of the cardiomyocytes with near confluency was found and the cells were paced at 1 Hz to determine viability. They cells were deemed viable if they contracted at the pacing frequency. Once a region of the cells was found, the field of view was focused down to the optode and an image was taken. The image was then binned to achieve a scan time of 50 ms which allows for an imaging rate of 20 Hz. A time series was acquired over 50 s while the cells were being simultaneously paced at 1 Hz. At 25 s, haloperidol, a known inhibitor of HERG (and the prototype of a drug with unintended HERG blockade), was added to the extracellular solution to achieve a final concentration of 2×10^{-6} M in the bath. Because the drug was introduced from the side into the bath, effects of solution addition were not seen. Diffusion of the drug was assumed to occur within 1–2 s after addition.

A region of interest with a consistent frequency of contraction of 1 Hz over the first 20 s was defined in the time series of images. The average intensity values for this region were then plotted against time for the first 20 s of the run (Figure 3 (A)). The data was mean-normalized and smoothed with a running average. For visualization purposes, a fast Fourier Transform (FFT) amplitude threshold filter was then applied to isolate all high amplitude harmonic components (red line, Figure 3(A)). A FFT was then applied to the data and the power spectrum was plotted, as shown in Figure 3(C). The dominant frequency, the frequency with the highest power, was found to be 1 Hz. There were no other frequencies that were above the noise threshold in the power spectrum. This shows that a 1 Hz beating frequency was the only frequency found in the potassium flux data, which correlates to the pacing frequency and hence the frequency of action potentials.

The intensity values for the same region of interest were then plotted for the last 20 s of the time series following addition of haloperidol (Figure 3(B)). This data was also normalized, and smoothed with a running average. A FFT was taken and the power spectrum was plotted (Figure 3(D)). After addition of the drug, the dominant frequency is still 1 Hz. However, the power of the frequency at 1 Hz is reduced suggesting that potassium efflux in the presence of this HERG blockade was decreased as expected, and this decrease was successfully detected by our optode. There was an average decrease in the power spectrum of $30 \pm 20\%$ ($n = 3$). In addition we did see a smaller peak at a frequency of 1.3 Hz. No alteration in the power spectrum was observed in control experiments in which DMSO with no drug was added to the cells. The slight decrease in the power spectrum at 1 Hz and the appearance of a second peak at 1.3 Hz suggest that HERG blockage alters the function of the beating cardiomyocytes. This may corresponded to a population of cardiomyocytes that had been perturbed by the addition of the drug and now were spontaneously contracting at a higher frequency than the pacing frequency. The exact effect of haloperidol on cardiomyocyte function is a complicated matter and was not explored in this report. However, the detection of potassium channel inhibition and consequent decrease in potassium ion efflux, which is essential to the drug screening process, was easily detected.

Conclusion

We have shown that optical potassium optodes can be used to determine the potassium concentration changes in the extracellular environment of cardiomyocytes during normal cardiomyocyte action potentials. The addition of a known blocker of the potassium channel HERG decreased the power of the dominant beating frequency without changing the dominant frequency, thereby suggesting that this platform could be successfully used to detect inhibition of the HERG channel. Our platform is easily adaptable to a high throughput platform and represents an exciting advance in the field of drug development.

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Figure 1.

Optode response to potassium and immobilization. A) An emission scan of optode fluorescent response to potassium concentration. Fluorescent intensity (excitation 485 nm) is plotted over the emission wavelength for potassium concentration ranging from 0 to 1000×10^{-3} M. B) Ratiometric response of potassium optode to potassium concentration. The normalized value α is plotted against the log of potassium concentration, in Molar. Data are an average of $n = 8$, with standard deviation. A sigmoidal curve was fit (blue line). C) Fluorescent image of a spun coat potassium optode on a glass coverslip, $5 \times$ ex/em 485/514 nm. D) Fluorescent image of deposited, immobilized potassium optode microparticles on a glass coverslip, ex/em 485/514 nm.

Figure 2. Mounting device. A) Solidworks image of mounting device in which all the key components are labeled. B) 2-dimensional cross section of the device in which the cardiomyocytes and optode microparticles are mounted. The orientation of the optics is also shown.

Figure 3. Potassium flux during cardiomyocyte beating. A) 20 s of mean normalized fluorescent emission (670 nm) intensity prior to Haloperidol addition. Data are smoothed (dotted line) and low pass filtered (solid line). B) 20 s of mean normalized fluorescent emission (670 nm) intensity following Haloperidol addition. Data are smoothed (dotted line) and low pass filtered (solid line). C) Fast Fourier Transform of the low pass filtered data in A. D) Fast Fourier Transform of the low pass filtered data in B.