Suppression of Neuronal and Cardiac Transient Outward Currents by Viral Gene Transfer of Dominant-Negative Kv4.2 Constructs*

(Received for publication, July 7, 1997, and in revised form, August 28, 1997)

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To probe the molecular identity of transient outward (A-type) potassium currents, we expressed a truncated version of Kv4.2 in heart cells and neurons. The rat Kv4.2-coding sequence was truncated at a position just past the first transmembrane segment and subcloned into an adenosine shuttle vector downstream of a cytomegalovirus promoter (pE1Kv4.2ST). We hypothesized that this construct would act as a dominant-negative suppressor of currents encoded by the Kv4 family by analogy to Kv1 channels. Cotransfection of wild-type Kv4.2 with a β-galactosidase expression vector in Chinese hamster ovary (CHO)-K1 cells produced robust transient outward currents (Ito) after two days (14.0 pA/pF at 50 mV, n = 5). Cotransfection with pE1Kv4.2ST markedly suppressed the Kv4.2 currents (0.8 pA/pF, n = 6, p < 0.02; cDNA ratio of 2:1 Kv4.2ST:wild type), but in parallel experiments, it did not alter the current density of coexpressed Kv1.4 or Kv1.5 channels. Kv4.2ST also effectively suppressed rat Kv4.3 current when coexpressed in CHO-K1 cells. We then engineered a recombinant adenovirus (AdKv4.2ST) designed to overexpress Kv4.2ST in infected cells. A-type currents in rat cerebellar granule cells were decreased two days after AdKv4.2ST infection as compared with those infected by a β-galactosidase reporter virus (116.0 pA/pF versus 281.4 pA/pF in Ad β-galactosidase cells, n = 8 each group, p < 0.001). Likewise, Ito in adult rat ventricular myocytes was suppressed by AdKv4.2ST but not by Adβ-galactosidase (8.8 pA/pF versus 21.4 pA/pF in β-galactosidase cells, n = 6 each group, p < 0.05). Expression of a GFP-Kv4.2ST fusion construct enabled imaging of subcellular protein localization by confocal microscopy. The protein was distributed throughout the surface membrane and intracellular membrane systems. We conclude that genes from the Kv4 family are the predominant contributors to the A-type currents in cerebellar granule cells and Ito in rat ventricle. Overexpression of dominant-negative constructs may be of general utility in dissecting the contributions of various ion channel genes to excitability.

Eukaryotic cells express a rich tapestry of potassium channel genes. Indeed, the expressed genes often outnumber the recognized ionic currents in any given cell type (1, 2). Because different genes can produce channels with very similar phenotypic properties and overlapping pharmacologic sensitivities, the functional role of each gene often has proven difficult to assign. The conundrum is epitomized by the transient outward potassium current. This current (abbreviated Ito,1 but commonly known as the A-type current (IA) in neurons) has attracted considerable attention given its dynamic regulation in disease states including epilepsy and heart failure (3–6).

Various members of two separate potassium channel gene families have been implicated in the formation of Ito. The first was Kv1.4, a member of the Shaker family which, when expressed in oocytes, exhibits roughly appropriate kinetics (fast activation and fast inactivation) during single depolarizing voltage stimuli (7, 8). However, the recovery kinetics of the heterologously expressed Kv1.4 channel are much slower than those of Ito in native heart cells, and there is no correlation between the amount of current and the message levels for Kv1.4 (9). More recently, Kv4.2 and Kv4.3 have emerged as stronger candidates (10, 11). When expressed alone, these members of the Shal family exhibit plausible kinetics (although again, the match with native cells is imperfect) (12). These genes are richly expressed in ventricular myocytes and in neurons that express IA (4, 10, 12–14); furthermore, antisense oligonucleotides targeting Kv4.2 suppress Ito in rat ventricular myocytes (15). Nevertheless, there is a paucity of evidence at the protein level that Kv4 genes underlie Ito. The picture is further confused by the fact that many tissues express both Kv4.2 and Kv4.3 (9, 11, 12), which differ when expressed individually but are thought to be capable of forming heteromultimers that obscure such differences.

Selective gene suppression has become an attractive method for elucidating protein function experimentally. The two most commonly used strategies to accomplish this include antisense methodologies (16) and dominant-negative constructs (17). Both have been used to manipulate functional expression of ion channels (15, 18), but the latter has proven to be particularly useful in manipulating potassium channels due to the fact that they form multimers in the cell membrane (19–27). One dysfunctional K channel subunit (e.g. with a missense mutation in the pore region) can suffice to cripple an otherwise normal tetrameric complex; indeed, such dominant-negative interactions underlie various forms of the inherited long QT syndrome (28, 29). The introduction of a dominant-negative ion channel construct into native cells also has been used to map the levels of specific channel families in neurons from Xenopus embryos (30). Unfortunately, such a technique generally is not applicable to mammalian systems without manipulating the germline.

In the present study, we have used somatic gene transfer

* This work was supported by a Markey Foundation Training Grant (to D. C. J.), the Leonard Horowitz Fellowship in Cardiac Pacing and Electrophysiology (to H. B. N.), and the Tanabe Seiyaku Co. Ltd. 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.

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1 The abbreviations used are: Ito, transient outward current; CHO, Chinese hamster ovary; CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced GFP; AraC, cytosine arabinoside; β-galactosidase, E. coli lacZ gene; pF, picofarad.
methods to introduce dominant-negative Kv4 constructs into various cell types. We first demonstrate that a truncated version of Kv4.2 (Kv4.2ST) acts to suppress Ito encoded either by Kv4.2 or Kv4.3 in CHO cells. For gene delivery into myocytes and neurons, which are resistant to conventional transfection methods, we use a recombinant adenovirus that overexpresses Kv4.2ST. Recombinant adenoviruses have previously been used to express functional ion channels, as well as to deliver dominant-negative constructs of other genes into primary cells of both cardiac and neuronal origin (31–33). Our results support the idea that Shal potassium channels represent the major constituents of the A-type current in neuronal cells and of Ito in adult cardiac myocytes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The coding sequence for the rat Kv4.2 potassium channel gene was provided by M. Tamkun (Vanderbilt University) (34). The portion of the sequence coding for the first 206 amino acids (Fig. 1A) was amplified by polymerase chain reaction using primers that contained unique restriction sites on the 5’ and 3’ ends. The product (Kv4.2ST) was cloned into pSL301 (Invitrogen, San Diego, CA) and sequenced to confirm the absence of polymerase chain reaction-induced mutations. The Kv4.2 ST sequence was then cloned into the adenovirus shuttle vector pE1CMV (Fig. 1B) (31). Constructs were made in both the sense and antisense orientations with respect to the promoter, designated pE1Kv4.2ST and pE1CKv4.2AS respectively. An additional vector, pE1RKv4.2AS, contains the Rous sarcoma virus long terminal repeat as the promoter and was used to generate AdRKv4.2AS. The 4.2ST sequence was also fused in frame to the enhanced green fluorescent protein (EGFP) sequence in the vector pEGFP-C3 (CLONTECH, Palo Alto, CA) to make the construct pEGFP-4.2ST. The full-length Kv4.2 sequence was subcloned into pREP4 (Invitrogen, Carlsbad, CA) and into the pEGFP-C3 backbone to make pRep4.2FL and pE-Kv4.2FL respectively. The full-length rat Kv4.3 sequence was obtained from B. Rudy (New York University Medical Center) and cloned into the expression vector pGFPIRS. This vector is a modified version of pEGFP-C3, which contains the polio virus internal ribosomal entry site, obtained from G. Ketner (Johns Hopkins School of Public Health) (34) and cloned between the EGFP sequence on the 5’ side and the polycloning site on the 3’ site. This vector (pGFPIrKv4.3)

**FIG. 1. Schematic representation of AdKv4.2ST.** A, topology diagram depicting the structure of the full-length Kv4.2 channel (left) next to the predicted structure of the Kv4.2ST protein (right). B, cotransfection of the adenoviral shuttle vector pE1Kv4.2ST and the bacterial plasmid pJM17 in HEK293 cells results in a recombinant adenovirus. This recombination replaces the E1 region of the adenovirus with Kv4.2ST expression cassette.
produces a single transcript, encoding both the EGFP protein and the Kv4.3 protein. The human inward rectifier cDNA (Kir2.1) containing the inactivating mutation of GYG to AAA (27) was provided by G. Tomaseelli (Johns Hopkins University) and was fused to the EGFP sequence in pEGFP-C3 (pGFPKir2.1-AAA). The CMV-β galactosidase plasmid pLacZ was a gift from F. Kirchhoff (University of Pittsburgh) and L. Philipson (University of Chicago) (36), respectively.

Adenovirus Vector Preparation—The strategy is outlined in Fig. 1B. The various adenovirus shuttle plasmids were cotransfected with pJM17, containing the full human adenovirus serotype 5 genome (37) into HEK293 cells using LipofectAMINE (Life Technologies Inc.). As described previously, homologous recombination between the shuttle vector and pJM17 replaces the region of the adenovirus between map units 1.0 and 9.8 with the expression cassette containing the desired DNA. Successful recombinations were screened either by direct visualization (AdmGFP and AdhGFP) or by Southern blot analysis of small scale infections. Adh4.2AS, AdhC4.2AS, and Adh4.2AS followed by RNase protection analysis of RNA made from infected cells as described below. AdCMVβ-Gal contains the β-galactosidase gene driven by the CMV promoter and was provided by G. Wilkinson (University of Wales College of Medicine, Cardiff, U.K.).

Cerebellar Granule Cell Isolation—Cerebellar granule cells were isolated as described previously (38). Plates were plated on dishes that had been coated with poly-L-lysine (5 μg/ml for 10 min). If coverslips were needed they were sterilized by immersion in 95% EtOH followed by flaming in a bunsen burner. Coverslips were then placed in the dishes prior to coating with polylysine. Cells were cultured in Basal Eagle’s medium with Earle’s salts free of glutamine (Life Technologies Inc.) supplemented with 10% fetal bovine serum for 2 h prior to coating with polylysine. Cells were cultured in Basal Eagle’s medium supplemented with heat-inactivated 2% fetal bovine serum for 2 h (39). Coverslips were then placed in the dishes flaming in a bunsen burner. Coverslips were then placed in the dishes prior to coating with polylysine. Cells were cultured in Basal Eagle’s medium with Earle’s salts free of glutamine (Life Technologies Inc.) that had been supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml gentamicin, 25 μM KCl, and 2 mM L-glutamine. The medium was removed after one day and replaced with fresh medium containing 10 μM cytosine arabinoside. Under these culture conditions, expression of the A-type current is stable throughout the course of the experiment (39).

Ventricular Myocyte Cell Isolation—Adult rat ventricular myocytes were isolated by enzymatic dissociation as described previously (40). Cells were cultured on laminin-coated coverslips in medium 199 with 100–500. The AdhGFP allowed visual inspection of the percent infected cells. Infections were carried out in a minimal volume of culture medium with Earle’s salts free of glutamine (Life Technologies Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Ribonuclease protection assays were performed using the RPAII kit (Ambion, Austin, TX). 1 μg of total RNA was hybridized to both sense and antisense RNA probes for the Kv4.2ST sequence. Protected fragments were separated on a 5% denaturing polyacrylamide gel.

Confocal Microscopy—CHO-K1 cells (CCL-61, American Type Culture Collection, Rockville, MD) plated on glass coverslips were transfected with pGFP-Kv4.2ST or pGFP-Kv4.2ST and pE-Kv4.2FL using LipofectAMINE (Life Technologies, Inc.). After 36 h, cells were washed with phosphate-buffered saline and placed upside down on a microscope slide over a drop of phosphate-buffered saline. The edges of the coverslip were sealed with rubber cement to prevent drying. Images were taken on a laser scanning confocal microscope (PCM 2000, Nikon Inc., Melville, NY) with a 60× objective lens (numerical aperture 1.2).

Statistical Analysis—Pooled data are shown as the mean ± S.E. Comparisons of means between groups were performed using one-way analysis of variance. P values less than 0.05 were deemed significant.
been infected with either AdKv4.2ST, AdCKv4.2AS, or AdRKv4.2AS, revealed that only sense and antisense (respectively) RNAs were expressed from these constructs in detectable amounts (Fig. 3). Based on preliminary experiments in isolated dog cardiac myocytes, AdKv4.2ST was expanded for further analysis.

Cerebellar granule neurons were chosen to test the idea that the A-type current is encoded by *Shal* family genes (12, 39, 43), because these cells are easily maintained in culture with minimal changes in A-type current density over time. Infection of cultures of granule neurons using low multiplicities of infection (range 10–50) of AdhGFP resulted primarily in infection of surrounding glial cells, as had been previously observed in other neuronal cultures (32). Granule cells are easily distinguished from glial cells by their size and morphology. When multiplicities of infection were increased to 100–500, infection of neuronal cells could be observed at 24–48 h. To positively identify infected neurons for electrophysiologic study, AdhGFP was included in all infections and the duration of expression was calculated from the first appearance of GFP-positive neurons. Fig. 4A shows representative A-type currents elicited in an Adβ-Gal-infected control cell upon depolarization to +40 mV following prepulses to two different potentials (−90 mV, −40 mV, 500 ms). Infection with AdKv4.2ST suppresses the A-type current in the cerebellar granule neurons without affecting the maintained component of outward current. Results of eight experiments are summarized in panel C.
rents elicited by test pulses to +40 mV in a myocyte infected with Adβ-galactosidase (panel A) and in another infected with AdKv4.2ST (panel B). The pooled data in Fig. 5C (n = 6 in each group) confirm the significant suppression of native rat cardiac I_{to} by infection with AdKv4.2ST. As was the case with the cerebellar granule cells, the suppression of current was substantial but not complete at 42–50 h. Nevertheless, the results indicate that Kv4 genes constitute the major contributors to I_{to} in heart cells and to A-type currents in cerebellar granule cells.

**GFP-Kv4.2ST Constructs**—To probe the mechanism of action of Kv4.2ST, fusion constructs were generated with EGFP so that the expressed truncated protein could be localized within living cells with confocal imaging. We first confirmed that the fusion protein GFP-Kv4.2ST acted similarly to Kv4.2ST. Fig. 6, A and B, shows currents recorded in a CHO-K1 cell cotransfected with pRCCMVKv1.4 and pCMVβ-Gal (A) or pGFPKv4.2ST (B). As was the case for Kv4.2ST and Kv1.5, there is no suppression of Kv1.4 by GFP-Kv4.2 (Fig. 6C). Nevertheless, the fusion protein could suppress Shal family currents (either Kv4.2, Fig. 6, D–F, or Kv4.3, Fig. 6, G–I). The summary data in panels F and I show comparable levels of suppression for Kv4.2 and Kv4.3 (p < 0.005). Kir2.1-AAA is an unrelated inwardly rectifying potassium channel with a pore mutation designed to suppress Kir2.1 channels (27) and was added as a control in these experiments to ensure that suppression of Kv4.2 current was not simply attributable to coexpression with another membrane protein. This control is particularly apt because GFP Kir2.1-AAA does suppress the functional expression of inwardly rectifying currents encoded by wild-type Kir2.1 (44). Confocal imaging of CHO-K1 cells transfected with pGFP-Kv4.2ST (Fig. 7A) or cotransfected with pGFP-Kv4.2ST and pE-Kv4.2FL (Fig. 7B) reveals that the fusion construct is richly concentrated in the perinuclear region of the cells. In families of Z-plane images, fluorescence intensity was also detected on the surface of the cells (not shown). These findings suggest that at least some of the suppression of functional current may be due to premature degradation of heteromeric channel complexes, and/or to effects on the processing of the mature protein prior to externalization. However, this apparently abnormal localization of a membrane protein does not seem to be restricted to the truncated version of this protein, as fusion constructs containing full-length channels also have similar localization patterns despite the fact that robust membrane currents can be readily detected in such cells (45). Therefore, it is not possible at this time to state unequivocally whether the suppression of current occurs as a result of pre-
mature degradation, as the result of the formation of nonfunctional tetramers in the surface membrane, or as a combination of the two effects.

Summary—The use of a dominant-negative Kv4.2 ion channel construct specifically suppresses the transient outward current of rat ventricular myocytes as well as the A-type current of cerebellar granule neurons. These two cell types have previously been shown by other methods to express Kv4 family genes (9–12, 15). We show here that Kv4.2ST specifically suppresses members of the Shal family. In addition, this is the first experimental demonstration that Shal family members can form heteromultimers with each other. This strategy provides a unique way of determining the molecular identity of macroscopic ionic currents in native cells and may provide a useful tool in understanding the exact role these currents play in cellular physiology. The use of the adenovirus vector also allows the potential use of this strategy in vivo as well as in vitro. Whereas the introduction of dominant-negative constructs may also be achieved by transgenic approaches, developmental adaptation or possible lethal effects may complicate the interpretation of such experiments.

Acknowledgments—We thank M. H. Montrose and B. O’Rourke for help in confocal imaging, and G. Ketner for help in adenovirus techniques.

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Fig. 7. GFP-Kv4.2ST is distributed in various cell membranes. A confocal image of pGFP-Kv4.2ST transfected into CHO-K1 cells (A) shows concentration of the fusion protein in the intracellular membranes. This distribution is similar in a cell that was cotransfected with pGFP-Kv4.2ST and pE-Kv4.2FL (B).