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Automated fast perfusion of *Xenopus* oocytes for drug screening

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Abstract

Fast ('concentration jump') applications of neurotransmitters are crucial for screening studies on ligand-gated ion channels. In this paper, we describe a method for automated fast perfusion of neurotransmitters (or drugs) during two-microelectrode voltage-clamp experiments on *Xenopus* oocytes. The oocytes are placed in a small bath chamber that is covered by a glass plate with two channels for the microelectrodes that are surrounded by a quartz funnel serving as a reservoir for test solutions. The oocytes are perfused in a vertical direction via the two channels in the plate. Automation of compound delivery is accomplished by means of a programmable pipetting workstation. A mean rise time for 10–90% current increase through muscle-type nACh channels of 55.0 ± 1.3 ms (30 μ M acetylcholine) was estimated. Automation, fast perfusion rates, and economical use of compounds (≈ 100 μ l/data point) make the system suitable for screening studies on ligand- and voltage-gated ion channels.

Keywords

Automation; Drug screening; Fast perfusion; Concentration jump; Ion channels; *Xenopus* oocytes

Introduction

Ligand- and voltage-gated ion channels are targets for a large number of therapeutic drugs and are a focus for drug discovery [12]. The effects of new compounds on ion channels expressed heterologously in *Xenopus laevis* oocytes are frequently studied by means of the two-microelectrode voltage-clamp technique [1, 8, 13]. The development of automated procedures to increase throughput and to standardize drug application to oocytes during voltage-clamp experiments is, therefore, of prime interest.

In 'conventional' two-microelectrode experiments, the oocytes are placed in a circular or elongated bath with one or more tube inlets and a drain on the opposite side of the chamber. Drugs or neurotransmitters are applied via individual tubes that are connected to reservoirs. These perfusion systems have a number of limitations making them unsuitable for automation. The number of tested drugs in such systems cannot exceed the number of perfusion tubes. Plastic tubes are contaminated by the applied drugs and must be replaced frequently. Furthermore, tubes have to be filled and this 'dead volume' may result in waste of valuable compounds.

Fast perfusion of oocytes is a *conditio sine qua non* for studies of ligand-gated ion channels. Large bath volumes and slow perfusion rates prevent fast and timed applications of neurotransmitters resulting in apparently slow activation of the channels and substantial receptor desensitization during the chamber perfusion leading to underestimation of the peak current values.

Several automated systems for voltage-clamp experiments on *Xenopus* oocytes have been developed [9, 11, 14]. The rate of solution exchange of these systems is, however, either not exactly specified or slow (1–2 s, see Schnizler et al. [11] and Trumbull et al. [14], for a review).

In this paper, we describe an improved oocyte perfusion chamber for ‘concentration jump’ applications [2, 4]. We illustrate how compound delivery to the perfusion chamber can be automated by means of a standard pipetting robot.

Materials and methods

Expression of nACh receptors and K_v1.1 channels

Stage V–VI oocytes from *X. laevis* were prepared and cRNA was injected as previously described by Khom et al. [4]. Female *X. laevis* (NASCO, USA) were anaesthetized by exposing them for 15 min to a 0.2% MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester, Sandoz, Germany) solution before their ovaries were surgically removed. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (Type 1A, Sigma, Germany). Synthesis of capped off run-off poly(A⁺) cRNA transcripts was obtained from linearized cDNA templates (pCMV vector). One day after enzymatic isolation, the oocytes were injected with 10–50 nl of DEPC-treated water (diethyl pyrocarbonate, Sigma, Germany) containing the different cRNAs of α -, β -, γ -, and δ -subunits containing nACh receptors (gift of V. Witzemann). K_v1.1 channels (gift of B. Fakler) were expressed to evaluate the speed of solution exchange. The oocytes were stored at 18°C in ND96 solution [7].

Two-microelectrode voltage-clamp studies

Electrophysiological experiments were performed by the two-microelectrode voltage-clamp method making use of a TURBO TEC amplifier (NPI Electronic, Tamm, Germany) at a holding potential of –80 mV (nAChR and K_v1.1). Currents were low-pass-filtered at 1 kHz and sampled at 3 kHz. Unless otherwise stated, the bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5 mM HEPES (pH 7.4).

Construction of the perfusion chamber

A schematic representation of the chamber is shown in Fig. 1a,b (cross-sectional and top view, respectively). As previously described [2], the voltage clamp experiments on *Xenopus* oocytes are performed in a small ($\approx 15 \mu\text{l}$) bath that is covered by a glass plate (7 in Fig. 1a,b). Two sloping inlet channels in the glass cover (diameter <1 mm) enable access of the two microelectrodes (1 and 2 in Fig. 1a–d) to the oocyte. A funnel reservoir (6 in Fig. 1a,b and d) for drug application surrounds the two microelectrodes. The principal modification compared to the previous chamber constructions is the placement of the oocyte on a cylindrical holding device (15 in Fig. 1a) incorporated into the chamber bottom. The chamber outflow occurs via a circumferential gap (annular gap) around the cylinder (Fig. 1c). The perfusion gap with a ring-shaped cross-section is connected to the chamber outflow (12 in Fig. 1a,b). Rapid vertical perfusion of the oocyte occurs when a suction pulse of variable strength is applied to the outlet via a syringe pump (9 in Fig. 1a) of the pipetting workstation. The oocytes reside in a small well at the cylinder and two microelectrodes are

inserted into the chamber via the glass plate. The vertical perfusion through the two symmetrically arranged channels for the microelectrode in the glass plate guarantees a high degree of stability during solution exchange. Neurotransmitters are rapidly washed by applications of control solutions (see, for example, Fig. 4a). Contamination of wash solution by remaining neurotransmitters or dilution of drugs or neurotransmitters by residual control solution is prevented by emptying the funnel via two funnel outlets (4 and 5 in Fig. 1a) before the next sample is applied.

Automation of drug application and washout

To automate the oocyte perfusion, we connected the chamber outlet (D) to a syringe (P2) of a pipetting workstation (Miniprep 60, TECAN, Germany) (Fig. 2a). The different fluid handling procedures performed by the workstation include: (1) agonist/drug application to the funnel (F) by means of the liquid handling arm (LHA), (2) application of suction to the drain (D), and (3) removal of residual solution from the funnel via two additional funnel outlets (FO) by means of a MiniWash pump module (MW) (TECAN, Germany) (Fig. 2a). Simultaneous data recording was organized by corresponding software and hardware adaptations. To prevent mechanical disturbances during two-microelectrode voltage-clamp measurements, the pipetting workstation and perfusion chamber were mounted on separate tables.

The software enabled a user-defined adjustment of the speed of solution exchange via the stepping motor of the suction pump (P2) at the chamber outlet (D, Fig. 2a). We conventionally apply 120 μl to the funnel and perfuse the chamber at 100–700 $\mu\text{l/s}$ for rapid application and washout of neurotransmitters (drugs). The 120 μl will exchange the chamber volume (15 μl) about eight times. After the fast perfusion step, the chamber is continuously perfused at a slower rate (1–8 $\mu\text{l/s}$) to ensure steady-state conditions throughout the experiments (see Fig. 3).

Software for operating the pipetting workstation

To adapt the pipetting robot for the needs of oocyte perfusion, we developed software for the TECAN workstation that defines parameters such as (1) the number of chamber wash cycles, (2) perfusion volume, (3) perfusion speed, (4) timing of drug application, and (5) the positions of the compound reservoirs and chamber funnel. The software enables the design of individual protocols for screening studies on ligand- or voltage-gated channels (Fig. 2c).

Results

Rate of solution exchange

To compare the rate of oocyte perfusion in the chamber shown in Fig. 1 with previously described systems, we expressed muscle-type nicotinic ACh receptors and estimated the 10–90% current rise times during fast perfusion with agonist-containing solution. To elicit ACh-induced cationic currents (I_{ACh}), 120 μl of ACh (30 μM)-containing solution were applied to the funnel (6, Fig. 1a,b,d) and the chamber was perfused by a subsequent suction pulse applied to the channel outlet (12, Fig. 1a,b). To minimize diffusion of compound from the funnel into the chamber, we used glass covers with small openings for micro-electrodes and applied the suction pulse immediately (<1 s) after agonist delivery to the funnel.

The rise time of I_{ACh} (30 μM acetylcholine) is dependent on the speed of chamber perfusion. Two examples are shown in Fig. 4b,c. Mean current rise times from 10 to 90% of 89.9 ± 5.7 ms ($n=7$) (perfusion speed 300 $\mu\text{l/s}$) and 55.0 ± 1.3 ms ($n=5$) (perfusion speed 600 $\mu\text{l/s}$) were estimated. Higher perfusion rates did not significantly accelerate current rise times (data not shown).

After an initial fast perfusion step for a ‘concentration jump’ application of an agonist, the chamber is continuously perfused at rates between 1 and 8 $\mu\text{l/s}$ (Fig. 3). During this period, the funnel can be refilled by the workstation depending on the experimental design (see Fig. 2c, for details). Before washout of agonist and/or drug or, alternatively, before a new compound is applied, the funnel is emptied via the two funnel outlets (4, 5 in Fig. 1a).

To estimate the rate of solution exchange independent of the kinetics of ligand–receptor interactions, we expressed $K_v1.1$ channels in *Xenopus* oocytes, applied voltage clamp steps from -80 to $+20$ mV, and analyzed the time course of current decay during a rapid increase of the extracellular potassium concentration from 1 to 30 mM. Figure 5a illustrates a typical experiment. The current decrease at a higher time resolution is shown in Fig. 5b. The estimated mean current decline time $t_{10-90\%}$ of 51.1 ± 5.1 ms ($n=7$) at a perfusion rate of 600 $\mu\text{l/s}$ was similar to the rise time of the agonist-induced current (Fig. 4c). A mean current decline time $t_{10-90\%}$ of 81.2 ± 12.1 ms ($n=5$) was observed at a perfusion rate of 300 $\mu\text{l/s}$. Faster perfusion rates (700 $\mu\text{l/s}$) did not significantly accelerate the current decrease time ($t_{10-90\%}$ 46.5 ± 2.8 ms; $n=5$).

Timed ligand applications

The time between two ligand applications is defined by the time required for moving the motorized pipetting arm of the workstation (e.g., from the chamber to the reservoir and back to the chamber funnel; Fig. 2a). Shorter drug application intervals can be achieved, however, if several test and control solutions are aspirated consecutively (separated by air gaps) into the tip of the liquid handling arm (Fig. 2b). In Fig. 6, this approach is illustrated by applying consecutively a short (2 s) pulse of ACh followed by a wash step and subsequently 5-, 10-, and 20-s ACh (300 nM) pulses.

Discussion

Automated ‘concentration jumps’

The automated voltage-clamp of *Xenopus* oocytes is an efficient approach in drug discovery. Our main goal was, therefore, to develop a screening system for oocytes, enabling automated applications of neurotransmitters in ‘concentration jumps’ to *Xenopus* oocytes. In the chamber construction illustrated in Fig. 1, test solutions can be injected directly into the funnel without disturbing the measurements. The link between the perfusion chamber and the liquid handling system (illustrated in Fig. 2a) that is operated by custom-designed software permits consecutive fast (‘concentration jump’) and slow chamber perfusion modes (Fig. 3).

The speed of solution exchange is illustrated by the rise time of currents through ligand-gated channels (Fig. 4b,c) and the decrease of potassium outward currents through $K_v1.1$ channels upon rapid increase of the extracellular potassium concentration (Fig. 5a,b).

An increase of the onset rates of ligand-gated ionic currents was observed upon increasing the speed of chamber perfusion from 300 $\mu\text{l/s}$ (89.9 ± 5.7 ms) to 600 $\mu\text{l/s}$ (55.0 ± 1.3 ms) (see Fig. 4b,c). Further increasing the speed of solution exchange did not lead to significantly faster onset rates, suggesting that invaginations at the oocyte surface and/or the vitelline membrane cause a diffusion barrier. The measurements of ligand-gated currents and potassium current decay yielded comparable time courses (Figs. 4c and 5b).

The diffusion from the funnel reservoir through the two inlets was negligible. This was achieved by optimizing the size of openings for microelectrodes in the glass cover and the vertical position of the oocyte in the chamber and by immediate perfusion of the chamber after filling the funnel (Fig. 1). Identical $K_v1.1$ current traces before, during, and after a

‘concentration jump’ with a high-potassium solution (compare early current traces 1 and 2 and late current traces 2 and 3 in Fig. 5a) confirm that the diffusion of high-potassium solution from the funnel into the chamber is insignificant. Furthermore, as illustrated in Fig. 4b,c, we observed fast-rising phases of I_{ACh} , suggesting that solution exchange occurs with a sharply segregated liquid front.

Progress compared to previous methods

Previously described ‘concentration jump’ techniques permit oocyte perfusion times in the range of 800 [5], 120–200 [2, 4], and 100 ms [10] or even less than 100 ms [6]. Even faster perfusion rates are achieved by means of the chamber described in Fig. 1.

Earlier chamber constructions [2, 4] had the disadvantage such that the solution was applied laterally to the oocyte. The abrupt liquid flow during a ‘concentration jump’ resulted in a ‘liquid hammer’ effect, that is, the cell was hit from the side by the pressure impulse. Depending on the speed and duration of the liquid flow, this lateral pressure impulse can lead to translocations of the oocytes and disturbances of the measurements (Baburin and Hering, unpublished data). The new construction overcomes this problem by enabling vertical flow of test solution from the funnel reservoir through the two symmetrically arranged access channels towards the outlet under the oocyte chamber. Placing the oocyte on cylindrical pedestal substantially increased oocyte stability.

Thus, the cylindrical holder for the oocytes and the outflow through an annular gap by operating precise pumps of a programmable pipetting robot are principal advantages compared to a previous design [2]. The solution flow perpendicular to and towards the support surface (see also [10]) enabled faster exchange rates compared to [2] (Figs. 4b,c and 5b).

In the automated oocyte perfusion system described by Joshi et al. [3], test solutions are delivered from the bottom of the chamber to the oocyte. Test solutions and control solution are applied to a common inlet tube that is incorporated in the chamber holder. The exchange rate of 400 ms is one order of magnitude slower than that achieved by the system described here. Our system permits timed and short drug applications (Fig. 6), which is particularly helpful if short periods of ligand applications are essential to prevent channel desensitization.

Suitable for ‘medium-throughput’ drug screening

Compared to conventional systems where drug application is limited by the number of inlet tubes, the use of a pipetting platform enables testing of a larger number of compounds. Once the oocyte has been placed manually in the chamber, the experiment runs automatically. The throughput will depend, however, on the physico-chemical properties of the tested compounds (lipophilicity, etc.) and the corresponding length of periods required for washout between consecutive applications. The time for desensitization (in case of ligand-gated channels) or recovery from inactivation (voltage-gated channels) has also to be taken into account.

At perfusion rates of 300 $\mu\text{l/s}$, a single oocyte can tolerate more than 50 ‘concentration jump’ applications without substantial increase in leak current. Between 10 and 20 ‘jumps’ are usually tolerated at higher rates (400–600 $\mu\text{l/s}$). The use of the system is simple and requires only little training as placement of the oocyte in the chamber and microelectrode impalements are performed in a similar way as in the conventional oocyte perfusion chambers. Automation and precise timing of the experiment reduce inter-operator variability and improve the reproducibility of the data.

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Fig. 1.

Cross-section view (a) and top view (b) of the oocyte perfusion chamber. Two microelectrodes (1 and 2) are inserted via the sloping access inlets (8) through a glass cover plate (7) into the small ($\sim 15 \mu\text{l}$) oocyte chamber. Drug is applied by the tip of the liquid handling arm (3) of a TECAN Miniprep 60 to a funnel reservoir made of quartz (6) surrounding the microelectrode access holes. Perfusion of the oocyte (10) that is placed on a cylindrical holding device (15) is enabled by means of the syringe pump (9) of the Miniprep 60 connected to the chamber body (11) via the outlet (12). Residual solution is removed from the funnel before drug application via the funnel outlets (4 and 5). In addition to the ground reference electrode (13), the cylindrical holder for the oocyte contains a reference electrode (14) that serves as an extracellular reference for the potential electrode. Salt bridges can be inserted into the side outlet for the ground electrode (13). c Schematic drawing of the solution flow inside the perfusion chamber and in the annular gap around the cylinder with oocyte. d Photo of the oocyte perfusion chamber. An oocyte (10) is placed on a cylinder and impaled with two microelectrodes (1, 2) surrounded by the funnel (6).

Fig. 2.

a Schematic diagram of the liquid handling system connected to the perfusion chamber. Neurotransmitters or drugs are applied via the tip of the movable liquid handling arm (LHA). Dashed lines and arrows illustrate LHA movements. LHA is connected to a pump (P1) allowing aspiration and delivery of the desired volumes from the rack with tested solutions (R) to the funnel reservoir (F) of the perfusion chamber. The second syringe pump (P2) is connected to the chamber drain (D). This pump is also connected to a distilled water station (DW). Pump P2 provides a suction pulse to the outlet, permitting fast perfusion of the chamber. Residual volumes of test solutions are removed from the chamber by means of a MiniWash pump module (MW) (TECAN). This module provides a standard suction pulse to the two funnel outlets (FO) that are operated ≈ 0.5 s before a new test solution is applied to the funnel that excluded mixing of test and wash solutions in the funnel. The outlets of the MiniWash pump module and the pump (P2) are connected to a waste container (W). Arrows indicate the direction of liquid flow in the tube system. **b** Schematic drawing of the tip of the liquid handling arm (left panel) and fragment of the tip with solutions inside (right panel). To shorten time between applications of different drugs or time between application of drug and washout, the drugs (D1 and D2) and the wash solution (WS) can be aspirated into the tip one after the other and separated from each other by air gaps (AG). **c** Block diagram of the protocol used for studies on ligand-gated channels. Each cycle (Cycle 1) starts with the standard cleaning step (Wash Tip) of the LHA tip in distilled water (Wash Tips procedure, see TECAN Gemini handbook). Control solutions for washout of drug and test solution (containing a drug or neurotransmitter) are subsequently aspirated [ASP(1) and ASP(2)] into the tip of LHA. Immediately before the test solution is applied, the residual solution is removed via the funnel outlets (residual solution removal, RSR) by means of MW. Application of test solution to the funnel (ATS) and immediate fast perfusion (FP) of the chamber are followed by the subsequent application of wash solution to the chamber (AWS) and rapid washout (FP). The next sequence of commands (Cycle 2) can then be executed. Control bath solution is aspirated and applied to the funnel, and the chamber is slowly perfused (SP, at 1–8 μ l/s) to guarantee steady-state conditions throughout the experiment. Cycle 1 can be repeated user-defined depending on the number of drugs to be tested

Fig. 3. Subsequent oocyte perfusion by 1 μ M (*left trace*) and 300 nM (*right trace*) ACh at different perfusion rates. ACh application (1) and washout (2) were performed at 350 μ l/s ('concentration jump mode'). Between the neurotransmitter applications, the chamber was perfused at low speed 8 μ l/s ['slow perfusion mode', (3)]

Fig. 4.

a Receptor-activated ionic currents in oocytes expressing nACh receptors (α -, β -, γ -, and δ -subunits). I_{ACh} were induced by subsequent applications of 100 nM, 300 nM, and 1 μ M ACh to the same oocyte. **b** Upon application of 30 μ M nACh with a chamber perfusion speed of 300 μ l/s, I_{ACh} increased from 10 to 90% within 85 ms. **c** Upon application of 30 μ M ACh with a chamber perfusion speed of 600 μ l/s, I_{ACh} increased from 10 to 90% within 55 ms

Fig. 5.

Estimation of the rate of solution exchange by 'concentration jump' application of high-potassium solution during $K_v1.1$ recordings. **a** The extracellular potassium concentration was rapidly increased (chamber perfusion speed of $600 \mu\text{l/s}$) from 1 to 30 mM during a potassium outward current (voltage step from -80 to +20 mV) (*thick line, 2*). Superimposed current traces before (*1*) and after (*3*) the 'concentration jump' are shown as *dashed lines*. **b** The time of current decrease from 10 to 90% ($t_{10-90\%}$) was taken as a measure of the volume rate of oocyte perfusion

Fig. 6. Timed applications of neurotransmitters. Fast perfusion of an oocyte expressing nACh receptors channels with 300 nM ACh for different time periods. I_{ACh} was induced by consecutive ACh applications for 2, 5, 10, and 20 s. Short periods of ACh application (<20 s) were achieved by successive application of neurotransmitter and wash (control) solution as illustrated in Fig. 2b