THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 48, Issue of November 29, pp. 30524–30528, 1996
© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Subunit Stoichiometry of a Heteromultimeric G protein-coupled Inward-rectifier K^+ Channel*

(Received for publication, June 5, 1996, and in revised form, July 23, 1996)

Scott K. Silverman‡, Henry A. Lester§¶, and Dennis A. Dougherty‡

From the Divisions of ‡Chemistry and Chemical Engineering and §Biology, California Institute of Technology, Pasadena, California 91125

We investigated the stoichiometry of the heteromultimeric G protein-coupled inward-recitfier K^+ channel (GIRK) formed from GIRK1 and GIRK4 subunits. Multimeric GIRK constructs with several concatenated channel subunits were expressed in *Xenopus* oocytes. Coexpression of various trimeric constructs with different monomers clearly showed that the functional channel has stoichiometry $(GIRK1)_2(GIRK4)_2$. Efforts to establish a preferred arrangement of subunits around the channel pore suggest that more than one arrangement may be viable.

Since the landmark cloning of the *Shaker* K^+ channel in 1987 (1–3), many selective K^+ channels have been characterized, leading to a number of structural families. Unlike Na^+ and Ca^{2+} channels, which are monomeric but contain four repeats of a highly homologous sequence, K^+ channels are assembled from multiple copies of smaller subunits. As anticipated based on the Na^+ and Ca^{2+} channels, *Shaker* and other voltage-gated K^+ channels are tetramers of identical subunits (4–7). More recently, two members of the inward-rectifier class, IRK1 (8) and ROMK1 (9), have also been found to be tetramers.

We are interested in a class of heteromultimeric K^+ channels, the G-protein-coupled inward rectifiers (GIRKs).[1] In particular, coexpression of GIRK1 and GIRK4 (CIR) produces a heteromultimeric channel in oocytes and in other cells (10). It seems likely that this channel is tetrameric, as its subunits are highly homologous to those of the known tetramers IRK1 and ROMK1. However, the issues of stoichiometry and subunit arrangement have yet to be addressed. We describe here studies designed primarily to establish the stoichiometry of the GIRK1/GIRK4 channel. Our results clearly indicate a preference for the $(GIRK1)_2(GIRK4)_2$ stoichiometry. We have been unable to establish a preferred arrangement of subunits around the pore, and the evidence suggests that more than one arrangement may be viable.

For heteromultimeric systems like the GIRKs, channels are typically expressed by co-translation of several mRNA sequences, each coding separately for a single copy of a necessary subunit. For the GIRK1/GIRK4 channel, for example, two mRNAs are translated. An alternative approach is to use multimeric constructs, as has been done with a number of homomultimeric ion channels (8, 11–16). With this method, a single protein is formed by translation of one mRNA coding for several concatenated channel subunits, *i.e.* two or more complete subunits connected by linkers of variable (and typically small) size. Multimeric constructs have proven useful in a variety of contexts, and we have investigated multimeric constructs of GIRK1 and GIRK4 to address the issues of stoichiometry and subunit arrangement.

For the stoichiometry question, the most revealing experiments involve coexpression of trimeric constructs, either alone or with appropriate monomer subunits. A trimer, expressed alone, should give at most small signals (16), because the functional channel requires four subunits. Upon coexpression of a trimer and a monomer, one can envision two limiting outcomes. If the trimer and monomer can coassemble to provide a functional tetramer, a large increase in signal is expected relative to the trimer or monomer alone. If the particular trimer plus monomer combination results in a nonfunctional tetramer, or if the trimer cannot coassemble with the monomer, no increase in signal over the trimer alone is expected. By judicious choice of trimer composition and coexpressed monomer, the subunit stoichiometry of functional heteromultimers may be determined.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—A two-step PCR procedure was employed as follows. Two complementary oligonucleotides incorporating the desired point mutations were synthesized and paired with appropriate outer primers in a first round of PCR, using *Pfu* polymerase (Stratagene), or in some cases, Expand (Boehringer Mannheim). The PCR products were purified on agarose gel, then combined with each other and the two outer primers from the first round of PCR, and a second round of PCR was performed. The second PCR product was gel-purified and trimmed on each end with an appropriate restriction enzyme. This product was gel-purified and ligated into the parent construct, previously digested with the same two restriction enzymes and dephosphorylated. All sequences originating in PCR were verified by automated sequencing over the entire amplified region and over the ligation sites.

DNA Clones—GIRK1 (KGA) and GIRK2 were available from previous studies (17, 18). GIRK4 was obtained from J. Adelman (19). The m2 acetylcholine receptor was obtained from E. Peralta (20), and was in the pGEM3Z vector. All GIRK constructs were subcloned into the pMXT vector, obtained from L. Salkoff (21). This vector is pBluescript KS II+ (Stratagene) with *Xenopus* β-globin 5′- and 3′-untranslated regions on appropriate ends of the polylinker, to enhance expression in oocytes. The m2 receptor was linearized with *Hin*dIII, and mRNA was transcribed using the T7 polymerase mMessage mMachine kit from Ambion (Austin, TX). All GIRK constructs were linearized with *Sal*I, and mRNA was transcribed using the T3 polymerase mMessage mMachine kit. mRNA concentration was estimated by both UV absorption (A_{260}) and intensity on ethidium bromide-stained agarose gel.

Multimeric GIRK Constructs—To allow simple construction of a variety of multimers, a modular approach was taken. All the GIRK monomers have a unique *Sal*I restriction site (used for linearization) after the poly(A) tail. Two new unique restriction enzyme sites were introduced in both GIRK1 and GIRK4, one each at the 5′- and 3′-ends, such

* This work was supported by National Institutes of Health Grants MH49176, GM29836, NS11756, and NS34407. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125. Tel.: 818-395-6872; Fax: 818-564-8709; E-mail: Lester@Caltech.edu.

[1] The abbreviations used are: GIRK, G protein-coupled inward-rectifier K^+ channel; PCR, polymerase chain reaction; ACh, acetylcholine.

This paper is available on line at http://www-jbc.stanford.edu/jbc/

that digest with appropriate restriction enzymes would provide compatible overhangs between the 3′-end of GIRK1 and 5′-end of GIRK4, and conversely. These sites were designed as follows (all nucleotide designations assume initial ATG = 1 to 3): GIRK1, 5′ *Bsp*EI, introduced by T6 → C, C8 → G, and 3′ *Nhe*I, introduced by T1489 → G, G1492 → A, A1493 → G; GIRK4, 5′ *Avr*II, introduced by G4 → C, C6 → T, G7 → A, T9 → G, and 3′ *Age*I, introduced by A1245 → C, A1246 → G, G1248 → T.

*Avr*II and *Nhe*I have compatible overhangs, as do *Age*I and *Bsp*EI. Ligation of the 3′-end *Nhe*I overhang of GIRK1 to the 5′-end *Avr*II overhang of GIRK4 provides a concatenated GIRK1-GIRK4 sequence, the translation of which has protein residue N496 of GIRK1 (5 residues removed) linked to D4 of GIRK4 (3 residues removed) through two new residues (AR). Ligation of the 3′-end *Age*I overhang of GIRK4 to the 5′-end *Bsp*EI overhang of GIRK1 provides a concatenated GIRK4-GIRK1 sequence, the translation of which has T415 of GIRK4 (4 residues removed) linked to L4 of GIRK1 (3 residues removed) through one new residue (G).

The dimer construct GIRK1-GIRK4 was thus prepared by ligating the *Avr*II-*Sal*I fragment from GIRK4 into the dephosphorylated *Sal*I-*Nhe*I fragment from GIRK1, and the dimer GIRK4-GIRK1 was prepared by ligating the *Bsp*EI-*Sal*I fragment from GIRK1 into the dephosphorylated *Sal*I-*Age*I fragment from GIRK4. By appropriate iterations, all possible alternating trimers and tetramers were then obtained. To prepare the **144** construct with two GIRK4s concatenated adjacent, two complementary oligonucleotides (5′-CCGGAGCACAAGGTG-3′ and 5′-CTAGCACCTTGTGCT-3′) were synthesized as a short adapter between the *Age*I and *Avr*II sites in a three-way ligation. The final construct has 6 new residues (GAQGAR) between T415 of the first GIRK4 subunit and D4 of the second. The tetramer **1441** was prepared from **144** as for the other constructs. The monomers used in coexpression experiments were the original constructs without 5′ or 3′ mutations, as these alter the coding region.

Truncated GIRK4 Mutant 4_{trunc}—The GIRK4 mutant Y348TAA was prepared, introducing an early stop codon which preempts synthesis of GIRK4 residues 349–419. When coexpressed with GIRK1, $\mathbf{4}_{trunc}$ gave <10% of the current of wild-type GIRK4.

Oocyte Preparation and Injection—Oocytes were removed from *Xenopus laevis* as described previously (22) and maintained at 18 °C in ND96 solution, changed twice daily. The ND96 solution consisted of (mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, supplemented with 2.5 mM sodium pyruvate, 50 μg/ml gentamicin, and 0.6 mM theophylline, at pH 7.5. Oocytes were injected with 50 nl of water solution containing ≤50 ng of total mRNA (including 3 ng of the m2 receptor) and, when appropriate, 12.5 ng of fully phosphothioated Xir antisense oligonucleotide KHA2 (5′-CTGAGGACTTGGTGCCATTCT-3′), prepared at the Biopolymer Synthesis facility of the Beckman Institute at Caltech.

Electrophysiology—Two-electrode voltage clamp recordings were performed 1–2 days postinjection at room temperature (~20 °C) using a GeneClamp 500 amplifier and pCLAMP software (Axon Instruments, Foster City, CA). Microelectrodes were filled with 3 M KCl and had resistances of 0.5–2 megohms. Oocytes were continuously perfused with a calcium-free bath solution of 98 mM NaCl or KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.5 with NaOH). Acetylcholine (ACh) was added (1 μM) when appropriate to the high K^+ solution. Currents were quantified at −80 mV and are reported as mean ± S.E.

RESULTS

All GIRK mRNAs were injected into *Xenopus* oocytes, along with mRNA for the m2 muscarinic acetylcholine receptor. After 1–2 days, oocytes were subjected to electrophysiological analysis by the two-electrode voltage clamp technique (Fig. 1). Successful channel assembly was evaluated by the total current in response to high external K^+ with 1 μM acetylcholine ($I_{K,ACh}$). Because expression levels in oocytes vary from batch to batch, absolute signal levels should be compared with caution. In contrast, relative responses within an oocyte batch are generally reproducible and offer more reliable data for interpretation.

Two GIRK trimeric constructs were evaluated first, **141** and **414**. These contain the indicated GIRK sequences concatenated in-frame; for details of construction, see "Experimental Procedures." Injection of **141** mRNA alone gave rise to a modest current ($I_{K,ACh}$ of several hundred nanoamperes, Fig. 2*A*), measurably above that from either **1** or **4** alone. (The monomeric GIRK constructs, when expressed alone, gave very small signals, not more than 200 nA and usually less under the conditions used.) Coexpression of **141** and **1** gave a slight *decrease* in total current relative to **141** alone. Coexpression of **141** and **4**, in contrast, reproducibly gave a large (approximately 6-fold) increase in current, and the electrophysiological signals were indistinguishable from those from coinjected monomers **1** + **4** (Fig. 1). Coexpression of **141** and GIRK2 also showed a large increase in current over **141** alone, as anticipated given the high homology between GIRK2 and GIRK4.

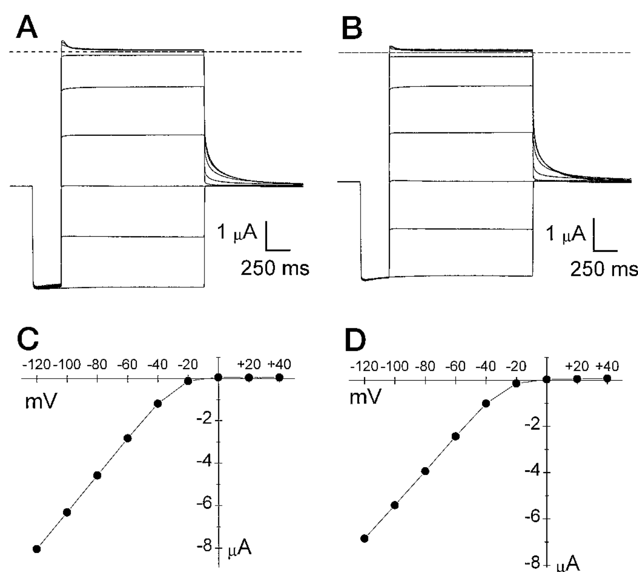

FIG. 1. **Representative voltage-step recordings for GIRK channels.** *A*, recordings on day 1 after injecting 1.25 ng each monomer **1** and **4** (along with 3 ng of m2 receptor). *B*, recordings on day 1 after injecting 5 ng of trimer **141** and 1.25 ng of monomer **4** (with 3 ng of m2). *C* and *D*, current-voltage relations for traces in *A* and *B*. Oocytes were held at a potential of −80 mV, stepped to −120 mV for 300 ms, then stepped to various test potentials between −120 and +40 mV in 20 mV increments for 1500 ms, at intervals of 7 s. The *dashed lines* indicate zero current. Current traces are those recorded in 98 mM K^+ with 1 μM ACh after subtraction of the leak currents recorded in 98 mM Na^+. After subtraction, the traces were corrected for desensitization (multiplicative scaling, factor of 1.00–1.09). Similar waveforms were obtained by subtracting the traces in 98 mM K^+ from those in 98 mM K^+ with 1 μM ACh.

The **414** trimer expressed alone gave relatively large currents, even with injection of 40-fold less **414** mRNA than **141** mRNA. Nevertheless, coexpression of **414** and **1** reproducibly gave an increase in signal relative to **414** alone (Fig. 2*B*). The magnitude of this increase (2–3-fold) was smaller than the 6-fold increase observed with **141** plus **4**, but was unchanged over the 40-fold range of injected mRNA. Therefore, the signals from coinjection of **141** and **4** were probably not limited by the oocyte's expression capacity. Coexpression of **414** and **4** gave no reproducible increase in signal over **414** alone.

Taken together, the results from the **141** and **414** trimer studies clearly point to a $(GIRK1)_2(GIRK4)_2$ stoichiometry. We have performed a number of additional experiments, described below, to expand and refine these basic observations.

Any GIRK signal from a trimer alone would presumably arise either from (*a*) coassembly of the trimer with the endogenous *Xenopus* inward-rectifier subunit Xir (GIRK5) (23) or (*b*) coassembly of two trimer molecules, with two of six subunits not contributing to the final, functional channel. Xir is highly homologous to GIRK4, and it has been shown to coassemble with GIRK1, forming a channel that functions similarly to the GIRK1/GIRK4 heteromultimer (23). To the extent that the signal from **141** alone is due to coassembly of the **141** trimer

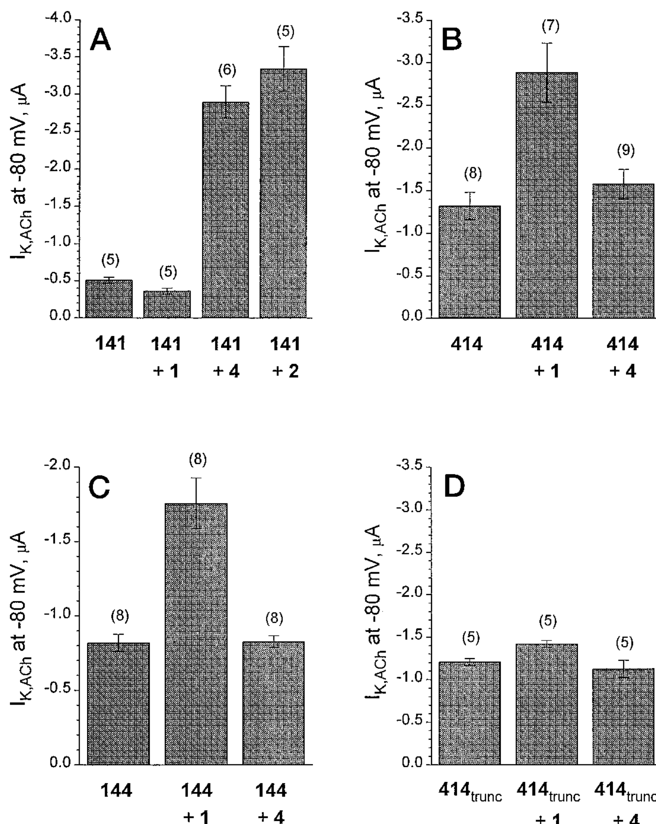

FIG. 2. **Coexpression of trimer constructs with GIRK monomers.** *A*, 5 ng of **141** mRNA and 1.25 ng of monomer mRNA were coinjected, and currents were recorded on day 1. In two experiments, the ratio of currents for **141** + **4** *versus* **141** alone were 6.2 ± 1.7 and 5.7 ± 1.0. *B*, 5 ng of **414** mRNA and 1.25 ng of monomer mRNA were coinjected, and currents were recorded on day 1. In three experiments, with 0.125, 1.25, and 5 ng of **414** mRNA, the ratio of currents for **414** + **1** *versus* **414** alone were 2.4 ± 0.3, 2.7 ± 0.7, and 2.2 ± 0.6; the average currents for **414** alone in these experiments were (nA) −649 ± 30 (n = 6), −1942 ± 241 (n = 5), and −1322 ± 160 (n = 8). *C*, 0.5 ng of **144** mRNA and 1.25 ng of monomer mRNA were coinjected, and currents were recorded on day 1. In another experiment in which 5 ng of **144** mRNA was coinjected, the currents were −3268 ± 403 nA (n = 9) for **144** alone and −5057 ± 481 nA (n = 8) for **144** + **1.** *D*, 5 ng of $\mathbf{414}_{trunc}$ mRNA and 1.25 ng of monomer mRNA were coinjected, and currents were recorded on day 1.

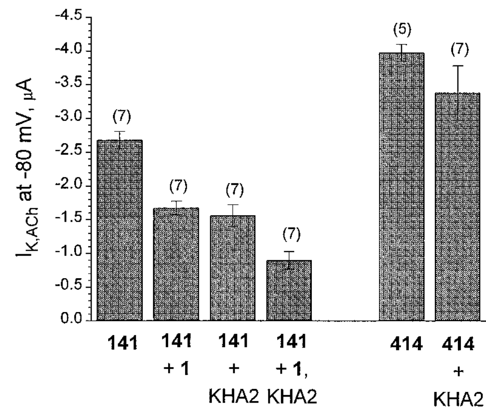

FIG. 3. **Antisense suppression of Xir differentially affects 141 and 414.** *A*, combinations of 5 ng of **141** mRNA, 1.25 ng of monomer **1** mRNA, and 12.5 ng of Xir antisense oligonucleotide KHA2 were coinjected, and currents were recorded on day 2. In two other experiments where recordings were done earlier, on day 1, the currents from **141** alone *versus* **141** + **1** were (nA) −369 ± 47 (n = 5) *versus* −233 ± 48 (n = 4) and −510 ± 42 (n = 5) *versus* −362 ± 41 (n = 5). *B*, 0.125 ng of **414** mRNA and 12.5 ng of antisense oligonucleotide KHA2 were coinjected, and currents were recorded on day 2. On day 1, the currents were −1033 ± 93 nA (n = 6) for **414** alone and −679 ± 147 nA (n = 6) for **414** + KHA2.

with Xir, an antisense oligonucleotide (23) (KHA2) directed against Xir should suppress the **141** signal. Trimer **141** was injected by itself, with **1**, with KHA2, and with both **1** and KHA2 (Fig. 3). Separately, **1** and KHA2 suppressed the **141** signal by a similar amount (~40%), while together, **1** and KHA2 suppressed the **141** signal by a larger amount (66%). In contrast, KHA2 did not significantly suppress the current from **414**.

To address whether trimers may themselves dimerize, the trimer $\mathbf{414}_{trunc}$ was made by concatenating **4**, **1**, and $\mathbf{4}_{trunc}$, where $\mathbf{4}_{trunc}$ is a nonfunctional GIRK4 truncated in its C-terminal tail (see "Experimental Procedures"). Large currents were observed from $\mathbf{414}_{trunc}$ alone, comparable to those from untruncated **414**, but the signal was not significantly affected by coexpression of **1** or **4** (Fig. 2*D*).

The dimeric constructs **14** and **41** were examined, and each gave large signals when expressed alone (see Fig. 5). Dimer **14** was not significantly suppressed by **4**, while **41** was suppressed slightly by **4**. In contrast, both **14** and **41** were significantly suppressed by **1**. Dimers **14** and **41** did not suppress each other; coinjection of **14** and **41** showed no decrease in signal relative to each dimer alone.

In order to evaluate the question of subunit arrangement, the **144** trimer was prepared. As observed with **414**, this trimer gave robust signals when expressed alone and showed an increase in signal when coexpressed with **1**, but not with **4** (Fig. 2*C*). Several tetrameric GIRK constructs were also prepared. Both **1414** and **4141** gave robust signals, and both showed a *decrease* in signal upon coexpression of either **1** or **4** (Fig. 4). The **1441** tetramer also gave large signals, while the **1444** tetramer produced noticeably smaller currents (Fig. 5).

DISCUSSION

The primary goal of the present work was to establish the stoichiometry of the GIRK1/GIRK4 channel as expressed in oocytes. Our major tool has been the evaluation of specific multimeric constructs. As with other studies involving multimers, one should be concerned that the channel properties, either structural or kinetic, may be altered by linking the subunits, especially given the short linkers used. Were this so, one might expect unusual characteristics for channels formed from multimeric constructs. With this in mind, we evaluated voltage-ramp and voltage-step recordings for all the multimer combinations and found them indistinguishable from those of the wild-type channel formed after coinjection of GIRK1 and GIRK4 monomers (Fig. 1). We also examined the effect of Cs^+ on currents from the **141** + **4**, **141** + **2**, **414** alone, and **414** + **1** mRNA combinations, and all showed voltage-dependent block with 1 mM external Cs^+, as for wild type (data not shown). These observations support the general claim that we have not grossly perturbed the channel kinetics or structure by using multimers, although it is possible that subtle changes would not be revealed by these experiments. Another possible concern is that when only monomers are injected, the channel stoichiometry varies depending on the relative amounts of mRNAs, as suggested by Slesinger *et al.* (24). Multimeric constructs may then artificially favor the programmed stoichiometries. Similarly, as discussed further below, constraining the order of subunits within multimers may enforce arrangements that otherwise would not form naturally from monomers. This may be further compounded if the oocyte is relatively tolerant of less favorable channel compositions, compared with the mammalian cells in which GIRK channels are naturally expressed. Despite these possible complications, however, some definite

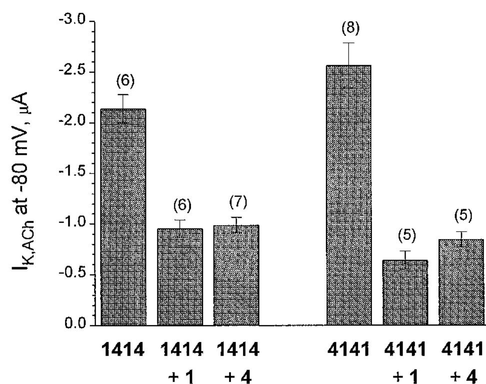

FIG. 4. **Expression of tetramers 1414 and 4141, and suppression by monomers.** 0.5 ng of tetramer mRNA and 1.25 ng of monomer mRNA were coinjected, and currents were recorded on day 1. In a similar experiment, 5 ng of mRNA for the tetramer **1441** gave −1499 ± 65 nA (n = 8), while **1444** gave −508 ± 44 nA (n = 6).

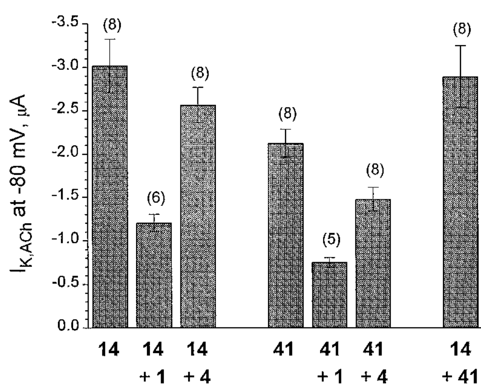

FIG. 5. **Dimers 14 and 41 express well alone and do not suppress each other.** 0.25 ng of dimer mRNA and 1.25 ng of monomer mRNA were coinjected, and currents were recorded on day 1. When both dimers were injected, either 0.25 or 0.125 ng of each gave a quantitatively similar signal (data shown for 0.25 ng of each dimer). In a separate experiment in which 1.25 ng of each dimer was injected separately, **14** gave −3305 ± 271 nA (n = 5) and **41** gave −6708 ± 504 nA (n = 6).

conclusions concerning the GIRK channels may be drawn.

Disregarding subunit arrangement, three stoichiometries are possible for a GIRK1/GIRK4 (**1**/**4**) heterotetramer: $\mathbf{1}_2\mathbf{4}_2$, $\mathbf{1}_1\mathbf{4}_3$, or $\mathbf{1}_3\mathbf{4}_1$. The expected results for these possibilities are quite different, and the experiments with the **141** and **414** trimers (Fig. 2) convincingly establish the $\mathbf{1}_2\mathbf{4}_2$ stoichiometry. It is important to note that the experiments with **141** and **414** are complementary; both are required to allow a complete interpretation.

After establishing the channel stoichiometry, we made an effort to identify the arrangement of subunits around the pore. A tetrameric channel composed of two each of two different subunits may assemble in two possible ways: identical subunits across from or adjacent to each other. Our results did not distinguish these possibilities, and in fact suggest that both arrangements are possible. In particular, the **1414**, **4141**, and **1441** tetramers all give strong signals (Fig. 4). While it is conceivable that these tetramers all fold to give the same arrangement of subunits around the pore, this would be surprising. We used short linkers between subunits in an attempt to constrain the possible arrangements; however, the C-terminal tails of the GIRK's are relatively long and their structural flexibility is unknown. An alternative explanation of the data, which we cannot rule out, is that signals we see from injections of tetrameric constructs arise from "dimers of tetramers" with only some of the concatenated subunits contributing to the channel, as we propose for the signals seen from injection of trimers alone. Other observations support the view that both arrangements are possible, including the facts that both the **414** and the **144** trimers give a specific increase in signal upon coexpression of **1** but not **4** (Fig. 2*C*), and that the **14** and **41** dimers do not suppress each other when coinjected (Fig. 5). Of course, we do not imply that both arrangements are formed under "natural" conditions, when monomeric subunits are expressed, only that when perhaps forced into an unnatural arrangement, the channel still functions.

A number of additional observations deserve comment. A potential complication in these studies is the presence of the endogenous oocyte inward-rectifier subunit, Xir (GIRK5) (23). For example, the small but clearly measurable currents seen on injection of **141** alone presumably arise at least in part because this trimer can form a functional tetramer with Xir.[2] Interestingly, we observe a reproducible decrease in signal upon coexpression of **1** with **141** (Fig. 2). We conjectured that this may arise because **1** competes with **141** for Xir, producing a **1**-Xir dimer that is not present in high enough concentration to dimerize to a functional tetramer. The role of Xir may be evaluated, at least qualitatively, using an antisense oligonucleotide (KHA2) directed against the 5′-untranslated region of Xir (23). The results show that KHA2 suppresses the **141** signal about as well as **1**, but in a partially independent manner (Fig. 3). This is sensible if KHA2 and **1** act at different time points in the lifetime of Xir: the antisense oligonucleotide binds to the mRNA and prevents new Xir protein synthesis, but does not affect any Xir present before injection, whereas **1** binds to the already synthesized protein. To the extent that these suppressions are imperfectly efficient, KHA2 and **1** should have a combined effect greater than each alone, as observed. We cannot rule out other roles for **1** with **141** (*e.g.* direct coassembly to form nonfunctional $\mathbf{1}_3\mathbf{4}_1$), but the given explanation appears plausible.

A second possible complication for these studies is that availability of endogenous G protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) may in some cases limit the observed signals. In fact, we have evidence for this in the GIRK1 + GIRK4 channel.[3] However, in the present studies we concentrate less on absolute signals and more on relative responses to different mRNA combinations. The experiments with the **414** trimer, which show a reproducible 2–3 fold increase in signal upon coexpression of monomer **1** over a 40-fold range of injected mRNA, demonstrate that availability of $G_{\beta\gamma}$ is unlikely to distort the results obtained here.

Unlike the **141** trimer, the **414** trimer unexpectedly gives large currents when injected alone. Two observations suggest that the signal is due to dimerization of this construct. First, the signal from **414** alone is not influenced by either coinjection of **4** or the antisense oligonucleotide KHA2, indicating that the endogenous Xir subunit is not involved. Second, the signal from **414** increases 2–3-fold (but not more) on coinjection of **1**, consistent with a (trimer + trimer) going to two copies of (trimer + monomer). Both the magnitude of increase and the fact that that it is unchanged over a range of expression levels are as expected for a trimer able to dimerize efficiently (12). While the **414** trimer apparently dimerizes to give a functional channel, the much smaller signals from **141** suggest that the latter cannot.

Upon truncation of the second GIRK4 subunit of the **414** trimer to form $\mathbf{414}_{\mathrm{trunc}}$, the increase with coexpression of **1** is abolished (Fig. 2*D*). This is as expected if both outer subunits of the trimer are required for functional coassembly with added

[2] The signal observed upon injection of GIRK1 alone into oocytes has been attributed to coassembly with Xir (23).

[3] S. K. Silverman, unpublished data.

monomer, and supports assigning a $\mathbf{1_2 4_2}$ stoichiometry to the **414** + **1** channel. This result also help us to understand the mechanism of trimer dimerization. The large signals from $\mathbf{414}_{\text{trunc}}$ alone suggest that $\mathbf{414}_{\text{trunc}}$ (and by extension, **414** itself) can dimerize in 2 + 2 fashion, with the two terminal subunits not contributing to the channel.

In summary, within the constraints of the multimer strategy discussed above, the data present a clear view of the heteromultimeric GIRK channel as composed of two each of the GIRK1 and GIRK4 subunits. Perhaps surprisingly, the data suggest that both of the two possible arrangements of subunits around the pore may be viable.

Acknowledgment—We thank Paulo Kofuji for many helpful discussions.

Note Added in Proof—Adelman and co-workers recently described studies involving tetrameric constructs similar to the ones described here (Tucker, S. T., Pessia, M., and Adelman, J. P. (1996) *Am. J. Physiol.* **271,** H379–H385).

REFERENCES

1. Papazian, D. M., T. L. Schwarz, T. L., Tempel, B. L., Jan, Y. N., and Jan, L. Y. (1987) *Science* **237,** 749–753
2. Pongs, O., Kecskemethy, N., Müller, R., Krah-Jentgens, I., Baumann, A., Koltz, H. H., Canal, I., Llamazares, S., and Ferrus, A. (1988) *EMBO J.* **7,** 1087–1096
3. Iverson, L. E., Tanouye, M. A., Lester, H. A., Davidson, N., and Rudy, B. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5723–5727
4. MacKinnon, R. (1991) *Nature* **350,** 232–235
5. Hurst, R. S., Kavanaugh, M. P., Yakel, J., Adelman, J. P., and North, R. A. (1992) *J. Biol. Chem.* **267,** 23742–23745
6. Tytgat, J., and Hess, P. (1992) *Nature* **359,** 420–423
7. MacKinnon, R., Aldrich, R. W., and Lee, A. W. (1993) *Science* **262,** 757–759
8. Yang, J., Jan, Y. N., and Jan, L. Y. (1995) *Neuron* **15,** 1441–1447
9. Glowatzki, E., Fakler, G., Brändle, U., Rexhausen, U., Zenner, H.-P., Ruppersberg, J. P., and Fakler, B. (1995) *Proc. R. Soc. Lond. Ser. B* **261,** 251–261
10. Krapivinsky, G., Gordon, E. A., Wickman, K., Velimirovic, B. Krapivinsky, L., and Clapham, D. E. (1995) *Nature* **374,** 135–141
11. Gordon, S. E., and Zagotta, W. N. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10222–10226
12. Isacoff, E. Y., Jan, Y. N., and Jan, L. Y. (1990) *Nature* **345,** 530–534
13. Heginbotham, L., and MacKinnon, R. (1992) *Neuron* **8,** 483–491
14. Kavanaugh, M. P., Hurst, R. S., Yakel, J., Varnum, M. D., Adelman, J. P., and North, R. A. (1992) *Neuron* **8,** 493–497
15. Pascual, J. M., Shieh, C.-C., Kirsch, G. E., and Brown, A. M. (1995) *Biophys. J.* **69,** 428–434
16. Liman, E. R., Tytgat, J., and Hess, P. (1992) *Neuron* **9,** 861–871
17. Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W., Chavkin, C., DiMagno, L., Labarca, C., Kieffer, B. L., Gaveriaux-Ruff, C., Trollinger, D., Lester, H. A., and Davidson, N. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10235–10239
18. Kofuji, P., Davidson, N., and Lester, H. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6542–6546
19. Ashford, M. L. J., Bond, C. T., Blair, T. A., and Adelman, J. P. (1994) *Nature* **370,** 456–459
20. Lechleiter, J., Hellmiss, R., Duerson, K., Ennulat, D., David, N., Clapham, D., and Peralta, E. (1990) *EMBO J.* **9,** 4381–4390
21. Wei, A., Solaro, C., Lingle, C., and Salkoff, L. (1994) *Neuron* **13,** 671–681
22. Quick, M., and Lester, H. A. (1994) in *Ion Channels of Excitable Cells* (Narahashi, T., ed) pp. 261–279, Academic Press, San Diego, CA
23. Hedin, K. E., Lim, N. F., and Clapham, D. E. (1996) *Neuron* **16,** 423–429
24. Slesinger, P. A., Reuveny, E., Jan, Y. N., and Jan, L. Y. (1995) *Neuron* **15,** 1145–1156