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Research Report

The degradation of the inwardly rectifying potassium channel, Kir2.1, depends on the expression level: Examination with fluorescent proteins

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ABSTRACT

The expression of ion channels is regulated by their synthesis as well as degradation, and some ion channels are degraded in an expression level-dependent way. Recently, new techniques of fluorescent proteins have been developed and seem to be useful to study protein degradation. To examine the regulation of the degradation of strongly inwardly rectifying potassium channel (Kir2.1) and the usefulness of the fluorescent proteins, we constructed Kir2.1 fusion proteins with SNAP tag and fluorescent timer (FT). The SNAP tag, which covalently binds to a specific membrane-permeable fluorescent dye, enables a pulse-chase experiment with fluorescence. When the SNAP-Kir2.1 proteins were expressed in 293T cells by low and high expression plasmids, the half-life of the fusion protein expressed by a high-expression plasmid was shorter (18.2±1.9 h) than that expressed by a low-expression plasmid (35.1+2.3 h). The addition of Ba^{2+}, a selective blocker of Kir2.1, slowed the degradation, suggesting a current-dependency of degradation. Consistently, patch-clamp recording showed that cultivation in the presence of Ba^{2+} increased the whole cell conductance of SNAP-Kir2.1. Since the fluorescence of FT changes gradually changes from green to red, the green/red ratio should allow us to monitor the changes in the degradation rate of FT-Kir2.1. Using this method, we confirmed the slower degradation by Ba^{2+}. The results suggest a homeostatic regulation of the degradation of Kir2.1 in the 293T cells, and the usefulness of fluorescence-based methods for examining the degradation of ion channels.

1. Introduction

To maintain the excitability and ion balance of cells, the expression of ion channels is tightly regulated through synthesis, intracellular transport, posttranslational modification, and degradation. Recent reports showed dynamic and compensatory mechanisms of mRNA synthesis (Bergquist et al., 2010; Schulz et al., 2006) and surface delivery (Boyer et al., 2009; Dart and Leyland, 2001; Schachtman et al., 1992) of potassium channels in neurons. In addition to them, degradation also

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regulates the expression of ion channels. For instance, the impaired degradation of renal epithelial Na^+ channels results in Liddle syndrome (Rotin, 2008). The intrinsic excitability of neurons is regulated in a homeostatic way, in which intrinsic excitability and synaptic inputs change to maintain appropriate firings (Turrigiano et al., 1994). Indeed, temporal lobe epilepsy upregulated the Kir2 channels (Young et al., 2009), and neuronal activity elevated the surface expression of G-protein-activated inwardly rectifying K^+ channels (Chung et al., 2009). Ablation of auditory input decreased the expressions of Kv1.1 and Kv3.1 (Lu et al., 2004). Furthermore, degradation is shown to be involved in the activity-dependent regulation of expression of Na^+ channels (Paillart et al., 1996).

The 293T cells are derived from the kidney, which expresses several K^+ channels (Giebisch et al., 2003) including Kir2.1 (Leichtle et al., 2004; Raab-Graham et al., 1994). Interestingly, regulated degradation machinery seems to be retained in 293 cells. Indeed, human ether-a-go-go-related gene (HERG) K^+ channel was degraded in a K^+ conductance-dependent way in the HEK293 cells (Massaeli et al., 2010). Therefore, it is expected that 293T cells retain the regulated degradation mechanism.

Conventionally, protein degradation has been studied by radioisotope pulse-labeling followed by immunoprecipitation with a specific antibody against the protein of interest (pulse-chase experiment). This approach, however, requires costly radioisotopes and reliable antibodies, and is difficult to implement in vivo. Alternatively, cycloheximide (CHX) has been used to block the de novo synthesis of proteins, and so to estimate half-lives in vitro. This method also needs reliable antibodies, and the toxicity of CHX makes it impossible to examine proteins with long half-lives. Recently, new fluorescent proteins and methods of chemical labeling have been developed (Miller and Cornish, 2005). The SNAP tag, a mutant of a catalytic domain of a DNA-repairing protein, O^6-alkylguanine-DNA-alkyltransferase, can covalently bind to specific substrates including fluorescent dyes (Johnsson, 2009; Keppler et al., 2002). Therefore, the fusion of a SNAP tag to a protein allows for pulse-labeling with fluorescence instead of a radioisotope. In addition, fluorescent timer (FT), a mutant of red fluorescent protein, is initially synthesized as a protein with green fluorescence and gradually matures into a red fluorescent protein. The green-to-red conversion is spontaneous and very slow; it takes 10 h for half of FT proteins and 50 h for all FT proteins to convert (Terskikh et al., 2000). This spontaneous and slow conversion allows us to monitor the "youth" of FT-fused proteins. Given that the degradation is accelerated, proteins are degraded before turning red and so the green/red ratio should be higher. Thus, the green/red ratio of FT is expected to be useful for detecting the changes in protein degradation.

Strongly inwardly rectifying potassium (Kir2.1) channels are tetramers with each subunit having two transmembrane domains, a pore-forming region, and N- and C-terminal cytoplasmic domains (Kubo et al., 1993). Kir2.1 channels are expressed in heart, kidney, and brain, and play pivotal roles in intrinsic excitability. Their physiological relevance is evident from the severe phenotypes of mutants of Kir2.1. A loss of function mutation of Kir2.1 resulted in Andersen–Tawil syndrome with long QT syndrome, ventricular arrhythmia, and physical abnormalities of the head, face, and limbs (Andelfinger et al., 2002; Plaster et al., 2001). Curiously, a gain of function mutation of Kir2.1 also resulted in arrhythmia. Familial atrial fibrillation is linked to a mutation which increases conductance of Kir2.1 (Xia et al., 2005). The transgenic overexpression of Kir2.1 resulted in a slower heart rate and atrial fibrillation in mice (Li et al., 2004). These findings indicate the importance of accurate regulation of Kir2.1. Recent studies have shown that the channel is degraded through lysosomal pathway (Feliciangeli et al., 2010; Jansen et al., 2008; Vos and van der Heyden, 2011). Since the lysosomal degradation of Na^+ channels is regulated in an activity-dependent way (Paillart et al., 1996), degradation of Kir2.1 might be dependent on the current level.

In this report, to investigate the degradation of Kir2.1 with fluorescence, we constructed SNAP-Kir2.1 and FT-Kir2.1. Using these methods, we found that higher expression and larger currents accelerated the degradation of Kir2.1 and usefulness of the fluorescent proteins.

2. Results

2.1. *Change in the degradation rate of SNAP-Kir2.1 depending on expression level*

To test the hypothesis that the expression of Kir2.1 is regulated by degradation depending on the expression level, we constructed the SNAP-Kir2.1 fusion gene and cloned it downstream of the CMV or SV40 promoters, and expressed them in 293T cells (Fig. 1A). The CMV promoter is more potent than the SV40 promoter in 293T cells. To further enhance expression, we attached the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to the 3′ of SNAP-Kir in the CMV promoter plasmid. We first examined the whole cell conductance of the cells transfected using the SV40 and the CMV promoters (Fig. 2). Expectedly, 24 h after transfection, the whole cell conductance of SV40 plasmid cells was significantly lower than that of CMV promoter. Interestingly, 48 h after transfection, the whole cell conductance was comparable between high and low expression cells. If the abilities of these promoters did not change over time, this result suggests that the half-lives of Kir2.1 were different depending on the expression level.

We next attempted to measure the half-life. We pulse-labeled the SNAP-Kir2.1 with a membrane-permeable fluorescent substrate for the SNAP tag, SNAP-cell-TMR-Star, 24 h after the transfection. SNAP-cell-TMR-Star covalently binds to the SNAP tag domain (Fig. 1A). After the washing-out of unbound dye for 2 h, we examined it microscopically and found that the SNAP-Kir2.1 fusion protein was successfully labeled in both cells transfected using the SV40 and the CMV promoters (Fig. 3A). The fluorescence of the cells transfected with the CMV promoter plasmid was significantly higher than that of the cells transfected with the SV40 promoter plasmid as we observed in whole cell current. Reportedly, HEK293 cells endogenously express the O^6-alkylguanine-DNA-alkyltransferase (Keppler et al., 2004), but the background