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Ligand-Specific Temperature-Dependent Shifts in EC_{50} Values for the $GABA_A$ Receptor

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We introduce a system for temperature control of a commercial microfluidic superfusion device that, in combination with patch-clamp, enables rapid acquisition of dose−response data at different temperatures. We obtained dose−response curves for the $GABA_A$ receptor, a ligand-gated ion channel, for two different agonists at temperatures between 25 and 40 °C. For GABA, the dose−response curves shifted toward higher EC_{50} values as the temperature increased, whereas for β-alanine, the EC_{50} values were constant. This shows that temperature is an important factor for obtaining accurate estimations of EC_{50} values and also that such temperature effects can be ligand-specific. Using the EC_{50} values, we estimated the enthalpy of dissociation between the ligand and the receptor. Furthermore, the technology introduced here is generally applicable to all patch-clamp studies where temperature control is desirable, e.g., studies of kinetics and thermodynamics, drug screening, compliant ADME/Tox testing, and in studies of temperature-gated ion channels.

Ion channels are of fundamental importance for the generation and transduction of nerve signals.[1] They are also involved in many disease states,[2] making them very important as drug targets. However, as for many membrane-embedded proteins, they are not only difficult to purify and crystallize but also difficult to study functionally. The standard method for studying ion channel function is the patch-clamp technique,[3] allowing real-time measurements of ion channel currents, i.e., pore open−close dynamics, whereas information about other conformational states is more difficult to extract. In patch-clamp studies of ligand−receptor interactions, the affinity is often characterized by the EC_{50} and IC_{50} values, even though they actually mirror a complex interplay of both binding/dissociation and channel opening.[4] However, for the interaction between the $GABA_A$ receptor and γ-amino-*n*-butyric acid (GABA), which is the model system used here, the EC_{50} value coincides with the dissociation constant, k_{off}/k_{on}, where k_{on} and k_{off} are the estimated rate constants for ligand binding and dissociation, respectively.[5] The $GABA_A$ receptor is a ligand-gated ion channel that mediates synaptic inhibition in the CNS and is activated by, for example, GABA and β-alanine.[5] The kinetics of the $GABA_A$ receptor has been described by several kinetic models,[6−8] most of which contain binding and dissociation of ligand to two separate binding sites on the receptor, opening

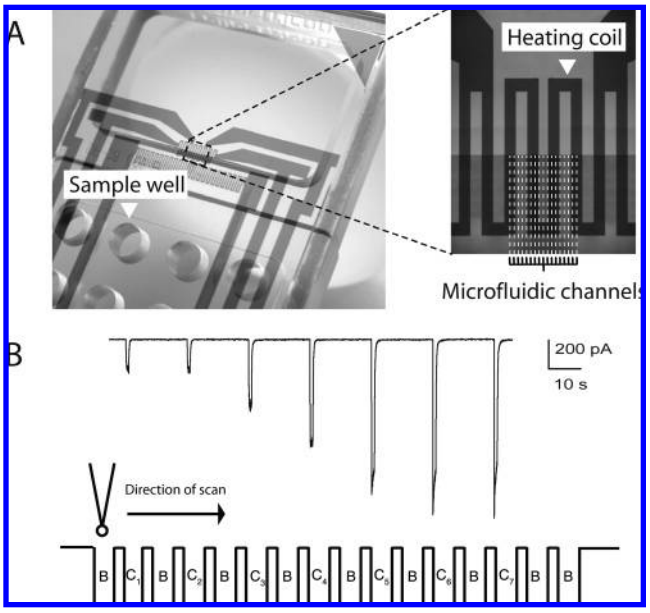

Figure 1. (A) Superfusion device used for dose−response experiments. Each microchannel originates in a sample well loaded with the appropriate solution. The chromium/gold heating coil is positioned directly under the microfluidic channel outlets (inset). (B) The lower image shows how the patch-clamped cell is scanned outside the channel outlets in the open volume. The upper image shows the resulting current trace for a scan with pulses of increasing ligand concentrations, C1−C7, separated with buffer, B, for washing.

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(gating) and desensitization, i.e., transfer into ligand-bound, but closed, long-lived states.

In this study, a novel temperature-controlled microfluidic superfusion device (Figure 1), combined with patch-clamp, is used to reveal temperature-dependent behavior of the $GABA_A$ receptor and estimate the dissociation enthalpies for two different ligands. Furthermore, the technology is generally applicable to all patch-clamp studies aiming at investigating the temperature-dependence of EC_{50} and IC_{50} values, which is of fundamental importance in drug screening and compliant ADME-Tox testing, and to directly study temperature-gated ion channels.[9]

Very few studies of ion channels specifically consider the importance of temperature. However, several temperature-dependent processes involving ion channels have been reported, e.g., the effect of volatile anesthetics on $GABA_A$ receptors,[10] the modulation of chloride channels by phorbol esters,[11] the affinity of benzodiazepine agonists,[12] single-channel conductance,[10,13] and mean channel open times.[14]

Temperature control was added to a commercially available microfluidic device for patch-clamp (Dynaflow 16, Cellectricon AB, Göteborg, Sweden) via a thin-film resistive heater evaporated onto the glass underside of the chip (manuscript in preparation). The microfluidic device comprises 16 microchannels, originating in solution reservoirs, and exiting into an open volume, as described previously.[15−17] As the patch-clamped cell is scanned across the microchannel exits, it experiences pulses of ligands, separated by buffer exposure for clearance. Hence, this device enables rapid acquisition of dose−response data. A small (~50 μm) thermocouple probe, positioned ~50 μm outside the channel outlets, was used to measure the temperature in real time during the scans. In this study, temperatures between 25 and 40 °C were applied, with an accuracy of ~±0.5 °C.

MATERIALS AND METHODS

Microfluidic System. All electrophysiology experiments were performed using a commercially available microfluidic device for patch-clamp (Dynaflow 16, Cellectricon AB, Göteborg, Sweden). The device used in this article has 16 channels of dimensions 50 × 60 μm (w × h) separated by 22-μm-thick walls at the point of exit into the open volume. The volume of the 16 sample reservoirs is 80 μL each, and the dimensions of the open volume are 20 × 35 mm. Prior to experiments, the device was loaded with different solutions using a micropipet. A 1-mm-thick polycarbonate lid was attached over the sample reservoirs using double adhesive tape in order to create a closed system. The lid was connected with PE tubing to an electronic pressure controller (Cellectricon AB, Göteborg, Sweden), which was used to compress the air enclosed by the lid to initiate a well-defined pressure-driven flow in the channels, forming a laminar patterned flow with individual well-defined solution compartments in the open bath, accessible for controlled delivery to a patch-clamped cell.[18,19] For the experimental conditions used, the solution exchange time was 10−12 ms, as determined by solution change-induced kinetics of hERG channel currents.

Temperature Measurement and Control. Computer-controlled, on-chip thin-film resistive heating was chosen over a heat stage setup in order to enable rapid local heating and cooling at the channel exits. A thin-film resistive heating structure with contact lines (2 nm of Cr followed by an 8-nm transparent Au film) was evaporated onto the glass bottom side of the commercial microfluidic chip, using a standard photolithography process with the LOR/S1813 resist system. The surface-printed coils were contacted by modified IC test clamps (ELFA AB, Sweden) on the underside of the microscope stage. Heating was achieved with a battery power supply (0−36 V), yielding temperatures between 25 and 40 °C (accuracy, ~±0.5 °C) at the channel outlets. Due to the small heater dimensions, fast and efficient cooling was achieved simply by dissipation after turning off the heating current. Temperatures in the liquid flow close to the patch-clamped cell were determined by means of an amplified, junction-compensated, and calibrated Cu/Ni thermocouple of micrometer dimensions (CHCO-0005, Omega Ltd.). The measured temperature was fed back into the heating circuit through an optimized PI controller.

Cell Culture. Adherent WSS-1 cells expressing the rat $GABA_A$ receptor (subunit composition $\alpha_1\beta_3\gamma_2$[20]) were cultivated in Petri dishes for 4−8 days in medium (DMEM, high glucose, with L-glutamine and sodium pyruvate) supplemented with penicillin/streptomycin (1%) and fetal calf serum (10%). All chemicals used in the cell culturing were from PAA Laboratories (PAA Laboratories GmbH, Pasching, Austria). Before the experiments, cells were washed and detached in Dulbecco's phosphate-buffered saline (Invitrogen Ltd., Paisley, UK); pH adjusted to 7.4.

Electrophysiology. The whole-cell patch-clamp configuration was used in all experiments. The resistances of the pulled patch pipets were 3−5 MΩ. All data were recorded using a HEKA EPC10 (HEKA Instruments Inc.) patch-clamp amplifier. The cells were clamped at −40 mV. Current signals were recorded at a sampling frequency of 5 kHz, low-pass filtered (1 kHz), and analyzed using the Clampfit 8 software. The cell bath solution (extracellular buffer) was Dulbecco's phosphate-buffered saline (Invitrogen Ltd., Paisley, UK) containing (in mM), 137.93 NaCl, 2.67 KCl, 0.901 $CaCl_2$, 0.493 $MgCl_2$, 1.47 KH_2PO_4, 8.06 Na_2HPO_4, 0.327 sodium pyruvate, and 5.56 D-glucose; pH was adjusted to 7.4 with NaOH. The patch-clamp electrode solution (intracellular buffer) contained (in mM) 120 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, and 10 HEPES; pH was adjusted to 7.2 with KOH. Channels 1, 3, 5, 7, 9, 11, 13, 15, and 16 were loaded with extracellular buffer, and channels 2, 4, 6, 8, 10, 12, and 14 were loaded with agonist solutions. For GABA, the concentration gradient used was 1, 5,

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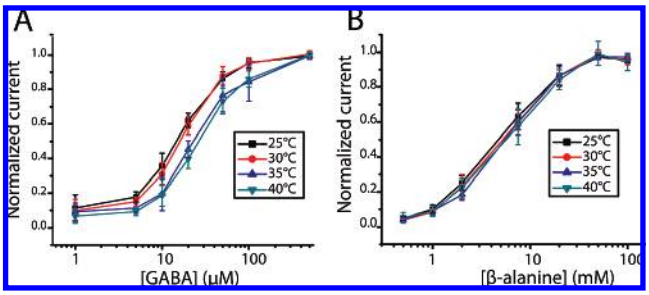

Figure 2. Dose−response curves for the $GABA_A$ receptor agonists GABA and β-alanine for 25, 30, 35, and 40 °C. (A) For GABA, the dose−response curves are shifted toward higher EC_{50} values for higher temperatures. Error bars do not overlap for 25 and 40 °C or 30 and 40 °C, except for the lowest and highest concentrations. (B) For β-alanine, the dose−response curves do not change with temperature. All error bars are SD.

10, 20, 50, 100, and 500 μM, whereas for β-alanine, the concentration gradient used was 0.5, 1, 2, 7.5, 20, 50, and 100 mM. The cells were scanned from channel 1 to 16. The time spent outside the ligand filled channels (t_{exp}) were 500 ms, and the washing time outside each buffer channel (t_{wash}) was 10 s for GABA and 15 s for β-alanine.

Data Analysis. GABA and β-alanine dose−response data at each temperature were fitted to the Hill equation

$$I = I_{min} + \frac{I_{max} - I_{min}}{1 + \left[\frac{EC_{50}}{[L]}\right]^{n_H}}$$

where I is the equilibrium current for any ligand concentration [L], I_{min} is the minimum and I_{max} is the maximum equilibrium current, and EC_{50} is the ligand concentration where half the maximum current response is reached. All current peak heights were then normalized to I_{max} and plotted against the concentration. The EC_{50} values obtained by fitting data to the Hill equation were used in the van't Hoff plots.

RESULTS AND DISCUSSION

Using our new temperature-controlled system, we have performed patch-clamp in the whole-cell configuration and obtained dose−response curves for the $GABA_A$ receptor with two different agonists, GABA and β-alanine, at temperatures between 25 and 40 °C. The dose−response curves for GABA display a shift toward higher EC_{50} values for higher temperatures (Figure 2A). In fact, between 25 and 40 °C, the EC_{50} value increases from 17.3 to 29.1 μM GABA. For β-alanine, however, no shift in the EC_{50} value was observed (Figure 2B). It can thus be concluded that temperature is an important factor for accurate estimation of EC_{50} values in ion channel−ligand interactions. Additionally, the effect of temperature on the EC_{50} value appears to be ligand-specific and therefore may reflect differences in ligand−ion channel interactions.

Jones et al. suggested that the $GABA_A$ kinetics of gating and desensitization induced by GABA and β-alanine are identical, whereas the affinities of these two ligands for the $GABA_A$ receptor are very different. GABA has a faster binding rate and a slower dissociation rate compared to β-alanine, corresponding to a much higher affinity.[5] Therefore, differences between $GABA_A$ currents induced by GABA and β-alanine should result only from the difference in affinities. This suggests that the temperature effect observed for GABA, but not for β-alanine, is linked to binding and dissociation. Since the EC_{50} value is a function of both binding and gating, changes in the EC_{50} value cannot, in general, be assumed to stem from changes in binding. However, for GABA it has been shown that the EC_{50} value coincides with the dissociation constant, k_{off}/k_{on}, where k_{on} and k_{off} are the estimated rate constants for ligand binding and dissociation, respectively.[5] Therefore, we estimated the dissociation enthalpy (ΔH_{diss}) for GABA using a van't Hoff plot of $\ln(EC_{50})$ against $1/T$ (Figure 3A). The obtained ΔH_{diss} was 26.1 kJ/mol.

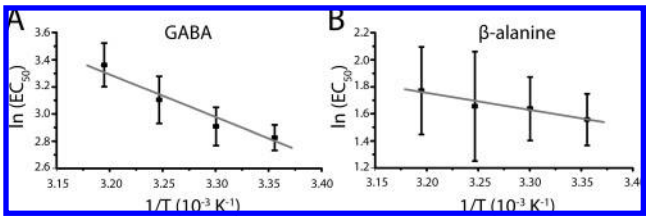

Figure 3. Van't Hoff plots of $\ln(EC_{50})$ against $1/T$ for (A) GABA and (B) β-alanine.

For β-alanine, a similar analysis would give a ΔH_{diss} value of zero (Figure 3B), since there is no significant change in the EC_{50} value. However, for β-alanine, the EC_{50} value deviates from the estimated k_{off}/k_{on} ratio,[5] and hence, this approximation ($EC_{50} \approx k_{off}/k_{on}$) is not as valid as for GABA. Jones et al. also estimated the activation energies for binding (E_{on}) and dissociation (E_{off}) for GABA and β-alanine to the $GABA_A$ receptor, using indirect methods.[5] Their estimations correspond to ΔH_{diss} values of 26.3 kJ/mol for GABA and 4.1 kJ/mol for β-alanine, given that ΔH_{diss} is equal to $E_{off} - E_{on}$. These values agree well with the values derived from our experimental data.

CONCLUSIONS

Using a microfluidics-based superfusion device supplemented with on-chip temperature control, we have obtained dose−response curves for the $GABA_A$ receptor with two different agonists at temperatures between 25 and 40 °C. For GABA, the dose−response curves are shifted toward higher EC_{50} values for higher temperatures, whereas the dose−response curves for β-alanine remain identical. By approximating the EC_{50} value with the dissociation constant, k_{off}/k_{on}, we estimated ΔH_{diss} values for GABA and β-alanine that are in agreement with values obtained using indirect methods.[5] Moreover, it is concluded that temperature is an important factor for accurate estimation of EC_{50} values in studies of ligand−ion channel interaction. This is a significant finding since EC_{50} values are of great importance in ranking the potencies of drugs, as well as for identifying, characterizing, and classifying ligands and receptors. Furthermore, the technology presented here is generally applicable to all patch-clamp studies where temperature control is desirable, e.g., studies of kinetics and thermodynamics, drug screening, compliant ADME/Tox testing, and studies of temperature-gated ion channels.[9]

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