The GXGXG motif in the pI_{Cln} protein is not important for the nucleotide sensitivity of the pI_{Cln}-induced Cl^- current in *Xenopus* oocytes

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Abstract It has been proposed that the pI_{Cln} protein forms a nucleotide-sensitive plasma membrane anion channel with a GXGXG motif being an essential component of the extracellular nucleotide-binding site. To evaluate this hypothesis, we have performed voltage-clamp experiments on *Xenopus laevis* oocytes injected with RNA encoding a rat mutant pI_{Cln} in which the three glycines of the putative nucleotide-binding site have been changed into alanines (G54A; G56A; G58A). The injected oocytes displayed outwardly rectifying anion currents, which were voltage-dependently blocked by extracellular cAMP, but which were not affected by removal of extracellular Ca^{2+}. Furthermore, the mutation did not affect the voltage-dependent inactivation. We therefore conclude that there is no evidence in favour of an extracellular nucleotide-binding site in pI_{Cln}.

Key words: Anion channel; Oocyte; Cyclic adenosine monophosphate

1. Introduction

Anion channels activated by cell swelling have been identified in a large number of mammalian and non-mammalian cell types [1–3]. These volume-regulated anion channels (VRACs) allow the efflux of Cl^- and organic osmolytes and thereby contribute to regulatory volume decrease [3]. Over the past years, several papers have appeared in which VRAC has been directly linked with the protein pI_{Cln} [4–6]. This protein was originally identified as a nucleotide-sensitive Cl^- channel due to its ability to induce a Cl^- current after its expression in *Xenopus* oocytes [7]. This current, also termed I_{Cln}, has the following characteristics: outward rectification; block by extracellular nucleotides; slow inactivation at positive potentials; independent of extracellular Ca^{2+} [7]. Phenotypic similarities between I_{Cln} and $I_{Cl,swell}$ as well as the finding that $I_{Cl,swell}$ in NIH/3T3 fibroblast was reduced after treatment with antisense pI_{Cln} oligonucleotides led Paulmichl and co-workers to conclude that pI_{Cln} corresponds to VRAC [5–7]. In contrast, Clapham and co-workers [4,8] proposed that pI_{Cln} is not VRAC itself but rather a critical cytosolic regulator of VRAC. Two lines of evidence supported this latter hypothesis. Firstly, *Xenopus* oocytes express pI_{Cln} endogenously and this protein is located in the cytosol. Secondly, *Xenopus* oocytes have an endogenous $I_{Cl,swell}$, proposed to be identical to I_{Cln} (but see [9]), which was inhibited by injecting a monoclonal antibody against pI_{Cln}.

An apparently conclusive experiment in favour of pI_{Cln} being an anion channel originated from the originally proposed structural model of pI_{Cln}. A GXGXG sequence (amino acids 49–53 in MDCK pI_{Cln}) was predicted to lie at the extracellular side and to form a 'consensus' nucleotide-binding site responsible for the block of I_{Cln} by extracellular nucleotides [7]. Experimental support for this contention was drawn from experiments in which a mutant MDCK pI_{Cln} (G49A/G51A/G53A) was expressed in *Xenopus* oocytes. Currents observed after expression of the mutant protein differed in three aspects from wild-type I_{Cln}: $I_{Cln-mut}$ was nucleotide-insensitive, Ca^{2+}-dependent and it activated slowly at positive potentials [7].

The mutagenesis data seemed to provide conclusive evidence for a channel function (see also [10,11]), but subsequent observations (cytosolic location; I_{Cln} being an endogenous *Xenopus* current) have questioned the channel model for pI_{Cln} [4,9,12]. In view of the ongoing controversy, we have repeated these crucial experiments by expressing mutant rat pI_{Cln} in *Xenopus* oocytes.

2. Materials and methods

Stage V–VI *Xenopus laevis* oocytes were isolated by partial ovariectomy under anaesthesia (Tricaine, 1 g/l). Anaesthetised animals were then kept on ice during dissection. The oocytes were defolliculated by treatment with 2 mg/ml collagenase in zero calcium ND-96 solution (see below). A cDNA clone encoding rat mutant pI_{Cln} (G54A/G56A/G58A) was obtained from K. Strange (Vanderbilt University Medical Center, Nashville, TN, USA). RNA transcription and purification were performed as described previously [9]. Oocytes were injected with 50 nl of 10–100 ng/μl of the rat mutant pI_{Cln} RNA. Oocytes were then incubated at 18°C for 2 or 3 days in ND-96 solution supplemented with gentamicin sulphate (50 mg/ml). The ND-96 solution contained (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.5 (210 ± 5 mOsm/kg). Expression of mutant pI_{Cln} was verified by Western blot analysis using a polyclonal anti-pI_{Cln} antiserum as described previously [13]. Whole-cell currents from oocytes were recorded using the two-microelectrode voltage-clamp technique by means of a home-made amplifier. Voltage and current electrodes were pulled from borosilicate glass and had DC resistances between 0.5 and 2 MΩ when filled with 3 M KCl. Current records were filtered using a four-pole low-pass Bessel filter. To eliminate the effect of voltage drop across the bath-grounding electrode, the bath potential was actively controlled. Experiments were performed at room temperature (~25°C). The standard bath solution during two-microelectrode voltage-clamp experiments was ND-96, which, when indicated, was supplemented with 1 mM cAMP (Sigma) or with 1 mM EGTA. In the latter case, $CaCl_2$ was omitted from the solution.

3. Results

Xenopus oocytes were injected with RNA encoding rat mutant pI_{Cln} (G54A; G56A; G58A). This mutation is equivalent to the G49A/G51A/G53A mutation in MDCK pI_{Cln} tested by Paulmichl et al. [7]. Two control experiments were performed to verify expression of the mutant protein. First, the mutant

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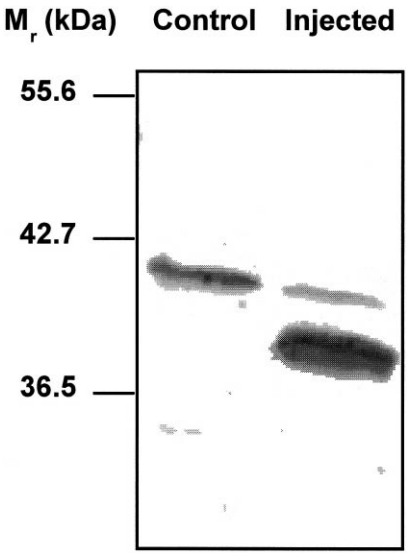

Fig. 1. Western blot analysis of protein extracts from control oocytes and oocytes injected with rat mutant pI$_{Cln}$ (G49A/G51A/G53A) RNA.

pI$_{Cln}$ cDNA clone was sequenced to confirm the introduction of the triple mutation (data not shown). Second, we performed Western blot analysis of lysates prepared from injected and non-injected *Xenopus* oocytes using a polyclonal anti-pI$_{Cln}$ antiserum [13]. Fig. 1 shows that the anti-pI$_{Cln}$ antiserum detected a 39-kDa protein in injected oocytes, but not in control oocytes. The 41-kDa band present in both lanes corresponds to the endogenous *Xenopus* pI$_{Cln}$, which migrates more slowly than mammalian pI$_{Cln}$ [13]. Current traces in response to a voltage-step protocol for both non-injected and mutant pI$_{Cln}$-injected oocytes are displayed in Fig. 2A. While non-injected oocytes had current amplitudes <1 μA at +100 mV ($n=18$), 17 out of 25 oocytes (68%) injected with mutant pI$_{Cln}$ mRNA displayed an outwardly rectifying Cl^- current, which inactivated slowly at positive potentials. This current amounted to 6.4 ± 0.6 μA at +100 mV and 0.8 ± 0.1 μA at −100 mV (Fig. 2B). Furthermore, it was sensitive to NPPB (87 ± 8% block with 100 μM at +80 mV) and had an $NO_3^- > I^- > Br^- > Cl^-$ > gluconate permeability sequence (data not shown).

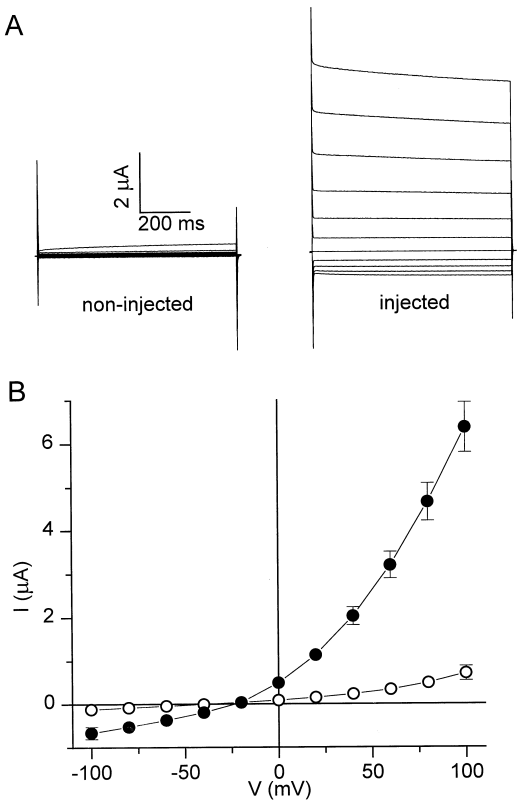

Fig. 2. Macroscopic currents in *Xenopus laevis* oocytes. Oocytes were held at −20 mV and stepped for 800 ms to potentials ranging from −100 to +100 mV. A: Current traces recorded from a non-injected (left) and a RNA-injected (right) oocyte. B: Average current-voltage relations for non-injected (○; $n=18$) and RNA-injected (●; $n=17$) oocytes, measured from step protocols as shown in A.

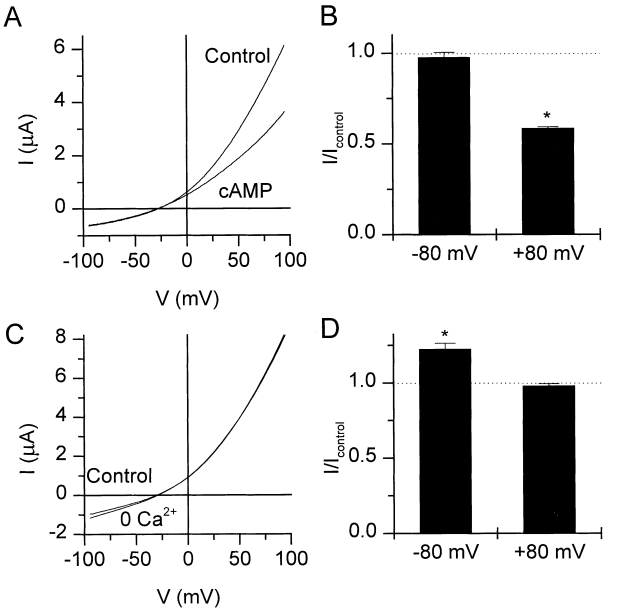

Fig. 3. Effect of cAMP and zero Ca^{2+} on the currents in oocytes injected with mutant pI$_{Cln}$ (G49A/G51A/G53A) RNA. A: Current-voltage relations, obtained from linear voltage ramps from −100 to +100 mV (0.4 V/s), in the absence and presence of extracellular cAMP (1 mM). B: Effect of 1 mM cAMP on the current measured at ±80 mV ($n=6$). C: Current-voltage relation in the presence and absence of extracellular Ca^{2+}. D: Effect of removal of extracellular Ca^{2+} on the current measured at ±80 mV ($n=6$). $^*P<0.05$ (Student's paired *t*-test).

The current in mutant pI$_{Cln}$ expressing oocytes was blocked by cAMP (1 mM) in a voltage-dependent manner (Fig. 3A,B). Removal of extracellular Ca^{2+} and addition of 1 mM EGTA did not affect outward currents, but activated a small inward current (Fig. 3C,D). This inward current presumably reflects the Ca^{2+}-inactivated anion current, which has been described in *Xenopus* oocytes [14,15].

4. Discussion

We have shown that expression of the mutant rat pI$_{Cln}$ induces an anion current in *Xenopus* oocytes. The properties of this current (outward rectification, inactivation at positive potentials, $NO_3^- > I^- > Br^- > Cl^- \gg$ gluconate permeability sequence and block by NPPB) are identical to those of I$_{Cln}$, the current which is induced by wild-type pI$_{Cln}$. Importantly, the triple mutation in the putative extracellular nucleotide-

binding site of pI_{Cln} did not affect the nucleotide sensitivity, Ca^{2+} dependence or kinetics of the expressed current in *Xenopus* oocytes. This is in contrast with the original data of Paulmichl et al. [7], where the same mutation had dramatic effects on the kinetics, nucleotide sensitivity and Ca^{2+} dependence of the expressed current. These results, together with the finding that pI_{Cln} is mainly located in the cytosol [4,12], do not support the hypothesis that pI_{Cln} forms a plasma membrane anion channel.

The simplest explanation of our data is that I_{Cln} is an endogenous current in *Xenopus* oocytes, whose expression is upregulated after expression of certain exogenous proteins. Indeed, I_{Cln} is also present in a small percentage ($\sim$5%) of non-injected or H_2O-injected oocytes [7,12]. Additionally, expression of mutant pI_{Cln} and of the unrelated protein ClC-6 also induces an anion current identical to I_{Cln} [12]. The mutant I_{Cln} phenotype reported by Paulmichl et al. [7] is in our opinion best explained by another endogenous Cl^- current, namely the slowly activating, nucleotide-insensitive, Ca^{2+}-dependent anion current, $I_{Cl,Ca}$. Indeed, the kinetics, the Ca^{2+} dependence and the nucleotide insensitivity of $I_{Cln-mut}$ are very similar if not identical to those of $I_{Cl,Ca}$ (for a discussion, see [12]).

Recently, we have provided evidence against the hypothesis that pI_{Cln} is a critical cytosolic regulator of VRAC. This regulator hypothesis was based on the contention that the pI_{Cln}-induced current in *Xenopus* oocytes is identical to the endogenous swelling-activated anion current $I_{Cl,swell}$. However, we could show that both currents can be clearly discriminated by biological, biophysical and pharmacological criteria [9]. We therefore conclude that there is no evidence in favour of an extracellular nucleotide-binding site in pI_{Cln} and, more generally, that any convincing evidence for a tight link between pI_{Cln} and VRAC is currently missing.

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