Novel voltage clamp to record small, fast currents from ion channels expressed in Xenopus oocytes

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ABSTRACT The present report describes a novel technique for voltage-clamping amphibian oocytes in which part of the membrane is isolated by a vaseline gap and the cytoplasmic fluid is exchanged by cutting or permeabilizing the remaining membrane. The main features of this open-oocyte, vaseline-gap voltage clamp are: (*a*) low current noise (1 nA at 3 kHz), (*b*) control of the ionic composition of both the internal and external media, (*c*) fast time resolution (20–100 μs time constant of decay of the capacity transient) and (*d*) stable recordings for several hours. These features allow reliable measurements of tail or gating currents and the new method is especially suitable when either of these currents must be measured to test the effects of mutations introduced into the cDNAs of cloned ion channels.

INTRODUCTION

In the last few years, complementary DNAs for voltage-dependent Na^+ (Noda et al., 1984); Ca^{2+} (Tanabe et al., 1987); and K^+ (Papazian et al., 1987) channels have been isolated. To determine whether the cDNA encodes a functional channel protein requires that it or its cognate cRNA be expressed in a suitable expression system and that currents from the expressed channels be measured electrophysiologically. For ion channels, the most widely utilized expression system is the Xenopus oocyte, which has the ability to synthesize exogenous protein when injected with foreign mRNA (Dascal, 1987). To measure currents, two different electrophysiological techniques are presently used: (*a*) the two microelectrode voltage clamp for the recording of whole-oocyte currents, and (*b*) the patch clamp for the study of single channel or multichannel currents.

These electrophysiological techniques, have several technical drawbacks including: (*a*) Resolution of small currents with fast kinetics. With a conventional two-microelectrode voltage clamp apparatus, currents of < 10 nA are difficult to resolve at 3 kHz. Furthermore, the capacity transient under this condition, lasting ~ 2 ms, can obscure the resolution of fast events like the activation and deactivation of Na^+ channels or of some fast K^+ channels like the Shaker channel (Dascal, 1987; Timpe et al., 1988). (*b*) Difficult access to the cell interior. Due to their large diameter (1–1.3 mm), it is extremely difficult to internally perfuse the oocyte. Nevertheless, it is possible to study the action of compounds on the internal face of the membrane by injecting low molecular weight compounds such as TEA (Taglialatela et al., 1991), IP_3 and Ca^{2+} (Miledi and Parker, 1984) or using inside-out patches. (*c*) Stability of the preparation. Particularly for patch clamp studies, the average survival time of the patch is generally < 1 h. Also, oocytes impaled with two microelectrodes usually do not allow recordings lasting for longer periods. For patch clamp, the usual signal/noise ratio limits the bandwidth to 1–2 kHz. The macropatch method allows faster clamping but has the requirement that channels be expressed at very high densities which is often not possible.

In this study we describe the development of a new open-oocyte vaseline-gap method which overcomes many of these limitations. This voltage clamp method has relatively low current noise (~ 1 nA at 3 kHz), can charge the membrane capacity in 20–100 μs and provides the possibility to control the intracellular milieu by internal perfusion of the oocyte. In addition, stable recording lasting for several hours could be obtained. These properties allow the adequate resolution of the time course of fast ionic and gating charge currents. A somewhat similar perfusion technique for oocytes had been previously described by Yoshii and Takahashi (1984).

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METHODS AND RESULTS

Oocytes injection and handling

cRNAs and mRNAs injected into oocytes were kindly given by Dr. A.M. Brown and Dr. R. Joho from this department. In brief cDNAs

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from type III brain Na^+ channel (Suzuki et al., 1988) and the delayed rectifier K^+ channel DRK1 (Frech et al., 1989) were cloned from a rat brain cDNA library. The engineered delayed rectifier K^+ channel DRK/NGK L374V was obtained as described elsewhere (Drewe et al., submitted for publication). cRNAs were transcribed from linearized cDNAs encoding for these voltage-dependent channels to obtain run-off transcripts (Joho et al., 1990). Stage V-VI Xenopus oocytes were injected with 75 nl of 50–200 ng/μl of cRNA solutions in 0.1 M-KCl. After the injection, oocytes were kept at 19°C in Barth solution. All the recordings were performed at room temperature (22°C) on manually defolliculated oocytes 2–7 days after the RNA injection.

Solutions

The solutions utilized in the present experiments were the following (mM): Barth: NaCl 88, KCl 1, $NaHCO_3$ 2.4, Na-HEPES 15, $Ca(NO_3)_2$ 0.3, $CaCl_2$ 0.4, $MgSO_4$ 0.8, pH 7.6; K-MES: K-CH_3SO_3 120, Na-HEPES 10, pH 7.3; Mg-TEA-MES: TEA-CH_3SO_3 107, Mg-$(CH_3SO_3)_2$ 5, Na-HEPES 5, pH 7.3; Ca-TEA-MES: TEA-CH_3SO_3 107, Ca-$(CH_3SO_3)_2$ 5, Na-HEPES 5, pH 7.3; Cl-free Na-MES: Na-CH_3SO_3 71.3, K_2SO_4 1.6, $Ca(CH_3SO_3)_2$ 1.8, Na-HEPES 10, pH 7. The abbreviations are TEA for tetraethylammonium, MES for methanesulphonate and HEPES for (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]).

Description of the experimental apparatus

A schematic drawing of the experimental chamber and electronics is shown in Fig. 1. The system is an adaptation of the cut-fiber vaseline-gap used for skeletal muscle (Campbell and Hille, 1976; Kovacs et al., 1983; Francini and Stefani, 1989). At the beginning of the experiment, the upper division (*A*) of a perspex chamber was lifted and the chamber was filled with extracellular Barth solution. A defolliculated oocyte was accommodated in the hole of the lower division (*B*) and a vaseline rim (~300-μm thick) was placed between the oocyte membrane and the edge of the hole. The size of this vaseline rim was not critical for the clamp performance. To improve the seal resistance, hydrostatic pressure was applied to the oocyte by adding solution to the upper chamber. Once the oocyte was secured, the upper part (*A*) of the chamber was positioned on top of the oocyte in such a way that the hole coincided with the upper pole of the oocyte. Before positioning the upper part of the chamber, a very thin vaseline rim (~50-μm thick) was placed along the edge of the hole. The thickness of this second vaseline rim was critical to achieve an optimal frequency response of the clamp (10–50 kHz) and to prevent the artifactual recording of currents arising in the oocyte membrane under this seal. This procedure established three electrically independent pools: (*a*) a lower pool (I) to inject current and to have access to the cell interior; (*b*) a middle pool (GS, guard shield) to connect an electronic guard shield and (*c*) an upper pool (P) which contained the oocyte membrane to clamp. Connections between pools and the electronic components were made via 1 M-NaCl agar bridges and nonpolarizable Ag-AgCl electrodes immersed in 3 M-KCl. The bridges (1–2-cm long, 1.8-mm outer diameter) were thread up to ~1 mm from the edges with 100 μm chlorided silver wire or platinized platinum to reduce their impedance. The seal resistance between the compartments varied between 0.5 to 2 megohms.

The electronic system consisted in two voltage-clamps: the first one clamped the external pool P (pulse pool) to the command potential whereas the second one clamped the oocyte interior facing pool P to ground. Due to the oocyte cytoplasmic resistance in series with the membrane resistance, simply grounding pool I would not be sufficient because a large systematic error in the recorded voltage would be generated by the IR (current *x* resistance) drop in the cytoplasm. The second intracellular clamp practically eliminates this error.

The speed of the clamp was monitored by following the time constant of decay of the capacity transient (20–50 μs, see Fig 2 *C*). Membrane currents were recorded from the voltage-clamped area of the oocyte membrane which faced the upper compartment *P* (radius between 200–350 μm, capacitance 20–100 nF). The circuit consisted of

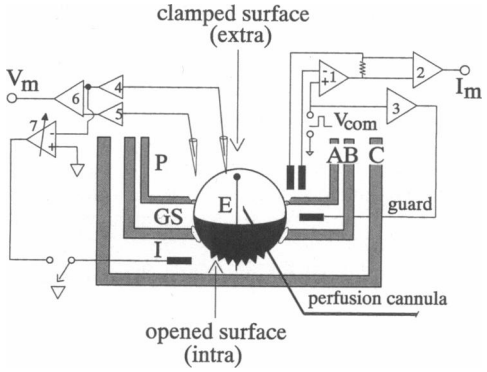

FIGURE 1 Schematic drawing of the experimental chamber and electronics. A transverse section of a Xenopus oocyte mounted in the experimental chamber is shown. Compartments A, B, and C of the perspex chamber established by means of two vaseline rims, three electrically separated pools: a lower compartment (I) to open and internally perfuse the oocyte, a middle compartment for guard shield (GS) and an upper compartment (P) where the oocyte exposed surface was voltage clamped. In compartment I, a perfusion cannula and a metal electrode E inserted into the oocyte to improve the clamp performance are also shown.

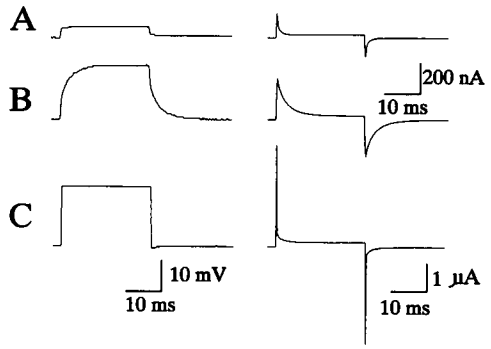

FIGURE 2 Passive electrical properties of the oocyte at different stages of the clamp procedure. Nonsubtracted record. *A* shows the oocyte membrane potential and membrane current in response to a 20-mV command pulse in a nonopened oocyte in Barth solution with the I pool connected to ground. In *B*, the same voltage pulse was applied after opening the oocyte membrane facing the I pool by K-MES solution containing 0.1% saponin. In *C*, the oocyte membrane potential and membrane current in response to the same 20-mV command pulse are shown, but the interior of the oocyte was clamped to ground with the intracellular microelectrode by connecting the feedback amplifier 7 to pool I.

a current to voltage transducer (I-V; 1) with variable resistances (0.1–2 megohms) in the feedback loop that can be selected via an electronic switch. The upper compartment P was connected by two electrodes which prevented artifacts due to electrode polarization. One of the electrodes in pool P was connected to the negative input of the I-V (1), and the other to the feedback resistance of the same amplifier. The command pulse was fed into the positive input of the I-V amplifier. Membrane current (I_m) was measured via the differential amplifier 2 as a voltage drop across the feedback resistance. To improve electrical isolation between the recording (P) and the current injecting pool (I), the middle compartment (GS) was clamped to the command potential via amplifier 3. Thus, this compartment acts as a guard shield and, ideally, no current should flow between GS and P because both pools are maintained at the same potential. A conventional microelectrode voltage-clamp was used to clamp to ground the interior of the oocyte facing the compartment P. To this end, a 3–6 megohm resistance microelectrode filled with 3 M-KCl was used. This microelectrode sensed the intracellular potential in the center of the membrane in P. The microelectrode signal was fed via amplifier 4 into the negative input of a high gain differential amplifier (7) whose positive input was grounded. The output of the amplifier was connected to the lower pool (I) in the voltage-clamp configuration. The membrane potential across the clamped oocyte membrane was differentially recorded via amplifier 6 and was obtained as the potential difference between the intracellular microelectrode (4) and an external one (5) positioned close to the external oocyte surface. The bottom of the oocyte was opened to inject current intracellularly. The opening of the oocyte membrane facing pool I (intracellular pool) was achieved either by superfusing an internal solution with saponin (0.1%) for ~1 min, or by cutting a small hole with iridectomy scissors in the oocyte surface before mounting the oocyte in the chamber. The opening of the oocyte allowed the dialysis of the intracellular medium as described by Yoshii and Takahashi (1984). However, this is a slow process due to the oocyte dimensions (~1 mm in diameter), and, for example, it can take hours to replace the intracellular K^+. To obtain a faster and better controlled exchange of the internal milieu a 200-μm polyethylene tube connected to a conventional perfusion pump was introduced into the oocyte via its lower cut end. In this way, with a flow rate of 1–50 μl/h, we could eliminate outward K^+ currents by perfusion with a K^+-free medium in <5 min. In addition, the clamp had conventional features such as series resistance compensation and capacity compensation at the negative input of amplifier 1 to prevent the amplifier saturation during large capacity transients.

An IBM compatible personal computer was used for data acquisition (D/A-A/D conversion by TL-1 DMA interface; Axon Instruments Inc. [Burlingame, CA]) and processing (P-CLAMP software, [Axon Instruments Inc.] and custom made software). Analog signals were filtered at ¼ the sampling frequency. To record nonlinear currents (Fig. 3), linear capacity and resistive components were digitally subtracted by scaled control currents obtained with small negative pulses of ¼ test pulse amplitude.

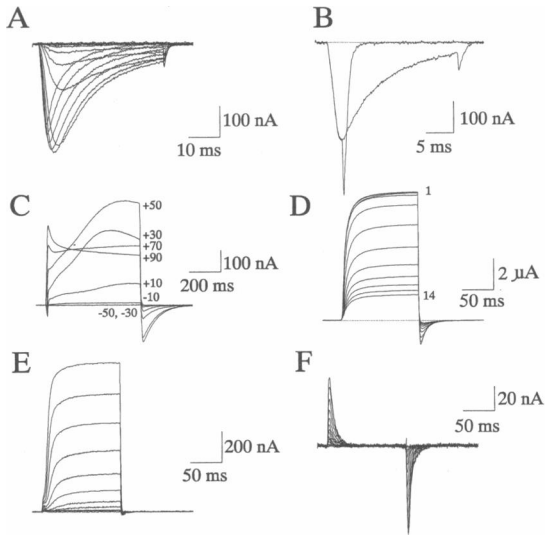

FIGURE 3 Examples of ionic and gating currents from channels expressed in oocytes recorded with the open-oocyte vaseline-gap method. Subtracted records for linear components. (*A*) current traces in response to depolarizing pulses of 40 ms duration from an oocyte injected with type III Na^+ channel cRNA. Holding potential −90 mV, depolarizing pulses from −60 mV to +15 mV, in 5 mV steps. (*B*) Na^+ tail currents in the same oocyte of *A*. Two depolarizing voltage-clamp pulses of 3 and 28 ms duration to +10 mV from a holding potential of −90 mV were delivered. Both in *A* and *B*, the internal solution was K-MES, whereas the external was Barth. (*C*) current traces in response to depolarizing pulses of 900 ms length from an oocyte injected with total rat brain mRNA. Holding potential: −70 mV, depolarizing pulses from −50 to +90 mV, in 20-mV steps. The internal solution composition was K-MES, whereas the external was Cl-free Na-MES. (*D*) current responses from a DRK1 cRNA-injected oocyte to 14 consecutive depolarizing pulses to +40 mV repeated every 20 s. The external solution was Barth, whereas the internal one was K-MES. After recording the first four control traces, the solution in pool I was replaced with Mg-TEA-MES. (*E*) current traces in response to depolarizing pulses of 125 ms length from an oocyte injected with DRK/NGK L374V cRNA. Holding potential −70 mV, depolarizing pulses from −50 to +50 mV, in 10 mV steps. The internal solution was K-MES, whereas the external one was Barth. (*F*) gating currents (10 averages) from the same oocyte shown in *E* recorded upon complete blockade of the ionic currents with the exchange of the internal and external solutions with Mg-TEA-MES and Ca-TEA-MES, respectively. Holding potential −70 mV, depolarizing pulses from −60 to +5 in 5 mV steps.

Experimental results

Fig. 2 shows the oocyte passive electrical properties at different stages of the clamp procedure. *A* shows the oocyte membrane potential and membrane current in response to a 20 mV command pulse in a nonopened oocyte with the I pool connected to ground. In this configuration there is no voltage control because the oocyte membrane in region P is passively charged via the capacity and the resistance of the intact membrane facing pool I and the cytoplasmic resistance in series. In *B*, the same voltage pulse was applied after saponin-induced opening of the oocyte membrane facing the I pool. The membrane opening improved the electrical access to the oocyte interior. The voltage response followed more closely the command pulse and the recorded membrane capacity increased due to the removal of the in series capacity of the membrane facing compartment I (see Yoshii and Takahashi, 1984). However, the speed of the clamp was limited by the product of the cytoplasm resistance and the clamped membrane capacity which resulted in the slow rise of the recorded transmembrane potential and in the slow decay of the capacity transients. This limitation was overcome by clamping to ground the interior of the oocyte close to the P membrane. In fact, the voltage recorded by the intracellular microelectrode was connected to the negative input of feedback amplifier 7. The positive input of amplifier 7 was grounded and its output was connected to pool I via low resistance electrodes. In some experiments we tested the spatial clamp homogeneity of the

surface facing pool P. To this end we monitored the transmembrane potential close to the edge by a third microelectrode. The recorded transmembrane potentials measured by both microelectrodes (one in the center and the other close to the edge of a 250-μm hole) were indistinguishable indicating adequate clamp control during passive membrane property measurements. To further avoid the speed limitation due to the access resistance of the oocyte cytoplasm, in some experiments, we introduced a metal electrode (platinized platinum or chlorided silver) 100 μm in diameter (*E*) along the oocyte vertical axis. The upper end of this metal electrode was positioned as close as possible to the clamped surface. This electrode short circuits the cytoplasm resistance and made the charging process of the membrane capacitance much faster. With these improvements, the voltage response in *C* closely followed the command pulse and had a rise time of one sampling point (20 μs). The decay of the capacity transient had two time constants: a predominant fast one of ~30 μs and a smaller slow one of 1.2 ms. The amplitude of this slow component was usually ~10% of the fast one and was directly correlated to the thickness of the vaseline rim around the oocyte in the upper compartment A. In fact, when the oocyte was mounted using a very thin rim of cured sylgard instead of vaseline positioned on the edge of the upper chamber hole, the slow component became undetectable. These results indicate that the slow component results from currents arising from a poorly clamped membrane area located under the seal. The use of sylgard was ideal to achieve optimal clamp speed, however, it was time consuming because, due to its instability with the perspex of the chamber, it required a frequent coating of the upper chamber hole. For most practical purposes, with a thin vaseline rim the clamp was fast enough to record fast currents (see Fig. 3).

Different examples of ionic and gating currents recorded under the described conditions are shown in Fig. 3. *A* shows Na^+ currents from oocytes injected with type III brain Na^+ channel cRNA. The graded increase in the Na^+ current in response to small depolarizations and the absence of notches in the current traces is an indication of the good quality of the voltage control. This is in contrast to what has been previously reported for oocyte expressed Na^+ currents (Methfessel et al., 1986). The good clamp performance is further illustrated by the optimal resolution of the Na^+ channel tail currents (*B*). To assess the fraction of the total Na^+ current that may arise from channels located under the upper vaseline seal we added a high concentration of tetrodotoxin (3.2 μM) to the recording pool P and immediately (1–2 min) measured the ionic current. The unblocked Na^+ current would give an estimate of the current arising from channels unaccessible to the drug located under the seal. This value was ~2% of the total current and further indicated the lack of major artifacts arising from unclamped ionic currents.

As previously mentioned, another advantage of this method is the possibility to exchange the oocyte internal milieu. *C* shows expressed ionic currents in a saponin-opened oocyte previously injected with mRNA extracted from whole rat brain. A bell-shaped sustained outward component was recorded in presence of external Ca^{2+} and in the absence of both internal and external Cl^- ions. Therefore, this current cannot be carried by Cl^- ions via the endogenous Ca^{2+}-dependent Cl^- channels (Barish, 1983) and, most likely, may result from the activation of Ca^{2+}-dependent K^+ channels. This view was confirmed by the ability of charybdotoxin as well as Ca^{2+}-free media to reduce this component (data not shown). The removal of internal and external Cl^- made possible the identification of expressed Ca^{2+}-dependent K^+ currents. The endogenous Ca^{2+}-dependent Cl^- current present in intact oocytes would have made uncertain this identification.

The capability to exchange solutions close to the internal side of the clamped membrane is also shown in *D*. Current responses to voltage steps to +40 mV repeated every 20 s from a saponin-opened oocyte injected with DRK1 cRNA are shown. The first four current traces were recorded under control conditions (intracellular K-MES and extracellular Barth), whereas the subsequent traces were recorded after the beginning of the superfusion in pool I with a solution containing Mg-TEA-MES. The relatively rapid block of the delayed rectifier current is a good indication of the internal diffusion of Mg-TEA-MES.

In *E*, currents recorded from oocytes expressing an engineered delayed rectifier K^+ channel, DRK/NGK L374V, are shown. *F* shows the corresponding K^+ gating currents which could only be recorded after complete blockade of the ionic currents by internal and external equilibration with Mg-TEA-MES and Ca-TEA-MES, respectively. The high frequency response of the clamp and the low current noise allowed the measurements of gating currents which are fast and small events.

CONCLUSIONS

In the present study we described a novel method for voltage clamping Xenopus oocytes, which overcame some of the limitations of the two microelectrode voltage clamp or the patch clamp methods. The advantages of the present method are: (*a*) High frequency response and low noise recording. This allowed the accurate description of small gating currents at channel densities of 1–2/μm^2. Furthermore, fast activation and deactivation of ionic currents can be adequately resolved. (*b*) Stable recording conditions lasting for several hours. This is a clear advantage over the gigaseal patch clamp method in which the patches are difficult to maintain for long periods of time. Furthermore, the spontaneous increase in microelectrode resistance which severely limits the duration of the experiment and the size of the currents that can be successfully clamped with the two microelectrode voltage clamp, is no longer a problem with the open-oocyte vaseline-gap technique. (*c*) Access to the cell interior. The intracellular medium can be exchanged with various solutions. This was of invaluable help in recording K^+ gating currents, an experimental condition which required the complete blockade of all ionic currents. This advantage will make the present method suitable for the study of channel modulation by second messengers and drugs. In addition, channel selectivity properties can be defined by ion substitution experiments. Furthermore, exogenous channels expressed at very low densities can be discriminated from native oocyte channels, as in the case of Ca^{2+}-dependent K^+ channels. (*d*) Channel localization. A study of the membrane compartmentalization of channels can be easily obtained with this method, because it allows to voltage-clamp different regions of the oocyte surface.

(Note: a complete circuit diagram including the mechanical layout and the scale drawing of the chamber will be sent upon request.)

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