Max Planck International School

Neurosciences

Göttingen

Primož Pirih

2nd Lab Rotation

January 18th – March 28th 2003

Supervisors:

PD Marjan Rupnik & Simon Sedej

European Neuroscience Institute Göttingen, Neuroendocrinology group

Project

Analysis of macroscopic currents and current noise in whole-cell patched single beta cells of mouse pancreatic islets

Final version

Summary

Beta cells of pancreatic islets are blood glucose level sensors and effectors of insulin release. One of the possible key players in metabolism-membrane excitation coupling, which leads to exocytosis of hormone-filled vesicles, are ATP-sensitive K^+ channels. There are strong objections against the prevailing role of these channels in coupling because of their almost complete inactivation at physiological ATP level. We measured macroscopic currents in whole-cell patched isolated beta cells and tried to assess whether the changes in these channels' activity upon ATP depletion with dialysis could be detected with stationary and non-stationary analysis of current noise.

Introduction

Beta cells of Langerhans' islets of pancreas are endocrine cells that regulate the systemic sugar metabolism by means of insulin hormone excretion. Beta cells secrete insulin in response to raised blood glucose concentration (and also to raised levels of other energy-rich compounds). One of the key steps in stimulus-secretion coupling is the depolarisation of the membrane and the appearance of bursts of calcium action potentials (**Figure 1**; Gomis et.al., 1996).

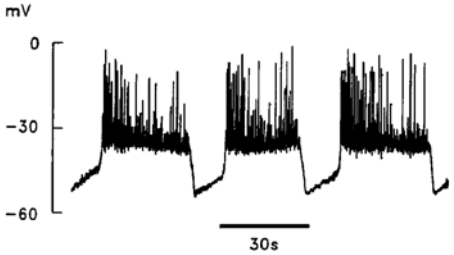

Figure 1: Rhythmical electrical activity patterns of a pancreatic beta cell at physiological blood glucose concentration 7.2 mM, recorded in vivo with sharp micro electrodes from anaesthetised mice. Taken from (Gomis et.al., 1996).

Voltage traces recorded from whole-pancreas preparations under physiological glucose levels (<10 mM) reveal the following stereotypical and very stable characteristics of the electrical activity: after a slow depolarisation from about -55 mV to -45 mV, a half-minute burst of spikes that can reach to 0 mV, appears with plateau at about -35 mV, then cell is repolarised again (Gomis et.al., 1996).

Similar experiments performed on isolated islets, where glucose levels were experimentally changed, show that at non-physiologically high glucose levels (25 mM), cell membrane potential remains at plateau, and the frequency of spikes goes up to 5 Hz. Proportion of time, spent at the plateau, and the frequency of spikes follow a similar sigmoid curve with half-maximum activation at about 15 mM glucose, while at glucose levels under 5 mM, both transition to plateau and spiking activity are abolished (Meissner, 1976).

The key players of the mechanism that underlies the oscillatory pattern are ion channels. The following types of channels have been shown to exist in beta cells:

- voltage-activated Na^+ channels, which in case of mice, have the Boltzmann sigmoid parameters of half inactivation at -110 mV and slope factor of about 10 mV (Lou et.al., 2003). At -50 mV, they are almost completely inactivated;
- voltage activated Ca^{2+} channels, which start activating at about -45 mV and are the main mechanism of spike initiation and exocytosis of insulin-containing vesicles;
- K^+ delayed rectifiers, which cease the spike and bring the potential down to the plateau level;
- Ca^{2+}-activated K^+ channels, which activate upon build-up of $[Ca^{2+}]_{int}$ during spiking (**Figure 2**), and probably bring the burst to an end;

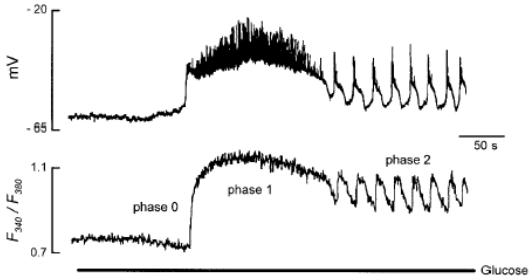

Figure 2: Simultaneous measurements of $[Ca^{2+}]_{int}$ with Fura-2, and electrical activity, upon stimulation with 12 mM glucose. Taken from (Aguilar-Bryan & Bryan, 1999).

- ATP sensitive K^+ inward rectifiers with molecular structure of four $K_{IR}6.2$ channel subunits and four SUR regulatory subunits (**Figure 3**). These channels were long thought to be the main coupling elements between blood glucose level and onset of activity of beta cells through glucose-breakdown increased [ATP], which in turn reduces the open probability of K_{ATP} channels and hence the repolarising inward current through these channels.

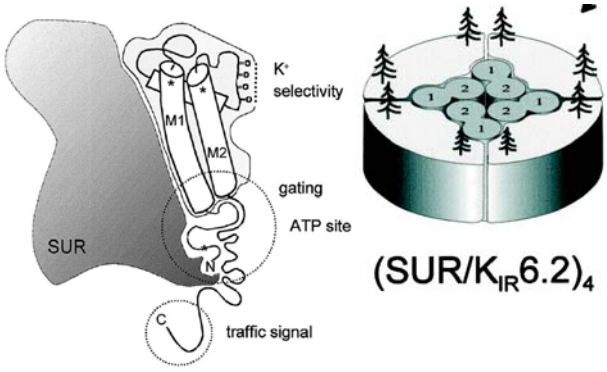

Figure 3: Molecular structure of a SUR/K_{IR} heterodimer, and the whole functional channel. Taken from (Aguilar-Bryan & Bryan, 1999)

Because K_{ATP} channels are almost completely closed at physiological (2-5 micromolar) [ATP] levels (**Figure 4**), a "spare-channel" hypothesis was proposed, where a large number of these channels could in ensemble sufficiently react to changes in [ATP] (Cook et.al., 1988). However, a biochemical experiment, where levels of [ATP], [ADP] and other phosphate compounds after glucose stimulation of whole pancreas were measured, showed that concentration changes of these compounds could

not cause sufficient decrease of K_{ATP} channels open probability to elicit the bursting activity (Ghosh et.al., 1991). Therefore, existence of other, K_{ATP} channel independent, metabolism-excitation coupling mechanisms was postulated.

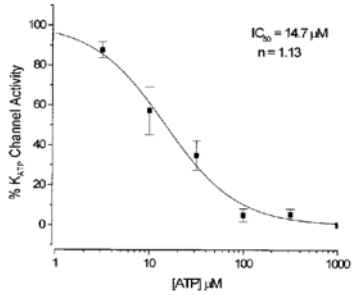

Figure 4: K_{ATP} channel activity as a function of ATP concentration. Note that 100% means saturating open probability (0.2 according to Cook et.al., 1988). Taken from (Aguilar-Bryan & Bryan, 1999)

Several other cellular elements could also be involved in the bursting activity regulation (Aguilar-Bryan & Bryan, 1999): cell-volume sensitive anion channels (Best, 1999), sodium channels, calcium-activated non selective cation channels, calcium store in endoplasmic reticulum and cytoplasmic micro domains with raised $[Ca^{2+}]$ (Goforth et.al., 2002).

An important cell element, that could contribute to controlling of the electric activity of beta cells, is the electrogenic pump (Na^+/K^+ ATP-ase) in the cell membrane; expenditure of [ATP] due to increased activity of the pump might open K_{ATP} channels and cease the bursting. (Aguilar-Bryan & Bryan, 1999)

On the other hand, it could also be hypothesed that reduced activity of the electrogenic pump (due to an unknown coupling with glucose intake) could be the mechanism of initial depolarisation of the cell that would lead to bursting activity.

It is also worthwhile to mention that physiology of beta cells strongly depends upon the extent of the analysed system (membrane patches; isolated cells; isolated islets; slices; whole preparation), for instance through electrical coupling of the cells (Aslanidi et.al., 2002), paracrine messengers such as NO (Krippeit-Drews et.al., 1996), or autonomous innervation.

The purpose of our experiment was to test whether it is possible to detect change of K_{ATP} channels open probability in whole-cell patch clamp through current noise analysis. For this purpose, we used experimental parameters which should deprive the cell of ATP, and so increase the probability of channel opening.

Materials and Methods

Electrophysiological measurements

NMRI mice were killed by cervical dislocation after anesthetization with CO_2. Enzyme liberaze was injected into the bile duct and then pancreas was isolated and incubated at 37°C. Tissue was washed in HBSS and centrifugated in FICOLL gradient. Islets were picked and digested for 5 minutes in $[Ca^{2+}]$=0, [EGTA]=5 mM extracellular medium with 0.1% trypsin. Cells were isolated with shaking and kept in perfusion with carbogen (95%O_2, 5% CO_2) bubbled, glucose-free extracellular solution at 25°-30°C with [NaCl]=150 mM, [KCl]=2 mM, [$CaCl_2$]=2 mM, [$MgCl_2$]=1 mM, [HEPES]=10 mM at pH=7.2. Fire-polished patch pippetes with resistance 2 to 4 MOhm were filled with calcium-chelating, ATP-free solution with [KCl]=150 mM, [$MgCl_2$]=2 mM, [HEPES]=10 mM, [EGTA]=5 mM at pH=2. Calculated equilibrium potential for potassium at 27°C is: E_k=-112 mV.

Patch-clamp recording was performed on HEKA EPC-9 amplifier. Upon formation of giga-seal in cell-attached mode, cell membrane was broken with gentle suction and measurements were performed in whole-cell current-clamp (membrane potential measurement) and voltage-clamp modes. Potential was monitored for five minutes; during this time, dialysis of small cell compounds (ATP and glucose) should have already come to a steady state.

As the pippete offset potential, pippete capacitance, cell membrane capacitance and cell access admittance were automatically compensated at the beginning of the experiment, the recorded capacitive transients were relatively small during the voltage steps and negligible during the voltage ramps. The current values should therefore reflect the real ionic current through the cell, except for the possible increase of the technical leak during at extreme command voltages due to the loss of giga-seal.

Current traces of 175 ms were sampled at 20kHz rate (sample interval 50 us) after on-line low-pass filtering to prevent aliasing. The voltage protocol that we used consisted of a hold at -70 mV, hold at E_K=-112 mV, hold at -200 mV, a ramp from -200 to +100 mV with slope 1mV/ms and a hold at -70 mV (**Figure 5**).

Current traces were then exported from HEKA Pulse software to Mathworks Matlab, where analysis and graphing were performed. Some

graphs were also plotted with KyPlot 2 beta 15.

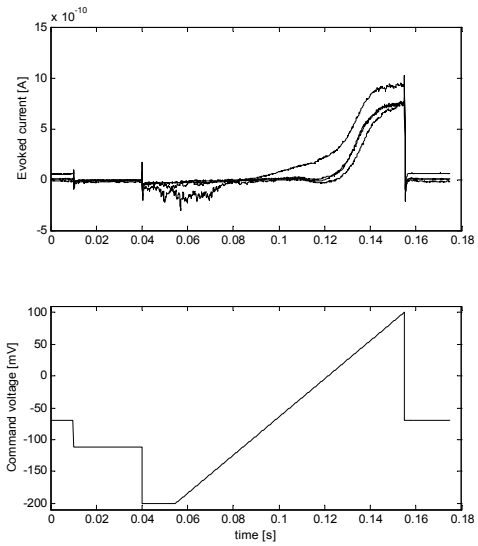

Figure 6: The protocol in voltage-clamp mode, and four representative current traces.

Noise analysis

The current noise in voltage-clamped cells reflects the subsiding single-channel events. Assumption is made that the cell has N channels of a single type with unitary current i, with their open probability P_o susceptible to changes, e.g. with application of an agonist. Then, the instanteous number of open channels and hence the derived ensemble current I follows binomial distribution with mean value $\langle I \rangle = N.P_o$ and variance $s^2(I) = N.P_o.(1-P_o)$.

Graphing variances of currents against their stationary means in several time windows ($t_1..t_2$), that cover a range of open probabilities P_o the following parabolic relationship (**Figure 6**) can be drawn:

$$s^2(t_1..t_2) = i .\langle I(t_1..t_2)\rangle - \langle I(t_1..t_2)\rangle^2/N \quad \ldots (1)$$

Initial slope of the parabola corresponds to unitary current i (Gray, 1994).

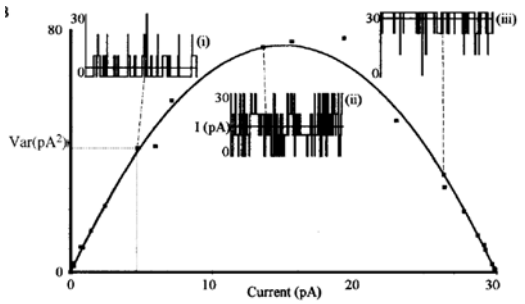

Figure 6: Plot of variance against mean current in a simulation. Taken from (Gray, 1994)

Since ion current depends upon electromotive force exerted upon the species, and since there are also other sources of noise (instrumentation and leak; it is very important that these noises be constant and uncorellated to channel noise throughout the recording), the equation (1) is expanded to

$$s^2(t) = g.(V-E).\langle I(t)\rangle - \langle I(t)\rangle^2/N + s^2_{base} \ldots (2)$$

where g is single channel conductance, V is the command voltage, E is the reversal potential of the current flowing through the channels and s^2_{base} is non-channel noise. (Traynellis & Wahl, 1997)

Assuming that each event (ion flowing through channel) produces an exponentially decaying "blip" of unitary amplitude, the open probability of a channel can be estimated:

$$\langle I(t)\rangle = N.P_o.i \ldots (3)$$

$$s^2(t) = N.i^2.P_o.(1-P_o) \ldots (4)$$

Generalising this equation in sense of equation (2), we obtain:

$$s^2(t) = N.g^2.(V-E)^2.P_o.(1-P_o) + s^2_{base} \ldots (5)$$

If the mean current is changing through the experiment, the variance of the current in a given time window should be calculated from sum of deviances of the signal from instanteous, rather than whole-window mean. This can be accomplished by subtracting the trace from:

- average of a number of records, provided that the rundown during the recording is negligible;
- a known theoretical relationship;
- from the same, but low-pass filtered signal (Gray, 1994).

As we could not predict a relationship, nor we had enough traces for averaging, we used the low-pass filtering method for the non-stationary part of noise analysis.

Since P_o of K_{ATP} channels does not depend upon the voltage, but on metabolic factors, noise analysis could yield estimates of N, g and P_o under different metabolic conditions.

Results

After establishment of whole-cell patch configuration and dialysis, we have observed a polarisation of membrane potential towards E_K from -20 towards -110 mV in about 120 seconds. During this time, voltage clamp experiments were performed in four groups: immediately (0 s), 50, 80 and 130 seconds after establishment of whole cell configuration. (**Figure 7**).

We analysed currents in the voltage ramp range (-

130..-50 mV) of the voltage ramps, where current is mostly conducted through K_{ATP} and "leak" channels. These parts of voltage traces were fitted with linear regression (I=A.V+B), where the slope (A) represents conductance (**Figure 8**), and quotient (-B/A) represents reversal potential of the current. (**Figure 7**)

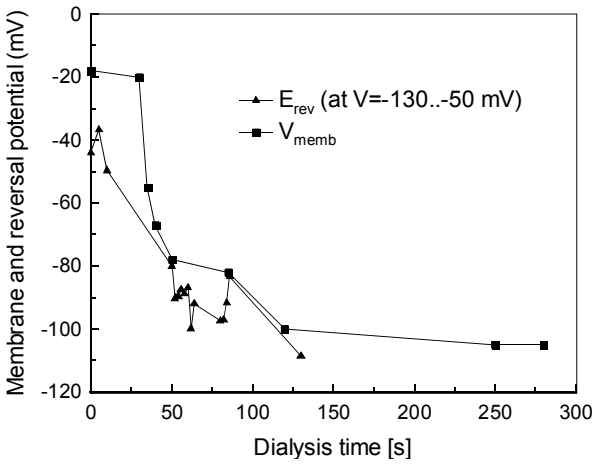

Figure 7: Measured membrane potentials in current clamp mode, and calculated reversal potentials for the "passive" (-130 .. -50 mV) regions of ramps in voltage clamp mode.

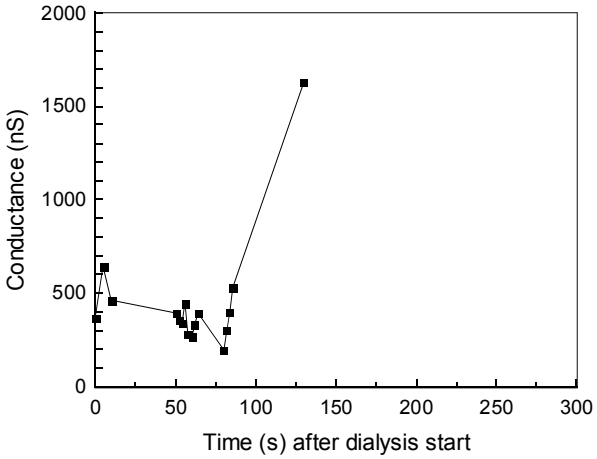

Figure 8: Conductances, calculated from slope of "passive" region of voltage ramp (-130..-50 mV).

The reversal potential of this "passive" current was -40 mV at the beginning of the dialysis and then hyperpolarised towards -110 mV. During the dialysis, the difference between membrane potential and the reversal potential diminished, indicating increase in the relative proportion of the K_{ATP} conductance. The corresponding conductance was in range (200 .. 600 nS) in the first three voltage clamp groups, while it markedly increased to 1600 nS at 130 s after dialysis (**Figure 8**).

To assess whether the indicated increase of relative proportion of K_{ATP} conductance was due to increase of their open probability, we first tried to perform current noise analysis in the stationary parts of voltage clamp traces. We took two 200-sample windows from V_{hold}=-70 mV (0..10 ms and 165..175 ms) and two at V_{hold}=-112 mV (15..25 ms and 30..40 ms).

For analysis, second trace was rejected because of variance discrepancy. In other records, good correlation between paired measurements was observed. Current at V_{hold} -112 mV stayed within the -30 to -10 pA range, while current at V_{hold}=-70 mV markedly reversed from -10..-20 pA towards +50 pA. (**Figure 9)**

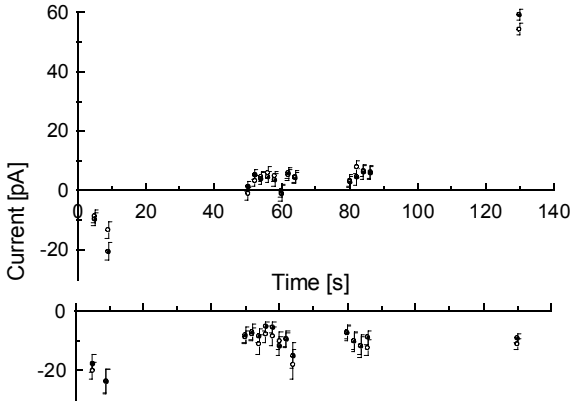

Figure 9: Plots of current and its standard deviation as function of dialysis time. Upper pane, currents at **-70 mV**. Lower pane, currents at **-112 mV**.

As the current at -112 mV remained fairly constant, assumption that current it can be attributed only to leak, its variance being of sources other than K_{ATP} channel noise, was tested. For this purpose, we averaged variances for paired trace windows, and compared their values at V_{hold} -112 at -70 mV. Under the assumption, variances at -112 mV should be lower than those at -70 mV. However, this assumption was invalidated (**Figure 10**).

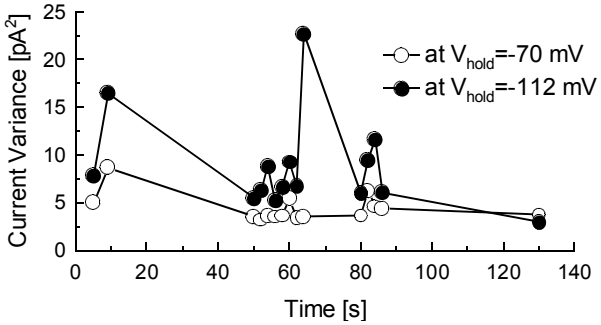

Figure 10: Comparison of variances at -70 at -112 mV.

The invalidated assumption might mean:

- that E_K was not -112 mV throughout dialysis;
- that non-K_{ATP} channel noise is correlated with hold voltage (violating the assumption of noise independence).

We therefore wanted to estimate the E_K at dialysis times with non-stationary analysis. For this purpose, current traces were low-pass filtered with two way, zero-phase 100th order digital filter with cut-off frequency at 10 Hz. Original traces were then subtracted from the filtered traces. (**Figure 11**)

20-sample windows were moved with 10-sample step through the region of records with the ramp from -130 to -60 mV (**Figure 6**). Window means were estimated from non-filtered traces and window variances from difference traces.

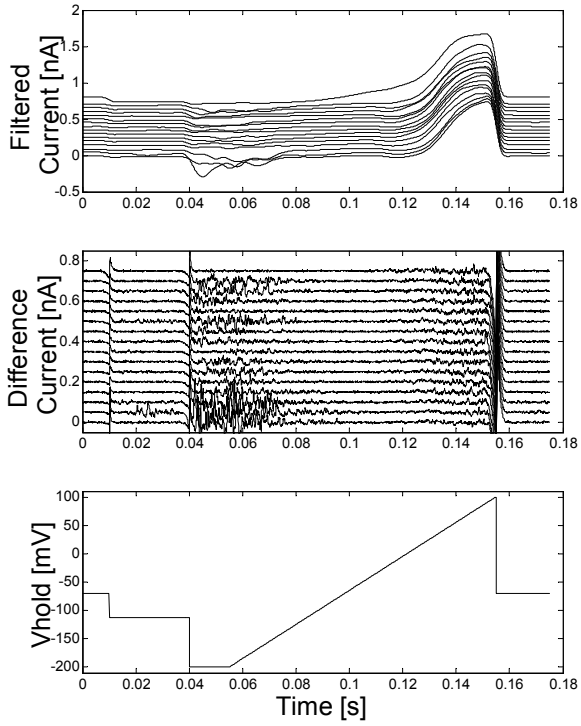

Figure 11: Low-pass filtered current traces (upper part) and difference between non-filtered and low-pass filtered traces (center part). Lower part shows voltage ramp. Chronological order of traces is from bottom to top.

Plotting window variance vs. clamped voltage for each trace should, according to equation (5), yield a parabolic relationship, where function minimum would correspond to equilibrium potential and base variance.

In this analysis, only the group of 8 voltage clamp traces, recorded 50 seconds after dialysis are shown. (**Figure 12**). The second order polynomial, fitted to the data, predicted reversal potential at around -65 mV. However, this estimate could not be regarded as valid due to the data scatter and huge increase of variance at more negative potentials.

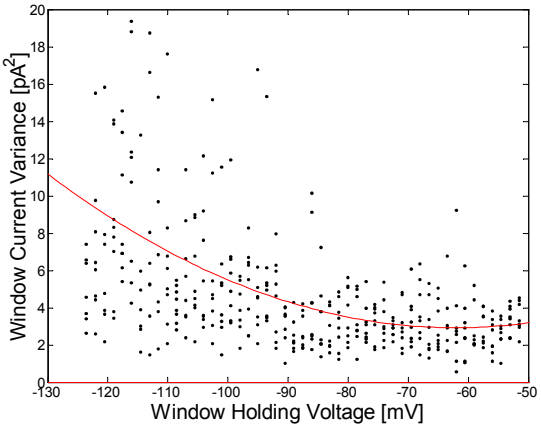

Figure 12: Relationship between holding voltage and variance, which could lead to estimation of reversal potential and base variance, for a group of 8 records at 50..60 seconds after dialysis. A quadratic polynomial is fit to data.

We did not try to further assess the open probability of K_{ATP} channels by relating variance to mean current, since a good estimate of reversal potential and base variance are essential for that.

Discussion

After transition to whole-cell mode and onset of dialysis of the cell content, membrane potential started shifting from -20 mV towards -100 mV. Reversal potential of currents, attributable mostly to potassium flow through K_{ATP} channels, was at the beginning lower than membrane potential, but the difference diminished during the course of experiment.

Calculated conductances, attributable to K_{ATP} channels, were at the beginning of dialysis at about 500 pS and remained at this level also at 50 and 80 seconds after its onset. However, the trace at 130 s showed a 3-fold conductance increase.

It is plausible to infer that K_{ATP} channels' open probability increased at 130s of dialysis, probably due to depletion of ATP (as extracellular fluid contained no glucose, and intracellular no ATP). The observed membrane hyperpolarisation also speaks in favour of this inference.

The difference between membrane potential and reversal potential for “passive” currents at the beginning of the dialysis is somewhat suspicious; especially because the calculated “passive current” conductance in the first three groups did not significantly change. This might mean that an netto inward current existed at the beginning of experiment and then diminished.

To directly prove this increase of K_{ATP} channel open probability, we tried to estimate single channel

parameters with noise analysis; however the available data did not allow us to do so, probably due to a non K_{ATP} channel noise source, which was voltage dependent and too big in comparison to channel noise, and due to unknown (and probably changing) reversal potential for K_{ATP} current.

In order to make the noise analysis possible, influence of erroneous estimation of reversal potential and non-channel variance should be theoretically treated.

Two cellular elements, that might affect the noise in the traces, should also be taken into consideration:

- the noise of the leak current and the current through voltage-activated Na^+ channels ;
- contribution of the electrogenic pump Na^+/K^+ ATP-ase, as its activity is also being changed upon concentration of ATP and the potential, at which the cell is held.

In terms of experimental protocols, we propose that the voltage protocols be adjusted to more "gentle" conditions, such as ramps and holds in a limited potential range, where mainly K_{ATP} channel activity is postulated (e.g. -130 to -50 mV only), together with membrane potential measurements in regular intervals.

Acknowledgment

I wish to thank Marjan Rupnik for introducing me to the principle of noise analysis of ensemble cell currents, and for giving me a good chance and a good mentor for learning the patch-clamp technique of acute pituitary slices, to Shibing Yang for providing me with data from patch-clamp experiments and to Simon Sedej, for taking me as a co-pilot in pituitary patch-clamp flights.

References

Aguilar-Bryan L, Bryan J (1999): Molecular Biology of Adenosine Triphosphate-Sensitive Potassium Channels. Endocrine Reviews 20(2): 101-135

Aslanidi OV, Mornev OA, Vesterager M, Sorensen MP, Christiansen PL (2002): A Model for Glucose-induced Wave Propagation in Pancreatic Islets of Langerhans. J.theor.Biol (2002) 215:273-286

Best L (1999): Cell-attached recordings of the volume-sensitive anion channel in rat pancreatic beta cells. Biochimica et Biophysica Acta 1419 (1999) 248-256

Cook DL, Satin LS, Ashford MLJ, Hales CN (1988): ATP-Sensitive K+ Channels – Spare-Channel Hypothesis. Diabetes (1988) May; 37(5):495-8

Ghosh A, Ronner P, Cheong E, Khalid P, Matschinsky FM (1991): The Role of ATP and Free ADP in Metabolic Coupling during Fuel-stimulated Insulin Release from Islet Beta-Cells in the Isolated Perfused Rat Pancreas. J.Biol.Chem. 266(1991) 34:22887-22892

Goforth PB, Bertram R, Khan FA, Zhang M, Sherman A, Satin LS (2002): Calcium-activated K^+ Channels of Mouse Beta-cells are Controlled by Both Store and Cytoplasmic Ca^{2+}: Experimental and Theoretical Studies. J.Gen.Physiol 120(2002):307-322

Gomis A, Sánchez-Andrés JV, Valdeolmillos M (1996): Oscillatory patterns of electrical activity in mouse pancreatic islets of Langerhans recorded in vivo. Eur J Physiol (1996) 432 : 510–515

Gray PTA (1994): Analysis of whole cell currents to estimate the kinetics and amplitude of underlying unitary events. In: Microelectrode Techniques: The Plymouth Workshop Handbook", editor David Ogden, 2nd edition (1994). Company of Biologists. ISBN 0 948601 49 3

Krippeit-Drews P, Welker S, Drews G (1996): Effects of the Nitric Oxide Sythase Inhibitor N-LAME on Electrical Activity and Ion Channels of Mouse Pancreatic B Cells. Biochem. Biophys. Res. Comm. 224 (1996):199-205

Lou XL, Yu X, Chen XK, Duan KL, He LM, Qu AL, Xu T, Zhou Z (2003): Na^+ channel inactivation: a comparative study between pancreatic islet beta cells and adrenal chromaffin cells in rat. J Physiol (2003); in press; DOI:10.1113:/jphysiol.2002.034405

Meissner HP (1976): Electrical characteristics of the beta cells in pancreatic islets. Journal Physiologie [Paris] (1976) 72:757-767

Traynellis SF, Wahl P (1997): Control of rat GluR6 glutamate receptor open probability by protein kinase A and calcineurin. J.Physiol 503.3.(1997):513-531