Comparison of Accumulative Inactivation between the Aplysia K Channel (AKv1.1a) and Its Amino-Terminal Deletion Mutant

Authors: Furukawa, Yasuo, and Takahashi, Toshio

Source: Zoological Science, 14(3) : 397-408

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.397

ZOOLOGICAL SCIENCE 14: 397–408 (1997)

Comparison of Accumulative Inactivation between the *Aplysia* K^+ Channel (AKv1.1a) and Its Amino-Terminal Deletion Mutant

Yasuo Furukawa[1]* and Toshio Takahashi[2]

[1]*Laboratory of Adaptation Physiology and Neurobiology, Department of Biological Science, Faculty of Science, Hiroshima University, Higashi-Hiroshima 739, Japan*
[2]*Physiological Laboratory, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739, Japan*

ABSTRACT—Accumulative inactivation of a cloned *Aplysia* K^+ channel (AKv1.1a) was examined in *Xenopus* oocyte expression system by the patch clamp technique. AKv1.1a inactivates by both N-type and C-type mechanisms. The amino-terminal domain of the channel is indispensable for N-type inactivation, whereas other parts of the channel is involved in C-type inactivation. The accumulative inactivation induced by repetitive pulses (0.2-0.5 Hz) was relatively insensitive to the pulse duration (10-900 msec). The accumulative inactivation was inhibited when the external K^+ concentration ($[K^+]_{out}$) was increased, or when tetraethylammonium (TEA) was added in the external solution. The accumulative inactivation of the amino-terminal deletion mutant (ΔN) which lacks N-type inactivation was dependent on the pulse duration such that it was less pronounced for short repetitive pulses (<100 msec). The accumulative inactivation of ΔN was also inhibited by high $[K^+]_{out}$ and external TEA. By contrast, the accumulative inactivation induced by pair-pulse protocol was not perturbed by external TEA, and was not observed in ΔN. The accumulative inactivation of AKv1.1a was enhanced when the membrane patch was excised out of the cell. Paradoxically, the macroscopic inactivation of AKv1.1a became slower in the excised patch. The accumulative inactivation of ΔN was less sensitive to the patch excision. Some synthetic peptides which were designed based on the amino-terminal sequences of K^+ channels induced a use-dependent block of ΔN which was apparently similar to the inactivation of AKv1.1a. Our results suggest that either N-type or C-type inactivation can induce the accumulative inactivation of K^+ channels, and that C-type inactivation coupled to N-type inactivation plays substantial roles in the frequency dependent accumulative inactivation of AKv1.1a.

INTRODUCTION

Voltage-gated K^+ channels share a basic molecular design, implying general mechanisms for their activation and inactivation gatings. The K^+ channels have six potentially membrane-spanning domains (S1-S6) as well as a presumed pore region (H5), and both amino- and carboxy-terminals of the channel protein are cytoplasmic domains (for review see Jan and Jan, 1992; Pongs, 1992). Following the activation, most K^+ channels inactivate with variable rates (Rudy, 1988). Pioneering exploration of molecular mechanisms for the inactivation of K^+ channels has been carried out in *Drosophila Shaker* K^+ channels (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990). *Shaker* channels show one of the fastest inactivation among voltage-gated K^+ channels, and the inactivation is shown to be a pore block by the amino-terminal cytoplasmic domain of the channel (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990). This type of inactivation is called N-type inactivation (Hoshi *et al.*, 1991), and the amino-terminal cytoplasmic domain is often referred to as the N-terminal inactivation ball since the mechanism is similar to the one which has been proposed for the inactivation of Na^+ channels as the "ball and chain model" (Armstrong and Bezanilla, 1977). Hoshi *et al.* (1991) have studied another inactivation of *Shaker* channels which is, for example, observed as a slower current decay of the *Shaker* currents. The inactivation is referred to as C-type inactivation although it does not necessarily mean the involvement of carboxy-terminal region (Hoshi *et al.*, 1991). Although a partial coupling between N-type inactivation and C-type inactivation is considered, C-type inactivation is a distinct process because it persists after the removal of the N-terminal inactivation ball from *Shaker* channels (Hoshi *et al.*, 1991). C-type inactivation involves at least some structural change near the outer mouth of channel pore (Choi *et al.*, 1991; López-Barneo *et al.*, 1993; Yellen *et al.*, 1994; Liu *et al.*, 1996).

AKv1.1a is an *Aplysia* homologue of *Drosophila Shaker* channels cloned from a cDNA library of *Aplysia* nervous system

* Corresponding author: Tel. +81-824-24-7437; FAX. +81-824-24-0734.

(Pfaffinger *et al.*, 1991). Functional K^+ channel has been expressed following either the injection of cRNA transcribed from a coding sequence of AKv1.1a into *Xenopus* oocytes (Pfaffinger *et al.*, 1991) or the injection of an expression plasmid containing a coding sequence of AKv1.1a into *Aplysia* neurons (Kaang *et al.*, 1992). Properties of the K^+ channels expressed by either methods are almost identical (Kaang *et al.*, 1992). A prominent property of AKv1.1a is an accumulation of the inactivation (Furukawa, 1995). Even during low frequency depolarizing pulses (<0.1 Hz), the channel currents cannot recover completely during the pulse-interval and the inactivated channels accumulate. The process is dependent on external K^+ concentration ($[K^+]_{out}$) such that the accumulation is highly enhanced in low $[K^+]_{out}$ (Furukawa, 1995). Although the main cause of accumulation of the inactivation appears to be N-type inactivation (Furukawa, 1995), it is less clear whether C-type inactivation of the channel contributes to the phenomena. This issue is important because in some K^+ channels the accumulative inactivation is entirely attributable to C-type inactivation (Marom *et al.*, 1993). Moreover, Baukrowitz and Yellen (1995) have recently shown that the frequency-dependent accumulative inactivation in *Drosophila Shaker* channels results from the interaction of N- and C-type inactivation. In the present study, several aspects of the inactivation in both AKv1.1a and its amino-terminal deletion mutant are compared to gain an insight into relative contribution of N-type and C-type inactivation in accumulative nature of the K^+ channel inactivation.

MATERIALS AND METHODS

Oocytes of *Xenopus laevis* were prepared, injected with cRNA, and cultured as described previously (Pfaffinger *et al.*, 1991). cRNA for the wild-type AKv1.1a and its amino-terminal deletion mutant (second to 61th amino acids were removed) were synthesized from pSPAK01 and pSPAK01ΔN, respectively (Furukawa *et al.*, 1995). In the rest of this paper, the amino-terminal deletion mutant of AKv1.1a is simply called ΔN.

All electrical recordings were made by the cell-attached or inside-out mode of the patch clamp technique as described previously (Hamill *et al.*, 1981; Furukawa, 1995). Oocytes were immersed in a high K^+ standard internal solution (KSIS) to make a resting potential zero. KSIS had a following composition: 80 mM KCl, 10 mM EGTA, 10 mM HEPES, 20 mM KOH (pH 7.5). Recording pipettes were usually coated near to the tip with silicone (Silpot 184, Dow Corning, Japan), and fire polished. Pipette resistance was around 1 to 2 MΩ. Following solutions were used to fill a recording pipette (mM): 3K solution, NaCl 97, KCl 3, $CaCl_2$ 1.8, $MgCl_2$ 1; 10K solution, NaCl 90, KCl 10, $CaCl_2$ 1.8, $MgCl_2$ 1; 10K-30TEA solution, NaCl 60, KCl 10, tetraethylammonium chloride (TEA-Cl) 30, $CaCl_2$ 1.8, $MgCl_2$ 1; 30K solution, NaCl 70, KCl 30, $CaCl_2$ 1.8, $MgCl_2$ 1; 100K solution, KCl 100, $CaCl_2$ 1.8, $MgCl_2$ 1. All solutions contained 10 mM HEPES and pH was titrated to 7.5 by NaOH. Small liquid junction potentials (a few mV) were not corrected. All experiments were carried out at room temperature (23-25°C).

Membrane currents were filtered at 0.5 to 2 kHz, digitized at sampling frequency of 0.2 to 50 kHz by a 12-bits AD converter (ADXM-AT10, Canopus, Japan), and stored on a harddisk. Data were analyzed off-line using a personal computer (Dell 433/L, Dell Computer) and home-made programs. To obtain the charge transfered through open channels, the current during a pulse was integrated numerically.

To apply substances to intracellular side of a patch, an 'air-gate' method was used. The design of experimental chamber was essentially the same to the oil-gate chamber developed by Qin and Noma (1989). Two chambers were separated by a small slit (about 1 mm in width) filled with air. An oocyte was placed in a main chamber having a volume of about 10 ml and continuously perfused with KSIS. Another chamber had a much smaller volume (about 70 μl) and was not perfused. Solution in the small chamber was replaced by aspiration and injection via a hand-held pipette. After the formation of inside-out patch in the main chamber, the pipette tip could be moved through the air-gate into the small chamber by a coarse manipulator which held a three-dimensional micro manipulator with a head stage of the patch clamp amplifier (CEZ-2300, Nihon Kohden, Tokyo, Japan). By quickly moving the manipulator, the pipette tip could be moved through the air-gate within 40 msec for several times without rupturing the patched membrane. The exchange rate of solutions by the method was examined by measuring the onset of channel block by TEA (a "fast" K^+ channel blocker) and was found to be almost instantaneous for the present purpose. Rapid exchange, however, was not attained in some experiments, which most probably due to the suction of patched membrane deep inside the pipette.

Based on the published amino acid sequences, several peptides corresponding to the amino-terminal (N-terminal) sequence of *Drosophila* K^+ channels (ShB and ShC, see Murrell-Lagnado and Aldrich, 1993a), or AKv1.1a (see Pfaffinger *et al.*, 1991) were synthesized by a standard solid-phase procedure using an automated peptide synthesizer (model PSSM-8, Shimadzu, Kyoto). All peptides were amidated, and were purified by a high-performance liquid chromatography. The amino acid sequences of synthetic peptides were confirmed by sequence analysis. N-terminal peptides of ShB and ShC are called ShB-p and ShC-p , respectively. Three different sized peptides were made based on the N-terminal sequence of AKv1.1a (AK10, AK18, and AK20): each number following "AK" indicates the number of amino acids. For AK10 and AK18, we also made mutant peptides, AK10EQ and AK18EQ, in which glutamate in the parent sequences was replaced with glutamine. The sequences of these peptides were as follows (amino acids were shown by one-letter code): ShB-p, MAAVAGLYGLGEDRQHRKKQ; ShC-p, MQMILVAGGSLPKLSS; AK10, MEVAMAGIEG; AK18, MEVAMAGIEGNGGPAGYR; AK20, MEVAMAGIEGNGGPAGYRDS; AK10EQ, MQVAMAGIQG; AK18EQ, MQVAMAGIQGNGGPAGYR. Peptides were dissolved in distilled water to make 10 mM stock solutions, except AK10EQ and AK18EQ which were dissolved in ethanol at the concentration of 10 mM. These stock solutions were stored at -20°C, and diluted in KSIS immediately before use.

In some experiments, Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP, Pierce) or 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB, Sigma) was applied to the inside-out patch. TCEP is a very effective reducing agent of S-S bonds (Gozlan *et al.*, 1994) and DTNB is an oxidizing agent. TCEP and DTNB were dissolved at the concentration of 500 mM in water and DMSO, respectively. The stock solution was stored at -20°C. Each stock solution was diluted in KSIS, and pH of the solution was checked before use.

RESULTS

Effects of pulse duration on the accumulative inactivation

AKv1.1a is a transient K^+ channel whose inactivation shows distinct accumulative nature (Furukawa, 1995). In response to repetitive pulses of relatively low frequency (<0.1 Hz), the peak currents of AKv1.1a decrease rapidly. To test a dependence of the accumulative inactivation on the pulse duration, AKv1.1a currents were recorded from a cell-attached patch in response to depolarizing pulses to +40 mV of variable duration from the holding potential of -80 mV (Fig. 1). When the pipette was filled with 10K solution and the repetitive pulses

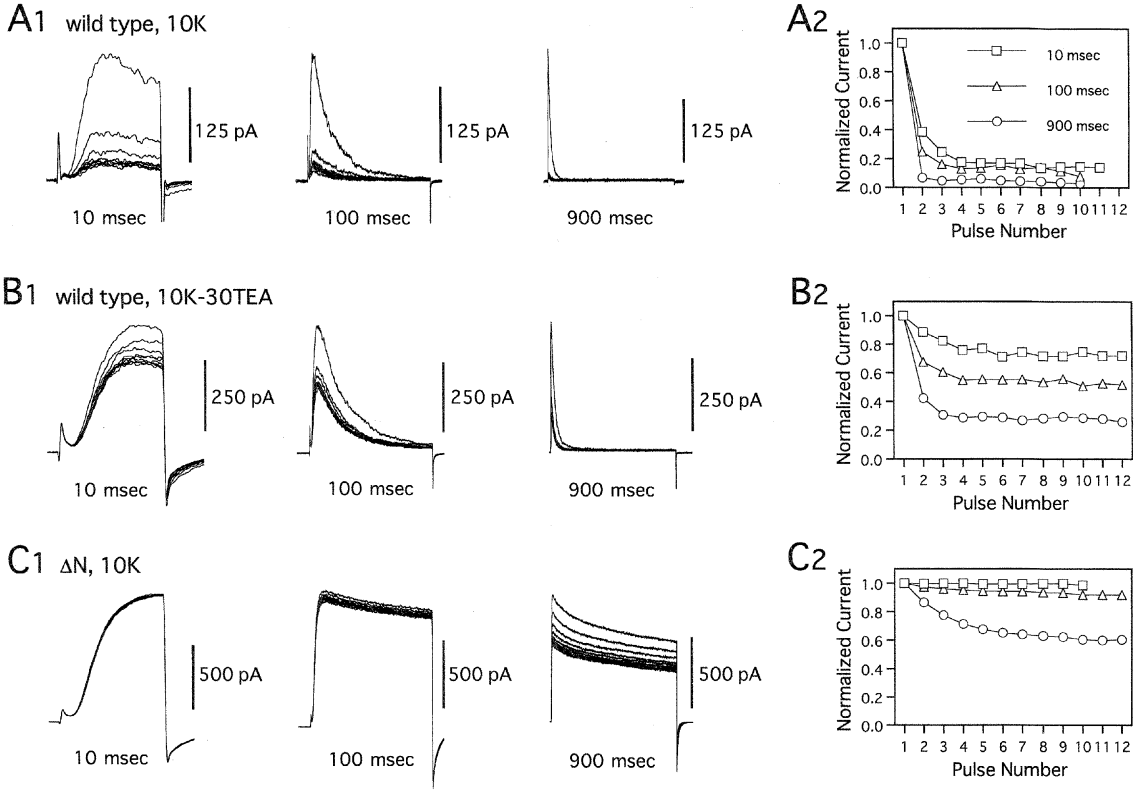

Fig. 1. The accumulative inactivation and its dependence on the pulse duration. (**A1**) AKv1.1a currents recorded by a pipette filled with 10K solution under the cell-attached condition. Holding potential was -80 mV, and the patched membrane was depolarized to +40 mV at a frequency of 0.5 Hz. In each column, 10 consecutive current traces are superimposed. The pulse duration were 10, 100 and 900 msec as indicated. (**A2**) Normalized peak currents of the data shown in **A1** plotted against the pulse number. A peak current was normalized to the peak amplitude of the first current. Different symbols indicate the data obtained by different pulse duration as shown. (**B1**) AKv1.1a currents recorded by a pipette filled with 10K-30TEA solution under the cell-attached condition. Pulse protocols were the same to **A1**. (**B2**) Normalized peak currents of the data shown in **B1** plotted against the pulse number. Meaning of each symbol is the same as in **A2**. (**C1**) ΔN currents recorded by a pipette filled with 10K solution under the cell-attached condition. Pulse protocols were the same to **A1**. (**C2**) Normalized peak currents of the data shown in **C1** plotted against the pulse number. Meaning of each symbol is the same as in **A2**.

were applied at a frequency of 0.5 Hz (Fig. 1A), 80-90% of the activatable channels in a patch was unable to open in response to 10th pulse because they entered the inactivated state(s) from which a recovery was quite slow. Figure 1A2 shows a time course of the development of accumulative inactivation by plotting the peak currents against the pulse number. Although there was a tendency that the steady state level of the accumulative inactivation was larger for the longer pulse, the difference was not so striking (Fig. 1A, see also Fig. 2). The accumulative inactivation was not affected by the pulse voltage in the range of 0 to +60 mV when the repetitive pulses of 10-90 msec were applied at 0.5 Hz in 10 mM $[K^+]_{out}$ (n = 3, data not shown).

In some K^+ channels (Grissmer and Cahalan, 1989; Choi *et al.*, 1991), external TEA is known to inhibit C-type inactivation. Thus, we next examined the effect of external TEA on the accumulative inactivation of AKv1.1a. Macroscopic inactivation and amplitude of AKv1.1a currents recorded by a pipette filled with 10K-30TEA solution were quite similar to those obtained without TEA. External TEA, however, greatly inhibited the accumulative inactivation (Fig. 1B). In the presence of TEA, less than 30% of activatable AKv1.1a was inactivated by repetitive pulses of 10 msec duration at 0.5 Hz. The accumulative inactivation in the presence of external TEA was more evident when longer pulses were applied (compare Fig. 1A2 and B2). The results suggest that C-type inactivation is likely to be involved in the accumulative inactivation of AKv1.1a.

N-type inactivation of K^+ channels can be removed by the amino-terminal deletion of the channels (Hoshi *et al.*, 1990). In such deletion mutants, isolated C-type inactivation can be examined (Hoshi *et al.*, 1991; López-Barneo *et al.*, 1993). In ΔN, the fast N-type inactivation of AKv1.1a is completely absent (Furukawa *et al.*, 1995), and the remaining inactivation (C-type inactivation) which was observed during a long depolarizing pulse was quite slow and variable. For the selected patches in which the ΔN currents showed substantial inactivation during a long pulse (0.8-4.0 sec) to +40 mV, the currents decayed with a time constant of 2.46 ± 0.34 sec (mean ± SE, n = 11). A macroscopic inactivation of ΔN during a long pulse had no consistent dependency on $[K^+]_{out}$. ΔN showed much less accumulative inactivation compared to the wild type

channels (Fig. 1C). Actually, if the pulse duration was less than several tens of msec, more than 70% of the activatable channels could be repetitively activated even at the frequency of 3 Hz in some patches. By longer pulses, ΔN did show a marked accumulative inactivation which developed slowly (Fig. 1C).

Effects of $[K^+]_{out}$ on the accumulative inactivation

We next examined the effect of pulse duration on the accumulative inactivation in different $[K^+]_{out}$. The recording pipettes were filled with 10K or 100K solution, and the currents in response to repetitive pulses were recorded as in Fig. 1. To quantify the accumulative inactivation, the membrane currents in response to repetitive pulses (0.5 Hz) were measured under the cell-attached condition until the currents reached a steady level. Currents at the steady level were normalized to the amplitude of the first currents, and plotted against the pulse duration (Fig. 2). In Fig. 2, the results obtained by 10K-30TEA filled pipettes are also shown. The accumulative inactivation of AKv1.1a showed little dependency on the pulse duration when $[K^+]_{out}$ was 10 mM: i.e., about 80-90% of the channels could not recover during the pulse-interval irrespective of the pulse duration (squares in Fig. 2A). When recording pipettes were filled with 100K solution, the accumulative inactivation of AKv1.1a was greatly inhibited. In 100 mM $[K^+]_{out}$, the accumulative inactivation for short repetitive pulses (100 msec or less) was so small that >80% of the channels were activatable (circles in Fig. 2A). By repetitive pulses of >100 msec in duration, however, the accumulative inactivation was clearly seen even in 100 mM $[K^+]_{out}$ and it was dependent on the pulse length (Fig. 2A). When the external solution contained 30 mM TEA, the accumulative inactivation was also inhibited in all the tested pulse durations (triangles in Fig. 2A).

The accumulative inactivation of ΔN was always dependent on the pulse duration (Fig. 2B). In 10 mM $[K^+]_{out}$, percentage of the inactivated channels by repetitive pulses was changed in a range of <5-70% depending on the pulse duration (squares in Fig. 2B). High $[K^+]_{out}$ inhibited the accumulative inactivation of ΔN although the effect was less drastic compared to the results in AKv1.1a (circles in Fig. 2B). Although a macroscopic inactivation of ΔN was not noticeably modified in the presence of TEA, external TEA clearly inhibited the accumulative inactivation of ΔN (triangles in Fig. 2B). The result suggests that external TEA inhibits C-type inactivation of AKv1.1a.

The accumulative inactivation by the pair-pulse protocol

In response to two identical depolarizing pulses which are too short to induce much inactivation (<20 msec), the current at second pulse is decreased by 40-60% compared to the current at the end of first pulse when the pulse interval is 30-60 msec (Furukawa, 1995). The phenomenon is not at all observed in ΔN (Furukawa, 1995). In a rat K^+ channel (Kv3), however, the inactivation depends solely on C-type mechanism (Marom and Levitan, 1994). The accumulative inactivation of Kv3 induced by the pair-pulse protocol occurs with a delay of >100 msec (see Fig. 1 of Marom and Levitan, 1994). Although we used longer inter-pulse interval (up to 900 msec), the

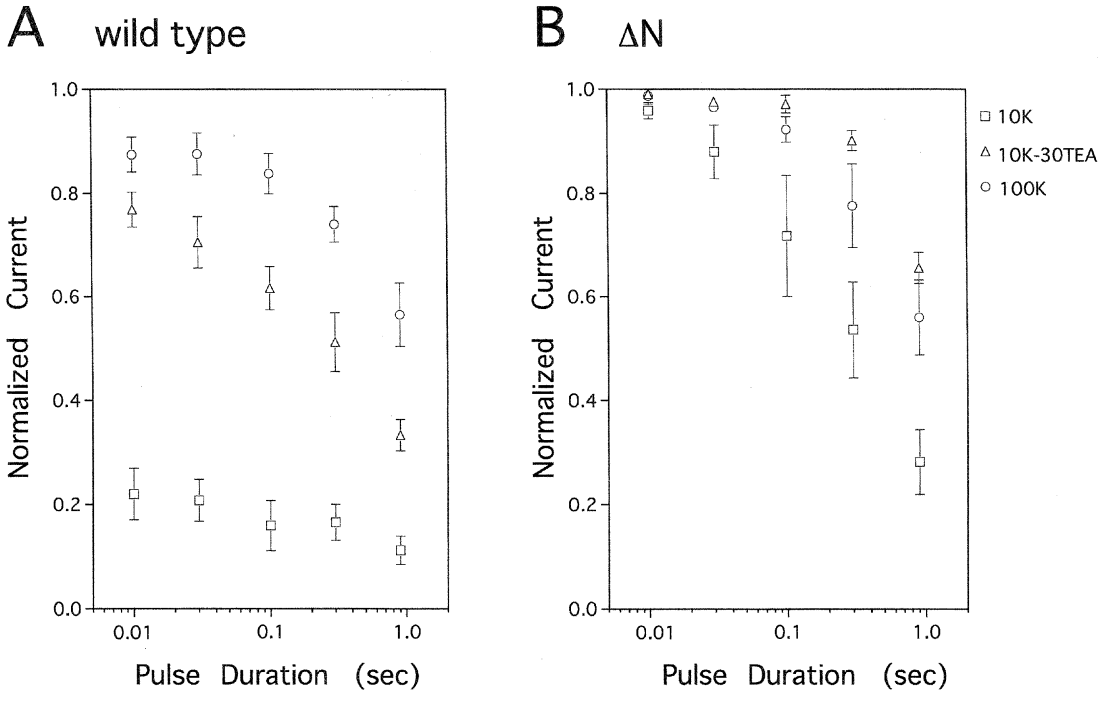

Fig. 2. Effects of $[K^+]_{out}$ and external TEA on the accumulative inactivation of AKv1.1a (**A**) and ΔN (**B**). The accumulative inactivation was quantified as described in the text, and the obtained normalized currents were plotted against the pulse duration. Holding potential was -80 mV, and the patched membrane was depolarized to +40 mV at a frequency of 0.5 Hz. Each symbol is a mean value of 3-5 different patches, and an error bar indicates SE of the mean. Recording pipettes were filled with 10K, 10K-30TEA, or 100K solution, as indicated in **B**.

accumulative inactivation was still not observed (n = 3, data not shown).

We also extended the pair-pulse experiments by using a longer first pulse during which the channels would inactivate by C-type mechanism. Figure 3A shows typical examples of such experiments in ΔN as well as in AKv1.1a. A 20 msec pulse to +40 mV was applied with a variable inter-pulse interval at -80 mV following a pulse to +40 mV of a variable duration (1P in Fig. 3A). In ΔN, no accumulative inactivation was detected irrespective of the duration of the first pulse in the range of 10-1000 msec (Fig. 3A1). In AKv1.1a, a clear accumulative inactivation was observed following the first pulse of 10 msec duration (Fig. 3A2). Even following a 2 msec pulse during which the current was rarely activated, the current at second pulse became smaller (Fig. 3A2). In the experiment of Fig. 3A2, the current elicited by second pulse following the 2 msec pulse with a 5 msec interval (the largest one in 1P = 2 msec) was actually 89% of the current which was activated without the first pulse (not shown). By contrast, the accumulative inactivation was less drastic if the first pulse was long enough to inactivate most of the channels in the patch (Fig. 3A2, 1P = 100 msec). Actually, a recovery from the inactivation was evident in this case. As described above, external TEA inhibited the accumulative inactivation of AKv1.1a by repetitive pulses. We, therefore, examined effects of external TEA on the accumulative inactivation by the pair-pulse protocol (Fig. 3B). Two identical depolarizing pulses (+40 mV, 10 msec) were applied with a variable inter-pulse interval. Membrane potential during the pulse interval was also changed. The accumulative inactivation of AKv1.1a by the pair-pulse protocol was not inhibited by external TEA at all. Extent of the accumulative inactivation was quantified by normalizing the peak current at second pulse to the current at the end of first pulse. Normalized minimum currents at different inter-pulse potentials were as follows (mean ± SE, n = 5): 0.398 ± 0.027 (-40 mV); 0.483 ± 0.036 (-80 mV); 0.583 ± 0.030 (-120 mV). These values were comparable to those obtained without TEA: 0.408 ± 0.035 (-40 mV); 0.474 ± 0.041 (-80 mV); 0.674 ± 0.036 (-120 mV).

Effects of patch-excision on the accumulative inactivation

We next examined the effects of patch-excision on the accumulative inactivation of AKv1.1a. The patch excision is known to enhance the accumulative inactivation in Kv3 (Marom

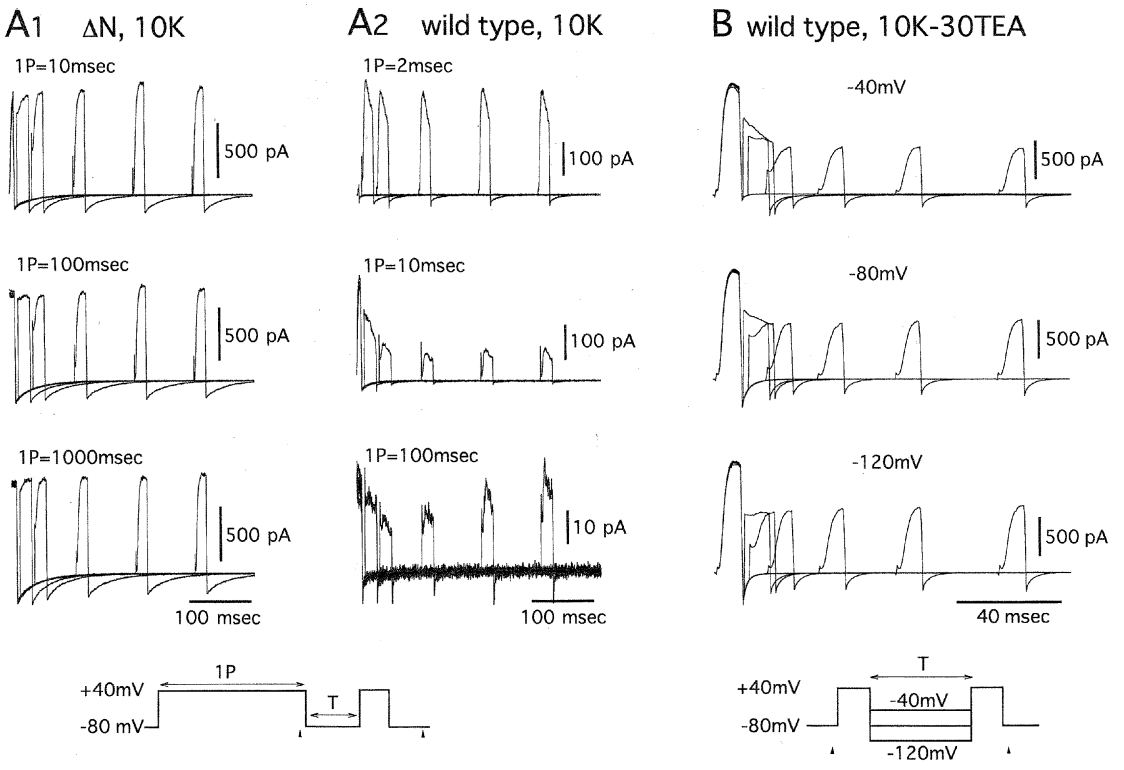

Fig. 3. The accumulative inactivation examined by the pair-pulse protocol. **A**: Superimposed currents of ΔN (**A1**) or AKv1.1a (**A2**), which were elicited by a 20 msec pulse to +40 mV following the first pulse of variable duration to +40 mV. The pulse protocol is shown underneath. Holding potential as well as potential during the pulse-interval were -80 mV. The currents displayed in each column start from the last 9 msec of the first current (a period between arrow heads in the pulse protocol) except for the top row in A2. Duration of the first pulse (1P) was as indicated. The pulse interval was 5, 30, 100, 200 or 300 msec. All measurements were carried out by 10K-filled pipettes in the cell-attached mode. (**B**) Superimposed currents of AKv1.1a which were elicited by a 10 msec pulse to +40 mV with a variable interval (T) at three different potentials. The pulse protocol is shown underneath. Holding potential was -80 mV. Membrane potential during a pulse-interval was as indicated. The currents displayed are those between arrow heads in the pulse protocol. The pulse interval was 1, 3, 10, 30, 60 or 100 msec. Measurements were carried out by 10K-30TEA filled pipettes in the cell-attached mode.

et al., 1993), while it promotes a recovery from the accumulative inactivation in another rat K^+ channel, RCK4 (Ruppersberg *et al.*, 1991). When AKv1.1a in the cell-attached patch was activated by 10 msec pulses to +40 mV at 0.5 Hz in 10 mM $[K^+]_{out}$, about 40-50% of the channels entered the inactivated states (Fig. 4A1). After the membrane patch was excised out of the cell, the same repetitive pulses induced a much larger accumulative inactivation, resulting in hibernation of nearly 80% of the channels in the patch (Fig. 4A1, B). Similar results were obtained when longer pulses were used (Fig. 4A2, B). Another noticeable change was that the macroscopic inactivation became slower in the inside-out condition (compare, for example, largest currents in Fig. 4A1 or A2). The issue will be described separately later.

The patch excision had similar but much less effect on ΔN. In the patch shown in Fig. 5, the ΔN currents activated by 30 msec pulses to +40 mV at 0.5 Hz in 10 mM $[K^+]_{out}$ were decreased by about 10% in the cell-attached mode, while they were decreased by about 20% in the inside-out (Fig. 5A1). The accumulative inactivation by 300 msec pulses was much larger but again not enhanced so much after the patch excision (Fig. 5A2): the currents before and after the patch excision were decreased by about 45% and 60%, respectively.

Because external TEA and high $[K^+]_{out}$ appeared to inhibit C-type inactivation (Fig. 2B), the effects of patch-excision on the accumulative inactivation were examined in the presence of external TEA or in high $[K^+]_{out}$. Figure 6 shows the results obtained by 10K-30TEA filled pipettes. The accumulative inactivation was quantified as described in Fig. 2. Even when the accumulative inactivation of AKv1.1a in the cell attached condition was highly depressed by external TEA, the patch excision promoted a dramatic enhancement of the accumulative inactivation (Fig. 6A). When similar experiments were repeated in ΔN, the patch excision had no effect if the pulse duration was less than a few hundreds of milliseconds (Fig. 6B). The accumulative inactivation of ΔN was increased slightly after the patch excision, however, when longer repetitive pulses were used (Fig. 6B). In 100 mM $[K^+]_{out}$, the accumulative inactivation of AKv1.1a was still enhanced by the patch excision: normalized steady levels of the currents induced by repetitive pulses of 100 msec at 0.5 Hz in cell-attached and inside-out patches were 0.838 ± 0.039 and 0.623 ± 0.026, respectively (n = 5).

Redox modification of N-type inactivation and its influence on the accumulative inactivation

When the AKv1.1a currents were elicited by depolarizing pulses at 0.05 Hz in the cell-attached condition, they were very stable (Fig. 7A1, B). Following the patch excision and concomitant exposure of an intracellular lumen of the patched membrane to KSIS, the macroscopic inactivation was gradually inhibited (Fig. 7A2) and the charge transferred through the

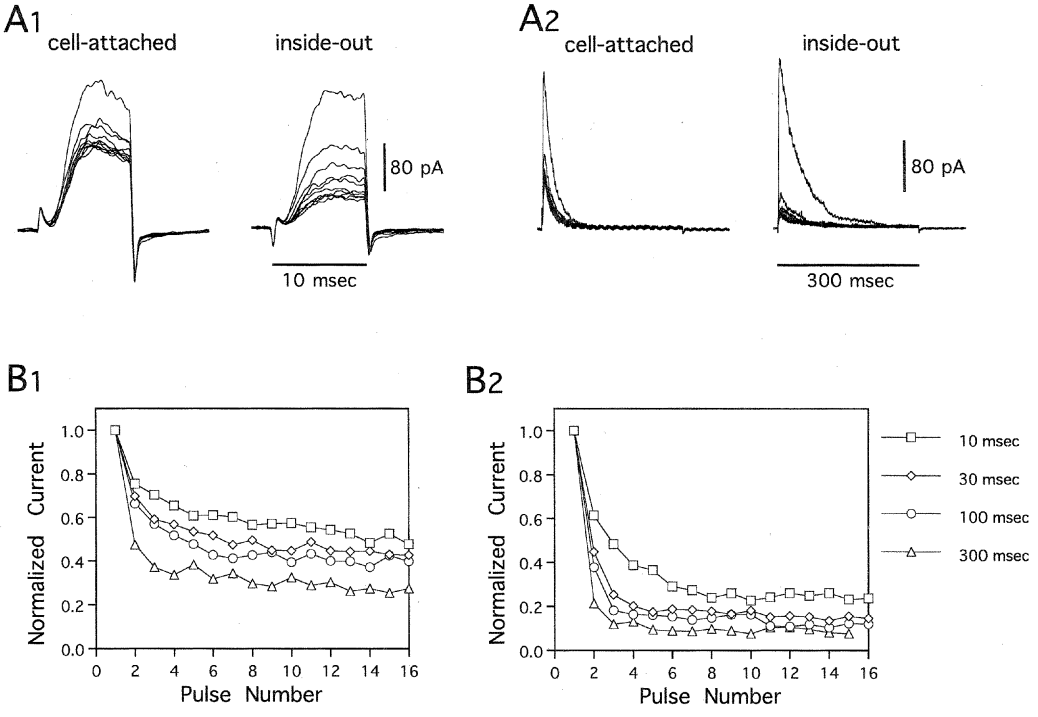

Fig. 4. Modification of the accumulative inactivation of AKv1.1a following the patch excision. **A**: Currents in response to repetitive pulses to +40 mV at a frequency of 0.5 Hz in the cell-attached or in the inside-out condition. 10 consecutive currents are superimposed in each column. Holding potential was -80 mV, and the pulse duration was either 10 msec (**A1**) or 300 msec (**A2**). Recording pipette was filled with 10K solution. **B**: Normalized peak currents plotted against the pulse number. A peak current was normalized to the peak amplitude of the first current. **B1** is a data from the cell-attached condition, whereas **B2** is from the inside-out condition. Different symbols indicate the data obtained by different pulse duration as shown. The data in **A** and **B** are from the same patch.

activated channels during each pulse was increased (Fig. 7B). Similar modification of RCK4 has been shown to result from the oxidation of cysteine residues of the channels which may block movements of the N-terminal ball by S-S bonds (Ruppersberg *et al.*, 1991). We, therefore, examined the effects of redox agents on the inactivation of AKv1.1a in the inside-out patch. After confirming reduction of the inactivation rate of AKv1.1a in the inside-out recording configuration, the tip of the pipette was moved through an air-gate into a chamber containing 500 μM TCEP. The macroscopic inactivation recovered quickly following the internal application of TCEP (Fig. 7A3, B), and the recovered currents were indistinguishable from the currents recorded in the cell-attached condition. The effect of TCEP was lost if an intracellular side of the patch was exposed again to TCEP-free KSIS (Fig. 7A4, B). Following the application of DTNB, the macroscopic inactivation of AKv1.1a became even slower in some patches (data not shown). These results suggest that N-type inactivation of AKv1.1a is also modified by redox state of the channel.

We next examined whether the accumulative inactivation of AKv1.1a in the inside out condition was modified by redox agents (Fig. 8). When depolarizing pulses were applied at 0.1 Hz in 10 mM $[K^+]_{out}$, there was no accumulative inactivation in the cell-attached patch (Fig. 8A1,2). After the patch excision, however, the channel currents showed a marked accumulative inactivation in response to the same repetitive pulses (Fig. 8B2) as well as the inhibition of N-type inactivation (Fig. 8B1). Internal application of 500 μM TCEP mostly restored N-type inactivation in response to a single pulse (Fig. 8C1), but had little effect on the enhanced accumulative inactivation (Fig. 8C2). DTNB was also ineffective on the accumulative inactivation in the inside-out patch. TCEP and DTNB had little effect on the inactivation of ΔN (data not shown).

Open-channel blockade by the N-terminal peptides simulates the inactivation

The results described so far as well as the previous ones (Furukawa, 1995) suggest that N-type inactivation is an essential step for accumulation of the inactivation in AKv1.1a. Because an essence of N-type inactivation is a pore block by a "N-terminal inactivation ball" (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990), it is interesting to test whether the accumulative inactivation of AKv1.1a is reproduced in ΔN via blockade by the N-terminal peptides of K^+ channels. Figure 9 shows the blockade of AKv1.1a by a N-terminal peptide of the *Drosophila Shaker* channel, ShB-p. Following the application of the peptide, the peak currents decreased due to the open channel blockade (an exceptionally slow onset of the block in this particular patch is most probably due to the formation of the patched membrane deep inside the pipette). After the peak currents diminished to about 30% of the control, the

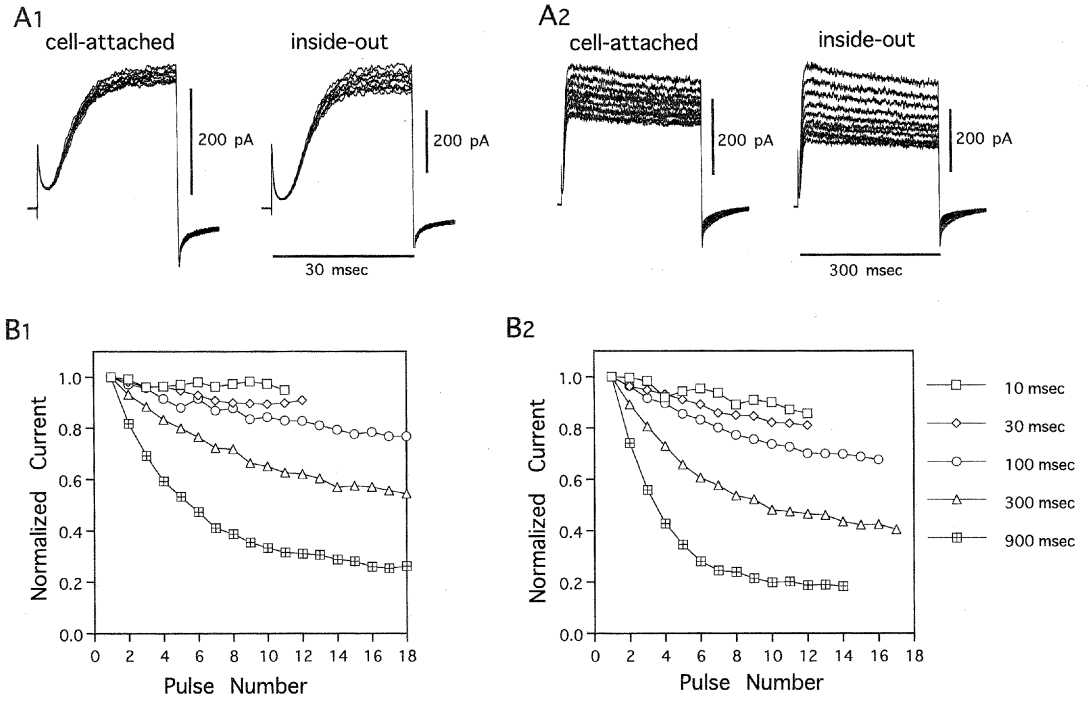

Fig. 5. Modification of the accumulative inactivation of ΔN following the patch excision. **A**: Currents in response to repetitive pulses to +40 mV at a frequency of 0.5 Hz in the cell-attached or in the inside-out condition. 10 consecutive currents are superimposed in each column. Holding potential was -80 mV, and the pulse duration was either 30 msec (**A1**) or 300 msec (**A2**). Recording pipette was filled with 10K solution. Note different vertical calibrations for the currents. Following the patch excision, the amplitude of ΔN currents was in most cases more enhanced compared to AKv1.1a currents. **B**: Normalized peak currents plotted against the pulse number. A peak current was normalized to the peak amplitude of the first current. **B1** is a data from the cell-attached condition, whereas **B2** is from the inside-out condition. Different symbols indicate the data obtained by different pulse duration as shown. The data in **A** and **B** are from the same patch.

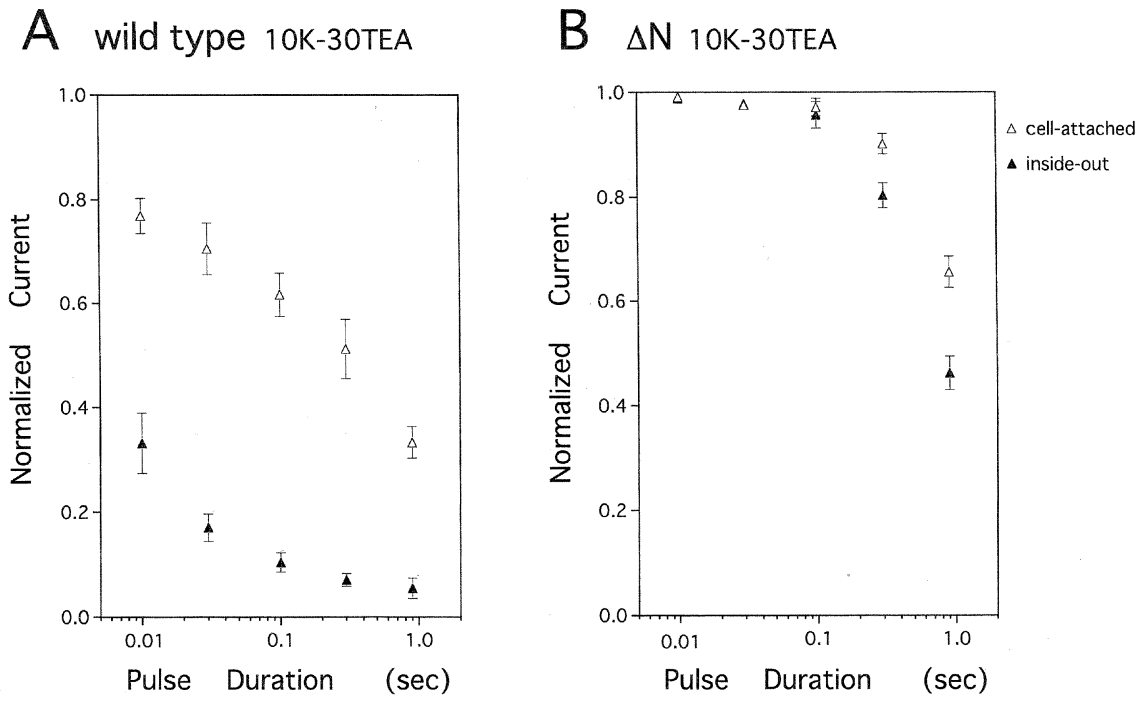

Fig. 6. Effects of the patch excision on the accumulative inactivation of AKv1.1a and ΔN in the presence of external TEA. The currents in response to repetitive pulses to +40 mV at a frequency of 0.5 Hz were measured under both the cell-attached and the inside-out conditions until the currents reached a steady level. Holding potential was -80 mV. Currents at the steady level were normalized and mean values were plotted as described in the legend of Fig. 2. Open and closed symbols indicate the results obtained in the cell-attached and the inside-out patches, respectively. Each symbol is a mean value of 5 different patches, and an error bar indicates SE of the mean.

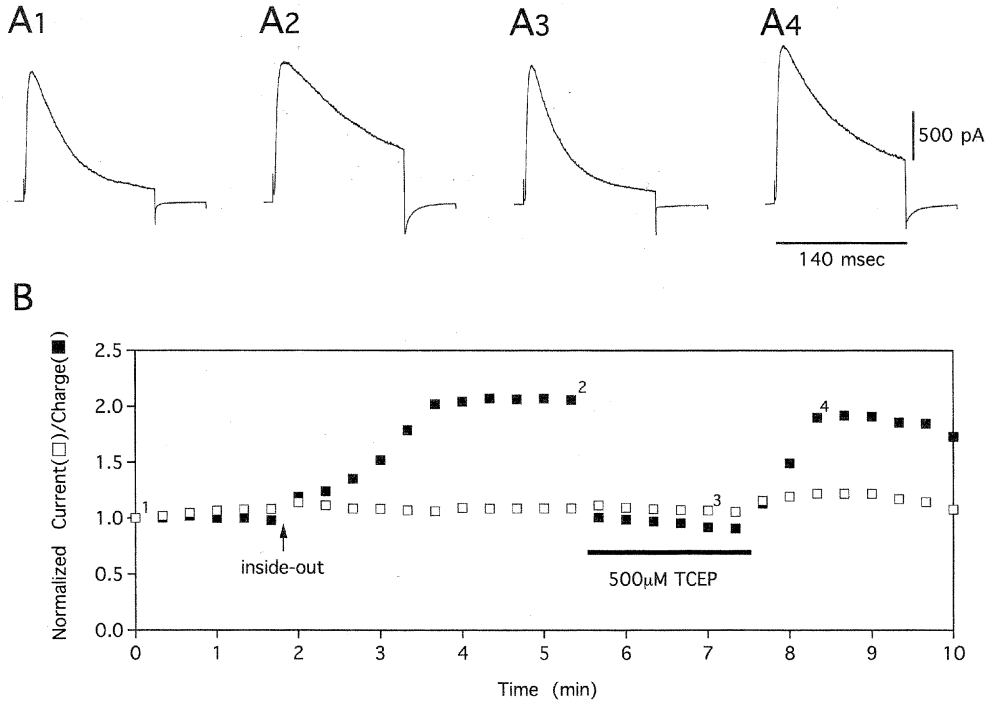

Fig. 7. Redox modification of the macroscopic inactivation of AKv1.1a. Currents in response to 140 msec pulse to +40 mV from the holding potential of -80 mV were elicited at a frequency of 0.05 Hz. Recording pipette was filled with 10K solution. **A**: Currents obtained at time points indicated in **B**. (**A1**) The current before the patch excision. (**A2**) The current in the inside-out condition. (**A3**) The current during internal application of 500 μM TCEP. (**A4**) The current after washing out of TCEP. (**B**) Time course of the change in peak current and time integral of the current (charge) following the patch excision and internal application of TCEP. Numbers near symbols indicate that the currents at the time points are shown in **A**.

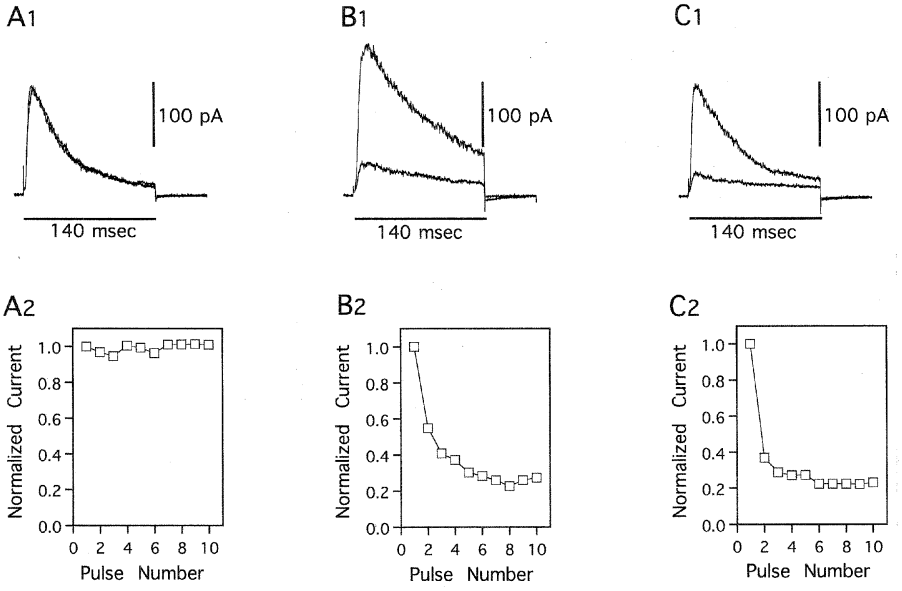

Fig. 8. Enhanced accumulative inactivation of AKv1.1a in the excised patch is not restored by internal application of TCEP. Currents in response to 140 msec pulse to +40 mV from the holding potential of -80 mV were elicited at a frequency of 0.1 Hz. Recording pipette was filled with 10K solution. The first and tenth currents are superimposed in **A1**, **B1** and **C1**. The peak currents were normalized as described in the legends of Fig. 1, and plotted against the pulse number (**A2**, **B2**, **C2**). **A**: The data obtained before the patch excision. **B**: The data obtained in the inside-out condition. **C**: The data obtained during internal application of 500 μM TCEP.

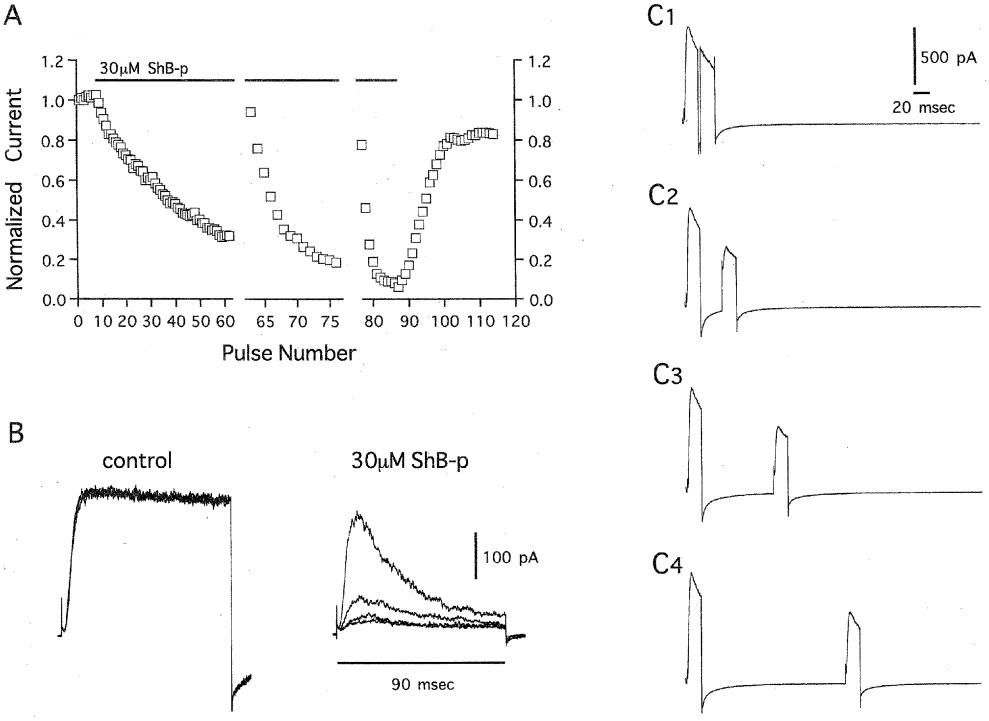

Fig. 9. Effects of ShB-p on ΔN. (**A**) ShB-p induced block of ΔN. ΔN currents in response to 30 msec pulse to +40 mV were elicited at a frequency of 0.25 Hz in the inside-out condition. Recording pipette was filled with 10K solution. Peak currents are plotted against the pulse number. 30 μM ShB-p was internally applied during a period indicated by the bar. Two breaks in the figure indicate resting periods (2 min each) during which no pulse was applied. (**B**) Use-dependent block by ShB-p. ΔN currents in response to four 90 msec pulses to +40 mV at a frequency of 0.2 Hz are superimposed. Recording pipette was filled with 30K solution. The currents were obtained with (30 μM ShB-p) or without (control) internal ShB-p. (**C**) ShB-p induced block examined by the pair-pulse protocol. ΔN currents were obtained in the inside-out patch with 30 μM internal ShB-p. Recording pipette was filled with 10K solution. Two identical pulses (20 msec, +40 mV) were applied with a variable interval. Holding potential and potential during the pulse interval were -80 mV. The pulse interval was as follows (in msec): 3 (**C1**); 30 (**C2**); 100 (**C3**); 200 (**C4**).

depolarizing pulses were stopped temporarily, and then the same repetitive pulses were applied again. The resting period permitted unblock of the channels, and the peak current immediately after the rest recovered to nearly 90% of the control. The currents elicited again by the repetitive pulses became smaller and smaller, showing a use-dependent block by ShB-p. The channel currents showed almost full recovery after washing out of the peptide (Fig. 9A). In Fig. 9B, the currents in response to the repetitive pulses at 0.2 Hz, with or without internal ShB-p, were superimposed. While there was no accumulative inactivation in control, ShB-p induced a clear time-dependent block in response to the first pulse, and the peak currents became smaller during succeeding pulses (Fig. 9B). The use dependent block of ΔN currents by ShB-p was quite reminiscent of the macroscopic inactivation and its accumulation observed in AKv1.1a. Figure 9C shows the pair-pulse experiments in ΔN with internal ShB-p. When the pulse-interval was as short as 3 msec, the current at the beginning of the second pulse was almost identical to the current level at the end of the first pulse (Fig. 9C1). For longer inter-pulse interval, however, the peak current at the second pulse became smaller than the current at the end of the first pulse, suggesting that the channel block by ShB-p proceeds during the pulse interval (Fig. 9C2,C3,C4). ShC-p had qualitatively similar blocking actions on ΔN (data not shown).

We, next, tried to simulate the inactivation of AKv1.1a by probing ΔN with the N-terminal peptides of AKv1.1a itself. This, however, was not successful. AK20 had no effect on the ΔN currents even at the concentration of 100 μM (data not shown). We also examined shorter peptides (AK18 and AK10) but they were also ineffective. Murrell-Lagnado and Aldrich (1993a,b) have shown that nonpolar residues within ShB-p are important for stable block and that the net positive charge of the peptide enhance its effectiveness by the electrostatic interactions. We therefor examined two mutant peptides, AK10EQ and AK18EQ. AK10EQ has no net charge in physiological pH, while AK18EQ has a net positive charge (Arg^{18}). The ΔN currents were not affected by 50 μM AK10EQ as well as 100 μM AK18 (Fig. 10A). By contrast, 50 μM AK18EQ induced a clear depression of the ΔN currents immediately following the application. AK18EQ showed a clear use-dependent block (compare Fig. 10B1 and B2). AK18EQ induced block of ΔN also simulated the accumulative inactivation by the pair-pulse (Fig. 10C). These results indicate that negatively charged amino acids in the peptides hampered the blocking potency of AK18 (and probably of AK20). Further experiments are

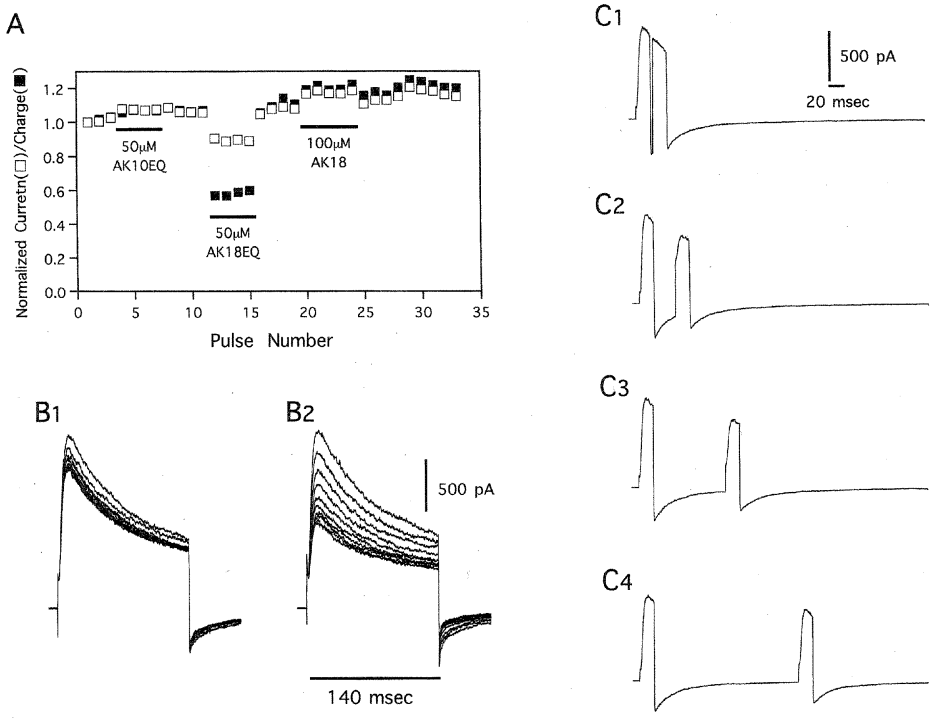

Fig. 10. Blocking effects of synthetic peptides derived from the N-terminal sequence of AKv1.1a. (**A**) Effects of AK10EQ, AK18EQ and AK18 on ΔN. ΔN currents in response to 140 msec pulse to +40 mV were elicited at a frequency of 0.1 Hz in the inside-out condition, and the peak currents as well as time integral of the elicited current (charge) were plotted against the pulse number. Recording pipette was filled with 30K solution. Synthetic peptides were internally applied during periods indicated by bars. (**B**) Use-dependent block by AK18EQ. ΔN currents in response to 140 msec pulse to +40 mV were elicited at a frequency of 0.1 Hz (**B1**) or 0.5 Hz (**B2**) in the inside-out condition with 50 μM internal AK18EQ. In each column, ten consecutive currents are superimposed. (**C**) AK18EQ induced block examined by the pair-pulse protocol. ΔN currents were obtained in the inside-out patch with 50 μM internal AK18EQ. Recording pipette was filled with 10K solution. Two identical pulses (20 msec, +40 mV) were applied with a variable interval. Holding potential and potential during the pulse interval were -80 mV. The pulse interval was as follows (in msec): 3 (**C1**); 30 (**C2**); 100 (**C3**); 200 (**C4**).

required to locate the "ball domain" of AKv1.1a.

DISCUSSION

The present study focused on the accumulative nature of the inactivation of AKv1.1a which shows a relatively fast macroscopic inactivation due to N-type inactivation (Furukawa *et al*., 1995). In the previous study (Furukawa, 1995), essential features of the inactivation of AKv1.1a have been documented based on the cell-attached patch recording. Among them, the accumulative nature of the inactivation by repetitive pulses of low frequency and by the pair-pulse protocol are distinct feature of the channel.

The accumulative inactivation of AKv1.1a was not so much dependent on the pulse duration that the steady level of the peak current obtained by the repetitive pulses at 0.5 Hz was about 10-20% of the initial peak current irrespective of the pulse duration (10-900 msec). Similar results were obtained at lower repetitive frequencies. In spite of the removal of N-type inactivation, ΔN do shows the accumulative inactivation by repetitive pulses although longer pulse duration and larger number of pulses were required to obtain a steady level of the accumulative inactivation. These results suggest that both N-type and C-type inactivation of the channels can contribute to the accumulative inactivation of AKv1.1a. In physiological conditions (in low $[K^+]_{out}$ and the short depolarization comparable to an action potential), however, the pure C-type inactivation is less likely to contribute to the process.

Hoshi and his colleagues have shown that C-type inactivation in a wild type *Drosophila Shaker* channel (ShB) is apparently faster than C-type inactivation of the N-terminal deletion mutant (ShB-ΔN), suggesting that C-type inactivation is partially coupled to N-type inactivation (Hoshi *et al*., 1991). The importance of the coupling in the accumulative inactivation of *Shaker* channels has recently been documented by Baukrowitz and Yellen (1995). They have shown that the slowly inactivating component of the *Shaker* channels is much faster in lower $[K^+]_{out}$, and have postulated an attractive hypothesis in which a K^+-binding site situated in outer mouth of the pore controls C-type inactivation: i.e., C-type inactivation does not proceed until K^+ on the site is removed (Baukrowitz and Yellen, 1995). In their model, C-type inactivation coupled to N-type inactivation is much faster and more stable in lower $[K^+]_{out}$ because N-type inactivation stops K^+-efflux through the open channels and the site has more chance to be unoccupied. Inhibitory effect of external TEA on C-type inactivation is also explained by binding of TEA to the site.

Although a slowly decaying component which reflects the interaction of N-type and C-type inactivation in ShB (Hoshi *et al*., 1991; Baukrowitz and Yellen, 1995) is not obvious in AKv1.1a, some evidences obtained so far may reflect the presence of such interaction in AKv1.1a. (1) There is a distinct slow component in recovery from the inactivation of AKv1.1a (Furukawa, 1995). (2) External TEA which is known to inhibit C-type inactivation (Grissmer and Cahalan, 1989; Choi *et al*., 1991) disturbed the accumulative inactivation of AKv1.1a as well as that of ΔN. (3) In high $[K^+]_{out}$ which enhances recovery from N-type inactivation (Demo and Yellen, 1993; Gómez-Lagunas and Armstrong, 1994; Furukawa, 1995) and from the block by ShB peptide (Murrell-Lagnado and Aldrich, 1993b), the accumulative inactivation of AKv1.1a was diminished such that the remaining accumulative inactivation was almost identical to the accumulative inactivation of ΔN (see Fig. 2). The effect of external TEA on the accumulative inactivation of AKv1.1a is easily explained by the hypothesis of Baukrowitz and Yellen (1995) because C-type inactivation is an immediate cause of the accumulative inactivation in the hypothesis. The results in high $[K^+]_{out}$ are also expected from the hypothesis since such high $[K^+]_{out}$ would saturate the K^+-binding site controlling C-type inactivation irrespective of N-type inactivation. Use-dependent block of ΔN by N-terminal peptides may also be explained in part by the hypothesis because some open channel blockers promote C-type inactivation of ShB-ΔN (Baukrowitz and Yellen, 1996). A presumed interaction of N-type and C-type inactivation in AKv1.1a, however, should not substantially contribute to the macroscopic inactivation during a single pulse, because high $[K^+]_{out}$ as well as external TEA do not modify the kinetics of the current decay (this study; Furukawa, 1995),.

On the other hand, ΔN showed no indication of the accumulative inactivation when the pair-pulse protocol was employed. The accumulative inactivation of AKv1.1a examined by the pair-pulse protocol was also not affected by external TEA. As described previously (Furukawa, 1995), the accumulative inactivation by the pair-pulse protocol is also not sensitive to $[K^+]_{out}$. These results suggest that the accumulative inactivation examined by the pair-pulse protocol is dominated by N-type inactivation. A presumed interaction of N-type and C-type inactivation in AKv1.1a may be a much slower process compared to the onset of N-type inactivation.

After the patch excision, the accumulative inactivation of AKv1.1a was dramatically enhanced. Even in the presence of external TEA, substantial enhancement of the accumulative inactivation was observed in AKv1.1a following the patch excision. On the other hand, the accumulative inactivation of ΔN was less sensitive to the patch excision. Kinetics of ΔN currents during depolarizing pulses in the inside-out recording mode was also not different from those in the cell attached mode. Because the TCEP or DTNB treatment did not affect the modified accumulative inactivation in the inside-out patch, redox state of the channel is not likely to be involved in the enhanced accumulative inactivation of AKv1.1a in the inside-out condition. Although some results may favor an idea that some modification of N-type inactivation following the patch excision (ex., slowed recovery from N-type inactivation) enhances the accumulative inactivation, further studies are required to elucidate the mechanisms. In a rat K^+ channel, Kv3, the accumulative inactivation by repetitive pulses of short duration at low frequency becomes apparent only after the patch excision (Marom *et al*., 1993; Marom and Levitan, 1994). Although a precise mechanism is also not known in Kv3, His^{401} which situates in the pore region of Kv3 is found to play an

essential role for enhancement of the accumulative inactivation after the patch excision (Kupper *et al.*, 1995).

Accumulative nature of the inactivation of K^+ channels should play important roles for their functions in neuronal excitability and cellular signaling. The principle determinant for the accumulative property appears to be different among K^+ channels. The interaction between N-type and C-type inactivation appears to play substantial roles for AKv1.1a as well as for *Drosophila* ShB channels to induce the accumulative inactivation under physiologically relevant conditions, while C-type inactivation can take every aspect of the inactivation in Kv3. The situation in endogenous voltage-gated K^+ channels may be more complex because N-type inactivation can be added even to delayed rectifier type K^+ channels by the association of β-subunits (Rettig *et al.*, 1994). To better understand molecular mechanisms underlying accumulation of the inactivation, specific domains in the channel proteins necessary for N-type and C-type inactivation as well as for their interactions should be located by future mutagenesis experiments.

ACKNOWLEDGMENTS

The authors thank Dr. T. Kubo for the generous gift of pSPAK01 and pSPAK01ΔN. The authors also thank Prof. Y. Muneoka for reading early version of the manuscript. This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to YF (No.06740630, No.07740647, No.08640865). YF was also supported by Nissan Science Foundation.

REFERENCES

Armstrong CM, Bezanilla F (1977) Inactivation of the sodium channel II. Gating current experiments. J Gen Physiol 70: 567–590

Baukrowitz T, Yellen G (1995) Modulation of K^+ current by frequency and external $[K^+]$: a tale of two inactivation mechanisms. Neuron 15: 951–960

Baukrowitz T, Yellen G (1996) Use-dependent blockers and exit rate of the last ion from the multi-ion pore of a K^+ channel. Science 271: 653–656

Choi KL, Aldrich RW, Yellen G (1991) Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K^+ channels. Proc Natl Acad Sci USA 88: 5092–5095

Demo SD, Yellen G (1991) The inactivation gate of the *Shaker* K^+ channel behaves like an open-channel blocker. Neuron 7: 743–753

Furukawa Y, Kim HN, Kubo T (1995) Up- and down-modulation of a cloned *Aplysia* K^+ channel (AKv1.1a) by the activators of protein kinase C. Zool Sci 12: 35–44

Furukawa Y (1995) Accumulation of inactivation in a cloned transient K^+ channel (AKv1.1a) of *Aplysia*. J Neurophysiol 74: 1248–1257

Gómez-Lagunas F, Armstrong CM (1994) The relation between ion permeation and recovery from inactivation of ShakerB K^+ channels. Biophys J 67: 1806–1815

Gozlan H, Diabira D, Chinestra P, Ben-Ari Y (1994) Anoxic LTP is mediated by the redox modulatory site of the NMDA receptor. J Neurophysiol 72: 3017–3022

Grissmer S, Cahalan M (1989) TEA prevents inactivation while blocking open K^+ channels in human T lymphocytes. Biophys J 55: 203–206

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391: 85–100

Hoshi T, Zagotta WN, Aldrich RW (1990) Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. Science 250: 533–538

Hoshi T, Zagotta WN, Aldrich RW (1991) Two types of inactivation in *Shaker* K^+ channels: effects of alterations in the carboxy-terminal region. Neuron 7: 547–556

Jan LY, Jan YN (1992) Structural elements involved in specific K^+ channel functions. Annu Rev Physiol 54: 537–555

Kaang BK, Pfaffinger PJ, Grant SGN, Kandel ER, Furukawa Y (1992) Overexpression of an *Aplysia* Shaker K^+ channel gene modifies the electrical properties and synaptic efficacy of identified *Aplysia* neurons. Proc Natl Acad Sci USA 89: 1133–1137

Kupper J, Bowlby MR, Marom S, Levitan IB (1995) Intracellular and extracellular amino acids that influence C-type inactivation and its modulation in a voltage-dependent potassium channel. Pflugers Arch 430: 1–11

Liu Y, Jurman ME, Yellen G (1996) Dynamic rearrangement of the outer mouth of a K^+ channel during gating. Neuron 16: 859–867

López-Barneo J, Hoshi T, Heinemann SH, Aldrich RW (1993) Effects of external cations and mutations in the pore region on C-type inactivation of *Shaker* potassium channels. Receptors and Channels 1: 61–71

Marom S, Goldstein SAN, Kupper J, Levitan IB (1993) Mechanism and modulation of inactivation of the Kv3 potassium channel. Receptors and Channels 1: 81–88

Marom S, Levitan IB (1994) State-dependent inactivation of the Kv3 potassium channel. Biophys J 67: 579–589

Murrell-Lagnado RD, Aldrich RW (1993a) Interactions of amino terminal domains of *Shaker* K channels with a pore blocking site studied with synthetic peptides. J Gen Physiol 102: 949–975

Murrell-Lagnado RD, Aldrich RW (1993b) Energetics of *Shaker* K channels block by inactivation peptides. J Gen Physiol 102: 977–1003

Pfaffinger PJ, Furukawa Y, Zhao B, Dugan D, Kandel ER (1991) Cloning and expression of an *Aplysia* K^+ channel and comparison with native *Aplysia* K^+ currents. J Neuroscience 11: 918–927

Pongs O (1992) Molecular biology of voltage-dependent potassium channels. Physiological Reviews 72: S69–S88

Qin D, Noma A (1988) A new oil-gate concentration jump technique applied to inside-out patch-clamp recording. Am J Physiol 255: H980–H984

Rettig J, Heinemann SH, Wunder F, Lorra C, Parcej DN, Dolly JO, Pongs O (1994) Inactivation properties of voltage-gated K^+ channels altered by presence of β-subunit. Nature 369: 289–294

Rudy B (1988) Diversity and ubiquity of K channels. Neuroscience 25: 729–749

Ruppersberg JP, Stocker M, Pongs O, Heinemann SH, Frank R, Koenen M (1991) Regulation of fast inactivation of cloned mammalian $I_K(A)$ channels by cysteine oxidation. Nature 352: 711–714

Yellen G, Sodickson D, Chen TY, Jurman ME (1994) An engineered cysteine in the external mouth of a K^+ channel allows inactivation to be modulated by metal binding. Biophys J 66: 1068–1075

Zagotta WN, Hoshi T, Aldrich RW (1990) Restoration of inactivation in mutants of *Shaker* potassium channels by a peptide derived from ShB. Science 250: 568–571

(Received January 16, 1997 / Accepted March 4, 1997)