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Potassium channels in synaptosomal membrane examined using patch-clamp techniques and reconstituted giant proteoliposomes

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Synaptosomes isolated from the rat cerebral cortex were mixed with sonicated phospholipid vesicles and subjected to freezing-thawing to acquire giant proteoliposomes. Membranes of these giant proteoliposome could thus be studied using patch-clamp techniques. Single-channel currents were measured with the inside-out patch of the membrane, in KCl solutions. Three different potassium channels were detected and unit conductances were 15.1, 28.6 and 91.0 pS, respectively, in a symmetrical 150 mM KCl solution. All these channels are more permeable to potassium than to sodium ions, the permeability ratio being about 2:1. Tetraethylammonium ions blocked these channels. The gating of these potassium channels is independent of the membrane potential. Presumably, these channels play a role in the resting membrane potential of presynaptic nerve terminals.

Introduction

Electrophysiological studies of presynaptic nerve terminals should provide detailed knowledge about ion channels which play an essential role in the mechanism of the neurotransmitter release and information processing at a chemical synapse. However, direct electrical measurement with a microelectrode of a nerve terminal from the vertebrate central nervous system is not feasible, as the size and inaccessibility are prohibitive. The synaptosome, a vesicular membranous preparation isolated biochemically from the brain, consists of pinched-off presynaptic nerve terminals. Much of our biochemical knowledge of nerve terminals has come from studies using synaptosomes. Membrane potentials of and ion fluxes across synaptosomal membranes have been investigated. Blaustein and Goldring measured the membrane potential of synaptosomes using a fluorescent probe [1] and Bartschat and Blaustein [2] determined the K^+ permeability of synaptosomes by measuring the efflux of ^{86}Rb, which simulates the efflux of K^+.

Single-channel currents in synaptosomal membranes were recorded using a planar membrane reconstituted by fusion of synaptosomes into the phospholipid bilayer membrane: the Na^+ channel was detected by Krueger et al. [3], while K^+ and Ca^{2+} channels were reported by Nelson and coworkers [4–6].

Another potent reconstituted system is a giant proteoliposome, a preparation expected to have advantages over the planar membrane in that protein is more readily incorporated and electrical measurements with higher time resolution and

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid; TEA, tetraethylammonium.

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higher signal-to-noise ratio are feasible when investigated using standard patch-clamp techniques. Methods for preparing giant proteoliposomes suitable for the application of a patch-pipet microelectrode have been reported [7–10], but these methods are not established. We reported optimal conditions for the formation of giant proteoliposomes using the freezing-thawing technique [11] and most recently, we further developed the freezing-thawing technique without use of detergent to prepare giant proteoliposomes [12]. In the present study, we applied this newly devised method to form giant proteoliposomes from rat brain synaptosomes, the objective being to characterize the potassium channels.

Materials and Methods

Membrane preparations

Synaptosomes were prepared from cerebral cortices of male Sprague-Dawley rats, 7–8 wks old, according to Dunkley et al. [13]. Briefly, the cortex dissected from six animals was homogenized in a solution of 0.32 M sucrose/10 mM Tris-HCl (pH 7.0) with a Teflon-glass homogenizer. The membranous P2′ fraction was obtained from the homogenate and placed on Percoll density gradient: 3, 10, 15, and 23% Percoll in 0.32 M sucrose. After ultracentrifugation at $32500 \times g$ for 5 min, the turbid band at the interface of 15 and 23% layers was taken as the synaptosome fraction. The synaptosomes thus obtained were suspended in a buffer of 150 mM KCl/10 mM Hepes-KOH (pH 7.0) and stored in liquid nitrogen until use. Sonicated phospholipid vesicles were prepared from asolectin (soy bean phospholipid), as described [12]. Asolectin (type II-S) was purchased from Sigma (St. Louis, MO, U.S.A.) and was partially purified, according to Kagawa and Racker [14].

Preparation of giant proteoliposomes

Giant proteoliposomes were prepared using a newly devised freezing-thawing method [12]. In a standard experiment, synaptosomes and sonicated asolectin vesicles were mixed in a solution containing 0.1 M KCl/10 mM Mops-KOH (pH 7.0) with the weight ratio of protein/exogenous lipid of 1 : 10. The mixture was frozen in liquid nitrogen and thawed on ice. During the freezing-thawing treatment, synaptosomes and sonicated phospholipid vesicles fused to form giant proteoliposomes ranging from 10 to 30 μm in diameter.

Single-channel recording

A 5–10 μl drop of a vesicle suspension was placed in a Corning plastic tissue culture dish 35 mm in diameter and covered with 2.5 ml of a bath solution of an appropriate composition. A standard patch-clamp technique was used [15]. A patch-pipet electrode with a tip diameter of about 0.5 μm was made from a Pyrex glass capillary of o.d. 1.5 mm with a pipet puller (model PP-83, Narishige, Tokyo, Japan). The pipet tip was fire-polished with a laboratory-built micro-forge. When the pipet was filled with 150 mM KCl, the resistance was 7–9 MΩ.

Current measurements were performed in the inside-out patch configuration, under voltage clamp conditions and using a patch-clamp amplifier (model CEZ-2200, Nihon Kohden, Tokyo, Japan). A video-tape recorder (Sony, SL-HF507) was used to record currents, which had been digitized with a PCM audio processor (Sony, PCM-501ES, resolution 16 bit) modified according to Itoh et al. [16]. The recorded data were retrieved through an active two-pole low-pass filter and digitized by a 12-bit A/D converter (model PCN-2198, Neolog Electronics, Tokyo, Japan) at an appropriate sampling rate (usually 1 kHz) and analysis was carried out using an NEC PC9801 micro-computer. Membrane potential was expressed, according to the standard convention, in terms of potential of bath solution against pipet potential, in the inside-out excised patch configuration.

Results

Freeze-thawing a mixed suspension of synaptosomes and sonicated asolectin vesicles in 150 mM KCl/10 mM Hepes-KOH (pH 7.0) leads to the formation of giant proteoliposomes ranging 10–30 μm in diameter, as shown in Fig. 1. A giant vesicle attached to the bottom surface of a plastic dish was approached with a patch-pipet filled with an internal solution identical to the bath solution. The pipet was kept under slight positive pressure,

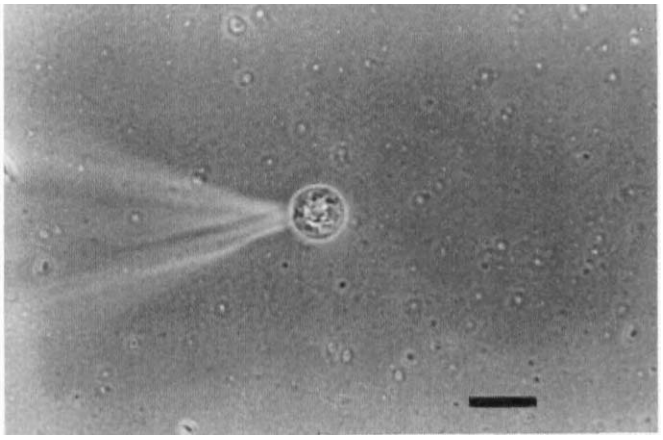

Fig. 1. Phase-contrast micrograph of a proteoliposome made by freeze-thawing a mixed suspension of rat brain synaptosomes and sonicated asolectin vesicles (protein/exogenous lipid = 1/10, w/w) in 150 mM KCl/10 mM Hepes-KOH (pH 7.0). A patch-pipet is attached to the giant vesicle. Bar, 20 μm.

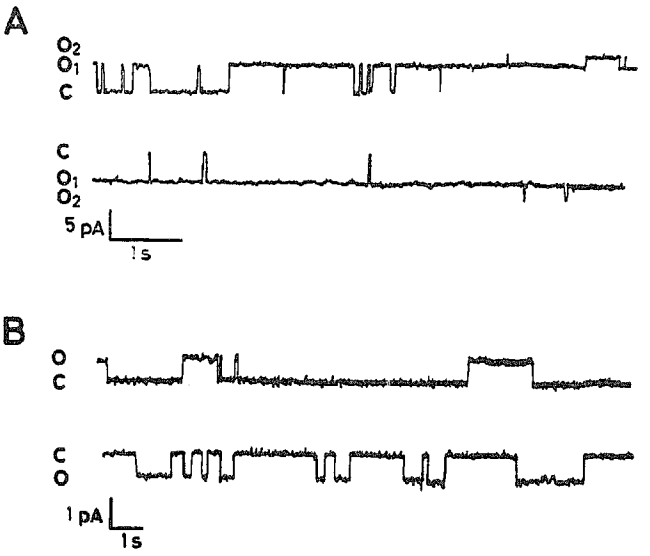

Fig. 2. Single-channel current traces obtained with an inside-out patch of membrane excised from a giant proteoliposome made by fusion of synaptosomes and sonicated asolectin vesicles. Currents were measured under voltage-clamped conditions in a symmetrical solution of 150 mM KCl/10 mM Hepes-KOH (pH 7.0). Current records were filtered at 320 Hz. (A) Letters C, O_1 and O_2 represent for closed and open levels of 90 and 30 pS channels, respectively. The upper trace was recorded at 60 mV, while the lower was at −60 mV. (B) Letters C and O represent closed and open levels of a 15 pS channel. Upper trace was recorded at 60 mV, lower trace at −60 mV.

until contact was made with the vesicle surface. Upon contact, a very slight negative pressure was applied to the pipet in order to obtain a gigaohm seal. A pipet attached to a giant vesicle is shown in Fig. 1.

Single-channel current fluctuations were recorded with a standard inside-out configuration [15] in a symmetrical solution of 150 mM KCl/10 mM Hepes-KOH (pH 7.0), and some traces are shown in Fig. 2. Fig. 2A indicates the presence of two types of channel with single-channel conductances of about 90 and 30 pS, respectively. In other experiments, only one of the two channels was observed, thereby indicating that the channels

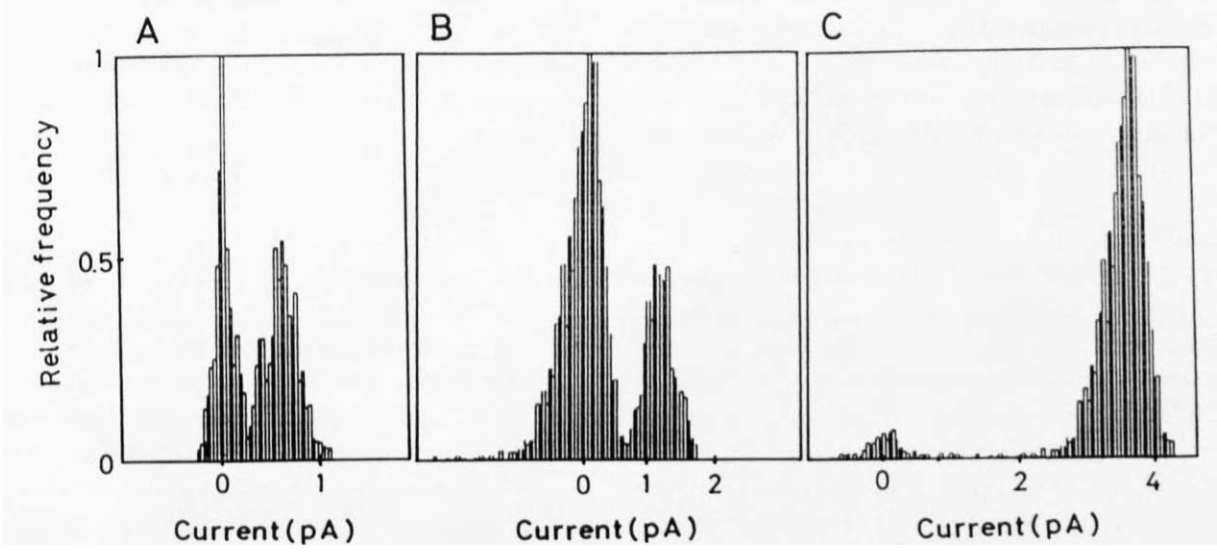

Fig. 3. Amplitude histograms from three types of channel. Number of points of digitized data are plotted against current amplitudes. (A) Current for the 15 pS channel measured at membrane potential of 40 mV. (B) 30 pS channel at 40 mV. (C) 90 pS channel at 40 mV.

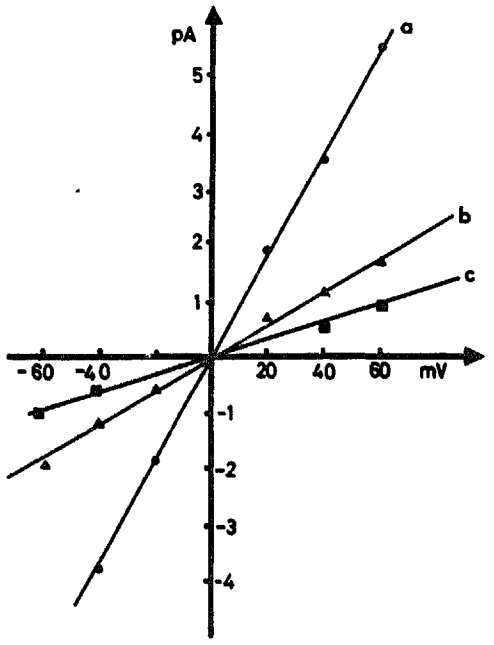

Fig. 4. Single-channel current–voltage relationship for three different potassium channels in rat brain synaptosomal membrane. Straight lines were obtained by a linear regression analysis of the data. Lines a, b and c correspond to 90, 30 and 15 pS channels, respectively.

differ and are independent. The two traces at two different voltages in Fig. 2A apparently indicate that the gating of these channels is independent of the membrane potential. Indeed, a study on the open probability of the two types of channel at various membrane potentials confirmed the conclusion: the open probability at membrane potential (in mV) indicated in parentheses was 0.8 (+60), 0.8 (+40), 0.8 (−40) or 0.7 (−60) for the 90 pS channel and 0.3 (+60), 0.2 (+40), 0.3 (−40) or 0.4 (−60) for the 30 pS channel. Fig. 2B shows the third type of channel, with a unit conductance of about 15 pS, in a patch of membrane different from that used for Fig. 2A. The gating of this channel was also independent of the membrane potential: the open probability was 0.5, 0.4, 0.5 or 0.6 at the membrane potential of +60, +40, −40 or −60 mV, respectively. Fig. 3 shows amplitude histograms for these three types of channel. For all the channels, two levels (open and closed) have been clearly resolved. No sublevels were detected.

Single-channel current–voltage relationships for these channels are shown in Fig. 4. Linear regression analysis of the data was performed to determine the slope of each straight line, which leads to single-channel conductances of 15.1, 28.6 or 91.0 pS, respectively. Current–voltage relationships were also measured in asymmetric KCl solutions (150 mM in bath and 50 mM in pipet) and the reversal potentials (mean ± S.D. (n)) were determined to be +24.3 mV ± 2.65 mV (4), 22.8 ± 1.9 (4) and 22.7 ± 1.84 (3) for the 90, 30 and 15 pS channels, respectively. These values were all very close to the K^+ equilibrium potential (27.7 mV at 20 °C). Therefore, we conclude these channels are far more permeable to K^+ than to Cl^-.

The current–voltage relationship was determined when a pipet solution contained 150 mM NaCl and a bath solution contained 150 mM KCl. From the reversal potentials determined under this bi-ionic condition, the permeability ratio for K^+ to Na^+ (P_K/P_{Na}) was estimated, using the Goldman-Hodgkin-Katz equation. The ratio was found to be about 2 for all three types of channel (Table I).

TABLE I

K^+ SELECTIVITY OVER Na^+ AND EFFECT OF 10 mM TEA ON OPEN PROBABILITY

Reversal potential (V_{rev}) was determined with a pipet solution containing 150 mM NaCl and bath solution containing 150 mM KCl. Permeability ratio (P_K/P_{Na}) was calculated from the reversal potential using the Goldman-Hodgkin-Katz equation. Values for single-channel conductance represent mean ± S.D. (n), where n stands for the number of the measurements of I–V relationship as shown in Fig. 4.

Single-channel conductance (pS)	V_{rev} (mV)	P_K/P_{Na}	Open probability		
			−TEA	+TEA	ratio (+/−)
15.1 ± 3.2 (5)	−21	2.3	0.53	0.08	0.15
28.6 ± 4.1 (4)	−18	2.0	0.35	0.11	0.30
91.0 ± 4.5 (4)	−15	1.8	0.85	0.01	0.01

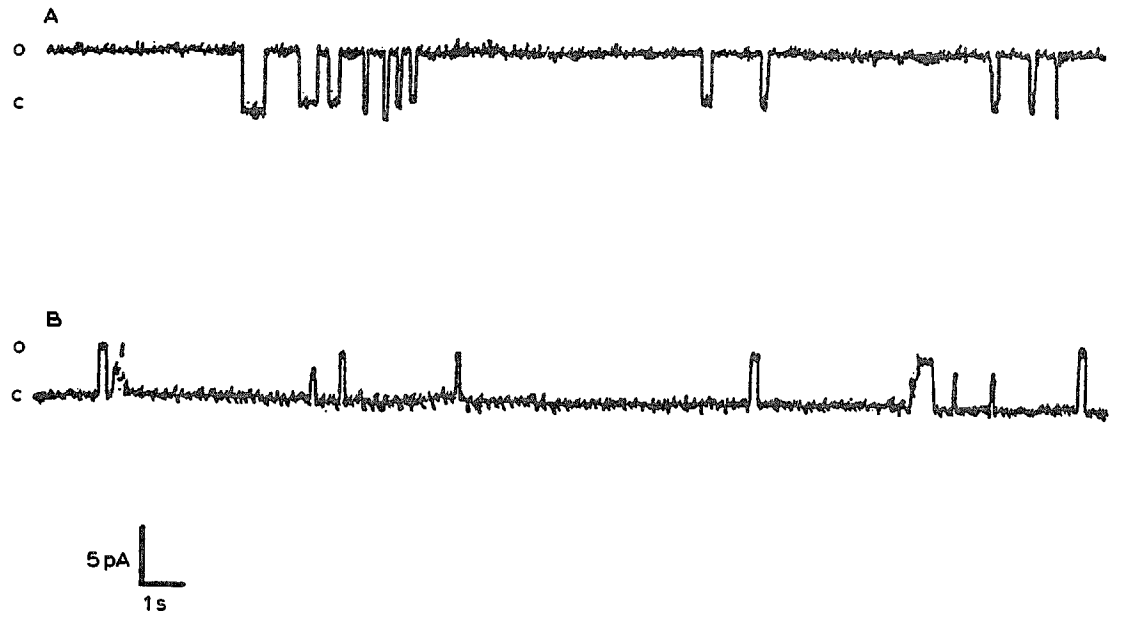

Fig. 5. Effects of TEA on the open probability of the 90 pS channel. Current traces at 40 mV in the absence (A) and presence (B) of TEA (10 mM) in the bath solution.

These channels were not blocked by 1 mM TEA ions. For the pharmacological examinations, results with the same patch before and after the addition of TEA to the bath solution were compared. For all three types of channel, the ratio of open probabilities after to before the addition of 1 mM TEA was 0.97–1.02. However, They were clearly blocked by 10 mM TEA. Fig. 5 shows the effect of 10 mM TEA on a 90 pS channel. The open probability decreased greatly and its open-channel conductance also decreased by about 10%. The other two types of channel were also blocked by 10 mM TEA, although the effect was less than that for a 90 pS channel (Table I).

Discussion

In the present study, synaptosomes from the rat brain were reconstituted into giant vesicles by a newly devised freezing-thawing method [12]. A giant vesicle thus prepared could be studied using the patch-clamp technique. Three different K^+ channels were detected in an inside-out patch excised from a giant proteoliposome and these were characterized. Their unit conductances were 15.1, 28.6 and 91.0 pS, respectively, in a symmetrical 150 mM KCl solution. The gating of these K^+ channels is independent of the membrane potential. They are not sensitive to the blockade by TEA, as a concentration as high as 10 mM was required for blocking. The permeability ratio of P_K/P_{Na} is about 2.

The membrane potential of resting synaptosomes could be depolarized by increasing external K^+ concentrations [1,17], thereby indicating that the synaptosomal membranes possess K^+ conductance in the resting state. Bartschat and Blaustein measured the $^{86}Rb^+$ efflux from synaptosomes and resolved the efflux curve into four components [2]. One component is independent of external K^+ concentrations, i.e., membrane potential, and is relatively insensitive to TEA. They attributed this component to ion flux through the resting K^+ channels.

Nelson and co-workers measured single K^+-channel currents of synaptosomes incorporated into the planar bilayer. The four different K^+ channels had unit conductances ranging from 8 to 40 pS in 500 mM KCl. There was no voltage-dependency, and the permeability ratio of P_K/P_{Na} was about 3–5. They reported that 1 mM TEA did not block those channels and they postulated that these channels may contribute to the resting K^+ conductance [4,5].

Therefore, the 15 and 30 pS channels found in the present study may be the same entities as two of those reported by Nelson and co-workers. The 90 pS channel seems to be a newly detected one. The characteristics of the channels in the present paper are similar to those reported by Nelson and co-workers [4,5] and by Bartschat and Blaustein [2]. Therefore, we also postulate that the channels in the present paper may contribute to the resting membrane potential.

The cation selectivity of the channels which we examined was somewhat smaller than that reported by Nelson and co-workers [4,5], perhaps for the following reason. When we determined a reversal potential to estimate the permeability ratio, we applied to patch-pipet filled with NaCl solution to a giant vesicle placed in KCl solution. This approach was used since perfusion easily damages the giga-seal in the vesicle-attached and excised-patch configuration. Thus, a small amount of K^+ ions might enter the pipet when suction, however weak, is applied in order to obtain the gigaohm seal. This can lead to an estimate of a smaller value for reversal potential and hence to a smaller permeability ratio.

Criado and Keller [18] prepared large proteoliposomes by partial dehydration and rehydration, in the presence of ethylene glycol, of the mixture of sonicated liposomes and biomembrane vesicles (sarcoplasmic reticulum membranes and postsynaptic membranes from electric organ) and they measured single-channel currents using the patch-clamp technique. Both their method and ours seem to be effective in preparing giant proteoliposomes suitable for studies with the patch-clamp technique. The simple and rapid technique we designed is most useful for reconstituting various biomembranes for which a patch-clamp pipet microelectrode cannot be used.

Acknowledgments

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