J. Exp. Biomed. Sci. 2012, 18(2): 96~103
pISSN : 1738-3226

Original Article

Novel Purification Method of Kv 4.2 Potassium Channel from Rat Brain Membrane

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Kv 4.2 ion channel protein has an ability to open at subthreshold membrane potentials and to recover quickly from inactivation. That is very important for neuronal signal transmission in vertebrate brain. In order to purify Kv 4.2 protein, the novel purification methods were experimented. The purification procedure utilized chromatography on DE-52 ion exchange column and affinity chromatography on a WGA-Sepharose 4B, and Kv 4.2 affinity column chromatography. It was found that 0.5% (wt./vol.) Triton X-100 detergent in lysis buffer worked well for Kv 4.2 protein solubilization from rat brain membrane. Protein quantitative determination was conducted by BCA method at 562 nm for each purification step to avoid determination interference of protein at 280 nm by detergent. The confirmation of Kv 4.2 existence and amount is performed using by SDS-PAGE/immunoblotting or 96-well dot blotting. The Kv 4.2 without interacting protein that contains carbohydrate, was purified from novel biochemical 3-steps purification method for further research.

Key Words: Potassium channel protein, Detergent, Purification, Immunoblotting

INTRODUCTION

Potassium channels are ubiquitous in the animal and plant kingdoms and in yeast and bacteria; they exhibit extraordinary heterogeneity among these organisms while preserving several salient features. These potassium channels contribute to the control of potassium flow, cell volume, release of hormones and transmitters, resting potential, and excitability of neurons and muscles. Potassium channels may be regulated by changes in the membrane potential or the metabolic state or the cell, or by transmitters and hormones (Hille, 1994). Such regulation contributes to signaling between neurons and mechanisms for cellular protection during stressful events, such as anoxia and ischemia. Indeed, pharmacological reagents that decrease or increase potassium channel activity have been characterized as potential anti-ischemic, anti-arrhythmic, anti-hypertensive, or anti-anginal agents as well as for treatment of bladder detrusor instability (Grover, 1994; Katz et al., 1993; Li et al., 1995; Lynch et al., 1992; Olsen, 1994; Quast et al., 1995). Venoms from snake, bee, scorpion, sea anemone, and marine snail often contain toxins that block potassium channel function (Castle et al., 1989; Hurst et al., 1991; Miller, 1995; Stanfeld et al., 1987).

In both hippocampal pyramidal neurons and ventricular myocytes, a voltage-dependent, A-type K^+ channel expressing a transient current is present in high density (Barry et al., 1995; Martina et al., 1998; Serodio et al., 1994). Hoffman et al. (1997) reported dense localization of voltage-dependent, A-type K^+ channel to the distal dendrites of hippocampal CA1 pyramidal neurons. In the last 20 years, rapidly inactivating, A-type K^+ currents have been found in a wide variety of mammalian neurons (Rudy, 1988). However, the biophysical properties of A-type K^+ currents have often diverged significantly from those found

*Received: 13 April, 2012 / Revised: 18 June, 2012
Accepted: 25 June, 2012

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initially in invertebrate neurons. For example, some A-type K^+ currents inactivate rapidly but recovery from inactivation very slowly, taking seconds at hyperpolarized membrane potentials. Other A-type K^+ currents activate only at suprathreshold membrane potentials, rather than at subthreshold potentials, predisposing them to a role in spike repolarization. This biophysical diversity is matched by the molecular heterogeneity of A-type K^+ channel revealed by molecular cloning. At least six genes code for K^+ channel subunits that form A-type channels in heterologous expression systems: Kv 1.4, Kv 3.4, Kv 4.1-3, and erg3 (Baldwin et al., 1991; Pak et al., 1991; Roberds and Tamkum, 1991; Schroter et al., 1991; Serodio et al., 1996; Shi et al., 1997; Stuhmer et al., 1989). The molecular picture is further complicated by the ability of auxiliary channel subunits to transform non-inactivating, delayed rectifier K^+ channels into inactivating, A-type channels (Heinemann et al., 1996; Rettig et al., 1994). In spite of this complexity, only channels composed of Kv 4 family subunits appears to have properties similar to those originally described in invertebrates that is, an ability to open at subthreshold membrane potentials and to recover quickly from inactivation (Serodio et al., 1994). Furthermore, the identity of the K^+ channel subunits responsible for transient A-type currents in hippocampal pyramidal neurons and ventricular myocytes is not conclusively known; however, several lines of evidence support the hypothesia that this current is mediated, at least in part, by channels containing Kv 4.2, the Shal-type, K^+ channel subunit proteins. 1) Kv 4.2, forms voltage-dependent, rapidly inactivating K^+ channels and is selectively localized and abundantly expressed on the soma and dendrites of dentate gyrus and CA1 and CA3 hippocampal neurons (Barry et al., 1995; Serodio and Rudy, 1998; Sheng et al., 1992). 2) Ultrastructural studies have demonstrated that Kv 4.2 is localized to the subsynaptic compartment (Alonso and Widmer, 1997). 3) In myocardium, Kv 4.2 is more abundant in ventricular versus atrial myocytes and is more abundant than other subfamily α-subunits known to express a transient current (Bary et al., 1995; Dixon and McKinnon, 1994). 4) In some mammalian species it has been shown that the transient current occurs in a gradient across the ventricular wall with expression being highest in the epicardial layers (Furukawa et al., 1990; Liu et al., 1993). As well, it has been shown in some species that the express of Kv 4.2 also follows a gradient in the ventricular myocardium (Dixon and McKinnon, 1994). Therefore, both the localization and biophysical properties of Kv 4.2 are consistent with the idea that this channel subunit contributes to A-type K^+ currents in ventricular myocytes and dendrites of hippoocampal pyramidal neurons.

In this study, we set out to identify novel purification method for Kv 4.2 as basic information for further studies that are involved in Kv 4.2 localization and function.

MATERIALS AND METHODS

Materials

All materials not specifically identified were purchased from Sigma Chemical Co. (MO, USA) or Roche Molecular Biochemicals (Quebec, Canada). Pre-stained molecular weight standards were from Sigma and Roche (IN, USA). Enhanced chemiluminescence (ECL) regents were from either Amersham or DuPont-New England Nuclear (PA, USA). DE-52 (pre-swollen) was purchased from Whatman (Kent, UK). Wheat germ agglutinin (WGA)-Sepharose 4B column and Kv 4.2-Sepharose 4B affinity column were prepared by coupling 8 mg of WGA (sigma) and monoclonal antibody (mAB) against to Kv 4.2 to 1 ml of CNBr-activated Sepharose 4B (Amersham Pharmacia, Uppsala, Sweden) each.

Antibodies

Antibodies generated on our laboratory, against the cytoplasmic and extracellular domains of potassium Channel especially Kv4.2-subunits have been described (Bekele-Arcuri et al., 1996; Manganas and Trimmer, 2000; Rhodes et al., 1995, 1996; Shi et al., 1996).

Rat brain membrane (RBM) preparation

A crude synaptosomal membrane fraction was prepared from freshly dissected adult rat brains by homogenization in 0.3 M sucrose/10 mM sodium phosphate, pH 7.4/1 mM EDTA containing 1 mM phenylmethylsulfonylfluoride, leupeptin at 1 μg/ml, aprotinin at 2 μg/ml, and pepstatin at

1 μg/ml. This homogenate was centrifuged at 3,000 × g for 10 min to remove nuclei and debris. The supernatant was centrifuged at 45,000 × g for 90 min to pellet the crude membranes. Membranes were suspended in the homogenization buffer, and protein was determined using the bicinchoninic acid (BCA) method (Pierce Biotechnology, IL, USA).

Preparation of detergent extract from RBM

To find optimal concentration of detergent on extracting buffer for Kv 4.2 protein, 0~2% (wt./vol.) Triton X-100 in lysis buffer were tested for extract efficiency of Kv 4.2. The immunoblotting evaluated quantity of extracted Kv 4.2 protein with Kv 4.2 monoclonal antibody. And then, membranes from rat brain were suspended in 20 mM Tris-HCl Buffer (pH 8) containing decided optimal concentration of Triton X-100, 10 mM EDTA, 0.15 M NaCl, 10 mM Iodoacetamide, and 10 mM Sodium Azide to a protein concentration of 1 mg/ml. Phenylmethylsulfonylfluoride (PMSF) was added to a concentration of 0.1 mM. The suspension was incubated at 4℃ for 1 h under slight stirring and then centrifuged for 1 h at 100,000 × g. The supernatant of this centrifugation is referred to as detergent extract.

SDS polyacrylamide gel electrophoresis and immuno-blotting

The pooled eluates from each purification step were size fractionated on 7.5% SDS polyacrylamide gels electrophoresis (SDS-PAGE; Maizel, 1971) and transferred to nitrocellulose membranes as described (Towbin et al., 1979). After electrophoretic transfer to nitrocellulose paper, the resulting blots were blocked in Tris-buffered saline (TBS) containing 4% low-fat milk (Blotto; Johnson et al., 1984), incubated in affinity-purified monoclonal or polyclonal antibodies diluted 1:50~1:2,000 in Blotto for 1 h or undiluted antibodies tissue culture supernatant (anti-Kv4.2), and washed three times in Blotto for 30 min total. Blots were then incubated in HRP-conjugated affinity purified goat anti-mouse and goat anti-rabbit secondary antibodies (Cappel, West Chester, PA; 1:1,000 dilution in Blotto) for 1 h and then washed in TBS three times for 30 min total. The blots were then incubated in substrate for enhanced chemiluminescence (ECL) for 1 min and autoradiographed on pre-flashed (to OD545=0.15) Fuji (Tokyo, Japan) RX or Kodak XAR-5 film (Monaghan et al., 2001).

Finally, the immunoblotting data were analyzed by densitometry with a GS-700 Densitometer 4, and Molecular Analyst Software (Bio-Rad, CA, USA). The specific activity of Kv 4.2 from densitometric analysis (OD) assay using the following equation;

$$Specific\ Activity = OD \times \frac{total\ volumn}{loaded\ volumn}$$

Dot blotting

The dot blotting was performed essentially as described by Panzeter et al. (1993) using with 96-well dot blot apparatus (minifold I, Schleicher and Schuell MicroScience). Peptides were dissolved in TBS containing 1% (wt./vol.) Triton X-100 (TBS-T) and 2 μg of each peptide was spotted on a 0.05 μm nitrocellulose membrane (Schleicher and Schuell). The membrane was air dried, rinsed 3 times with TBS-T and incubated for 1 h at room temperature with gentle agitation in TBS-T containing Kv 4.2 monoclonal antibody. It was then washed with TBS-T, 3 times at 10 min each. And blotted membrane was added by TBS containing HRP-conjugated secondary antibody. It was then washed with TBS-T again as same condition as mentioned above. The membrane was subsequently air dried and then incubated in substrate for enhanced ECL for 1 min and autoradiographed on preflashed (to OD545=0.15) Fuji (Tokyo, Japan) RX or Kodak XAR-5 film.

Chromatography on DE-52 column

DE-52 (15 ml) was equilibrated in a batch with RBM lysis buffer and poured into a column (1.5 × 20 cm). After passing several column volumes of same buffer with above through the column, the RBM lysate (250 ml) was loaded at a flow rate of 50 ml/h. The column was then washed with 3~6 column volumns of lysis buffer and the fraction with binding activity was eluted with a linear gradient to 300 mM NaCl in that buffer.

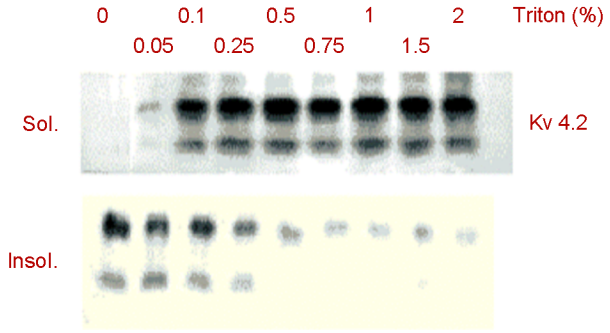

Fig. 1. Detergent concentration in lysis buffer for Kv 4.2.

Chromatography on WGA-Sepharose 4B affinity column

The eluate from the DE-52 column (15 ml) was directly loaded onto a column of WGA-Sepharose 4B (1.6 × 5.5 cm) equilibrated with RBM lysis buffer. The column was washed with 10 mM of buffer B and eluted with the lysis buffer containing 50 mM N-acetylglucosamine. The column retained usually about 20% of the binding activity.

Chromatography on Kv 4.2-Sepharose 4B affinity column

The eluate from the WGA affinity column was directly loaded onto a column of Kv 4.2-Sepharose 4B (1.6 × 5.5 cm) equilibrated with RBM lysis buffer. The column was washed with the same buffer and eluted with 0.1 M glycine, pH 2.5. And, analyze peek fraction for composition.

RESULTS

Optimum Triton X-100 concentration for Kv 4.2

Membrane proteins require detergents for extraction from membranes and for solubilization. In order to select a detergent for membrane protein, some criteria as followed must be considered; 1) hydrophilic-lipophilic balance values, 2) test for retention of structure and functional properties, 3) solubilization efficiency, 4) detergent compatibility 5) effect of charge 6) interference of aromatic detergents with optical detection of protein etc. Triton X-100 (TX-100) is one of excellent detergents that apt to fulfill mentioned 6 criterions. Kv 4.2 was extracted and permeabilized in lysis buffer containing 0%, 0.05%, 0.1%, 0.25%, 0.5%, 0.75%, 1%, 1.5% or 2.0% TX-100 detergent. Soluble (Sol.) and insoluble (Insol.) fractions were separated by centrifugation and analyzed by SDS-PAGE and immunoblotting (Fig. 1). I found that Kv4.2 was soluble even at the lowest concentration of TX-100 (0.1%). Nevertheless, because quantity of Kv 4.2 started to decrease definitely from 0.5% TX-100 on insoluble (Insol.) lane (Fig. 1), I used lysis buffer containing 0.5% TX-100 for the sequential experiment in this paper.

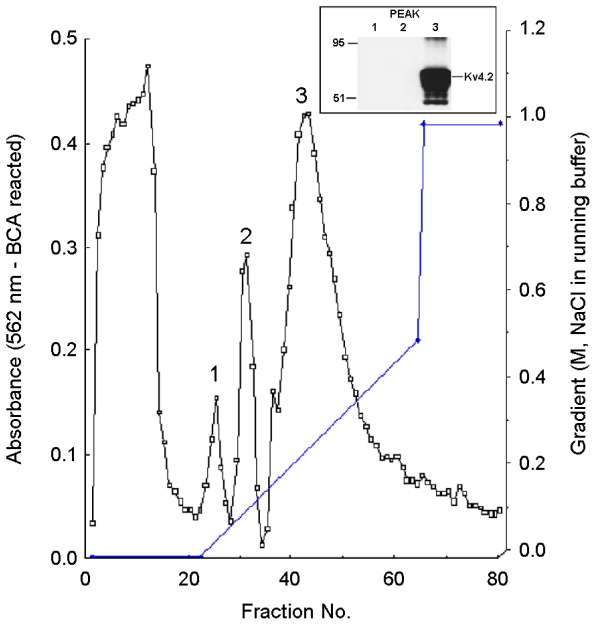

Fig. 2. Chromatograph of rat membrane protein on DE 52 ion exchange column. Total peaks fraction was separated on SDS-PAGE and blotted with anti-Kv4.2 as described in Materials and Methods.

Purification of Kv 4.2 on ion exchange chromatography

Fig. 2 shows the pattern of elution of the proteins from RBM lysate on DE-52 column chromatography. Absorbance was measured at 562 nm after BCA reaction using protein quantitative analysis kit. After elution was done, SDS-PAGE and immunoblotting methods identified protein peak fraction 1, 2, and 3 (Fig. 2). That data indicate that peak fraction 3 contained Kv 4.2 protein.

Chromatographic separation of Kv 4.2 on WGA-Sepharose 4B column

For further purification, the peak fraction 3 from DE-52 chromatography was put on a Wheat Germ Agglutinin

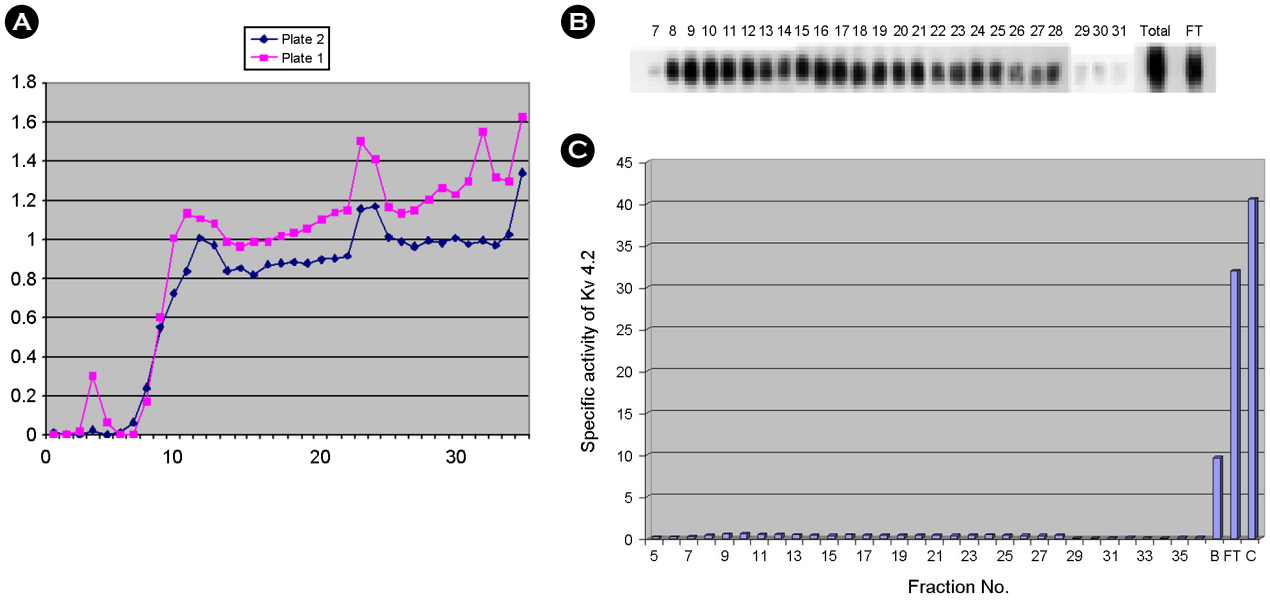

Fig. 3. WGA column chromatography and analysis. (A) WGA column chromatography. (B) Immunoblotting of protein fraction from WGA column. (C) Densitometry analysis from immunoblotting data. B, FT, C indicate binding fraction, flow through fraction, total amount of Kv 4.2 in fraction respectively.

(WGA)-Sepharose 4B column. According to the phenomenon concerning WGA could bind to the sugar chain of protein (Nagata and Burger, 1974), and Kv 4.2 doesn't hold sugar chain, non-specific protein or other membrane protein with Kv 4.2 should removed from fraction with Kv 4.2 on WGA-Sepharose 4B column. The chromatography of WGA-Sepharose 4B column shows several peaks that indicate lots of other protein still exist in peak fraction 3 from DE-52 chromatography (Fig. 3A). Autoradiogram of fraction from WGA-affinity column size fractionated by SDS/7.5% PAGE, transferred to nitrocellulose, and treated with 1:100 dilutions of immune sera from rat injected with the Kv 4.2 immunogen (Fig. 3B). Immunoblotting data indicate almost of Kv 4.2 (80%) remain in flow through (FT lane, Fig. 3B, and 3C), but some of Kv 4.2 was seen in WGA binding fraction although Kv 4.2 does not contain sugar chain. That means the binding sites between Kv 4.2 and Kv 4.2 specific protein with sugar chain were not cut out in WGA-Affinity column. Fig. 3C also shows that about 20% of Kv 4.2 remained in WGA-Sepharose 4B column till eluted of lysis buffer containing 50 mM N-acetylglucosamine. The specific activities of fraction from WGA-Sepharose 4B chromatography were calculated using the equation that is described in Materials. Specific activity means quantity of Kv 4.2 precisely.

Final purification procedure on Kv 4.2 affinity chromatography

The flow through fraction from the WGA affinity column was directly loaded onto a column of Kv 4.2 specific mAB-Sepharose 4B. After 0.1 M glycine (pH 2.5) elution, fractions were collected by fraction collector (FC-80K Gilson, Middleton, WI). Chromatography of Kv 4.2 affinity column revealed a sharp peak at early elution stage (Fig. 4A). Dot blot, an efficient technique for rapid detection of membrane proteins, was used for detection of Kv4.2 on Kv 4.2 affinity column fraction. The monoclonal antibody and polyclonal antibody against Kv 4.2, which generated from mouse and rabbit respectively were used in present study as heterologous immunoprobes for detection of Kv 4.2 using by 96-well dot blot apparatus (minifold I, Schleicher and Schuell MicroScience). The highest peak ranged from 1 to 7 of fraction number is well correspond with dot blotting results (Fig. 4B). Additionally, Fig 4B showing that fraction number 3 comparatively abounding in Kv 4.2.

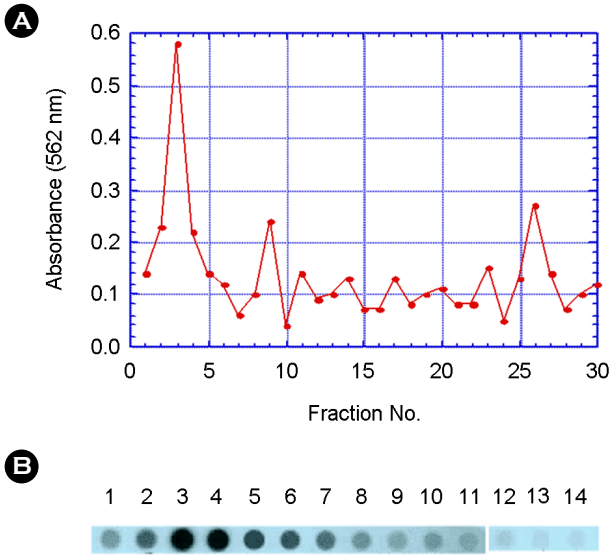

Fig. 4. Chromatograph on Kv 4.2 specific mAB-Sepharose 4B. (A) Chromatogram of Kv 4.2 affinity column. (B) Dot blotting

DISCUSSION

In the present study, we carried out the first trial of Kv 4.2 membrane protein purification from rat brain using ion exchange chromatography, WGA-Sepharose 4B chromatography, Kv 4.2 affinity chromatography respectively. Purification of membrane protein in general seems to be more problematic than purification of water-soluble proteins, although the methods used are principally the same. Membrane proteins tend to form aggregates even in the presence of detergents, which reduces the efficacy of all separation techniques. For instance, methods that differentiate according to molecular mass or size have lower resolution, salting out techniques show little discrimination, chromatofocusing and isoelectric focusing in the presence of anionic detergents do not work. It is therefore essential to select an unique detergent that does not dissolve denature protein but brain membranes. Detergents are amphiphilic molecules that form micelles in water. They solubilize proteins by binding to the hydrophobic parts of the proteins on one side and interacting with the aqueous phase on the other side. Properties and uses of detergents in biology and biochemistry have been reviewed (Deutscher, 1990; Neugebauer, 1988; Helenius et al., 1979). As a kind of polyoxyethylene detergents, Triton X-100 (TX-100) is low cost and very mild. In order to decide minimal concentration of TX-100 in lysis buffer for Kv 4.2 membrane protein, various concentrations of TX-100 were tested. The analysis about Kv 4.2 concentration on solubilized fraction and insolubilized fraction, suggesting that 0.5% (wt./vol.) TX-100 in lysis buffer is good for Kv 4.2 protein purification. In the case of water soluble protein, usually ultraviolet (UV) spectrophotometry at 280 nM is used for determination of protein concentration. This method is fast and nondestructive but is not very specific. UV spectroscopy is also sensitive to interference from other absorbing components as like buffer, nucleotides, and detergents, etc. In the term of membrane protein purification, UV spectroscopy method is not preferable. Then, we chose bicinchoninic acid (BCA) method to determine concentration of membrane protein during the each purification step. Wheat Germ Agglutinin (WGA) is not blood group specific, but has an affinity for N-acetyl-β-D-glucosaminyl residues, and N-acetyl-β-D-glucosamine oligomers. WGA contains no protein-bound carbohydrate (Nagata and Burger, 1974). By using WGA-Sepharose 4B affinity column, We could eliminate non-specific protein and specific protein with carbohydrate against Kv 4.2, because Kv 4.2 contains no sugar chain. For the final purification step, monoclonal antibody against Kv 4.2 was bound to Sepharose 4B bead in order to making Kv4.2 affinity column. Prepared Kv 4.2 affinity column was used for purified Kv 4.2 protein from WGA-Sepharose 4B column flow through fraction. So far, the research about purification method for Kv 4.2 ion channel protein was scanty as compared with its importance for neuronal signal transmission. The results presented here provide information on the biochemical purification in a field of membrane protein, especially ion channel protein by using an effective detergent at primary purification step.

Finally, the work described in this paper opens the way for further research concerning 1) finding novel protein which interacting with Kv 4.2 ion channel protein 2) interaction site between Kv 4.2 ion channel protein and Kv 4.2 interacting protein 3) structural approach about Kv 4.2 binding protein and 4) function of Kv 4.2-Kv 4.2 interacting protein complex.

Acknowledgements

The author thanks Professor James S. Trimmer (UC Davis), This work was supported by James S. Trimmer's lab, New York State University, Stony Brook, NY.

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