Published in final edited form as:
Biosens Bioelectron. 2014 April 15; 54: 448–454. doi:10.1016/j.bios.2013.11.038.

Application of Ion-sensitive Field Effect Transistors for Ion Channel Screening

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Abstract

Cell-based screening assays are now widely used for identifying compounds that serve as ion channel modulators. However, instrumentation for the automated, real-time analysis of ion flux from clonal and primary cells is lacking. This study describes the initial development of an ion-sensitive field effect transistor (ISFET)-based screening assay for the acquisition of K^+ efflux data from cells cultured in multi-well plates. Silicon-based K^+-sensitive ISFETs were tested for their electrical response to varying concentrations of KCl and found to display a linear response relationship to KCl in the range of 10 μM to 1 mM. The ISFETs, along with reference electrodes, were inserted into fast-flow chambers containing either human colonic T84 epithelial cells or U251-MG glioma cells. Application of the Ca^{2+} ionophore A23187 (1 μM), to activate Ca^{2+}-activated non-selective cation (NSC) channels (T84 cells) and large conductance Ca^{2+}-activated K^+ (BK) channels (U251 cells), resulted in time-dependent increases in the extracellular K^+ concentration ($[K^+]_o$) as measured with the ISFETs. Treatment of the cells with blockers of either the NSC or BK channels, caused a strong inhibition of the A23187-induced increase in $[K^+]_o$. These results were consistent with ion current measurements obtained using the whole-cell arrangement of the patch clamp procedure. In addition, K^+ efflux data could be acquired in parallel from multiple cell chambers using the ISFET sensors. Given the non-invasive properties of the probes, the ISFET-based assay should be adaptable for screening ion channels in various cell types.

Keywords

ISFET; K^+ efflux; screening assay; cell lines; ion channel modulators

1. Introduction

Ion channels play a critical role in a variety of physiological processes including cell signal transduction, muscle contraction, neurotransmitter release and enzyme activation. For this reason, ion channels have become important targets for the discovery of new therapeutic

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agents. Cell-based screening assays are now widely used for identifying compounds that serve as ion channel modulators (Zheng et al., 2004; Lu and An, 2008). Typically, these assays are conducted with immortalized cell lines (HEK293, CHO, etc.), expressing an ion channel of interest, that are cultured in multi-well plates (Zheng et al., 2004; Lu and An, 2008). While ion- and membrane potential-sensitive fluorescent dyes are most commonly utilized for screening ion channels, these assays do not provide a direct measurement of ion flux and are susceptible to a large number of imaging artifacts (Zheng et al., 2004). Automated patch clamp systems allow the direct measurement of ion current (Dunlop et al., 2008; Yajuan et al., 2012). However, planar patch clamp recording requires that the cells express high levels of a recombinant ion channel and that the cells be added to the wells in suspension (Dunlop et al., 2008; Yajuan et al., 2012). In addition, neither fluorescent dye-based assays nor automated patch clamp systems are applicable for the automated, real-time analysis of ion efflux from primary cells (myocytes, neurons, etc.) and stem cell-derived muscle and nerve cells. However, there is growing interest in academia and industry for using primary cells and stem cells in drug discovery programs (Eglen and Reisine, 2010; Möller and Slack, 2010) since these cells represent a more appropriate physiological and clinical target.

Field effect transistors (FETs) are devices that use an applied voltage (electric field) as a mechanism for electrical switching and amplification (Chaniotakis and Sofikiti, 2008; Bratov et al., 2010). FETs consist of three terminals called the “source”, “gate” and “drain” (Chaniotakis and Sofikiti, 2008; Bratov et al., 2010). With ion-sensitive field effect transistors (ISFETs), an ion-selective membrane is incorporated into the gate of the FET (Lee et al., 2009; Poghossian et al., 2009; Bratov et al., 2010). The ISFET-based sensor was introduced by Piet Bergveld in 1970 and first utilized as a biosensor for measuring penicillin (Caras and Janata, 1980). In recent years ISFET technology has been developed for detecting Na^+, K^+, Ca^{2+} and Cl^- as well as for measuring extracellular pH, glucose, DNA and a variety of enzymes, antibodies and neurotransmitters (Lee et al., 2009; Poghossian et al., 2009; Bratov et al., 2010; Sofue et al., 2011). ISFET-based sensors have a number of characteristics that make them desirable in designing an ion flux screening assay. Their miniature size, rapid response, durability, ion-selectivity and large-scale integrative capacity make them ideal for measuring ion concentrations in multi-well plate format. In addition, ISFET sensors can be fabricated in large numbers using standard lithographic processes. Recent studies have demonstrated the use of ISFETs for measuring ion flux from single cells plated and grown on coated (Straub et al., 2001; Wrobel et al., 2005; Chang et al., 2012) or uncoated (Yu et al., 2007; Gebinoga et al., 2010; Sakata et al., 2012) sensors. However, the application of ISFETs in multi-well plate, parallel measurement configurations for ion efflux analysis has not been determined.

The goal of the present study was to explore the use of ISFETs for ion channel screening. T84 epithelial cells and U251-MG glioma cells were cultured in multi-well plates and added to fast flow chambers that contained a K^+-sensitive ISFET and reference electrode. Application of the Ca^{2+} ionophore A23187, to activate Ca^{2+}-activated non-selective cation (NSC) channels (T84 cells) and large conductance Ca^{2+}-activated K^+ (BK) channels (U251-MG cells), resulted in time-dependent increases in the extracellular K^+ concentration ($[K^+]_o$). This K^+ efflux was inhibited by pretreatment of the cells with appropriate ion channel blockers. The results obtained using the ISFET-based system were consistent with those measured using the whole-cell arrangement of the patch clamp recording procedure. Thus, the ISFET assay system should be applicable in the future for screening ion channels in a number of clonal and primary cell types.

2. Materials and Methods

2.1 Chemicals

Charybdotoxin was purchased from Alomone (Israel) and dissolved in saline solution. All other chemicals were obtained from Sigma-Aldrich. Stocks of A23187, NPPB and DPC were prepared in DMSO.

2.2 Cell culture and plating

Human colonic T84 epithelial cells were obtained from ATCC and the human U251-MG glioma cell line generously supplied by Dr. Swapan Ray (University of South Carolina, School of Medicine). Cells were maintained in either DMEM/F12K (T84 cells) or DMEM (U251 cells) media supplemented with 10 % fetal bovine serum (FBS), 50 U/ml penicillin and 50 mg/ml streptomycin. Cells were platted on 12 mm glass coverslips (5,000 cells per coverslip) for patch clamp recording and 25 mm coverslips in 12-well plates (50,000 cells per well) for ISFET measurements. Cells were stored in an incubator at 37° C (5 % O_2 / 95 % CO_2) and used on days 1–4 after plating.

2.3 Patch clamp recording of NSC and BK ion currents

The patch clamp method (Hamill et al., 1981) was used to record whole-cell, Ca^{2+}-activated non-selective cation (NSC) currents (I_{NSC}) (T84 cells) and large conductance Ca^{2+}-activated K^+ (BK) currents (I_{BK}) using an Axopatch 200A (Molecular Devices) amplifier. Our procedure for measuring K^+ currents has been described (Walsh and Zhang, 2008). Pipettes were made from borosilicate glass capillaries (World Precision Instruments) and had resistances of 2–3 Mohms when filled with internal solution. All experiments were conducted on isolated, non-coupled cells at room temperature (22–24 °C). Cells were placed in normal saline solution consisting of; 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM dextrose, 5 mM HEPES, pH 7.4 (with NaOH) (280 mOsm.). In some experiments with the T84 cells this solution was replaced with Na^+-free solution containing: 140 mM N-methyl-D-glucamine (HCl), 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM dextrose, 5 mM HEPES,, pH 7.4 (with HCl). The internal solution consisted of; 50 mM KCl, 60 mM K^+-Glutamate, 2 mM $MgCl_2$, 0.5 mM EGTA, 3 mM ATP, 10 mM HEPES, pH 7.3 (with KOH) (280 mOsm.). Membrane currents were recorded with 12-bit analog/digital converters (Molecular Devices). Data were sampled at 5 KHz and filtered at 1 KHz with a low pass Bessel filter (Frequency Devices). Series resistance was compensated to give the fastest possible capacity transient without producing oscillations. I_{NSC} was activated in the T84 cells by the addition 1 μM of the Ca^{2+} ionophore A23187 using a rapid perfusion system. I_{BK} was activated in the U251-MG cells by applying depolarizing voltage steps to positive membrane potentials. Channel blockers were added when the currents reached their peak amplitude and the percent inhibition quantified. Averaged current values presented are means ± S.E.

2.4 ISFET electronics and recording procedure

K^+-sensitive silicon-based ISFETs were obtained from D+T Microelectronica (Spain). The gate of the ISFETs was coated with a polymeric membrane containing the K^+ ionophore valinomycin giving the probes K^+-sensitivity (Muñoz et al., 1997). The hardware interface consisted of a voltage-follower circuit that powered and buffered the sensor. It was determined during initial testing that the ISFETs have a 1.2 dB signal-to-noise ratio (SNR) near the end of their rated usable life. High frequency noise was removed using a low-pass 1 Hz 2^{nd} order Butterworth filter to achieve an SNR of 34 dB on the same sensors. This effectively increased the usable life of the ISFETs for the K^+ measurements. The interface was placed in an array on fabricated circuit boards to reduce noise and simplify

measurements. Data was logged using a data acquisition unit (DAQ) (Agilent 34972A). During calibration, the interface supply voltage (Agilent E3649A) was adjusted while observing the DAQ. The voltage used in the experiments was the highest allowable that did not produce signal oscillations.

For ISFET calibration the sensor and a 5 mm wide Ag/AgCl reference electrode (BASi) were inserted into a fast-flow recording chamber containing 1 ml of K^+-free saline solution (see above). Calibration curves were obtained prior to each experiment by measuring the response of an ISFET to KCl concentrations in the range of 10 μM to 200 μM. Coverslips (25 mm) containing either T84 or U251-MG cells were washed with K^+-free saline, to remove the culture media, and then placed into the chamber. Following a 3 min baseline recording period, A23187 was added to the chamber and the A23187-induced K^+ efflux determined by measuring the extracellular K^+ concentration ($[K^+]_o$) in the chamber.

3. Results and Discussion

3.1 Properties of ISFETs

K^+-sensitive silicon-based ISFETs used in this study were obtained from D+T Microelectronica (Figure 1). ISFET response curves were measured in 100 mM TRIS-Cl solution in the presence of various concentrations of KCl, NaCl, CsCl, LiCl and NH_4Cl (Figure 1C). In addition to the expected sensitivity to K^+, the sensors also displayed a response to Cs^+ and NH_4^+ in the concentration range of 10^{-5} to 10^{-3} M. Based on the size of the slope of the regression line fitted to the cation data, the sensitivity of the sensors was $K^+ > Cs^+ > NH_4^+$ (Figure 1C). No difference in the ISFETs output voltage was measured in TRIS-Cl solutions at pH 5, 6, 7 and 8 (data not shown). Small shifts (< 10 %) in the ISFETs baseline output were noted from one experiment to another. For this reason, KCl concentration versus ISFET response curves were obtained prior to each experiment (see below).

3.2 Expression of NSC channels in T84 cells

T84 cells have previously been reported to express Ca^{2+}-activated non-selective cation (NSC) channels as well as Ca^{2+}-activated Cl^- channels (Champigny et al., 1991; Braun and Schulman, 1995). NSC channels are equally permeable to Na^+ and K^+ (Champigny et al., 1991; Braun and Schulman, 1995). Figure 2A displays currents recorded from the T84 cells using the whole-cell arrangement of the patch clamp procedure. When the cells were bathed in normal saline solution containing 140 NaCl, application of the Ca^{2+} ionophore A23187 resulted in the activation of both large inward and outward currents (Figure 2A). In order to determine the contribution of NSC channels to these currents, the external Na^+ solution was replaced with N-methyl-D-glucamine (NMG) solution. Substitution of NaCl with NMG-Cl resulted in a complete elimination of the inward currents in the voltage range of −30 to −70 mV (Figure 2). This indicates that cation conductance through the NSC channels is responsible for the inward currents recorded in this voltage range. Thus, at the normal resting potential of the T84 cells (−40 to −60 mV), elevation of intracellular Ca^{2+} by treatment with A23187 results in the activation of the NSC channels with a resulting influx of Na^+ and efflux of K^+.

While there are no selective blockers of Ca^{2+}-activated NSC channels in the T84 cells, arylaminobenzoate compounds such as 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and diphenylamine-2-carboxylate (DPC) non-selectively block these channels (Champigny et al., 1991; Braun and Schulman, 1995). Addition of 20 μM NPPB caused a strong block of the A23187-induced inward and outward currents in the T84 cells (Figure 3). DPC (500 μM) also blocked the inward and outward currents but not as effectively as NPPB

(32 ± 2 % decrease with DPC versus 86 ± 4 % decrease with NPPB at −100 mV, n = 6 cells).

3.3 Development of an ISFET-based screening assay

The results shown in Figures 2 & 3 demonstrate that T84 cells express a Ca^{2+}-activated NSC channel that is blocked by NPPB and DPC. It was next determined if K^+-sensitive ISFETs could be used to measure K^+ efflux through these channels. The experimental design of the assay is outlined in Figure 4A. T84 cells were cultured on 25 mm coverslips and placed in a fast-flow chamber containing 1 ml of K^+-free saline along with an ISFET and reference electrode. Activation of NSC channels by addition of A23187 should increase K^+ efflux from the cells and therefore increase the extracellular K^+ concentration ($[K^+]_o$). Changes in $[K^+]_o$ were continuously monitored over a 20–25 min time period with the ISFET.

Immediately prior to each experimental assay a K^+ concentration (μM) versus ISFET (mV) calibration curve was obtained. This was measured by adding increasing concentrations of KCl to a chamber (without cells) containing K^+-free saline. Over the concentration range of 20 μM to 1 mM, the calibration curve displayed a linear relationship (Figure 4B). To provide a more rapid calibration procedure, final KCl concentrations of 10, 50, 100 and 200 μM were used for calibrating the ISFETs in this study.

The applicability of the ISFET assay system for measuring K^+ efflux from the Ca^{2+}-activated NSC channels was evaluated in Figure 4C. Each graph plots the $[K^+]_o$, calculated from the calibration curve, which was measured over time in a chamber containing T84 cells. As predicted by the experimental model, addition of A23187 to the cells caused a time-dependent increase in $[K^+]_o$. Overall, $[K^+]_o$ increased from a baseline value of 20 ± 3 μM to a stimulated value of 198 ± 7 μm (n = 12 assays) measured 20 min after the application of A23187. In contrast, addition of control solution (with the A23187 solvent dimethyl sulfoxide [DMSO]) resulted in only a small linear leak of K^+ (peak value = 43 ± 6 μM, n = 4 assays) (Figure 4C).

The utility of the ISFET assay for screening ion channel blockers was studied in Figure 5. Consistent with the results obtained with the patch clamp recordings, both NPPB and DPC were efficacious in inhibiting A23187-induced increases in K^+ efflux from the T84 cells. Of the two compounds NPPB was more effective in inhibiting the efflux (Figure 5A & B). In three assays NPPB caused the $[K^+]_o$ to be reduced to 55 ± 7 μM. In order to demonstrate the feasibility of using ISFETs for ion channel screening, A23187-induced K^+ efflux was simultaneously measured from three parallel chambers (Figure 5C). The K^+ efflux signals recorded from the three chambers displayed similar kinetics and amplitudes (Figure 5C). As expected the size of the averaged K^+ signal (215 μM) (Figure 5C) was consistent with that measured in single well experiments (Figures 4–5).

3.4 Measurement of K^+ efflux from U251-MG glioma cells

While the ISFET assay system was useful for studying Ca^{2+}-activated NSC channels in the T84 cells, it was important to determine if the assay was suitable for measuring K^+ efflux through other channels. A number of human glioma cell lines, including the U251-MG cell line, express large conductance Ca^{2+}-activated K^+ (BK) channels that are blocked by toxins such as charybdotoxin and paxilline (Ransom and Sontheimer, 2001; Abdullaev et al., 2010). Using the whole-cell patch clamp procedure, depolarizing voltage steps were applied to potentials from +20 mV through +120 mV in order to activate I_{BK} (Figure 6A) (Ransom and Sontheimer, 2001; Abdullaev et al., 2010). As described previously (Ransom and Sontheimer, 2001; Abdullaev et al., 2010), addition of 100 nM charybdotoxin (CHX)

(Figure 6A) caused an inhibition of the current (decrease at +120 mV = 60 ± 4 %, n = 5 cells).

To determine the use of the ISFET assay for screening BK channels, coverslips containing U251-MG cells were placed in the fast-flow chambers and K^+ efflux measured as described for the T84 cells. In the absence of a depolarizing stimulus, addition of A23187 was necessary for activating the BK channels in the U251-MG cells. Increases in intracellular Ca^{2+} caused by A23187 shift the voltage-dependence of BK channel activation to more negative potentials (Ransom et al., 2002) and thus allow channel opening at the resting membrane potential. As was the case for the T84 cells, addition of A23187 to the U251-MG cells caused a strong, time-dependent increase in $[K^+]_o$ (peak value = 183 ± 5 μM, n = 4 assays) (Figure 6B). This K^+ efflux was almost completely inhibited following pretreatment with CHX (peak value = 32 ± 3 μM, n = 3 assays) (Figure 6B).

The ISFET-based assay described in this report offers a number of advantages for ion channel screening. ISFETs provide an ideal sensor for measuring ion efflux and drug block in multi-well plates because of their small size (4–6 mm^2) and integrative electronic capacity. While ion-selective electrodes have been used for measuring ion flux, the large size of the electrodes (diameter = 15 mm) and cost ($600–$1000 per electrode) makes them unsuitable for parallel multi-well plate measurements. Although automated patch clamp systems directly measure ion current, these instruments require the use of cells expressing high levels of recombinant ion channels that are added in suspension to the recording wells (Dunlop et al., 2008; Yajuan et al., 2012). In addition, the invasive nature of the planar patch clamp technology makes these systems unsuitable for use with most primary and stem cell-derived cells. As demonstrated in the present study, the ISFETs provide a non-invasive approach for multi-channel, parallel measurements in cells that are cultured using standard plating procedures. Future ISFET assay development and optimization will allow for ion channel screening in a number of cell types.

4. Conclusions and Future Work

An ISFET-based screening assay has been developed and tested for the acquisition of K^+ efflux data from cultured mammalian cells. The assay provides a direct, real-time measurement of ion flux and allows for multi-channel, parallel data acquisition. The ISFET assay was successfully utilized to measure K^+ efflux from two different clonal cell lines that express two different types of K^+ conducting channels. The potential use of the ISFET system for screening ion channel modulators was demonstrated using known blockers of Ca^{2+}–activated NSC and BK channels. These channel blocking effects were consistent with the measurements obtained using the traditional whole-cell arrangement of the patch clamp procedure. In the future K^+-sensitive graphene ISFETs, with integrated Ag/AgCl reference electrodes, will be developed for direct insertion into 96-well plates. For this purpose an ISFET recording device will be designed that incorporates eight ISFETs and corresponding perfusion tubing for compound injection into one unit. Given the noninvasive properties of the ISFET sensor, this assay should be suitable for ion screening in a variety of cell types including primary and stem-cell derived cells.

Acknowledgments

This work was supported by US Public Health Service award HL-45789 (KBW) and an Aspire grant from the University of South Carolina.

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Highlights

- An ion-sensitive field effect transistor (ISFET)-based assay was developed for screening ion channels.
- The assay was utilized for measuring K^+ efflux from two mammalian cell lines cultured in multi-well plate format.
- The assay allowed for the screening of ion channel compounds using real-time, parallel measurement configurations.
- The ISFET screening assay provides several advantages over currently employed technologies in screening for modulators of ion channels.

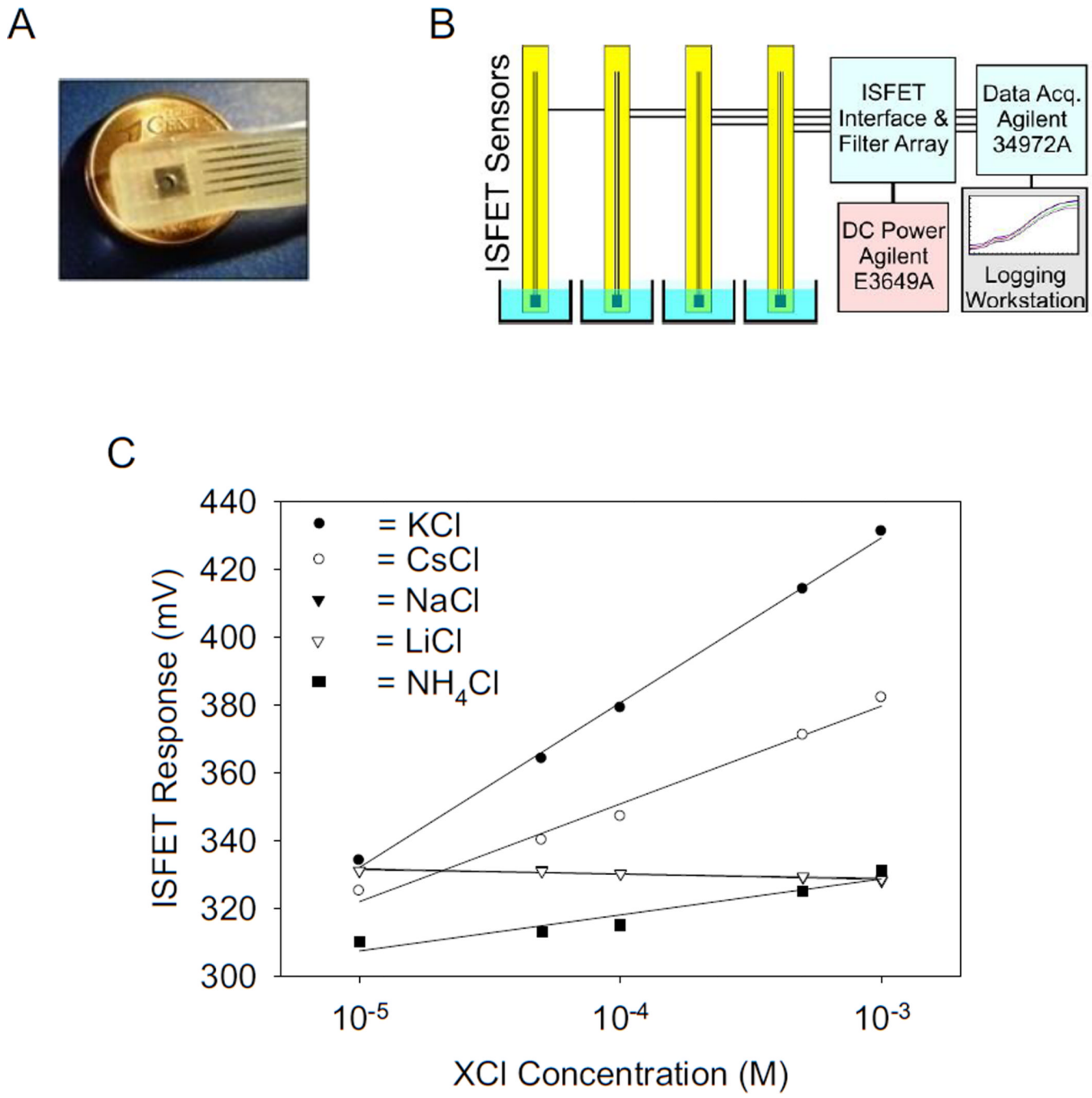

Figure 1.

Development of an ISFET-based assay for screening ion channels. **A**: K^+-sensitive ISFET encapsulated on a printed circuit board. **B**: schematic of the measurement set-up with 4 parallel ISFETs along with the DAQ and recording equipment. In some experiments three parallel recordings were simultaneously obtained (see Figure 5). **C**: cation sensitivity of the ISFET determined in the presence of KCl (m = 49 mV/decade), CsCl (m = 29 mV/decade), NH_4Cl (m = 11 mV/decade), NaCl and LiCl. The curves were measured in 100 mM TRIS-Cl solution and the slopes obtained using linear regression.

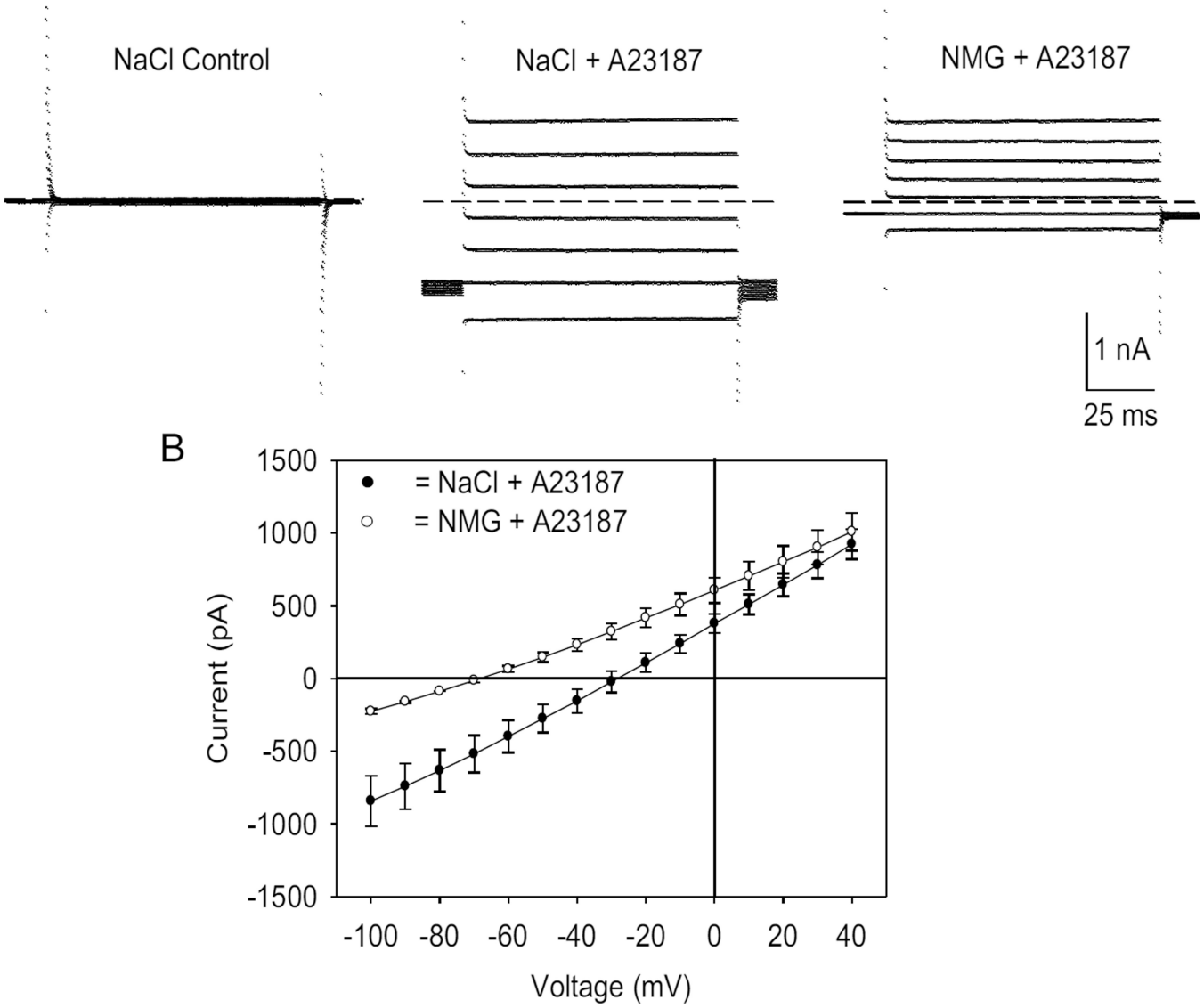

Figure 2.

Measurement of Ca^{2+}-activated NSC channels in T84 cells. **A**: ion currents recorded during voltage steps applied from a holding potential of −80 mV to −100 through +20 mV (in 20 mV increments) under control conditions (NaCl), in the presence of 1 μM A23187 and following substitution of NaCl with NMG-Cl. Dashed lines represent zero current. **B**: I/V relationship for A23187-sensitive current measured in NaCl or NMG-Cl solution. Each point represents the mean ± SE of the current measured in eight cells.

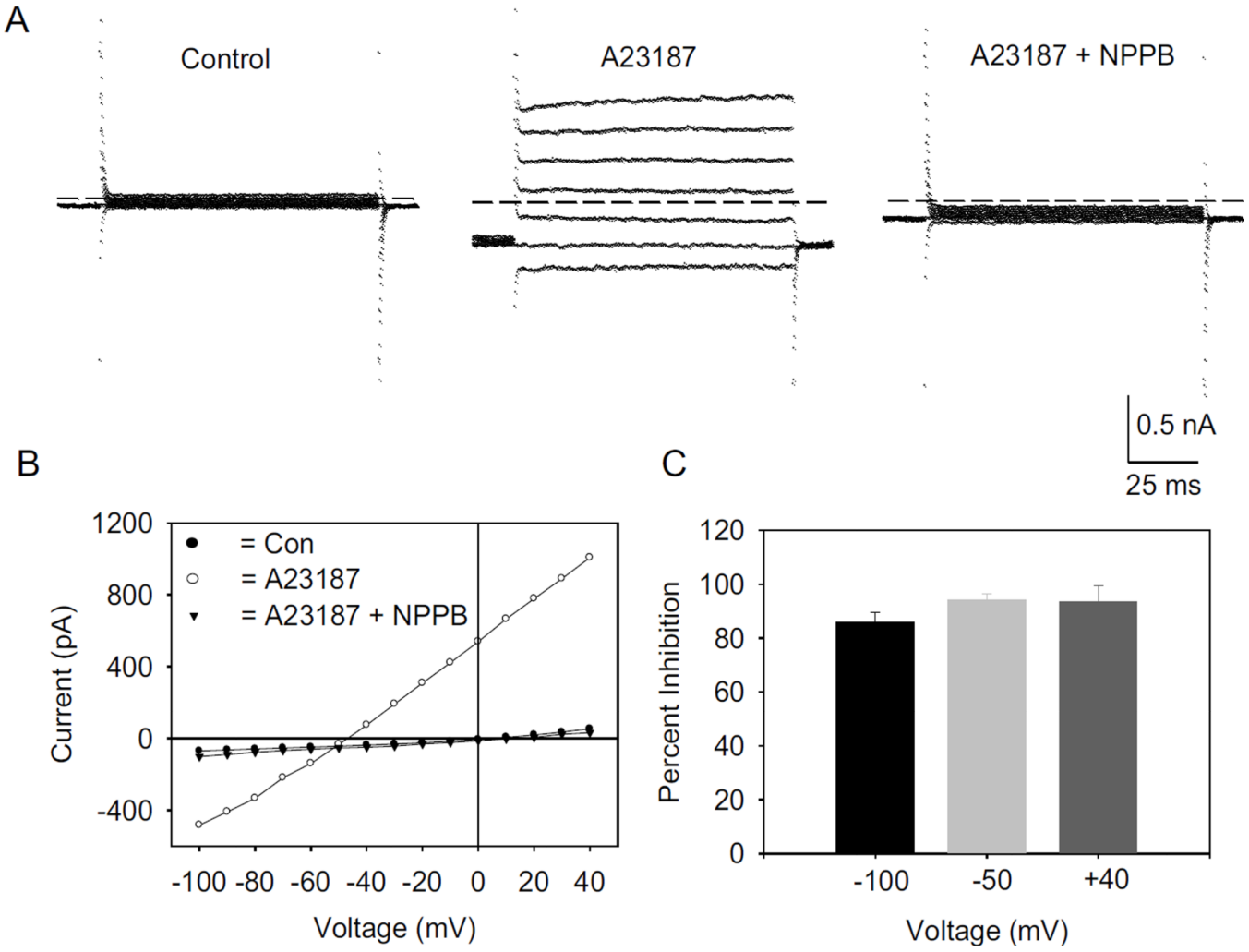

Figure 3.

NPPB block of I_{NSC}. **A**: I_{NSC} recorded during voltage steps applied to −100 through +20 mV under control conditions (NaCl), in the presence of 1 μM A23187 and following addition of 20 μM NPPB. Dashed lines represent zero current. **B**: I/V relationship for the A23187-sensitive currents shown in panel A. **C**: percent inhibition of I_{NSC} measured at −100 mV, −50 mV and +40 mV with NPPB. Each bar represents the mean ± SE inhibition measured in five T84 cells.

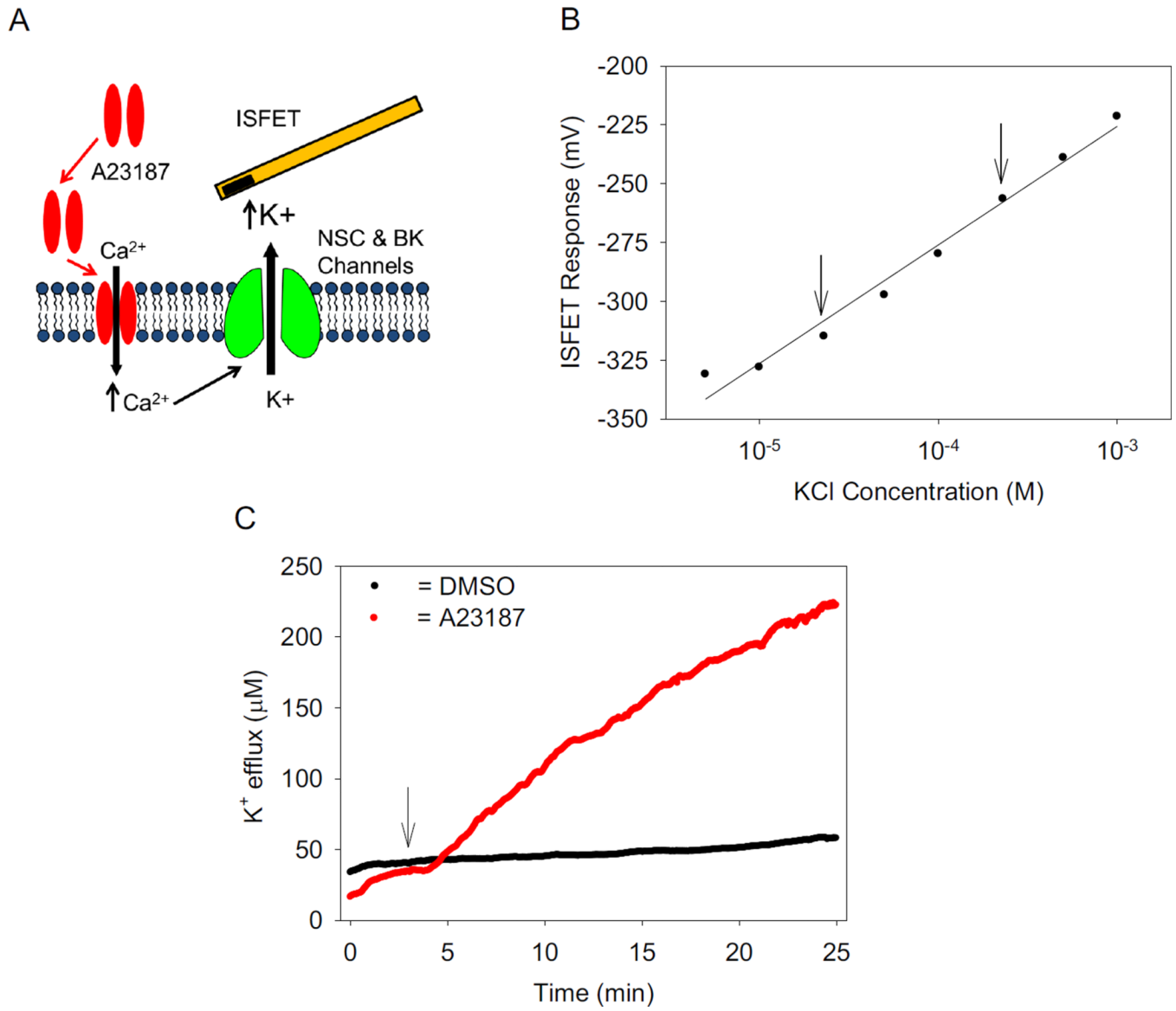

Figure 4.

K^+ efflux measured using the ISFET assay system. **A**: cell membrane model of K^+ efflux induced by the Ca^{2+} ionophore A23187. **B**: calibration curve for a K^+-sensitive ISFET. The arrows indicate the typical range of K^+ efflux (20 to 200 µM) measured from T84 cells. The slope of the curve measured over the linear portion of the graph was 42 ± 2 mV/decade (n = 8 calibrations in 140 mM NaCl saline solution). **C**: K^+ efflux from T84 cells recorded with an ISFET. Output voltage was sampled at 10 Hz and filtered at 1 Hz. Solution containing either 1 µM A23187 or control solvent (DMSO) was added following the basal efflux measurement (see arrow).

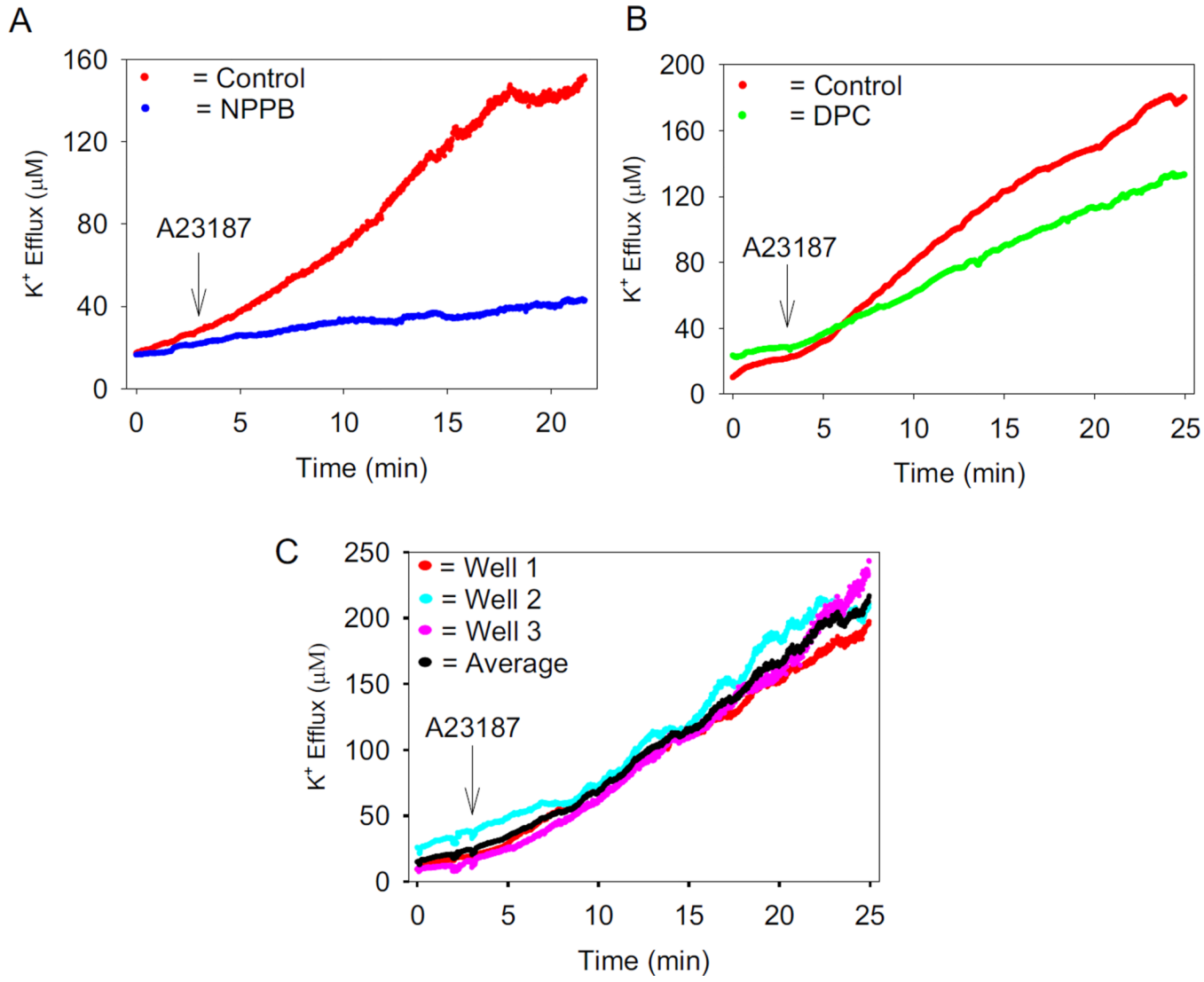

Figure 5.

Utility of the ISFET assay for ion channel screening. **A**: $[K^+]_o$ measured following pretreatment of the T84 cells with K^+-free saline containing either DMSO (Control) or NPPB. **B**: $[K^+]_o$ measured following pretreatment of the T84 cells with K^+-free saline containing either DMSO (Control) or DPC. **C**: $[K^+]_o$ measured in parallel from three recording chambers containing T84 cells. The black line is the average of the three K^+ efflux measurements.

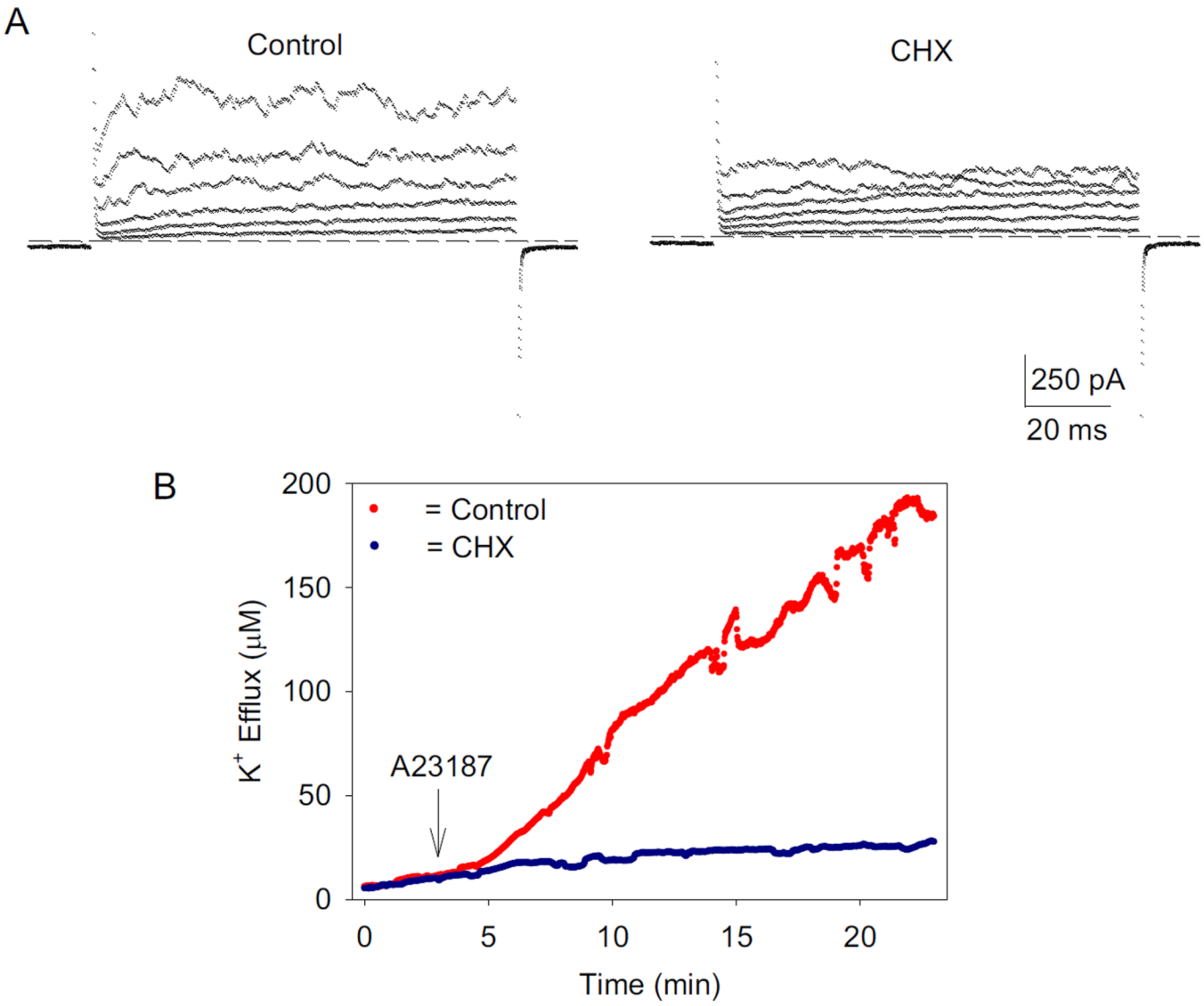

Figure 6.

Measurement of BK channels in U251-MG glioma cells. **A**: ion currents recorded during voltage steps applied from a holding potential of −80 mV to +20 through +120 mV (in 20 mV increments) under control conditions and in the presence of 100 nM charybdotoxin (CHX). Dashed lines represent zero current. **B**: $[K^+]_o$ measured following pretreatment of the U251-MG cells with K^+-free saline containing 100 nM CHX. A23187 was added to activate the BK channels.