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Injection of a K^+ channel (Kv1.3) cRNA in fertilized eggs leads to functional expression in cultured myotomal muscle cells from *Xenopus* embryos

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Abstract

The synthetic cRNA encoding for the major T lymphocyte K^+ channel (Kv1.3) was injected into *Xenopus* fertilized eggs. Somites from embryos of stage 20–22 (about 40 h post-fertilization at 19°C) were dissociated and myotomal muscle cells were cultured in vitro for 2 days. The whole cell configuration of the tight seal patch-clamp technique was used to record K^+ channel activity in cultured myocytes. These myocytes have two endogenous delayed-rectifiers (sustained and transient) and an inward-rectifier K^+ currents, all of which are insensitive to the scorpion toxin charybdotoxin. Cultured myocytes dissociated from embryos injected with the Kv1.3 cRNA expressed the exogenous Kv1.3 channel. The Kv1.3 channel was identified by its physiological (a very low recovery from inactivation) and its pharmacological properties (a high sensitivity to charybdotoxin). This work demonstrates that *Xenopus* cultured myotomal muscle cells represent a very efficient and practical assay system for the functional expression of cloned ion channels.

Key words: Cloned K^+ channel; Charybdotoxin; Fertilization, in vitro; Heterologous expression; Patch clamp; T lymphocyte

1. Introduction

The *Xenopus* oocyte is one of the best characterized preparation for studies of early development (for review see [1]). It is also widely used for the cloning of foreign ion channels and for the functional analysis of their mechanism and regulation (for review see [2]). One of the great advantages of the use of the non-fertilized *Xenopus* oocyte in the study of ion channels is the possibility of examining the expression of exogenous channels in the absence or quasi-absence of expression of the corresponding endogenous channels.

The present report presents a strategy to express ion channels in *Xenopus* myocytes by injection of synthetic cRNA directly into fertilized eggs (Fig. 1). As embryos develop, injected cRNA, and consequently the channels that it encodes, are expected to be distributed in the various embryonic cells, including the myotomal muscle cells constituting the somites (Fig. 1C–E).

Using this strategy, this paper describes the expression of the cRNA for a K^+ channel called Kv1.3 [3]. This channel is the major voltage-dependent K^+ channel in T lymphocytes where it controls the resting membrane potential (for review see [4]) and the osmotic regulation [5]. Kv1.3 is also an important K^+ channel sub-type in the brain [6,7]. This voltage-gated K^+ channel is blocked by nanomolar concentrations of charybdotoxin (Chtx) [3,4,8–11]. The Kv1.3 channel was chosen as a model because it had previously been expressed in cRNA-injected non-fertilized *Xenopus* oocytes and in cDNA-transfected IM9 B lymphocytes [3,9,10].

2. Materials and methods

Procedures for isolation, fertilization and mRNA injection of *Xenopus* eggs are described in detail by Moon and Christian [12]. Briefly, 1000 IU of human chorionic gonadotropin (HCG) was injected into the dorsal lymph sac of a female *Xenopus laevis* the night before spawning. The stock solution of hormone was made by dissolving lyophilized HCG in sterile external solution (see below) at a concentration of 5000 IU/ml and the unused stock was kept at 4°C. The morning after injection with HCG, the frog was squeezed (5 times every 30 min) to obtain mature eggs. Eggs were kept in modified 1 × Barth's solution (MBS) containing (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES 10, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, pH 7.5. Males were anesthetized by being placed on ice for 30 min and testes were removed. Testes were rinsed in 1 × MBS and minced with sharp forceps into 3 ml of the 1 × MBS solution. The sperm suspension was laid over the eggs and after 5 min fertilization the Petri dish was filled with aged tap water. The jelly coat then formed and the eggs were rotated within 20 min so that the dark animal pole faced up. At 30 min post-fertilization, water was removed and the dish was filled with a 2% cysteine solution. Cysteine was prepared fresh within 2 h of use and the pH was adjusted with NaOH to 7.8. The embryos were swirled for 3 min in the cysteine solution to remove the jelly coat. After removal of the cysteine solution, embryos were quickly rinsed 3 times with a rinse solution consisting of 10% glycerol, 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$. The third wash was removed and embryos were rinsed twice in 0.1 × MBS supplemented with 5% Ficoll (pH 7.5). During the 30 min following fertiliza-

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Abbreviations: 4AP, 4-aminopyridine; Chtx, charybdotoxin; DTX_I, dendrotoxin I; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethylether) *N,N,N',N'*-tetraacetic acid; HCG, human chorionic gonadotropin; HEPES, (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid]); MBS, modified Barth's solution; MCD peptide, mast cell degranulating peptide; TEA, tetraethylammonium.

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tion of the eggs, embryos were microinjected in the animal pole with 10 nl of the mRNA solution (0.3 μg/μl) using a pressure injection system (Fig. 1). About 12 h post-fertilization, before gastrulation, the Ficoll solution was removed and embryos were kept in 0.1 × MBS. The culture medium was then replenished with aged tap water as development proceeded. Methods for tissue culture of myoblasts from myotomes of stage 20 embryos are described in detail by Peng et al. [13]. Day 0 was defined as the day of plating. Somites from one embryo (stage 20 [14]) were dissected in Steinberg's solution containing (in mM): NaCl 60, KCl 0.67, $Ca(NO_3)_2$ 0.34, $MgSO_4$, 0.83, HEPES 10, pH 7.4. Cells were dissociated for 50 min in a Ca^{2+}-free Mg^{2+}-free Steinberg's solution containing 0.4 mM EDTA and cells were plated in a 35 mm Petri dish yielding to a specific myotomal muscle cell culture. The culture medium contained: 89% Steinberg's solution (Gibco), 10% Leibovitz L15-medium, 1% fetal bovine serum, 100 μg/ml gentamicin sulfate. Cell cultures were kept at 19°C in an incubator.

The patch clamp procedures and data analysis were previously described in [3,10]. The external solution contained in mM: 117.5 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES, pH 7.2 with NaOH. The K^+-rich external solution was made by substituting NaCl with KCl. The internal solution contained in mM: 115 KCl, 5 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.2 with KOH. Voltage-clamped isolated cells were exposed to different solutions by placing them in the mouth of a perfusion tube from which the rapidly exchanged solutions flowed. All chemicals were from Sigma unless otherwise stated. Preparation of capped synthetic poly-adenylated mRNA for the human Kv1.3 channel was previously described by Attali et al. [3]. Results are presented as means associated with their standard error and numbers of cells tested are indicated.

3. Results and discussion

Xenopus mature eggs were fertilized in vitro (Fig. 1A). During the 30 min post-fertilization, the fertilized eggs were microinjected in the animal pole with Kv1.3 cRNA (Fig. 1B). Then, embryos were allowed to develop to stages 20–22 (Fig. 1C–D). Somites were dissected and separated from the neural tube (Fig. 1E). Myotomal muscle cells were dissociated in a Ca^{2+}-free solution and cultured for 2 days.

First of all it was necessary to characterize the endogenous K^+ conductances in cultured control myotomal muscle cells. After plating, the first endogenous current that was recorded was a sustained delayed-rectifier K^+ outward current (I_{del}) activated upon depolarization (Fig. 2B inset). Although current amplitudes were small, it could be clearly seen that the tail currents reversed at a value close to −90 mV in the standard external conditions (not shown). Current densities measured at the end of the pulse during a voltage pulse to 20 mV from a holding potential of −80 mV were 5.4 ± 0.6 pA/pF (n = 15) at day 0, 8.8 ± 0.6 pA/pF (n = 32) at day 1 and 8.8 ± 0.9 pA/pF (n = 24) at day 2 of culture. Then, with a delay of a few hours, the expression of an hyperpolarization-activated inward rectifier K^+ channel current (I_{ir}) was observed (Fig. 2B, inset). I_{ir} densities measured at peak current with a test potential of −120 mV were 2.2 ± 0.5 pA/pF (n = 20) at day 0, 6.1 ± 0.5 pA/pF (n = 35) and 7.7 ± 0.8 pA/pF (n = 27) at day 2 of culture. Finally, a transient outward A-type delayed-rectifier K^+ current (I_{to}) activated upon depolarization was also recorded (Fig. 2B inset). I_{to} ($I_{peak} - I_{stable}$) densities were 2.1 ± 0.9 (n = 15) at day 0, 15.1 ± 1.4 pA/pF at day 1 and 20.8 ± 1.8 (n = 25) at day 2 of culture. Fig. 2A and B show the mean I–V relationships performed at day 0 and

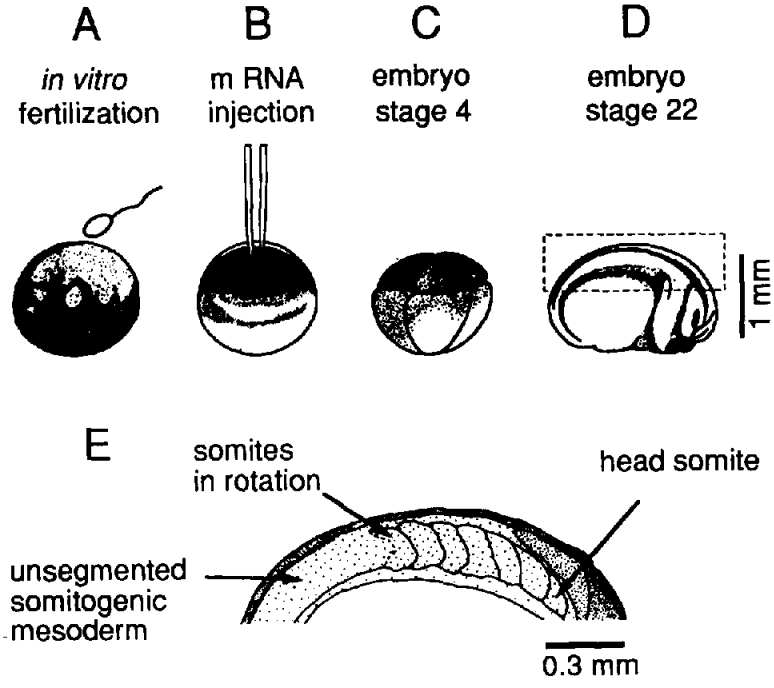

Fig. 1. Schematic representation of the protocol used to transfer cRNA into cultured myotomal muscle cells from *Xenopus* embryos. (A) Animal view (magnification × 24) of an egg with the white spot indicating maturation. Matured eggs were fertilized in vitro. The spermatozoid is not to scale. (B) Dorso-lateral view showing the microinjection of cRNA into the animal pole of a fertilized *Xenopus* egg. (C) Dorso-lateral view of a stage 4 embryo. (D) Lateral view of a stage 22 embryo. The dorsal part of the embryo was dissected as indicated by the dashed rectangle. (E) Somites were separated from both the neural tube and the notochord and dissociated in a Ca^{2+}/Mg^{2+}-free solution (lateral view).

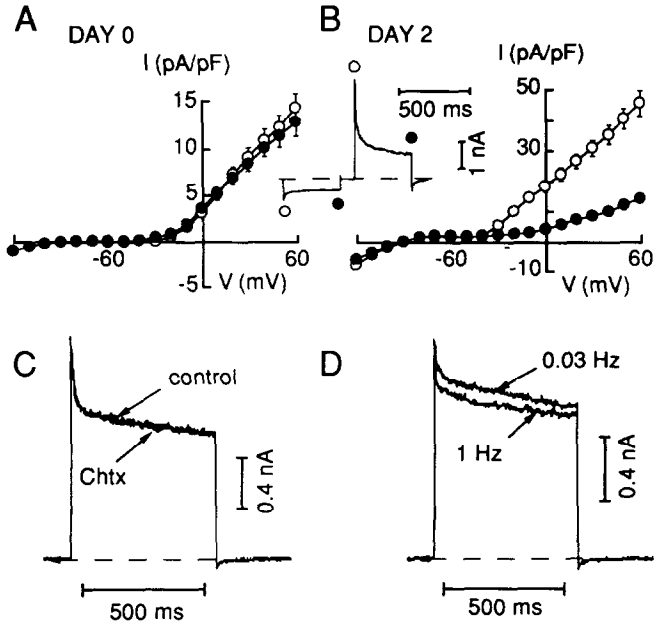

Fig. 2. (A) Current–voltage relationships measured at peak (○) and late (●) currents at day 0 of culture in control myocytes. Each data point represents the mean current density of 7 experiments associated with its standard error. (B) Same as in A for day 2 of culture. The inset shows the membrane current during a hyperpolarization to −120 mV and a depolarization to 40 mV from a holding potential of −60 mV in a cell at day 2 of culture. (C) Effects of 10 nM Chtx on membrane currents recorded during a voltage step to 20 mV from a holding potential of −80 mV at day 1 of culture in a control myocyte. (D) Effects of changing the frequency of stimulation from 0.03 Hz to 1 Hz at day 1 of culture in a control myocyte: same voltage protocol as C.

day 2 of culture. From these curves, it can be clearly seen that at day 0 (4–9 h post-plating), both I_{ir} and I_{to} had moderate amplitudes compared to I_{del}. The inward rectifier K^+ channel current recorded at −120 mV was reversibly and fully inhibited by 0.1 mM Ba^{2+}, although it was resistant to 1 mM TEA, 1 mM 4AP, 10 nM Chtx, 200 nM DTX_i and 200 nM MCD peptide (n = 7). Both I_{to} and I_{del} were depressed by 1 mM TEA (−41 ± 3%, n = 9; −39 ± 4%, n = 8, respectively) and 1 mM 4AP (−45 ± 2%, n = 7; −42 ± 2%, n = 7, respectively) while no effect was found with Ba^{2+}, DTX_i and MCD peptide (n = 7). Fig. 2C shows that 10 nM Chtx failed to affect either I_{to} or I_{del}. Increasing the frequency of stimulation had only a very slight depressing effect on both currents (Fig. 2D).

An important observation was that injection of Kv1.3 mRNA into fertilized eggs did not alter the normal development of the embryos (not shown). 35% of the cultured myotomal muscle cells expressed Kv1.3 channels at days 0–1 of culture (n = 17). The exogenous current was identified using both pharmacological and physiological criteria. The current was reversibly inhibited with 10 nM Chtx (Fig. 3A) and it displayed a typical slow recovery from inactivation (Fig. 3B). These properties are different from those of endogenous K^+ channels (Fig. 2C,D). The current voltage-relationship of the Kv1.3 current is presented in Fig. 3C. The voltage threshold for activation was −40 mV and the current rectified slightly in the inward direction for positive potentials. The Kv1.3 current density measured at 20 mV was 74 ± 18 pA/pF at day 1 of culture (n = 5). The addition of 10 nM Chtx depressed K^+ currents with a similar potency at all membrane potential (Fig. 3C). The Chtx-sensitive current measured at 20 mV was 51.2 ± 13.6 pA/pF (n = 5) at day 1 of culture. Steady-state activation and inactivation curves of Kv1.3 are shown in Fig. 4. Kv1.3 was half-activated at −15 mV and was half-inactivated at −37 mV.

These results clearly demonstrate that one can easily obtain the heterologous functional expression of a voltage-dependent K^+ channel in *Xenopus* cultured myotomal muscle cells. cRNA injection into the fertilized oocyte leads to the expression of the Kv1.3 channel with the following properties: (i) the channel has a voltage-dependent slow inactivation; (ii) it has a slow recovery from inactivation; (iii) it displays an inward rectification as seen in the I–V curve; (iv) it is very sensitive to nanomolar concentrations of charybdotoxin. All these properties are very similar to those found in T lymphocytes [8–10] as well as to those observed after expression of low cRNA concentrations in the non-fertilized *Xenopus* oocyte or after transfection in Kv1.3 defective B lympho-

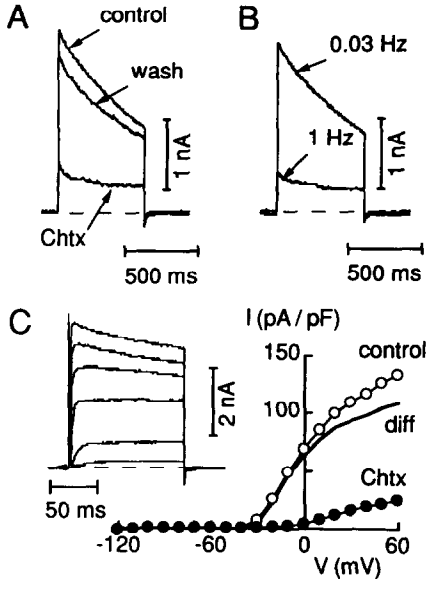

Fig. 3. (A) Effect of 10 nM Chtx on the exogenous Kv1.3 current elicited in a muscle cell 9 h post-plating. The holding potential was −80 mV and the cell depolarized to 20 mV every 30 s. (B) Effect of increasing the frequency of stimulation from 0.03 Hz to 1 Hz on the Kv1.3 current. Same voltage protocol as in A. (C) I–V curve of the Chtx-sensitive Kv1.3 current expressed in a muscle cell 8 h post-plating. The control current traces are shown for depolarizations to −30 mV, −20 mV, 0 mV, 20 mV, 40 mV and 60 mV from a holding potential of −80 mV. The I–V curves are shown under control conditions (○), in the presence of 10 nM Chtx (●) and the current difference is shown as a continuous thick line.

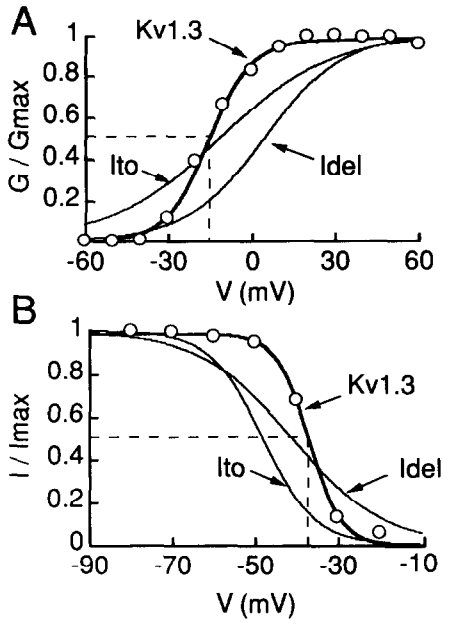

Fig. 4. (A) Steady-state activation of the exogenous Kv1.3 (same cell as Fig. 3C) and the endogenous I_{del} and I_{to} K^+ currents. Continuous lines were drawn using $G/G_{max} = 1/(1 + \exp((V_{0.5}-V)/k))$ with $V_{0.5} = -15.6$ mV and $k = 7.8$ mV for Kv1.3, $V_{0.5} = 5$ mV and $k = 14.3$ mV for I_{del} ($n = 7$) and $V_{0.5} = -11$ mV and $k = 20.2$ mV for I_{to} ($n = 7$). (B) Steady-state inactivation curves of the exogenous Kv1.3 (same cell as Fig. 3C) and endogenous I_{del} and I_{to} K^+ currents. Experimental points were fitted by $I/I_{max} = 1/(1+\exp((V_{0.5}-V))/k)$ with $V_{0.5} = -37$ mV and $k = -3.9$ mV for Kv1.3, $V_{0.5} = -41$ mV and $k = -11$ mV for I_{del} ($n = 7$) and $V_{0.5} = -48$ mV and $k = -6.3$ mV for I_{to} ($n = 7$).

cytes [3,9,10]. Therefore, it appears that the *Xenopus* embryo system could become a very efficient and practical system in which to express foreign ion channels (or membrane receptors) in well-differentiated cells, such as myotomal muscle cells from the somites, or neurons from the neural tube. The over-expression of ion channels in *Xenopus* embryos might also provide valuable information concerning their role during development.

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