Published in final edited form as:

J Neurosci Methods. 2011 July 15; 199(1): 35–42. doi:10.1016/j.jneumeth.2011.04.023.

A fast solution switching system with temperature control for single cell measurements

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Abstract

This article describes a perfusion system for biophysical single cell experiments at the physiological temperature. Our system regulates temperature of test solutions using a small heat exchanger that includes several capillaries. Water circulating inside the heat exchanger warms or cools test solutions flowing inside the capillaries. Temperature-controlled solutions are delivered directly to a single cell(s) through a multibarreled manifold that switches solutions bathing a cell in less than 1 s. This solution exchange is optimal for patch clamp, single-cell microamperometry, and microfluorometry experiments. Using this system, we demonstrate that exocytosis from pancreatic β cells and activation of TRPV1 channels are temperature sensitive. We also discuss how to measure local temperature near a single cell under investigation.

Keywords

Solution switch; Temperature control; Ca^{2+} measurement; Amperometry; Perfusion exchange system

1. Introduction

Over the past several decades, development of biophysical methods for single-cell experiments led us to new understanding of cellular physiology. Typically electrophysiological and optical measurements with single cells are performed under a microscope with a high magnification. Live cells positioned on a microscope stage inevitably experience room temperature (22–24 °C). The results acquired at room temperature are extrapolated to the physiological temperature for mammalian cells (i.e., 37 °C). However, many cellular processes, such as exocytosis in adrenal chromaffin cells, are highly temperature-sensitive and occur at much slower rate at room temperature (Dinkelacker et al., 2000). Several methods have been developed to control bath temperature of experimental chambers installed on microscopes. For long-term observation of single cells at 37 °C, the whole microscope or at least the microscopic stage are placed inside a

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temperature- and CO₂-controlled cage incubator (e.g., Okolab Co.). These incubators are expensive and, more importantly, use of devices such as a micromanipulator to handle cells is limited. Instead, peripheral heating of a chamber using surrounding heating blocks, has been an easy and common setting (e.g., Warner Instruments). Apparently this method suffers from a spatial temperature gradient across the bath, having the lowest temperature at the center of the chamber.

Another requirement for live-cell experiments is solution switch. Different agents are applied to cells in order to initiate or perturb cellular responses. If on-off kinetics of the perturbation are studied, the solution switch must occur faster than the cellular response itself. Several methods have been developed for solution exchange with different characteristics such as exchange time and number of available test solutions. A simple method is to select one solution fed into the chamber using a multiway stopcock valve or a combination of solenoid switches. This 'bath' perfusion changes solutions slowly (typically longer than several seconds), depending on flow rate and dead volume of the chamber. Time to switch solutions is considerably reduced by positioning a multibarreled manifold in the front of a cell (Koh and Hille, 1997). Such a 'local' perfusion system changes solution applied to a cell in less than 1 s. Additionally the local perfusion allows to use multiple test solutions and small volumes of test solutions for an experiment. The fastest exchange method available at present uses a sharp interface formed by two adjacent solutions flowing rapidly from a double-barreled pipette (Dudel et al., 1990). Shift of the interface by a few 100 μm by a piezo-electric element attached to the pipette achieves solution exchange time less than 1 ms. This ultrafast exchange system has been used to study the kinetics of rapidly desensitizing ligand-gated receptor channels in the excised patch membrane (Koh et al., 1995). When applied to a cell attached to a glass bottom, the exchange is slowed due to turbulence and malformation of the solution interface. This method of switching two solutions (control and a test solution) has a limit in number of solutions to be used: the test solution flowing in a barrel can be replaced by the second one within about 10 s.

Our aim was to develop a practical solution application system featuring the regulation of local temperature around single cells and fast solution switch in less than 1 s.

2. Materials and methods

2.1. Sources of materials

Stainless steel tubing (23 gauge, Type TW) was purchased from Eagle Stainless Tube and Fabrication (Franklin, MA). Polyethylene tubings (PE10, 50, and 200) and glass tubing (Cat. No. 3-000-275-G) were from BD Medical (Franklin Lakes, NJ) and Drummond Scientific (Broomall, PA), respectively. Miniature thermocouples were from Omega Engineering (Stamford, CT).

2.2. Preparation of pancreatic islet cells

Islets of Langerhans from 5 week-old Balb/c mice were prepared as described previously (Lernmark, 1974; Chen et al., 2003). Briefly, the pancreas was removed from mice euthanized with CO₂, according to the guideline for animal use provided by the University of Washington. The tissue was dissected into small pieces and treated with collagenase type P (5 mg/ml, Boehringer Mannheim) in a Hanks' balanced salt solution (HBSS, Gibco) containing 5 mM glucose, 20 mM HEPES, and 1 mg/ml bovin serum albumin. Islets were hand-picked and cells were dissociated by triturating islets in a Ca²⁺-free HBSS. Cells were maintained in an RPMI medium containing 10 mM glucose, 10% fetal bovine serum, 100 $\mu\text{g/ml}$ streptomycin, and 100 IU/ml penicillin in a CO₂ incubator for 1 day before experiments. Experiments for β -cells were performed in a saline solution containing (in

mM): 142 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 3 glucose, and 10 HEPES, pH 7.4. In a saline solution containing 15 mM glucose, NaCl was reduced to 136 mM to adjust osmolarity.

2.3. Ca²⁺ measurement

Intracellular free Ca²⁺ concentration ([Ca²⁺]_i) was measured using a Ca²⁺-sensitive fluorescent dye, indo-1, following preincubation of islet cells for 30 min with 2 μM AM-form of the dye and 0.01% pluronic F-127 in the saline solution containing 3 mM glucose. The excitation wavelength was 365 nm (100 W mercury lamp), and fluorescence signals were recorded at 405 nm and 500 nm, using a pair of photon-counting photomultiplier tubes. The sampling rate was 0.5 Hz and background fluorescence measured from a cell-free area was subtracted. [Ca²⁺]_i was estimated as a ratio of fluorescence obtained at 405 nm and 500 nm (F_{405}/F_{500}) (Grynkiewicz et al., 1985).

2.4. Carbon-fiber amperometry

Exocytosis from pancreatic β-cells was measured with microamperometry in real time. The method provides a high sensitivity necessary to detect molecules released from single secretory granules (Koh, 2006). Islet cells were incubated for 6 h with the culture medium containing 2 mM serotonin. Previous studies indicated that the neurotransmitter accumulates into insulin granules and is co-released with insulin upon exocytosis (Smith et al., 1995; Aspinwall et al., 1997; Chen et al., 2003). The released serotonin oxidizes at the tip of a carbon-fiber microelectrode (11 μm) polarized to +600 mV and generates pulses of electric current. The amperometric currents were recorded with an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) with filter and sampling frequencies of 100 and 500 Hz, respectively.

2.5. Expression of exogenous genes and patch clamp experiments

tsA 201 cells, a derivative cell line of HEK-293 cells, were used to express TRPV1 channels. The cells were cultured in a DMEM medium supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 IU/ml penicillin. For transfection, cells were incubated with 0.5 μg of cDNA coding for rat-TRPV1 channels (in a 3.8 cm² surface area well) using Lipofectamine® 2000 (Invitrogen) for 4 h. cDNA coding for green fluorescence protein (GFP) was coexpressed to select the transfected cells. Two days after the transfection, TRPV1 currents were measured using the standard patch clamp technique (Hamill et al., 1981). Extracellular solution contained (in mM): 160 NaCl, 2.5 KCl, 1 MgCl₂, 0.05 CaCl₂, 0.5 EGTA, 8 glucose, and 10 HEPES, pH 7.4 (Free [Ca²⁺]_i calculated with WEBMAXC STANDARD (<http://www.stanford.edu/~cpatton/webmaxcS.htm>) is 8.5 nM). Intracellular solution was composed of (in mM): 110 K-Asp, 30 KCl, 10 NaCl, 1 MgCl₂, 3 Mg-ATP, 0.1 Na-GTP, 0.05 EGTA, and 10 HEPES, pH 7.2. The numbers presented for patch clamp experiments are given as average ± s.e.m.

2.6. Construction of the heat exchanger

Our system was based on a local perfusion system that has been used to change multiple solutions for single-cell experiments at room temperature (Koh and Hille, 1997). The new system includes an additional heat exchanger to control temperature of test solutions (Fig. 1). The outer shell of the heat exchanger (the parts shown at the center of Fig. 1A) was fabricated from a plexiglass cylinder. It was made as two pieces so that later assembly with inner components would be possible. Two stainless metal tubes were installed to connect the heat exchanger with a water bath for water circulation (see Fig. 1C). The tubes together with stabilizing plexiglass rings were attached to the heat exchanger with epoxy glue.

Test solutions flow into the heat exchanger through polyethylene capillaries (PE10 from BD Medical, i.d. 0.28 mm; o.d. 0.61 mm). In order to maintain water tightness of the heat exchanger, the PE capillaries were combined into a bundle (denoted as 'PE capillaries A' in Fig. 1). To construct the capillary bundle, four PE10 capillaries were manually inserted into a bigger polyethylene tubing (PE200, i.d. 1.40 mm; o.d. 1.90 mm) and extended from both sides of PE200 for about 30 mm. Then the intratubular space between PE200 tubing and PE10 capillaries was filled with epoxy glue. Since filling of the mixed epoxy into a 1 ml syringe and injecting it between capillaries using a 24 gauge needle takes time, we recommend an epoxy glue with a long working time (e.g., 30 min). After overnight curing of the glue, PE10 capillaries were trimmed with a sharp blade, leaving equal lengths of capillaries extended out of PE200 tubing. The capillary bundle A was inserted into an O-ring (i.d. 1.70 mm; o.d. 4.50 mm) to the left end of the heat exchanger and tightened with the screw A. The right ends of the PE10 capillary were expanded with a warmed paper pin for the connection to stainless capillaries (i.d. 0.22 mm; o.d. 0.32 mm; length 20 mm) in which heat exchange occurred (Fig. 1B). The warmed (or cooled) solutions in stainless capillaries flowed into a manifold designed for rapid solution switch that was made of 'PE capillaries B' and a glass tubing. PE capillaries B was prepared in a similar way as PE capillaries A, but one side of PE200 tube was cut with a sharp blade leaving clean openings of capillaries imbedded in the epoxy glue. The left ends of capillaries were joined to stainless capillaries. Then the cut side of PE capillary B was sequentially inserted into the right part of the heat exchanger, the first O-ring (i.d. 1.70 mm; o.d. 4.50 mm), Screw B, second O-ring (i.d. 2.20 mm; o.d. 5.30 mm), and Screw C. Tightening of Screw B fixed capillary B to the heat exchanger. At this stage the two parts of the heat exchanger were combined and the connected site was sealed with a piece of Parafilm®. Next, a big glass tubing (Drummond Scientific Co., i.d. 2.10 mm; o.d. 2.75 mm; length 41 mm) was combined into the heat exchanger. The Drummond tubing was pulled with a vertical pipette puller beforehand and the tip was cut using a sharp diamond pencil and heat polished so that the diameter of the open tip became about 0.2–0.4 mm. It is important to have a tight seal between the cut end of the PE200 tubing and the Drummond glass tubing. The seal prevents back-filling of test solutions into the space between the tubings, which is a potential source of cross-contamination between test solutions. When the glass tubing was pushed toward the compressible PE200, it formed a tight seal against the wall of the narrowing part of the former. Tightening of Screw C fixed the glass tubing to the heat exchanger.

Next, each PE10 capillary extending from the back side of heat exchanger was connected to a solution reservoir through a solenoid switch (Fig. 1C). We recommend to warm up test solutions at 37 °C and shake before filling them into reservoirs. This step reduces the formation of air bubbles inside the capillaries by removing gases dissolved into cold solutions during refrigeration (note that gas solubility decreases as temperature increases). To fill solutions into empty capillaries we applied suction to the opening of the manifold while all solenoid valves were open. Further perfusion was driven by gravity and the flow rate of a solution was controlled at 0.20–0.25 ml/min by adjusting the height of reservoirs. At this flow rate, solution flowing out of the multibarreled manifold securely covered a single cell or a small cell cluster under investigation as tested using Lucifer yellow fluorescent dye. Flow rate ranging from 0.2 ml/min to 0.4 ml/min did not cause a dislocation of adherent cells, resulting in changes in access resistance of whole-cell patch clamp recording or amperometric signals with a carbon fiber probe.

The slim heat exchanger could easily be installed on a three-axis manual manipulator using a clamp (Fig. 1C). Via two Tygon tubes, the exchanger was connected to a water bath that was equipped with a circulating pump and temperature control system. The flow rate of circulating distilled water was set at about 200 ml/min in our system. At this flow rate and with the specific Tygon tubes, the temperature of water bath, circulating solution coming

into the heat exchange, and circulating solution returning to the water bath was 43.0, 38.6, and 34.8 °C, respectively, indicating that a significant amount of heat has been lost through the long circulation loop between water bath and heat exchanger (Fig. 1C) and to a lesser extent in the heat exchanger. Considering this heat loss, the temperature in the water bath was appropriately adjusted by measuring the temperature around the cell as described in Section 3.2 (Fig. 3). For electrophysiological experiments, we incorporated a copper tube in the circulating path and grounded it to remove the electrical noise transmitted from the environment including the water bath. In our system optimized for single channel recordings, the root-mean-square (rms) noise measured with a borosilicate patch pipette coated with Sylgard elastomer and giga-sealed with a cell membrane was ~0.3 pA at 1 kHz Bessel filtering. Without grounding of the circulation loop, the value was >5 pA of mainly 50 Hz noise.

3. Results

3.1. Optimal length of capillary for heat exchange

Since only one solution flows at a time in our multibarreled system, the flowing solution is not warmed as well as the other stationary solutions. To avoid this problem the flowing solution needs to be warmed up until its temperature is equilibrated to that of circulating water. We calculated the minimal length of the capillary for this heat equilibrium using basic principles of heat transfer. Assuming the internal solution flows slowly for complete heat exchange at each point of a capillary, the temperature of the internal solution (T) as a function of distance traversed in the capillary (x) is given by:

$$T(x) = T_c - (T_c - T_i) \exp\left(\frac{-x}{\lambda}\right),$$

where T_c and T_i denote temperatures of circulating water and incoming solution, respectively (see Appendix). The equation dictates that the temperature of test solution increases exponentially with a characteristic length (λ), finally reaching T_c (Fig. 2). λ is mainly determined by flow rate of solution inside a capillary, and geometry and thermal conductance of the capillaries (14.4 W/m K and 0.036 W/m K for stainless and polyethylene, respectively). We used flow rates of solutions around 0.25 ml/min since the rate ranging from 0.2 ml/min to 0.4 ml/min did not cause a dislocation of adherent cells which may result in changes in access resistance of whole-cell patch clamp recording or amperometric signals with a carbon fiber probe. At a flow rate of 0.26 ml/min, λ for a stainless capillary with i.d. 0.22 mm and o.d. 0.32 mm was calculated to be 0.51 mm, suggesting a minimum ~2 mm (4λ) of stainless steel capillary for the heat equilibrium (Fig. 2, line). In contrast, λ of polyethylene capillary (PE10, i.d. 0.28 mm and o.d. 0.61 mm, flow rate 0.22 ml/min) was 53 mm, about hundred times longer than stainless capillary, mainly due to lower thermal conductance and, to a lesser extent, thicker walls (Fig. 2, dotted line). When the flow rate becomes faster, λ increases.

We experimentally measured warming of solution inside capillaries. Stainless capillaries of different lengths were connected to polyethylene tubing at both ends immersed into a well-stirred water bath (35.3 °C). A saline solution (20.2 °C) was fed into a tubing by gravity at a flow rate of 0.26 ml/min, and the temperature of the solution coming out of the tubing on the other side was measured with a home-made thermocouple (tip size ~0.5 mm, K type, Fig. 2, circles). Heat exchange in polyethylene capillaries was similarly measured at a flow rate of 0.22 ml/min (Fig. 2, squares). The measured values are in a good agreement with the predicted temperature. This theoretical and experimental data suggest that beyond certain lengths of capillaries temperature of a flowing solution will be equilibrated with that of

circulating water in the heat exchanger. For stationary solutions in other capillaries will reach the same temperature without possible overheating.

It is advisable to have a capillary considerably longer than λ such as stainless capillary of 20 mm. If one wants to use PE capillary instead of stainless capillary, we suggest 200 mm capillaries. Longer capillaries than 200 mm will have a resistance too big for an optimal solution flow to cover an enough area around a cell. With this adjustment, the temperature of solutions flowing out the heat exchanger is relatively independent on the small fluctuation in the flow rate of solutions.

3.2. Measurement of local temperature around cells

We used a miniature thermocouple to measure the local temperature near a cell during perfusion (Fig. 3). The type K junction was made of 25 μ m chromel (nickel–chromium) and alumel (nickel–aluminium) wires (Cat. No. CHAL-001, Omega Engineering Inc., Fig. 3A). Because of its small size, the thermocouple sensitively measures the temperature of a small liquid volume. Additionally, due to small mass, the response time of the probe is also as fast as a few milliseconds according to the information sheet from the company. In order to position the junction precisely using a Sutter micromanipulator (MP 225), we installed the thermocouple in an electrode holder for patch clamp as follows (Fig. 3B).

First, we precasted a double-barreled PE tubing to insulate thermocouple wires. Two PE10 tubings inserted with 200 μ m tungsten wires were aligned in parallel, positioned into a circular heating coil, and heated until the tubings fused together. Then the tubings were removed quickly from the heating coil and slightly stretched. After solidification of the molten part, the tungsten cores were pulled out from the tubings. The fused region was cut with a sharp razor, leaving a fused double-barreled plastic tubing at one side and two original PE10 tubings at the other end (Fig. 3B). Since the wires are very thin, they were carefully inserted into the precast double-barreled tubing under a stereomicroscope. Subsequently, each free ending of the double-barreled tubing was inserted into each barrel of a theta glass tubing (Code No. 1401021, Hilgenberg GmbH, Malsfeld, Germany) and sealed with cyanoacrylate glue (Superglue). After combining the parts, the exposed thermocouple wires were coated with a thin film of Sylgard 184 elastomer (Dow Corning, Midland, MI) to insulate them. The thermocouple wires and glass theta tubing were inserted into an adaptor designed to fit to an electrode holder for the patch clamp headstage (Fig. 3B). The adaptor was made of the front part of 3 ml syringe (B-D Bioscience). Then the chromel and alumel wires coming out of the theta tubing were pulled out through a small window carved on the adaptor and further connected to thicker chromel and alumel wires. Finally, the miniature probe was positioned in a chamber using Sutter manipulator in a patch clamp setup. Temperature was recorded and digitized using a data acquisition system (TLOG-1000, Carvolab, Korea) at a sampling rate of 1 Hz.

Local temperature near a cell was monitored using the miniature thermocouple positioned 5 μ m above the glass bottom and 200 μ m away from the opening of the multibarrel manifold (Fig. 3C). Before recording, the temperature of the water bath was set as 43.0 °C and water was circulating for more than 15 min. Since no solution was flowing out of the solution exchange system, the thermocouple recorded a bath temperature equilibrated at room temperature initially. After solution 1 was turned on, the temperature in the bath increased slowly with a time constant of 1 min and reached a steady-state value (35 °C). Considering that a test solution entering the stainless capillary comes out the manifold within 2 s, this slow equilibration is apparently due to fast heat dissipation in the bath. As other areas of the chamber became warmed, heat dissipated less. Therefore a cell needs to be perfused with a control solution >1 min before the recording at this slow flow rate (about 0.2 ml/min). Importantly, after reaching the steady-state, temperature between different test solutions was

relatively constant and the variation was less than 1 °C. After the switch to a new solution the temperature transiently dropped by around 1 °C mainly due to the cooling of the solution that had stayed at the capillary beyond the heat exchanger. Insulation of the Drummond glass tubing with a shrinkable tube reduced the minor temperature drop. After turning off all solutions, bath temperature returned slowly to room temperature. A similar recording was obtained with a heat exchanger with polyethylene capillaries (not shown).

We also mapped spatial distribution of temperature over a wide area near the manifold tip (Fig. 4). Scanning of temperature using the miniature probe revealed that the temperature profile depends on flow rate and height from glass surface. With a normal perfusion rate (0.22 ml/min, Fig. 4A) and ~5 µm from the glass surface, temperature was highest at the manifold tip and dropped about 0.2–0.5 °C/100 µm. Therefore, for single cells of about 10 µm, we could control temperature with a precision of about 0.5 °C. However a small tissue or cell clumps such as islets of Langerhans of a few hundred µm may experience different temperatures. At a higher position (300 µm above the glass bottom) the peak temperature was bigger, probably due to upward convection of hot solution. The temperature gradient was steeper at the level. When the flow rate was approximately doubled (Fig. 4B), temperature gradient became smaller at both heights. This condition may be optimal for bigger cells or cell clusters. We also experienced that solution levels contributed to the temperature profile. For example, with a solution level lower than 1 mm, heat dissipated so quickly that the regional temperature variation was considerably large. Therefore we suggest to maintain the solution level higher than 2 mm if solutions are significantly warmer than the ambient temperature. In conclusion, it is recommended to study the temperature profiles to find the optimal parameters such as position of perfusion tip relative to cells, temperature of water bath, and flow rate.

3.3. Measurement of solution exchange

We tested the performance of our system with respect to solution exchange time (Fig. 5). Each solution was switched on and off by a solenoid valve (VC-6 M mini-valve systems, Warner Instruments, Hamden, CT) and TTL signals generated by the Pulse software for the EPC9 amplifier. The switch time was measured using a miniature carbon-fiber electrode (tip size 11 µm, Koh and Hille, 1999) during application of a saline solution containing 10 µM dopamine at a flow rate of 0.25 ml/min. Dopamine molecules colliding to the electrode oxidize immediately, releasing electrons. Therefore, the time course of the oxidative current faithfully reports the solution switch. It took about 30 ms to switch 10–90% of solution at 200 µm away from the manifold tip and longer in suboptimal regions (Fig. 5, inset).

3.3.1. Two application examples—First, we studied the effect of temperature on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and Ca^{2+} -dependent exocytosis in pancreatic islet cells (Fig. 6). Here we intended to measure the parameters at both room and more physiological temperatures. Since islets consist of different endocrine cells, we selected cells larger than 10 µm to measure insulin-secreting β cell (Rorsman and Trube, 1986). Increased level of external glucose causes the following sequential intracellular events in β cells; rise of ATP/ADP ratio by metabolism of glucose, block of ATP-sensitive K^+ channels, membrane depolarization, activation of voltage-gated Ca^{2+} channels, and Ca^{2+} influx from extracellular medium into the cells. Upon the application of 15 mM glucose, the ratio of fluorescence at 405 nm and 500 nm from indo-1 Ca^{2+} dye in a small cluster of 3–4 β cells increased with a time delay of 2 min at 22 °C (Fig. 6A), indicating the increase of $[\text{Ca}^{2+}]_i$. When the glucose level was decreased back to 3 mM $[\text{Ca}^{2+}]_i$ returned to the basal level. At 6 min, the pump for water circulation from the water bath was switched on. It was expected that the temperature around the cells reached 35 °C in 50 s (Fig. 3C). At the higher temperature $[\text{Ca}^{2+}]_i$ increased 1 min after addition of high glucose, suggesting a faster

glucose metabolism. Exocytosis from the same cells was simultaneously measured with a miniature carbon-fiber electrode (Fig. 6B). At room temperature and 3 mM glucose concentration, exocytotic events were rarely observed. Glucose elevation evoked a burst of amperometric peaks as well as $[Ca^{2+}]_i$ rise. At the higher temperature (35 °C), a similar increase in $[Ca^{2+}]_i$ evoked more pronounced $[Ca^{2+}]_i$ -dependent exocytosis than that at room temperature (Fig. 6B and C). Moreover, the first Ca^{2+} peak elicited the highest number of exocytotic events while less response was observed during the subsequent Ca^{2+} peaks, probably reflecting the depletion of the readily releasable vesicle pool during the first Ca^{2+} signal (Olofsson et al., 2002). Altogether, these results indicate that the reaction steps involved in exocytotic processes are sensitive to temperature in insulin-secreting β cells as many other biological processes with high Q_{10} values.

The second example examines heat-activation of transient receptor potential vanilloid type 1 channels (TRPV1). TRPV1 is a nonselective cation channel whose activity is highly modulated by temperature. Therefore these channels are commonly referred to as thermo-TRPs. They are activated as well by several agonists, including capsaicin, a pungent component of chili peppers (Welch et al., 2000). Whole-cell patch clamp experiments from tsA 201 cells overexpressing TRPV1 channels showed small leakage currents in response to a voltage jump from 0 mV to -60 mV at room temperature (Fig. 7). Upon a temperature increase by turning on solution flow from the heat exchanger, the current increased significantly, reaching a peak amplitude of 1.8 ± 0.6 nA ($n = 5$ cells). These currents decreased gradually even when the temperature was maintained high and only $62 \pm 12\%$ of current remained after 1 min of the heating period when compared to the peak current. TRPV1 currents returned back to baseline once the temperature dropped below 33 °C, which is consistent with what is known about the steep temperature sensitivity of TRPV1 channels. A second heat treatment was able to activate TRPV1 currents, but the amplitude of the second response was considerably lower (the peak current in response to the second heat stimulus was $35 \pm 14\%$ of the first). It has been reported previously that TRPV1 channels undergo Ca^{2+} -dependent desensitization (Rosenbaum et al., 2004). Even though the Ca^{2+} concentration in the extracellular solution was low (as buffered with 50 μ M Ca^{2+} and 100 μ M EGTA and calculated free $[Ca^{2+}]_o$ of 630 nM at pH 7.0 and 37 °C), we cannot rule out that the desensitization observed during elevated temperature is due to Ca^{2+} influx through TRPV1. For comparison, we used a commercial heating device from Warner Instruments (Model TC-324B) for an excised patch clamp experiment (Fig. 7C). In the outside-out excised patch recording, a temperature increase evoked a small inward current of about 0.3 nA at -100 mV. This system records the temperature of the solution flowing out of the heating element (continuous line in Fig. 7C) and uses this measurement to regulate the solution temperature to a set value. Even though the temperature in the heating element was reporting a temperature jump to 40 °C, only a small increase in TRPV1 current was observed. This suggests that the local temperature near the cell is far below the reading. Apparently, the solution entering the recording chamber experiences cooling to some extent. The system also measures the temperature in the recording chamber using a relatively large thermoprobe (a bead thermistor of 1 mm diameter and 5 mm housing, Model TA-29, Warner Instruments) (broken line). Therefore the local temperature around the cell, which was not known, is assumed to be a value between the heating element and bath measurements, e.g., 33–39 °C). At the end of the experiment, the same patch reversibly exhibited a current increase of 3.3 nA upon application of 1 μ M capsaicin, suggesting TRPV1 channels were minimally activated by the temperature elevation (may be lower than the threshold). This result argues for the necessity to measure local temperature near the cell using a miniature thermal probe. Note that the maximal TRPV1 currents of nA activated by heat or capsaicin in an excised patch are of similar amplitude to those measured in whole cell configuration (C.A. Ufret-Vincenty and S. Gordon, unpublished observation).

Considering the large difference in membrane area, this observation is unexpected and the underlying mechanism for this phenomenon remains unknown.

4. Discussion

4.1. Comparison of temperature controller systems

Some commercial systems heat bath solution using a heating element that makes contact with the bath (e.g., Warner Instruments and Harvard Apparatus). As the exact temperature of the bath solution is not known, they use a small thermoprobe of 1–2 mm in diameter to measure bath temperature and the value is fed into a heater controller. This type of temperature control still would not guarantee the precise regulation of local temperature due to the bulk size of the sensor and temperature gradient across the chamber. To reduce the temperature gradient, the chambers from Biopetechs and Cell MicroControls use a thin-film coating of transparent and electrically conductive materials on glass bottom that warm bath solutions evenly upon current flow. This system has a relatively even temperature over the glass surface while the temperature gradient across *z*-direction (i.e., height) still exists.

Other systems warm the inflowing bath solutions using heating elements (Warner Instruments). For these systems, solution exchange is slow due to a large dead volume of chambers. Since glass bottom and aqueous solution have high heat conductivity, heat dissipation is another major problem in controlling temperature around a cell. Some companies (for example, Zeiss and Bioscience Tools) sell a heating system for objective lens to minimize heat dissipation through chamber glass and lens. Combined use of our local perfusion and warming of glass surface and objectives will significantly reduce the temperature gradient in a recording chamber.

Our system has been developed to meet two demands; fast solution exchange and relatively constant temperature control near cells. It can be used for a variety of single-cell experiments for which temperature plays a critical role. It is worthwhile to mention that our system excludes the possibility of overheating of cells, since the temperature of the circulating water is the upper limit the test solutions possibly reach. Our system can be used for cooling of test solutions when connected to a cooling water bath. We have used this device successfully in several projects during the last seven years (Chen et al., 2003; Cho et al., 2010; Duman et al., 2008; Kim et al., 2008). For a biological mechanism very sensitive to temperature, it is advisable to measure local temperature around the cell using a miniature thermoprobe for every experiment. We plan to feed the measurements obtained from the miniature temperature probe into the temperature control unit of water bath for a better regulation of local temperature near a cell.

4.2. Alternative configurations of the heat exchanger

Our prototype version of the heat exchanger used a PE10 capillary bundle (i.e., “PE capillaries A and B” connected with continuous PE10 capillaries of about 180 mm, see Fig. 1A). The PE capillaries B side was combined with the Drummond glass tubing as described above and inserted into a heat exchanger made of a 5 ml disposable plastic syringe, protruding out through the front Luer taper that was drilled to enlarge the inner diameter. To seal PE capillaries A and the end of the syringe, we used a small plastic disc which had a central hole for the PE capillaries A. All potential leak areas were sealed with epoxy glue. Two pre-installed plastic adaptors on the syringe were connected to the water circulation. This prototype worked quite well and could be an alternative solution for investigators who do not have support from a fine mechanical facility.

Our results defined the length of capillaries for sufficient heat exchange; 4 mm for a thin-wall stainless steel capillary and 200 mm for PE10 capillaries. In terms of efficient heat

exchange the stainless tubings are superior. A user who has concern with any release of metal from stainless steel (in spite of its chemical stability) can use the PE10 capillaries as described for the prototype heat exchanger instead. Solutions flowing through PE tubings of 180 mm experience high resistance and therefore the solution reservoirs (Fig. 1C) need to be positioned high, e.g., >50 cm to achieve a reasonable flow rate, e.g., 0.25 ml/min.

With minor modifications, our system with heat exchanger can be connected to a robotic patch clamp, an imaging setup, and a lab-on-a-chip type chamber built for efficient screening of chemicals (Milligan et al., 2009). In such applications, the capillary bundle ('Capillary bundles B' in Fig. 1A) could be connected to a perfusion line to bring different warm (or cold) solutions to a small space where cells reside.

4.3. Consideration on rapid temperature change

Our system was designed for rapid solution exchange but not rapid (e.g., <1 s) temperature transition. However it allows us to measure biological responses at different temperatures. We observed that temperature of a warm solution dropped when it was kept for a while in the multibarrel manifold beyond the heat exchanger. This heat dissipation caused small dimples in the temperature trace when new solutions were switched on (Fig. 3C). Based on this consideration we did not attempt to modify our system for the experiments requiring temperature jump. For that purpose, we suggest to use multiple solution streams having different temperatures. The array of flowing solutions needs to be precisely shifted so that one solution with a specific temperature is perfused onto the cell. Mechanical shift of the solutions can be achieved using a piezo-electric element or a step motor system (e.g., Warner Instruments, SF-77B). The temperature of solutions will decrease as they propagate from the openings. Our method to measure local temperature will be useful for the calibration of temperature of multiple solution streams. Recently an innovative optical heating with 1460 nm laser diode has been developed for a temperature jump within submilliseconds (Yao et al., 2010).

Acknowledgments

The authors thank Drs. B. Hille and E. Dickson for comments on the manuscript, Dr. S. Gordon for helpful discussion, and Mr. E. Martinson for the construction of heat exchanger. This work was supported by the R&D Program of Advanced Technologies (to D.S.K.), NSFC 30871225 (to L.C.) and NIH R01-GM83913 (to B. H.).

Appendix A: Heat exchange in a hollow tube

To calculate temperature of fluid flowing along a tube ($T(x)$), we first consider warming of the fluid in the tube by the heat (ΔQ) delivered from the circulating warm water (T_c). The initial temperature of the fluid entering the tube is T_i . The heat delivered to the fluid is, by definition, $\Delta Q_{\text{fluid}} = mc\Delta T$, where m is mass, c is specific heat capacity of the wall substance

of the tube, and ΔT is temperature change of the fluid. Rate of heat transfer (H_{fluid}) defined as heat transferred through the wall per unit time ($\Delta Q/\Delta t$) is $H_{\text{fluid}} = \rho c F \Delta T$ ($\Delta t = \Delta V/F$, $m = \rho \Delta V$), where ρ and F are density and flow rate of fluid, respectively. Rate of heat transfer through the wall of the hollow tube is described as follows (Incropera and Dewitt, 2004):

$$H_{\text{tube}} = \frac{-2\pi k (T_c - T(x)) dx}{\ln(R_o/R_i)},$$

where k is thermal conductivity of the tube, dx is the infinitesimal change of distance. R_i and R_o indicate inner and outer radii of the tube, respectively. Provided that the heat delivered through the tube is fully and immediately conducted to the fluid, then H_{tube} is equal to H_{fluid} .

$$\rho c F \Delta T = \frac{-2\pi k (T_c - T(x)) dx}{\ln(R_o/R_i)}$$

Solving the equation as $T(x)$ yields:

$$T(x) = T_c - (T_c - T_i) \exp\left(\frac{-x}{\lambda}\right), \quad \text{where} \quad \lambda = \frac{\rho c F \ln(R_o/R_i)}{2\pi k}$$

In conclusion, the temperature of a flowing fluid approaches that of the circulating water as an exponential function with a characteristic length constant, λ . The value of λ was 0.51 and 53 mm for the stainless and PE10 plastic tubings we used, respectively. Parameters used in the calculation were $\rho = 10^3 \text{ kg/m}^3$, $c = 4.2 \text{ kJ/kg/K}$ for the fluid, $F = 0.22 \text{ ml/min}$, $R_i = 0.14 \text{ mm}$, $R_o = 0.31 \text{ mm}$, $k = 0.036 \text{ W/m K}$ for PE10 tubing and $F = 0.26 \text{ ml/min}$, $R_i = 0.22 \text{ mm}$, $R_o = 0.32 \text{ mm}$, $k = 14.4 \text{ W/m K}$ for stainless tubing.

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Fig. 1. Multibarrelled heat exchanger system. (A) Scale drawing of components of the heat exchanger. An expanded side view of PE capillary view is shown. (B) Combined heat exchanger. Stainless capillaries were marked as black. (C) A schematic drawing of the exchanger connected to water bath and solution reservoirs. Not drawn to scale. The heat exchanger is attached to a manipulator for positioning of the manifold tip near a cell.

Fig. 2.

Determination of capillary length for heat exchange. Temperature of solution flowing inside stainless (line) or polyethylene (broken line) capillaries was simulated using the equation in Appendix. Circles and squares indicate the values measured with stainless and polyethylene, respectively. Temperatures of solution entering the capillaries and water in the warming bath were 20.2 (T_i) and 35.3 °C (T_c), respectively.

Fig. 3. Quality check of temperature control. (A) Photograph of the tip of miniature thermocouple (K type). (B) Installation of the miniature thermocouple on an electrode holder. See text for details. (C) Temperature change after turning on solution 1 (downward arrow) and subsequent change of solutions (arrow heads). At 390 s all solutions were turned off (upward arrow) and the local temperature slowly returned to the room temperature. Temperature was measured every second. This heat exchanger had stainless capillaries of 20 mm.

Fig. 4. Spatial temperature profile of a solution coming out from the multibarrel local perfusion tip. Temperature is color-coded. Flow rate of the solution was 0.22 ml/min (A) or 0.38 ml/min (B). The miniature thermocouple was positioned either 5 or 305 μ m above the glass surface and measured the local temperature by scanning more than 1 mm $\times$ 1 mm area with 0.1 mm increment using a Sutter micromanipulator. Arrow indicates the opening of the local perfusion. Temperature at the water bath was set as 45.5 $^{\circ}$ C and 42.5 $^{\circ}$ C for (A) and (B), respectively. The chamber had a bottom of No. 1 glass (about 150 μ m thick).

Fig. 5.

Quality check of solution switch. Switching of saline solution (designated as '1') to the solutions containing 10 μ M dopamine ('2, 3, and 4') induced reversible oxidative currents at a carbon-fiber electrode. The electrode was insulated with polypropylene layer except the tip (11 μ m). The record indicated with a box is shown at an extended time scale (inset). Holding potential at the electrode was +400 mV.

Fig. 6.

Temperature sensitivity of exocytosis of insulin granules in pancreatic β cells. A representative recording out of three similar experiments. (A) Intracellular Ca^{2+} level expressed as the ratio of fluorescence at 405 and 500 nm was detected with indo-1 Ca^{2+} sensitive dye. (B) Simultaneous measurement of exocytosis with a carbon-fiber microelectrode from the same cells as A. High glucose solution (15 mM) was applied for 3 min at room temperature (22 °C, pump for water circulation off) and at 35 °C. Drift of the base line in the amperometric record was resulted by the difference of oxidation–reduction reaction between the solutions and by the change of temperature. Inset: an exocytotic event, marked with an asterisk, is displayed on an expanded time scale showing a typical quantal release of oxidizable molecules from a single vesicle. All solutions in this experiment contained 10 μM forskolin to potentiate Ca^{2+} -dependent exocytosis (Ding and Gromada, 1997). (C) Rate of exocytosis as analyzed as number of amperometric events per second. In this experiment, clustered exocytotic events synchronized with $[\text{Ca}^{2+}]_i$ oscillation are observed at both temperatures.

Fig. 7.

Activation of TRPV1 channels by heat. (A) Whole-cell patch clamp currents measured in a tsA 201 cell overexpressing TRPV1 (circles). Local temperature near the cell was measured using the miniature probe (broken line). Initially no solution was perfused and the probe reads the room temperature. Temperature was elevated at 1 min and 6 min by allowing a warm external solution to flow for 1 min at a rate of 0.4 ml/min. (B) Individual current traces at different time points as indicated in A. The currents were measured by voltage jump to -60 mV from a holding membrane potential of 0 mV for 200 ms. (C) A patch clamp recording using a Warner heating apparatus (TC-324B/344B) combined with a slow bath perfusion. Outside-out patch excised from a tsA 201 cell expressing TRPV1. Current was measured by voltage jump to -100 mV in this experiment (circles). Temperature of the solution was measured inside the heating element (line) and in the recording chamber (broken line).