

Direct injection of cell-free Kir1.1 protein into *Xenopus* oocytes replicates single-channel currents derived from Kir1.1 mRNA

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The development of integral membrane protein cell-free synthesis permits in-vitro labeling of accessible cysteines for real-time FRET and LRET measurements. The functional integrity of these synthetic ion channel proteins has been verified at the whole oocyte level by direct injection into, and recording from, *Xenopus* oocytes. However, the microscopic single-channel properties of cell-free translated protein have not been systematically examined. In the present study, we compare patch-clamp currents originating from cell-free protein with currents derived from mRNA injection, using the same (single-Cys) inward rectifier DNA template (C189-Kir1.1b). Results indicate that cell-free Kir protein, incorporated into liposomes and injected into oocytes, is trafficked to the plasma membrane where it inserts in an outside-out orientation and exhibits single-channel characteristics identical to that derived from a corresponding mRNA.

Early work by Miledi demonstrated the feasibility of injecting acetylcholine receptors (AChRs)¹ and proteoliposomes containing AChRs² into *Xenopus* oocytes where they were efficiently incorporated into the oocyte plasma membrane. Additional studies with reconstituted paddle chimera Kv channels³ and a novel insect aquaporin channel⁴ established the feasibility of trafficking injected reconstituted protein channels to the oocyte plasma membrane for functional study.

More recently, this method of integral membrane protein injection and incorporation into *Xenopus* oocytes has been elegantly refined to include membrane channel protein synthesized in a wheat-germ cell-free translation system.⁵ In this study, macroscopic oocyte currents derived from injected *Shaker* K protein were characterized pharmacologically and compared with whole oocyte currents derived from *Shaker* K mRNA.⁵ Although this work³⁻⁵ established the feasibility of direct injection of integral membrane proteins into *Xenopus* oocytes, only whole-cell macroscopic currents were reported. In the present study, we compare the properties of channel currents from oocytes injected with cell-free translated Kir1.1 protein with channel currents derived from oocytes injected with Kir1.1 mRNA.

Inward rectifier channel protein for injection into oocytes was prepared using a cell-free translation system.⁶ We chose a single Cys variant of Kir1.1b (C189-Kir1.1b) in which 5 normally occurring cysteines had been removed, leaving a single accessible

cysteine in the C-terminal domain at 189. This mutant has the same physiological properties as wild-type, but can be readily labeled at the single Cys locus for FRET and LRET experiments. The C189-Kir1.1b open reading frame was subcloned into a pEU plasmid used for in vitro transcription of RNA that directs cell-free protein translation in a wheat-germ dialysis reaction (WEPRO2240, CFS, Japan), supplemented with POPE, POPG extruded liposomes to increase yield.⁵ During the 23hr cell-free translation reaction, C189-Kir1.1b channel protein was incorporated into liposomes and recovered from the pellet fraction by centrifugation (18,000g for 3 min at ambient temperature). The single band at 40kDa in the pellet lane of the SDS gel (labeled Kir) is close to the predicted size of a single Kir1.1b subunit (Fig. 1). A western blot confirmed that this band reacted with anti-Kir1.1 antibody.

The C189-Kir1.1b proteoliposome suspension for oocyte injection was obtained by centrifuging and washing the cell-free pellet twice in buffer (vol = 0.6 × translation vol) containing 25mM HEPES-NaOH, 100mM KCl at pH 7.5 and resuspending in 1/20 volume of the original translation buffer. Prior to injection into stage IV *Xenopus* oocytes, the proteoliposome suspension was treated with 0.01mg/ml RNase A to eliminate any residual RNA, followed by a second wash to eliminate the added RNase.

Twenty-four hours after injection of C189-Kir1.1b proteoliposomes into oocytes, patch-clamp recordings revealed K

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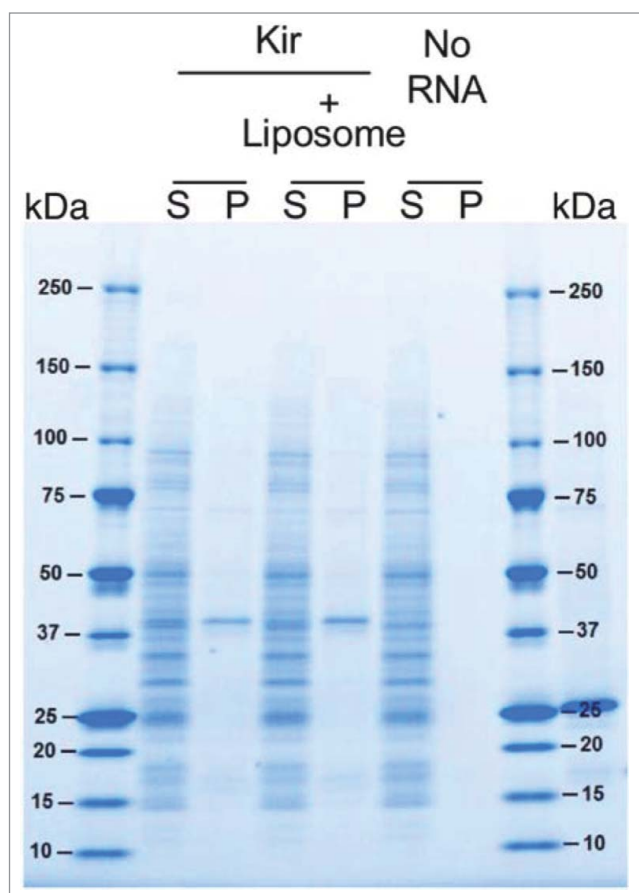


Figure 1. SDS gel of C189-Kir1.1b cell-free reaction products. Proteins were synthesized in a cell-free dialysis reaction with purified RNA (0.7mg/ml) using WEPRO2240 at ambient temperature for 23 hrs. Fractionation of supernatant (S) and pellet (P) were obtained by centrifugation at 18,000g for 3 min at ambient temperature. SDS-PAGE samples were prepared without prior heating. A single C189-Kir1.1b subunit appears as a 40kDa band in both pellet and supernatant lanes.

channels with an average inward conductance (g_K) of 36 ± 3 pS ($n=4$), an average open probability (P_o) of 0.8 ± 0.1 ($n=4$), an average open time of 14 ± 2 ms ($n=4$), and an average closed time of 4 ± 3 ms ($n=4$). An example is shown in **Figure 2**. In contrast to previous studies with *Shaker* cell-free protein injection, where peak expression occurred after 12 hrs,⁵ optimal C189-Kir1.1 channel density at the oocyte plasma membrane required 24 hrs incubation at 18°C. We do not understand the reason for this different time course, although it may be related to differences between Kir and Kv.

We also confirmed that oocytes injected with C189-Kir1.1b proteoliposomes exhibited pH gated channels (**Fig 3**) similar to what has been extensively reported for oocytes injected with Kir1.1b mRNA.⁷⁻¹³ In excised patches, a decrease in cytoplasmic-side pH from 7.4 to 6.0 blocked inward C189-Kir1.1b currents within a few secs (**Fig. 3**).

The above results with cell-free protein injection were compared to patch-clamp currents obtained from *Xenopus* oocytes injected with C189-Kir1.1b mRNA. Oocytes expressing C189-Kir1.1b displayed channel currents with an average inward g_K of 34 ± 3 pS ($n=12$), an average open probability (P_o) of 0.7 ± 0.1 ($n=4$), an average open time of 12.5 ± 3 ms ($n=4$), and an average closed time of 6 ± 2 ms ($n=4$). An example is illustrated in **Figure 4**. These channel currents were almost identical to currents arising from direct injection of C189-Kir1.1b proteoliposomes into oocytes (**Fig. 2**). In addition, whole oocyte currents, derived from proteoliposome injection, were also indistinguishable from whole oocyte currents derived from mRNA injection (not shown), further substantiating equivalence of the 2 techniques.

In summary, the present study confirms that injection of either cell-free protein or mRNA (derived from the same C189-Kir1.1b DNA template) produce nearly equivalent single-channel currents in *Xenopus* oocytes. This also demonstrates (as proof of principle) that proteoliposome injection into *Xenopus* oocytes can be used to conveniently screen integral membrane proteins derived from in vitro, cell-free translation.

Despite the fact that our C189-Kir1.1b protein was not glycosylated during cell-free translation, it was nonetheless trafficked to the plasma membrane after cytoplasmic injection where it consistently appeared in an outside-out orientation. Even when patch pipette pH was raised above 7 (allowing inside-out channel activation), channels

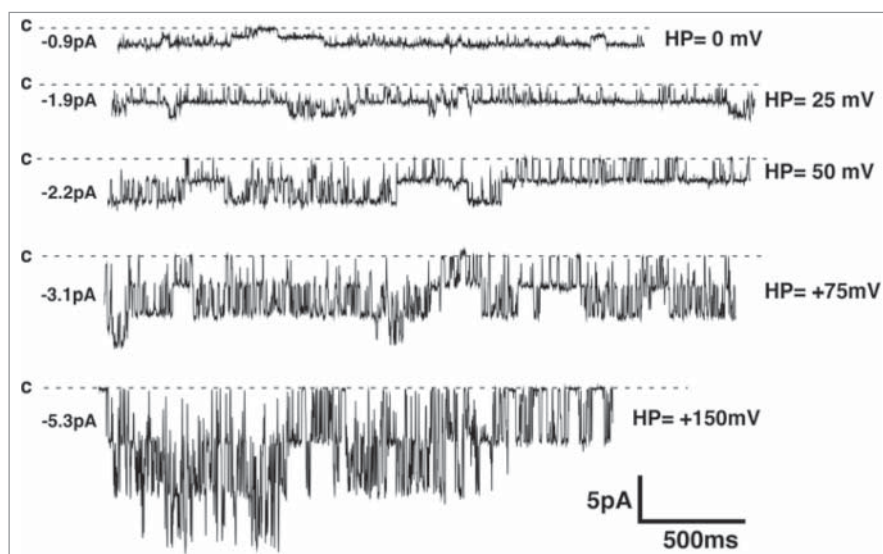


Figure 2. Inward rectifier channel currents recorded from a cell-attached patch on an oocyte, 24hrs after injection with C189-Kir1.1b cell-free synthesized protein incorporated into POPG (25%), POPE (75%) liposomes. Patch pipette: 50mM KCl, 1mM CaCl₂ at pH 6.5. Bath solution: 5mM KCl, 45mM NaAcetate, 0 Ca at pH 8. Oocyte $V_m = -75$ mV. HP(mV)=pipette minus bath. Inward single channel conductance = 32pS. Records filtered at 900Hz; closed current level denoted by c.

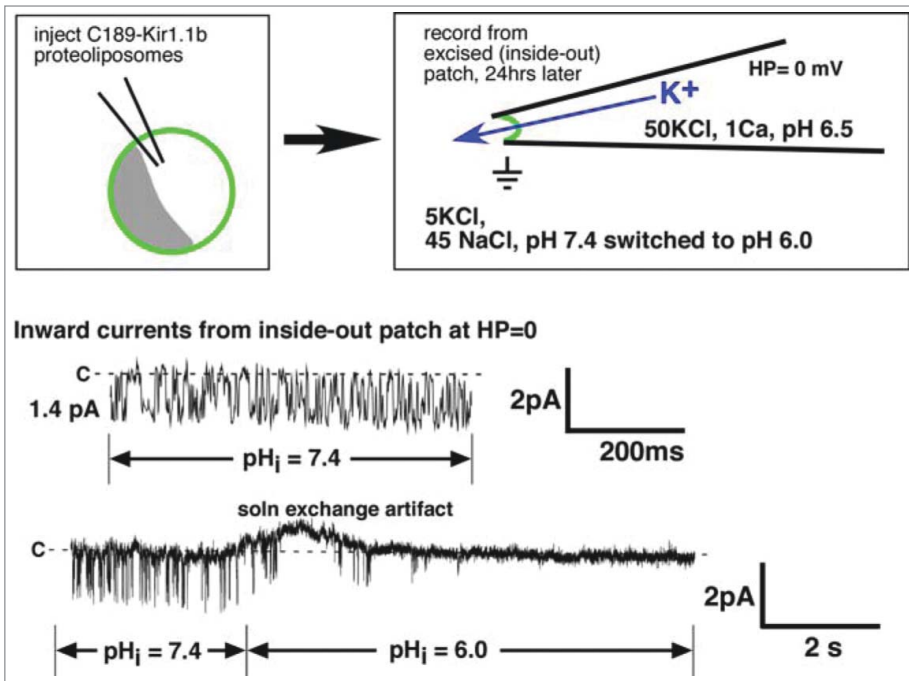
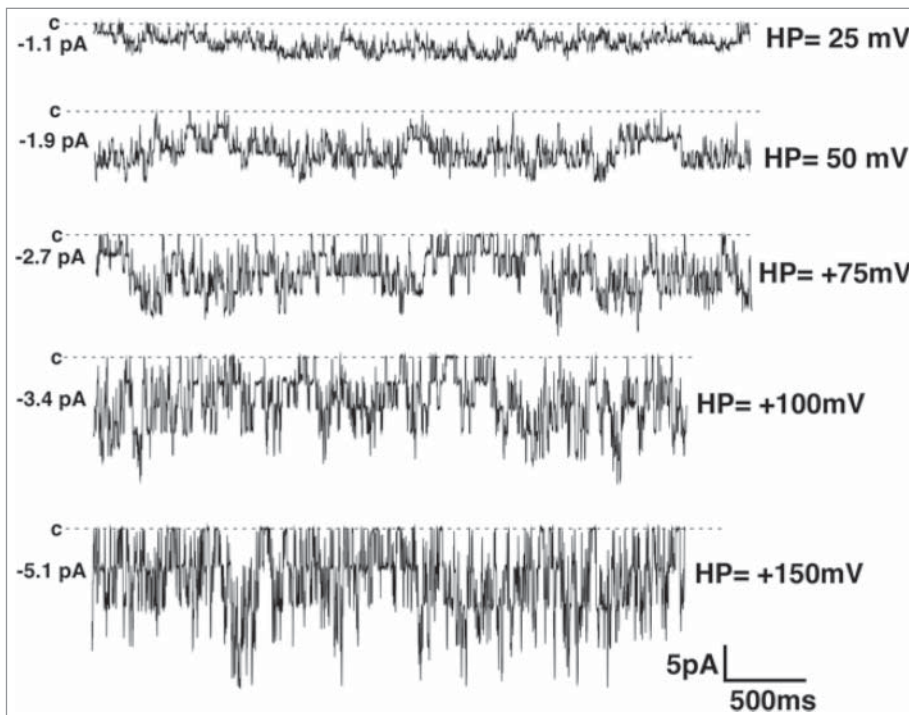


Figure 3. pH gating of C189-Kir1.1b inward rectifier channel currents, recorded from an inside-out excised oocyte patch, 24hrs after injection of cell-free C189-Kir1.1b proteoliposomes (25% POG, 75% POPE) into an oocyte. Patch pipette: 50mM KCl, 1mM CaCl_2 at pH 6.5. Bath solution: 5mM KCl, 45mM NaCl, 0Ca changed from pH 7.4 to pH 6, causing channel shutdown. Records filtered at 900Hz; closed current level denoted by c.



were never detected in a reverse (inside-out) orientation. This suggests that the oocyte may recognize the orientation of exogenous channel proteins and insert them correctly into the membrane. However, we cannot exclude the possibility that some cell-free C189-Kir1.1b channels were incorporated in a reverse (inside-out) orientation, but remained silent since the PIP2 required at the internal side of Kir1.1 for activation is absent from the outer leaflet of the oocyte plasma membrane.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

MN constructed the mutant DNA and produced and verified the cell-free K channel protein; SM designed the cell-free protocol and produced the cell-free K channel protein; HS designed the research, performed the patch-clamp expts, analyzed the data and wrote the paper.

Figure 4. Inward rectifier channel currents recorded from a cell-attached patch on an oocyte, 24hrs after injection with C189-Kir1.1b mRNA. Patch pipette: 50mM KCl, 1mM CaCl_2 at pH 6.5. Bath solution: 5mM KCl, 45mM NaAcetate, 0 Ca at pH 8. Oocyte $V_m = -75\text{mV}$. HP(mV) = pipette minus bath. Inward single channel conductance = 30pS. Records filtered at 900Hz; closed current level denoted by c.

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