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Original article

Minimized cell usage for stem cell-derived and primary cells on an automated patch clamp system

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ABSTRACT

Introduction: Chip-based automated patch clamp systems are widely used in drug development and safety pharmacology, allowing for high quality, high throughput screening at standardized experimental conditions. The merits of automation generally come at the cost of large amounts of cells needed, since cells are not targeted individually, but randomly positioned onto the chip aperture from cells in suspension. While cell usage is of little concern when using standard cell lines such as CHO or HEK cells, it becomes a crucial constraint with cells of limited availability, such as primary or otherwise rare and expensive cells, like induced pluripotent stem (IPS) cell-derived cardiomyocytes or neurons.
Methods: We established application protocols for CHO cells, IPS cell-derived neurons (iCell® Neurons, Cellular Dynamics International), cardiomyocytes (Cor.4U®, Axiogenesis) and pancreatic islet cells, minimizing cell usage for automated patch clamp recordings on Nanion's Patchliner. Use of 5 µl cell suspension per well for densities between 55,000 cells/ml and 400,000 cells/ml depending on cell type resulted in good cell capture.
Results: We present a new cell application procedure optimized for the Patchliner achieving > 80% success rates for using as little as 300 to 2000 cells per well depending on cell type. We demonstrate that this protocol works for standard cell lines, as well as for stem cell-derived neurons and cardiomyocytes, and for primary pancreatic islet cells. We present recordings for these cell types, demonstrating that high data quality is not compromised by altered cell application.
Discussion: Our new cell application procedure achieves high success rates with unprecedentedly low cell numbers. Compared to other standard automated patch clamp systems we reduced the average amount of cells needed by more than 150 times. Reduced cell usage crucially improves cost efficiency for expensive cells and opens up automated patch clamp for primary cells of limited availability.

1. Introduction

In recent years, automated patch clamp systems have become an indispensable asset in drug development and safety pharmacology (Möller & Witchel, 2011; Polonchuk, 2012) and led to a significant increase in efficiency in basic and applied research (Milligan et al., 2009). Automation of the experimental procedure and parallel processing of currently up to 384 single cell measurements allow for high throughput and standardized experimental conditions compared to manual patch clamp. This progress has become possible through the advent of planar patch clamp, in which the glass pipette used to approach cells in conventional patch clamp is replaced by a planar glass chip with a micrometer-sized hole. Instead of selecting and targeting specific cells with a movable pipette, planar patch clamp is based on flushing a suspension of cells over a static chip, with cells being caught by negative pressure applied through the apertures in the recording chip (Milligan et al., 2009). Although this approach overcomes former limitations of a tedious manual procedure requiring extensive practical training, it bears the disadvantage that a substantial amount of cells is irretrievably lost with each attempt to patch a single cell. The success rate of catching a cell for recording critically depends on the volume and density of the cell suspension flushed over each chip aperture. While the amount of cells needed is usually not a constraint for studying ion channels in standard cell lines like CHO or HEK cell lines, it is critically restricting the use of primary cells derived from limited amounts of tissue or cells designed and produced at high cost, such as induced pluripotent stem (IPS)

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cardiomyocytes, which play a crucial role in safety pharmacology. Differentiable stem cells constitute a significant advancement in the constant endeavor to replace in vivo animal research with cell based assays. Recently, an increasing number of human IPS cells have become commercially available, meeting a rapidly growing demand for differentiated human cells and creating the need for reliable and cost-efficient high throughput assays, as required for drug safety screening. Likewise, studies of primary cells are of crucial biological significance for target identification and black box screening, and hence would benefit substantially from compatibility with automated patch clamp approaches.

To this end, we set out to minimize the cell usage for an automated patch clamp system well-suited for cell lines, primary cells and stem cell-derived cells, the Patchliner, by optimizing the cell application protocol for minimal volume at minimal density of the cell suspension. Here, we present a protocol achieving > 80% success rate using only 5 µl of cell suspension at a density of 55,000 to 400,000 cells/ml per well depending on cell type, amounting to a total of just 300 to 2000 cells per well. Using these protocols, we successfully recorded high quality data from IPS cell-derived cardiomyocytes and neurons, as well as primary cells from pancreatic tissue. Furthermore, other automated patch clamp systems use between 8000 and 150,000 cells per well at 1.5 Mio. cells/ml density, making the Patchliner 10 to more than 150 times more economical in terms of cell usage. The Patchliner was shown before to be ideally suited for recordings from a wide range of cell types (Milligan et al., 2009). The optimization for minimized cell usage now makes it accessible for high throughput screening of expensive cells, which will significantly improve the efficiency and applicability of automated patch clamp in drug development and safety screening.

2. Materials and methods

2.1. Cell culture and harvesting

2.1.1. CHO cells

Cells were harvested according to a standard procedure (Brüggemann et al., 2008) and suspended at densities varying between 1000 and 250,000 cells/ml (see Table 1). The cells were then placed in the cell hotel of the Patchliner, where they were periodically pipetted up and down to ensure viability and minimize clustering.

Table 1
Summary of data analyzed to determine minimal ideal density of the cell suspension (n = number of attempts).

Cell type	Density (×1000 cells/ml)	Volume/well (µl)	Cells/well	Catch rate (%)	n
CHO cells	120	5	600	96	24
	110	5	550	96	24
	80	8	640	97	31
	80	5	400	94	32
	60	5	300	87	31
	55	5	275	83	24
	40	8	320	94	32
	40	5	200	50	16
	30	5	150	56	32
	20	5	100	58	24
	10	5	50	38	24
	5	5	25	29	24
	2.5	5	13	16	32
	1	5	5	0	8
Neurons	250	5	1250	94	31
	125	5	625	100	8
	62.5	5	313	68	22
	31.3	5	156	46	24
	15.6	5	78	25	8
Cardiomyocytes	200	5	1000	75	24
	50	5	250	75	28
	25	5	125	41	32
	12.5	5	63	25	16
	10	10	100	40	20

2.1.2. Neurons

Frozen stocks of iCell® Neurons (Cellular Dynamics International (CDI), Madison, WI, catalogue number: NRC-100-010-001) containing at least 2.5 million plateable cells per unit, were thawed as per the manufacturer's instructions at a density of 50,000 cells/cm^2. Cells were cultured for at least 4 days after thawing and were used on the automated patch-clamp platform 4 to 7 days after plating. Detailed culture and harvesting protocols have been described previously (Haythornthwaite et al., 2012).

2.1.3. Cardiomyocytes

Frozen vials of 1 million Cor.4U® cardiomyocytes were obtained directly from the manufacturer (Axiogenesis, Cologne, Germany). One vial sufficed for seeding two T-12.5 flasks. Before seeding, flasks were coated with fibronectin by applying 2 ml of 1:100 diluted fibronectin solution (e.g. Sigma F1141) in PBS to each flask. Flasks were then incubated for 3 h at 37 °C or overnight at 4 °C. A vial of cells was thawed in a 37 °C water bath and transferred into 8 ml Cor.4U® medium (in the following called medium) kept at 37 °C in a 50 ml tube. The cell vial was rinsed with 1 ml medium, added to the 50 ml tube (total volume of 10 ml). Cells were immediately spinned at 200 × g for 5 min. After discarding the supernatant, careful tapping loosened the cell pellet. A total of 500 µl of 37 °C medium was added and carefully pipetted up and down to resuspend the cells. For seeding, the fibronectin solution was removed from the flask and replaced by 2 ml medium. After centrifugation, cells were resuspended in 2 ml medium and 1 ml cell suspension was transferred into a T-12.5 flask. Cells were cultured at 37 °C, 5% CO_2 and 95% humidity. After 24 h the majority of cells were attached and medium was exchanged. Forty-eight hours later, medium was exchanged and supplemented with 1.5 µg/ml Puromycin. Twenty-four hours later, cells were washed twice and cultured for another 4 to 7 days, with medium exchanges every 48 h. For harvesting, cells were washed twice with 3 ml cold (4 °C) PBS/EDTA and then incubated in 3 ml cold PBS/EDTA for 10 min at 4 °C. Then, PBS/EDTA was removed and 2 ml Tryp/LE express (1×, Invitrogen) was applied. Most of the Tryp/LE express was removed leaving a thin film of liquid in which cells were incubated at 37 °C for 2 min. Gentle tapping helped detaching the cells. A total of 1.5 ml of DMEM medium + FCS and 1.5 ml external solution were added to the cells before transfer into one well of a 6-well uncoated plate to let them recover for 15 min at 4 °C. After recovery, cells were mixed until no more cell clusters were observed (~90% single cells) and then transferred to the cell hotel of the Patchliner.

2.1.4. Pancreatic cells

Mice where anesthetized with isoflurane. Collagenase (13.5 U/ml in Hank's balanced salt solution) was injected via the pancreatic duct. After a few minutes the animal was killed by removing the heart, after that the pancreas was removed and collagenase digestion commenced for 19 min at 37 °C. The digested pancreas was washed twice with ice-cold Hank's balanced salt solution and islets picked and transferred into the RPMI1640 medium. Before use on the Patchliner islets were dispersed into single cells by aspirating several times in Ca^{2+}-free HBSS. Cells were kept in RPMI1640 medium until use at a density of ~200,000 cells/ml.

2.2. Patch-clamp solutions

For automated patch clamp of all cell types, internal solution contained 50 mM KCl, 10 mM NaCl, 60 mM KF, 20 mM EGTA, and 10 mM HEPES/KOH, pH 7.2. External recording solution contained 140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 2 mM $CaCl_2$, 5 mM D-glucose monohydrate, and 10 mM HEPES/NaOH, pH 7.4. Kainic acid (10 µM;

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Sigma Aldrich, St. Louis, MO) was prepared in external solution. BayK 8644 (10 μM; Sigma Aldrich) was prepared in external solution.

2.3. Automated patch-clamp electrophysiology

All cells were recorded in whole-cell patch clamp configuration on Nanion's Patchliner (Nanion Technologies GmbH, Munich, Germany) and the PatchControlHT software (Nanion Technologies GmbH, Munich, Germany) was used for cell capture, seal formation, whole-cell access, and programming of the experiments. The Patchliner Quattro or Octo was used to record up to four, respectively eight cells simultaneously. The Patchliner uses one or two EPC-10 Quadro patch clamp amplifiers respectively (HEKA Elektronik, Lambrecht/Pfalz, Germany), with Patchmaster software (HEKA) for data acquisition and basic analysis. Borosilicate glass NPC-16 chips (Nanion Technologies) were used for all recordings; medium resistance (1.8–3 MΩ) for CHO and pancreatic cells, high resistance (3–5 MΩ) for neurons and low resistance (1.5–2 MΩ) for cardiomyocytes. The catch rate was calculated by dividing the number of successfully captured cell by the number of wells we attempted, expressed in percentage.

3. Results

3.1. Optimization of cell usage for automated patch clamp

Aiming at making automated patch clamp a viable approach for all cell types including expensive cells and low-yield preparations, we optimized the cell application procedure of the Patchliner (Fig. 1A) for minimal cell usage. We first minimized the volume of cell suspension that needs to be applied to each well. For single cell recordings, chips with one hole per recording well are used. Therefore, after catching a cell, the remaining cell suspension in a well is discarded unused. For simplicity, we used a standard CHO cell line for the optimization procedure before testing the protocol on more delicate cell types. We drastically reduced the amount of cell suspension by determining the absolute minimum of solution needed per well and by replacing part of this volume by external solution (Fig. 1A,B). With our new procedure, we cut the use of cell suspension from 35 μl down to 5 μl per well by stacking the cell suspension with 8 μl external solution in the pipette. The cell suspension is applied first, then followed by the external solution to push the entire volume of the cell suspension past the chip hole (Fig. 1B) and thereby maximizing the chance of catching a cell. In a second step, we determined the minimal density of the cell suspension required to achieve good success rates for catching a cell (Table 1; Fig. 1C). The data were best described by a logarithmic fit, with a correlation coefficient of $r^2 = 0.94$ (Fig. 1C). Solving the equation of the fit for an aspired success rate of 80% for catching a cell results in an absolute number of cells of less than 300 cells required per well for CHO cells (Fig. 1C). This corresponds to a density of about 55,000 cells/ml for CHO cells. Based on these numbers, we then optimized the protocol for human IPS (hIPS) cell-derived neurons (iCell® Neurons) and hIPS cell-derived cardiomyocytes (Cor.4U® cells). Roughly 500 iCell® Neurons at a density of 100,000 cells/ml were needed for 80% success in catching cells (Fig. 1D) and about 850 cardiomyocytes at a density of 170,000 cells/ml (Fig. 1E). Applying the protocol on mESC-derived cardiomyocytes at a density of 10,000 cells/ml and 100 cells per well exactly matched the fit for the Cor.4U cells (Fig. 1E, gray diamond).

3.2. Cor.4U® hIPS cell-derived cardiomyocytes exhibit primary-like electrophysiological characteristics

In previous studies, we showed that primary-like cardiomyocytes derived from mouse embryonic stem cells (mESCs) as well as hIPS cell-derived cardiomyocytes can be successfully electrophysiologically

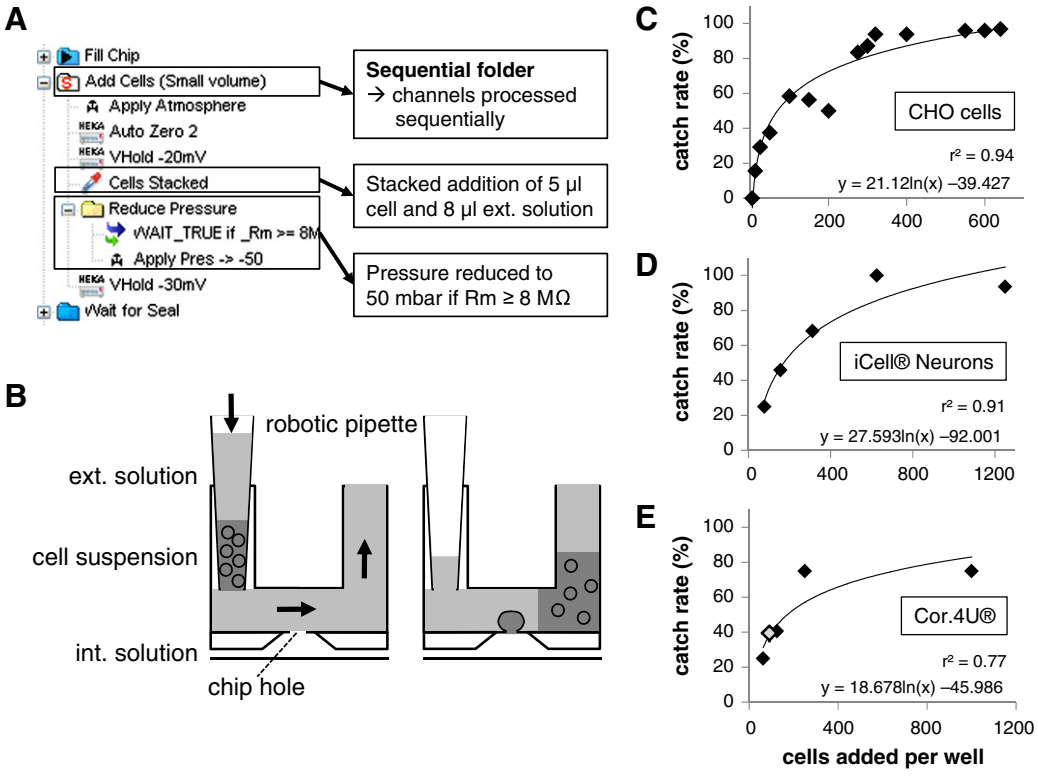

Fig. 1. Procedure for minimized cell usage and catch rates for different cell types. (A) Section of the PatchControlHT software depicting the cell application procedure. (B) Schematic illustration of applying the cell suspension stacked with external solution to a single well of the chip. (C, D, E) Catch rate as a function of absolute numbers of cells added per well for CHO cells (C), hIPS cell-derived iCell® Neurons (D) and hIPS cell-derived Cor.4U® cardiomyocytes (E). Gray diamond in E represents a single data point obtained from mEPS cell-derived cardiomyocytes.

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characterized on the Patchliner and Port-a-Patch (Stoelzle, Haythornthwaite, et al., 2011; Stoelzle, Obergrussberger, et al., 2011). To confirm that low densities of hIPS cell-derived cardiomyocyte cell suspensions are suited for automated patch clamp experiments, we set out to record voltage-dependent currents and induced action potentials (APs) in Cor.4U® cells. Using the application protocol for optimized cell usage, we found that Cor.4U® cells show voltage-dependent Na^+ (Fig. 2A) and K^+ currents (Fig. 2B), a prerequisite for AP generation, and that APs can be induced (Fig. 2C,D). For AP recordings, we ran protocols in the current clamp mode on the Patchliner, depolarizing cells by applying brief current pulses just above threshold for AP generation. Application of 10 μM BayK 8644 (Fig. 2C), an L-type Ca^{2+} channel agonist (Heubach et al., 2004), increased the amplitude as well as the duration of the AP (Fig. 2C). The demonstrable Ca^{2+} component strongly indicates the development of a typical cardiomyocyte AP feature. When blocking Na^+ channels with 10 μM TTX, no more APs could be induced (Fig. 2D). A subsequent washout fully reversed the inhibition.

3.3. hIPS cell-derived iCell® Neurons displayed voltage-dependent currents potentiable by kainic acid

iCell® Neurons were shown before to display primary-like expression of ion channels in conventional patch clamp as well as in automated patch clamp recordings (Haythornthwaite et al., 2012). As for cardiomyocytes, using the optimized cell application procedure on iCell® Neurons resulted in high quality recordings (Fig. 3). iCell® Neurons expressed voltage-dependent Na^+ (Fig. 3A) and K^+ channels (Fig. 3B), of which the latter were blocked by 5 μM quinidine (Fig. 3C). Furthermore, 10 μM kainic acid, a neuron-specific glutamate receptor agonist, reversibly increased positive currents in a voltage-dependent manner in a voltage ramp protocol (Fig. 3D).

3.4. Primary pancreatic cells showed Na^+ channel inactivation and K^+-delayed rectifier currents typical for B-cells

The optimized cell application protocol allowed high quality recordings from mouse primary pancreatic islet cells (Fig. 4A). We recorded voltage-dependent currents using a voltage protocol consisting of prepulses from − 120 to + 30 mV (10 mV increments) for 100 ms followed by a test pulse to 0 mV for 100 ms after a 2 ms delay at − 120 mV (Fig. 4A,B). Inward currents in B-cells are carried by voltage-gated Na^+ and Ca^{2+} channels (Nav, Cav), whereas outward currents mainly flow through K^+-delayed rectifier channels (Kdr). In this study we did not attempt to separate the different ion channel activities so the recorded currents represent the sum of all ion channel activities. Cav channels exhibit slower kinetics than Nav channels and are overlaid by outward Kdr currents. Hence, inward peak currents mainly reflected Nav channel activity. Nav activation and inactivation curves depict the peak inward currents of pre- and test pulse, respectively, plotted against the prepulse voltage (Fig. 4B), their voltage dependence being identical to previous reports (Göpel, Kanno, Barg, Galvanovskis, & Rorsman, 1999). IV-curves for outward currents, mainly reflecting Kdr channel activity, represent the maximal outward current during the last 30 ms of the prepulse at the respective voltages (Fig. 4B). At this time point Nav channels are completely inactivated whereas a small residual Cav current leads to a slight underestimation of the K^+ currents. However, as for the Nav currents, the voltage dependence of Kdr currents is similar to that previously reported (Göpel et al., 1999).

4. Discussion

Automated patch clamp systems performing parallel recordings aim for high throughput of highly complex measurements. High throughput often comes at the expense of restricting experimental conditions. Besides ample advantages of automated patch clamp systems, the amount of cells required per well can be a restricting parameter when working with expensive, non-proliferating cells or preparations from native tissue. With many stem cell-derived cell types developed at high costs on the rise, cell usage has become a factor of particular interest. At present, most automated patch clamp systems require a substantial amount of volume of cell suspension and of absolute numbers of cells added per recording site. For instance, a typical amount of cells needed on a comparable system is 8000 cells per well for cardiomyocytes (Timm Danker, NMI TT GmbH, Reutlingen, personal communication), while other reports using cardiomyocytes on different automated patch clamp systems vary between 25,000 and 150,000 cells per well (Ma et al., 2011, Schrøder, Christensen, Anson, & Sunesen, 2012), which we undercut by one to two orders of magnitude for Cor.4U® hIPS cell-derived

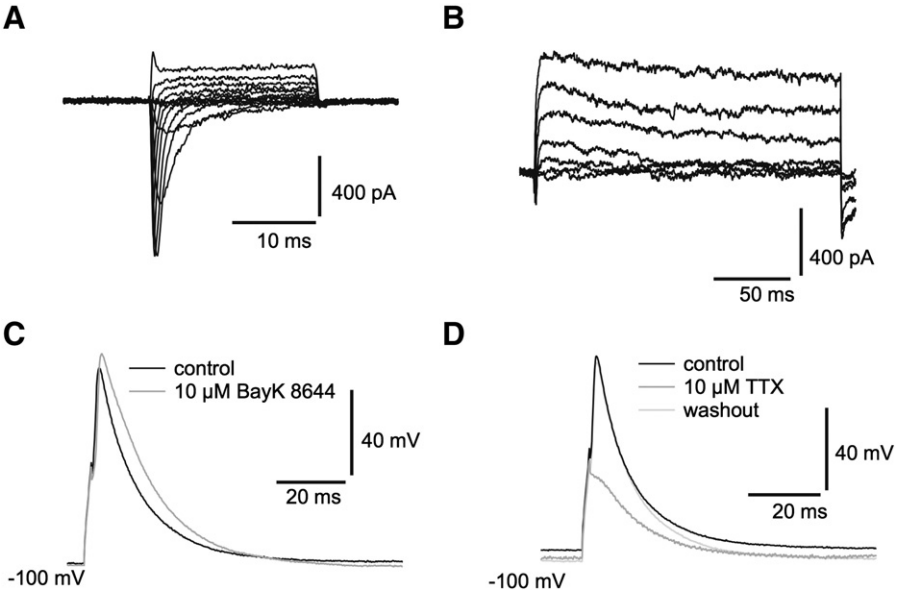

Fig. 2. Electrophysiological characteristics of Cor.4U® cardiomyocytes. Raw data traces of voltage-dependent Na^+ inward currents (A) and K^+ outward currents (B). (C) BayK 8644 (10 μM) increased the amplitude and duration of inducible APs. (D) APs were reversibly blocked by 10 μM TTX.

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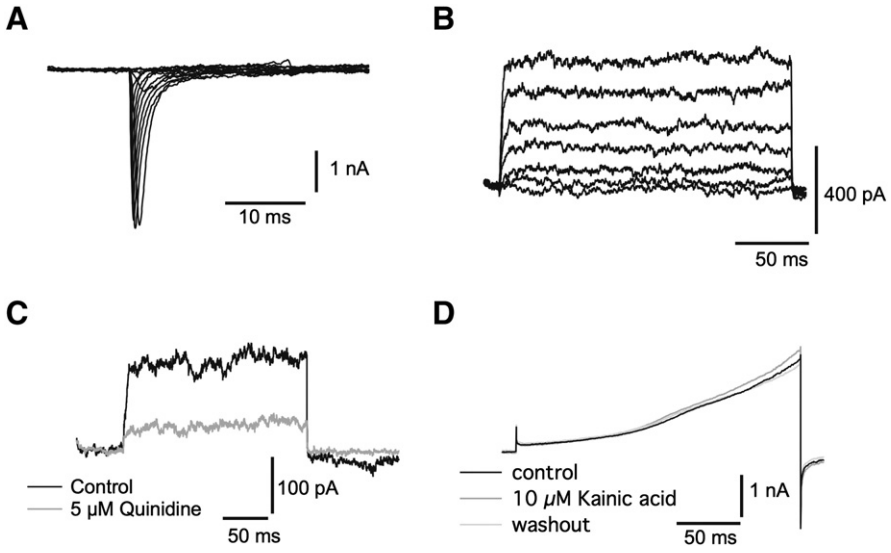

Fig. 3. Voltage-dependent currents of iCell® neurons. Raw data traces of voltage-dependent Na^+ inward currents (A) and K^+ outward currents (B), which were inhibited by quinidine (C). (D) Reversible enhancement of positive current by application of 10 µM kainic acid.

cardiomyocytes and iCell® Neurons, hIPS cell-derived neurons. Thus, the new cell application procedure on the Patchliner makes it the most economical automated patch clamp system in terms of cell usage to date. However, optimizing the volume and density of the cell solution should be directly transferable to other automated patch clamp systems, while stacking different solutions in the pipette is currently only possible with Nanion's Instruments.

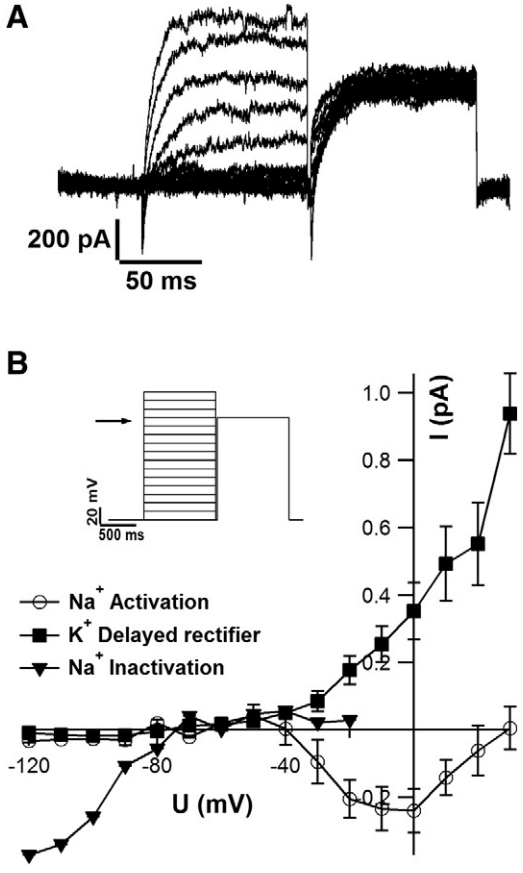

Fig. 4. Voltage-dependent currents in mouse primary pancreatic cells. (A) Raw traces of voltage-dependent currents. (B) Currents measured at different time points in response to voltage step protocol (inset).

We tested our new protocol on a range of distinct cell lines, including hIPS cell-derived Cor.4U® cardiomyocytes and iCell® Neurons, as well as pancreatic islet cells prepared from native tissue. Induced pluripotent stem cells have the substantial benefit of allowing the study of ion channel properties and their responses to substances in their native environment. Thus, hIPS cell-based assays improve accuracy and predictive value of compound screenings at an early stage and reduce the need for in vivo studies. To fully deploy the potential of the increasing range of available hIPS cells, high throughput electrophysiology assays are in great demand. Using our new protocol, we succeeded in recording high quality data from Cor.4U® cells expressing voltage-dependent channel activity similar to that of mESC-derived cardiomyocytes (Stoelzle, Haythornthwaite, et al., 2011; Stoelzle, Obergrussberger, et al., 2011), confirming that they exhibit crucial features of differentiated cardiomyocytes (Robertson, Tran, & George, 2013). Furthermore, we obtained high quality recordings of voltage-dependent channel activity in hIPS cell-derived iCell® Neurons, resembling recordings of a previous study (Haythornthwaite et al., 2012). Hence, we demonstrate that high recording quality of stem cell-derived cell types is achievable even with small amounts of cells at high throughput.

Equally important, the procedure proved successful when applied to pancreatic islet cells prepared from native tissue. Hormone secreting pancreatic islet cells are the subject of intense research due to their significant role in the development of diabetes. Since hormone secretion is regulated by their electrical activity, detailed electrophysiological studies on primary islet cells are highly desirable. Due to the limited amount of primary cells available and the insufficient data quality provided by most automated patch clamp systems this has been entirely restricted to manual patch clamp with all its drawbacks. By using automated patch clamp with minimized cell usage and data quality identical to that achieved on manual setups, it is now possible to increase the throughput substantially. We report here that voltage-gated currents from primary insulin-secreting B-cells can be recorded on an automated patch clamp system. The Nav, Cav and Kdr channel properties are identical to those previously reported. Besides higher throughput, a strictly standardized experimental flow and improved seal stability in planar automated patch clamp most likely increase efficiency by more than the mere number of recordings compared to manual patch clamp. This will allow for functional screening of small subsets of compounds directly on primary islet cell function. With the improved throughput it will also be possible to follow B-cell functionality and the effect of different treatments

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during short term culture. Due to the extremely limited throughput on manual setups this has in the past been restricted to very few experimental conditions.

5. Conclusions

In this study, we present an optimized cell application protocol minimizing the amount of cells required for an automated patch clamp system, the Patchliner. We demonstrate that this procedure is applicable to hIPS cell-derived neurons and cardiomyocytes playing a particular role in safety screening, as well as primary pancreatic islet cells. Our protocol makes automated patch clamp recordings compatible with the study of expensive cells and low-yield primary cell preparations. We think that this optimization will substantially enhance the efficiency and applicability of automated patch clamp in drug development and safety screening.

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