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Up- and Down-Modulation of a Cloned *Aplysia* K^+ Channel (AKv1.1a) by the Activators of Protein Kinase C

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ABSTRACT—Modulation of a cloned *Aplysia* K^+ channel, AKv1.1a, by protein kinase C (PKC) activators was examined in *Xenopus* oocytes expression system. Following the application of phorbol esters (phorbol 12-myristate 13-acetate, PMA; phorbol 12,13-dibutyrate, PDBu), or a diacylgrycerol analogue (1-oleoyl-2-acetyl-sn-glycerol, OAG), the fast inactivation of the AKv1.1a became slower and the peak current increased (up-modulation). However, the effect was transient. The expressed current was decreased even below control level about 15 to 20 min after the treatment (down-modulation). Both effects by PMA was blocked by the kinase inhibitor, H7, suggesting that phosphorylation by PKC is involved. The amino acid sequence of AKv1.1a contains three putative phosphorylation sites by PKC (Ser^{24}, Thr^{345}, Ser^{349}). We tested their contributions to the PMA-induced modulation by site-directed mutagenesis. The results suggest that the up-modulation by PKC activators is due to the inhibition of the fast inactivation by the amino-terminal domain (N-type inactivation), thereby increase the time the channels are conductive. Phosphorylation of Ser^{24} may enhance the PKC-induced down-modulation, while phosphorylation of Thr^{345} may inhibit the down-modulation. By contrast, mutation of Ser^{349} did not affect the modulation. The N-type inactivation were not indispensable for the down-modulation because the amino-terminal deletion mutant also showed some down-modulation although its onset was quite slow. Thus, the down-modulation of AKv1.1a may be heterogeneous. Because some modulation was still observed even in a mutant which lacks all putative phosphorylation sites mentioned above, additional mechanisms such as the regulation by other phosphorylated protein(s) exist endogenously in oocytes and/or recruitment of other kinases by PKC-activation may also be involved in the observed modulation of the AKv1.1a.

INTRODUCTION

The gating properties of voltage-dependent ion channels are not fixed but can be modulated by neurotransmitters. K^+ channels are well known target for such modulation. The resultant change in activities of K^+ channels can determine excitabilities of cells and the properties of neuronal signaling [10, 25]. In the central nervous systems of some marine molluscs (*Aplysia* and *Hermissenda*), modulation of K^+ channels can modify signaling for more than one day, and therefore contribute to the neuronal mechanisms underlying short- and long-term memory [2, 6, 17, 19, 27]. A similar mechanism may be operative in certain region of the mammalian brain [26]. Thus, molecular mechanisms underlying the modulation of K^+ channels are likely to be important for understanding the higher order function of the central nervous system.

There are many pharmacological evidences suggesting that neurotransmitters can modulate the function of ion channels via phosphorylation [10, 30]. However, few studies have focused on phosphorylation sites and their functional meanings. The cloning of many K^+ channels now opens the way to probe the function of the channels directly by a site-directed mutagenesis and reconstitution. Modulation of cloned ion channels by protein kinase C (PKC) are shown in a Na^+ channel [31] and various K^+ channels [4, 20, 23]. In the Na^+ channel [31] and the slowly activating K^+ channel [4], a site-directed mutagenesis successfully located an amino acid residue which is involved in the observed modulation.

In *Aplysia* nervous system, one of the K^+ currents that appears to be involved in a short-term presynaptic facilitation is a transient component of delayed outward currents (I_{Kv}) [3], which resembles the transient K^+ current, I_{Adepol} [7]. A cloned *Aplysia* K^+ channel, AKv1.1a express a transient K^+ current which most closely resembles I_{Adepol} [16, 24] (AKv1.1a was originally named as AK01a, see Shen *et al.* [28]). Because I_{Kv} as well as I_{Adepol} is known to be modulated by activators of PKC [29] (Furukawa, unpublished observation), we are interested in whether AKv1.1a is modulated by PKC activators. In the present study, the modulation of AKv1.1a by PKC activators was investigated using *Xenopus* oocytes as an expression system, and the contribution of putative phosphorylation sites located in the amino acid sequence of AKv1.1a was examined by site-directed mutagenesis.

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MATERIALS AND METHODS

Construction of AKv1.1a and mutant cDNAs

The DNA fragment containing the coding region of AKv1.1a

was obtained by recombinant polymerase chain reaction (PCR) as described [9]. The cloned DNA encoding AKv1.1a [24] was used as template in the following two primary PCRs. One was the PCR between the primers SalI-S;
5′-CGCGTCGACAAACCCTGTATTGTTAGTTCT-3′ [correspond to nucleotide residues (nt) -241— -221 in AKv1.1a with a SalI sequence at the 5′ end], and
5′-CGTTTCGAAGTGGGTCGTAAT-3′ (nt 302-322; a mutation to generate a BstBI restriction site without a change of translation is underlined). The other was between the primers 5′-CACTTCGAAACGAGTATTTCTTTGA-3′ (nt 311–335) and BamHI-A; 5′-CGCGGATCCAATTCTGAGATCCTCTTCAC-3′ (nt 1669-1688 with a BamHI sequence at the 5′ end). Amplification was performed for 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min with GeneAmp PCR system 9600 (Perkin-Elmer). After removal of the primers, two overlapping fragments of 560 bp and 1.38 kb from each reaction were mixed and the secondary PCR was conducted between the primers SalI-S and BamHI-A described above. Amplification condition was the same as above, except that the reaction at 72°C was held for 3 min in the last 10 cycles. After digestion with SalI and BamHI, the resulting 1.94-kb fragment was subcloned into pSP64(polyA) vector (Promega) to yield the pSPAK01.

The mutants S24A (amino-acid substitution from serine to alanine at the residue 24 in AKv1.1a) and R26Q were constructed by the procedures of recombinant PCR described above. In the primary PCR, the pSPAK01 and the following sense/antisense primer pairs were used: S24A, SalI-S/S24A-A (5′-GGCCGTTGGGCTGAATGG-3′, nt 63–80) and S24A-S (5′-ACCATTCAGCCCAACGG-3′, nt 62–78)/SmaI-A (nt 164–181); R26Q, SalI-S/R26Q-A (5′-AAAAGTGGCTGTTGGGATGA-3′, nt 67–86) and R26Q-S (5′-CCCAACAGCCACTTTTACGC-3′, nt 71–90)/SmaI-A. Recombinant PCR was performed using the primer pair SalI-S/SmaI-A for both mutant constructions, followed by digestion with SalI and SmaI. The resulting 417-bp SalI/SmaI fragment harboring the S24A or R26Q mutation in the AKv1.1a coding region was ligated with the 1.52-kb SmaI (nt 172)/SmaI (on vector) fragment of the pSPAK01 and the SmaI-SalI fragment of the pSP64(polyA).

For the N-terminal deletion construct (ΔN), DNA fragment was amplified by PCR from the pSPAK01 using the primers PstI-S (5′-CGCCTGCAGAAACCCTGTATTGTTAGTTCT-3′, nt -241— -221 with a PstI sequence at the 5′ end) and ΔN-A (5′-CACAGCTGCAGTCCATTTTACTGCC-3′,
nt -9–3 and 184–196), followed by digestion with PstI. The resulting 252-bp fragment was ligated with the 4.53-kb fragment of PstI-digested pSPAK01.

The rest of the site-directed mutagenesis were performed using the pSELECT mutagenesis system (Promega). The 1.99-kb SalI/EcoRI fragment of the pSPAK01, which contains AKv1.1a coding sequence and polyA, was subcloned into the pSELECT (Promega) to yield the pSELAK01. All procedures were as described in the manual (Promega, Cat. No. Q6210). Antisense mutagenic oligonucleotides were as follows:
T345A-A, 5′-GGCTTTGAGGGCCTGGCCTA-3′ (nt 1025–1044);
S349A-A, 5′-AGCGGCTTTGAGGGTCTGGCCTA-3′ (nt 1025–1047);
S349C-A, 5′-CTCGCATACAGGCTTTGAGG-3′ (nt 1035–1054);
S349P-A, 5′-CTCGCATAGGGGCTTTGAGGG-3′ (nt 1034–1054);
R351Q-A, 5′-CCCAGTTCTTGCATACTGGC-3′ (nt 1042–1061);
T345A·S349A-A: 5′-AGCGGCTTTGAGGGCCTGGCCTA-3′ (nt 1025–1047). To obtain triple mutants, the 440-bp SalI (on vector)/PstI (nt 191) fragment from the pSPAK01(S24A) and the 1.56-kb PstI (nt 191)/EcoRI (on vector) fragment from the pSELAK01(T345A) were ligated with the 5.7-kb EcoRI/SalI fragment of pSELECT to yield the pSELAK01(S24A·T345A). Single-stranded DNA rescued from the plasmid was used as template and the site-directed mutagenesis at the third position was performed using the antisense mutagenic oligonucleotide S349P-A or R351Q-A in the pSELECT mutagenesis system.

All the region amplified by PCR and the site-directed mutagenized region were sequenced for confirmation using the Sequenase kit (US Biochemicals). Two or more independent clones were isolated for each construct and were found to have essentially the same electrophysiological phenotype.

in vitro RNA synthesis

The plasmids containing cDNA encoding wild-type or mutant channels were linerlized by EcoRI to serve as template. The transcripts were synthesized and capped *in vitro* by SP6 RNA polymerase using the MEGAscript kit (Ambion). The resulting cRNA was shown by electrophoresis on 1.5% agarose gel to be homogeneous and to have expected size of~2.0 kb (~1.8 kb for ΔN).

Electrical measurements

Xenopus oocytes were prepared, injected with appropriate cRNA, and voltage-clamped as described previously [24]. The injection volume was fixed to 50 nl and the concentration of cRNA was determined empirically as described in Results. External solution for electrical measurements was ND-96 having a following composition (mM): NaCl 96.0, KCl 2.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 5.0, pH 7.5. Gating parameters of the expressed channels were examined as described previously [16, 24]. The peak conductance was calculated by dividing the peak current measured at each test potential, with the driving force (E_{rev} = −83 mV was used; [24]). The relationship between the resultant conductances and the membrane potentials was approximated by a Boltzmann function to estimate the midpoint ($V_{1/2}$) and the slope (k). To examine the steady state inactivation of channels, the peak current at +20, +30 or +40 mV following a two second conditioning pulse of variable amplitudes was measured. The currents were plotted against the conditioning voltages, and the relationship was fitted with a Boltzmann function. Recovery from the inactivation was examined by a double-pulse protocol. Channels were inactivated by a prepulse of 200 msec to +20 or +30 mV, and a second pulse to the same voltage was applied following hyperpolarization to −80 mV of variable duration. The relationship between the peak current at the second pulse and the pulse interval was fitted with an exponential function having two time constants (τ1 and τ2). Onset of the inactivation was also examined by a double-pulse protocol. A test pulse to +20 or +30 mV was applied 10 msec after a prepulse to −5 mV of variable duration. The relationship between the peak current at the test pulse and the duration of the prepulse was fitted with a single exponential having a time constant (τ). Most results were expressed as mean±SD and the statistical significance was examined by a one-way analysis of variance (ANOVA) and a Q-test. All measurements were done at room temperature (23–25°C).

All drugs were dissolved in ND-96 and applied by bath perfusion. Following drugs were used: phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 4α-phorbol, 1-oleoyl-2-acetyl-sn-glycerol (OAG), H7-dihydrochloride (H7). OAG and phorbol esters were obtained from Sigma. H7 was obtained from Seikagaku (Japan). Phorbol esters were dissolved in dimethyl sulfo-

xide (DMSO) as a 1 mM stock and H7 was dissolved in distilled water as a 10 mM stock. Both of them were stored in freezer (−20°C), and the stock solutions were diluted with ND-96 just before use. A final concentration of DMSO was less than 0.1% and had no effect on the membrane current.

RESULTS

Aplysia Shaker potassium channel (AKv1.1a) express a fast transient potassium current which inactivates almost completely by a time constant of around 20 to 30 msec in *Xenopus* oocytes as well as in *Aplysia* neurones [16, 24]. When a relatively large amount of cRNA of AKv1.1a was injected, however, the expressed current showed very slowly inactivating components (i.e. substantial currents can still flow even at the end of one second command pulse, data not shown). Similar observations were reported in other K^+ channel clones expressed in *Xenopus* oocytes and human B-lymphocyte cell line [12, 18] although an exact mechanism to produce such heterogeneous phenotypes from a single species of cRNA is still not known. To circumvent such problems, we first diluted each cRNA (wild-type as well as its mutants) to determine the appropriate amount of injection for each clones until the macroscopic inactivation rate approached to an asymptotic value. Based on the results, oocytes were injected with 0.3 to 30 ng of cRNA depending on the species of cRNA in the following experiments.

Modulation of the AKv1.1a current by the activators of protein kinase C

To determine whether protein kinase C (PKC) modulates the function of AKv1.1a, we examined the effects of several activators of PKC, phorbol esters (PMA and PDBu) and a synthetic diacylgrycerol (OAG), on the AKv1.1a currents expressed in oocytes. Figure 1 shows a dose-dependent modulation of AKv1.1a by PMA. Following a treatment with PMA, the inactivation of the AKv1.1a current became slower, and the current amplitude was enhanced (Fig. 1A), which resulted in a drastic increase of the amount of charge transferred by channel opening during a test pulse. However, the effect was transient and the current was decreased later even below the control level (Fig. 1B). During this late depression of the current, the inactivation rate was still slow (see also Fig. 5A). Thus, the AKv1.1a current was both up- and down-modulated by PMA. The onset of up-modulation was usually 2 to 3 min after the start of PMA treatment, while the onset of down-modulation was 5 to 10 min after the treatment. PMA had no prominent effect on the membrane currents of non-injected oocytes. Because of the extremely slow recovery from inactivation of AKv1.1a [24] and the time-dependent development of down-modulation, it was practically impossible to obtain a current-voltage relationship for modified channels. In a few oocytes in which the onset of the down-modulation was slow, however, a current-voltage relationship and a steady state inactivation of modified channels could be obtained. In such

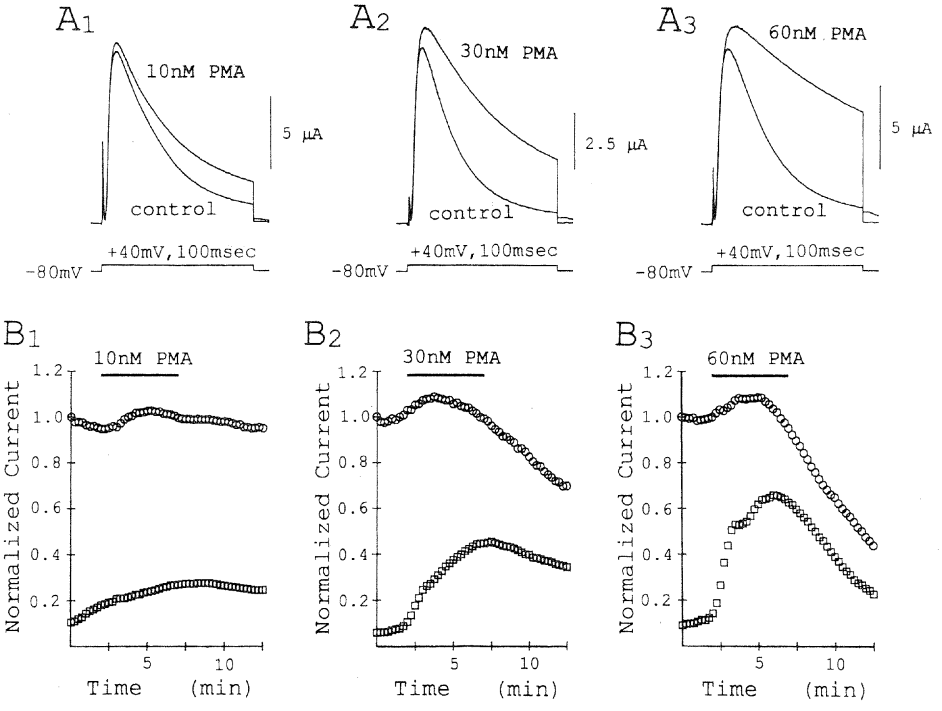

FIG. 1. Effects of PMA on the AKv1.1a currents. A: The AKv1.1a currents before and 3 to 4 min after onset of the PMA application. The AKv1.1a currents were obtained by 100 msec pulses to +40 mV every 30 sec. PMA was applied externally for five minutes. B: Time dependent changes of the AKv1.1a currents by the PMA treatment. The currents measured at the peak (circle) and at the end of 100 msec pulse (square) are plotted against time.

cases, there was essentially no difference in measured parameters before and after the PMA treatment (data not shown).

The effects of PMA were somewhat variable among oocytes from different donors. To circumvent such variation, any comparison of the effect of given drugs was done on oocytes from the same donor. Another phorbol ester, PDBu, and a synthetic diacyl grycerol, OAG, induced similar modulation. Modulation of the AKv1.1a current by the activators of PKC was quantified by comparing a time integral of the current (charge) before and after the drug treatment. A maximum increase of the charge by PKC activators was as follows (n=4): 62.4±18.0% (60 nM PMA), 32.3±11.2% (60 nM PDBu) and 54.3±21.3% (100 μM OAG). An inactive phorbol ester, 4α-phorbol, had no effect even at 1 μM (n=3).

The modulatory effects of PMA was depressed by a kinase inhibitor, H7. In control, 100 nM PMA increased the charge transferred by 68.2±13.9% (n=5), whereas the increase was 16.9±13.5% (n=5) after 30 to 60 min pretreatment with 100 μM H7. The result suggests that PKC-mediated phosphorylation of AKv1.1a and/or its regulatory protein(s) exist in oocytes is necessary for the modulation observed.

Effects of PMA on an amino-terminal deletion mutant of AKv1.1a

In a *Drosophila* Shaker K^+ channel, the amino-terminal domain is necessary for its fast inactivation (N-type inactivation) [13]. Thus, we first made an amino-terminal deletion mutant (ΔN, second to 61th amino acids were removed), and the effects of such deletion on the inactivation of AKv1.1a and the PMA-induced modulation were examined. As expected from a highly homologous amino-acid sequence and kinetic properties between AKv1.1a and *Drosophila* Shaker channel [24], the N-type inactivation of AKv1.1a was also disrupted by the amino-terminal deletion (Fig. 2A). The ΔN current did not show any accumulating inactivation which is a prominent feature of AKv1.1a [24]. As might be expected from the complete lack of the N-type inactivation, PMA did not increase the ΔN current. The result suggests that the inactivation machinery present in the amino-terminal domain of AKv1.1a is necessary for the up-modulation we described above. The ΔN current usually show little change until 15 to 20 min after the application of PMA. After that, however, the ΔN current was clearly decreased (Fig. 2B,C). This extremely slow onset of the current decrease was not due to a general run-down of the current because the current was stable if PMA was not applied (Fig. 2A,C). On average, the ΔN current became 89.6±12.8% of the control 15 min after the onset of PMA treatment, while the current was decreased to 58.6±15.7% 30 min after the treatment (n=6).

Kinetic properties of phosphorylation mutants

There are multiple putative phosphorylation sites by protein kinase C (PKC) in the amino-acid sequence of AKv1.1a. Figure 3 illustrates such sites, which were located by a consensus sequence (Thr/Ser-Xaa-Arg/Lys) [1, 32]. This figure also shows mutations we will describe later. One of the putative phosphorylation sites, Ser^{24} is in the cytoplasmic domain near the amino-terminal. Two other sites,

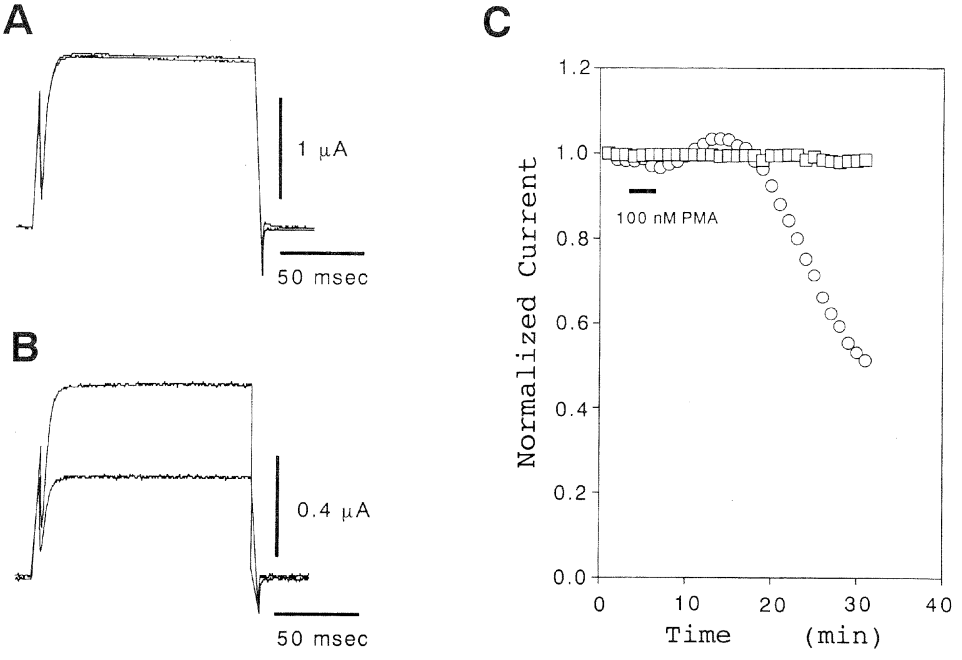

FIG. 2. Effects of PMA on the ΔN currents. Membrane currents were elicited by depolarizing command pulses to +40 mV from a holding potential of −80 mV every one minutes, and PMA was applied externally for 3 min. A: the ΔN currents at first and 30th trials without PMA. B: the ΔN currents before (1st) and after the PMA treatment (30th). The current was depressed to about 50 %. C: Time dependent changes of ΔN currents with (circle) or without (square) the application of PMA. Note a very slow onset of the effect of PMA compared to Figure 1.

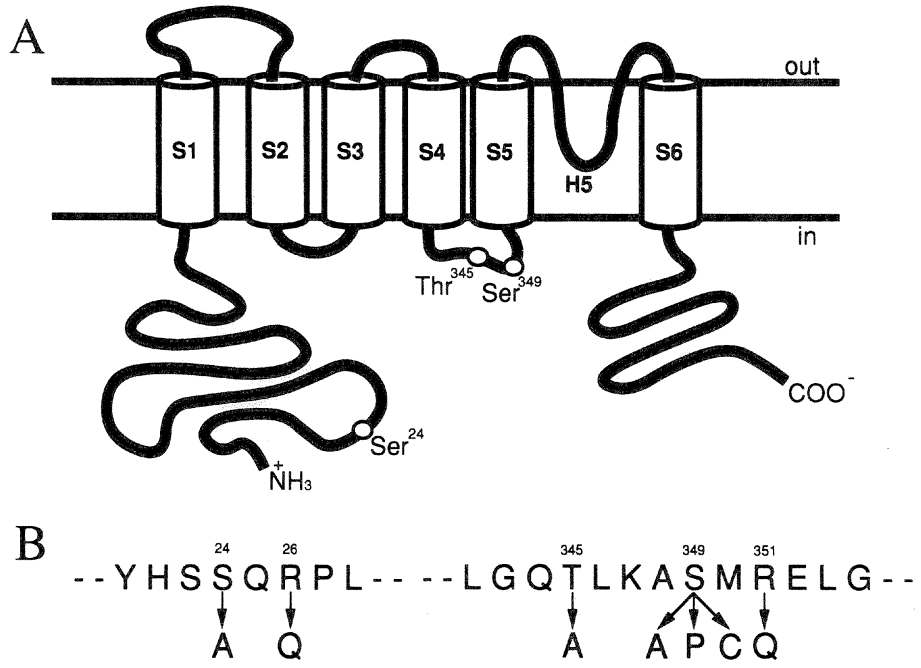

FIG. 3. Schematic drawing of a presumed membrane topology of the AKv1.1a and potential phosphorylation sites by protein kinase C (PKC). A: Location of potential phosphorylation sites by PKC in the amino acid sequence of AKv1.1a. S1-S6, putative transmembrane segments. H5, hydrophobic segment which is hypothesized to form a part of pore of the channel. B: Amino-acid sequences surrounding to the putative phosphorylation sites and the substitutions examined are shown by one-letter code.

Thr^{345} and Ser^{349}, are located in a cytoplasmic linker between putative transmembrane segments, S4 and S5. In homologous K^+ channels of *Drosophila*, the linker between S4 and S5 as well as the amino-terminal domain have been shown to be involved in the inactivation of the channel [13, 15, 33]. Thus, we next tried to examine the contribution of such putative phosphorylation sites by site-directed mutagenesis. For this purpose, we mutated a corresponding amino acid residue to alanine, cystein or proline to make mutants, S24A, T345A, S349A, S349C, S349P (see Fig. 3B). To disrupt the consensus sequence around Ser^{24} and Ser^{349}, we made other mutants (R26Q and R351Q) in which a basic amino acid (arginine) was changed to glutamine (Fig. 3B). We also made double and triple mutants as T345A·S349A,

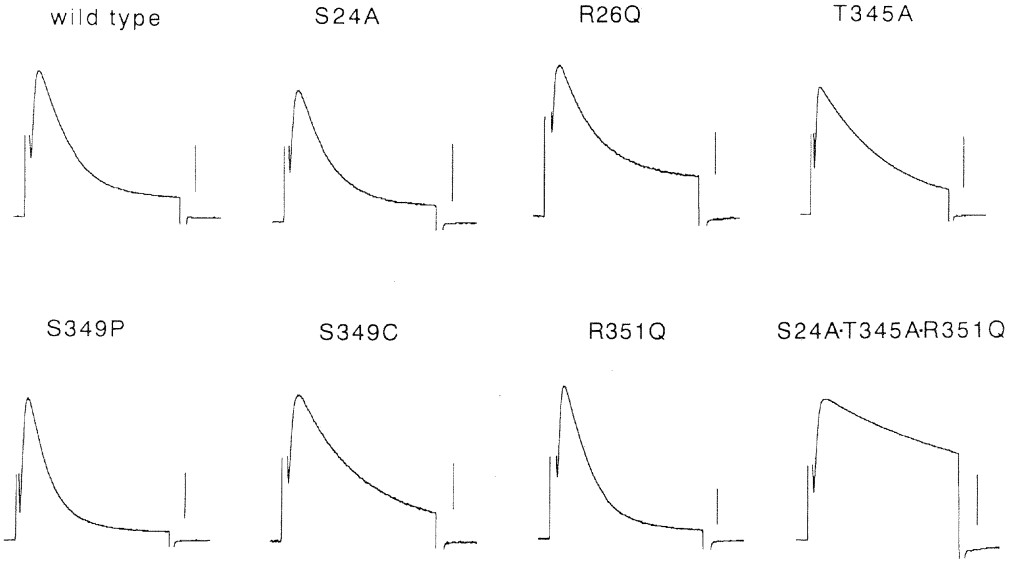

FIG. 4. Membrane currents of oocytes expressed AKv1.1a or its phosphorylation site mutants. Each current was elicited by a depolarizing pulse to +40 mV from a holding potential of −80 mV. Horizontal calibration is 50 msec, and vertical calibration is 1 μA (wild type, S24A, S349P, S349C, R351Q, S24A·T345A·R351Q), 2 μA (T345A) or 400 nA (R26Q).

TABLE 1. Comparison of kinetic parameters among AKv1.1a and its mutants

	Activation		Inactivation					
					Recovery		Onset	
	$V_{1/2}$ mV	k mV	$V_{1/2}$ mV	k mV	τ_1 sec	τ_2 sec	τ sec	N
AKv1.1a	5.2±2.0	−12.4±0.8	−19.1±0.9	2.1±0.2	0.479±0.211	5.476±0.961	0.108±0.055	4
S24A	6.2±2.2	−12.7±0.2	−17.6±2.1	2.3±0.4	0.621±0.319	6.523±3.211	0.205±0.212	5
R26Q	3.9±3.4	−16.6±2.2*	−17.4±1.5	2.2±0.1	0.712±0.214	4.658±0.651	0.156±0.071	4
T345A	7.8±3.7	−15.4±2.8	−16.8±3.9	2.5±0.2	0.216±0.048	2.142±0.430*	0.257±0.068	4
S349C	−4.2±2.1**	−13.4±1.0	−26.1±0.8**	2.8±0.2*	1.802±0.158**	4.837±0.467	0.108±0.017	3
S349P	8.5±2.4	−11.3±0.8	−18.6±1.5	2.8±0.2	0.440±0.223	6.720±1.780	0.108±0.060	3
R351Q	3.9±0.6	−9.6±0.6	−18.2±1.9	2.2±0.1	0.881±0.710	3.447±0.797	0.103±0.034	3
S24A·T345A·R351Q	0.5±1.8	−14.2±2.0	−23.1±2.3	2.6±0.2*	0.402±0.196	4.613±1.992	0.276±0.063	5

All data are mean±SD for N oocytes (number of tested oocytes for recovery of S24A and S24A·T345A·R351Q were 3, and that for onset and recovery of T345A were 5). Significantly different values compared to AKv1.1a are indicated by asterisk. *; $P<0.05$, **; $P<0.01$.

S24A·T345A·S349P, and S24A·T345A·R351Q. The expression of each mutant was examined in oocytes from at least three different donors. All mutants except three mutants (S349A, T345A·S349A, and S24A·T345A·S349P) expressed functional channels in *Xenopus* oocytes.

Figure 4 illustrates the membrane currents of oocytes injected with mutant-cRNAs or the wild type AKv1.1a-cRNA. All of them expressed the inactivating K^+ currents, although the inactivation rate was clearly different in some mutants. Table 1. compares some kinetic parameters of the mutants with those of the wild-type. In most cases, there was no significant difference in the parameters examined. However, the midpoints for the activation and inactivation of S349C were shifted to more hyperpolarized potentials, and early recovery from the inactivation of S349C was slower than others. Because the decay time constant of the expressed current was very sensitive to the amount of expressed channels, the parameter was not included in Table 1. However, the time constants (in msec) at +40 mV of T345A (68.2± 23.3, n=4), S349C (48.1±7.0, n=3), and S24A·T345A·R351Q (62.0±11.6, n=5) were consistently larger than those of other mutants as well as the wild-type. Similar mutation in a cytoplasmic linker between S4 and S5 of a *Drosophila* Shaker K^+ channel has been shown to affect the inactivation [15].

Effects of PMA on phosphorylation mutants

Effects of PMA was routinely tested as follows. The expressed currents were recorded every 60 sec by depolarizing test pulses to +30 or +40 mV from the holding potential of −80 mV, and 100 nM PMA was applied for 3 min by bath perfusion. To check the variability of oocytes described above, the modulation of the wild-type AKv1.1a was always examined as well.

The effects of 100 nM PMA on the wild-type AKv1.1a and S24A are shown in Figure 5. A time integral of the current during a test pulse (denoted as charge), which showed the modulation more clearly, was also plotted against time as well as the currents at its peak and at the end of each pulse (Fig. 5A2,B2). The charge of S24A was increased to 176.5 ±25.3% of the control following the PMA treatment (n= 15), which was comparable to that of the wild type AKv1.1a (163.3±17.3%, n=12). Although the early effect of PMA was quite similar, the S24A current was apparently less susceptible to a late decrease of the current which was clearly observed in the wild-type. In oocytes from two different donors, the charge increase of the wild type AKv1.1a was depressed to 114.1±32.3% (n=8) about 20 min after the PMA application, while that of S24A was still high (170.0± 25.1%, n=9). A similar tendency was observed in oocytes from another donor. Thus, phosphorylation of Ser^{24} appears to be important for a late decrease of the expressed current following PMA treatment.

The effects of PMA on other mutants are compared in Table 2. The up- and down-modulation were estimated by maximum increase of the charge and the charge about 20 min after the PMA treatment, respectively. The down-modulation of T345A was significantly larger than others, suggesting that the phosphorylation of Thr^{345} may inhibit the PKC-induced down modulation. By contrast, Ser^{349} are not considered to be involved in the observed modulation because S349P as well as S349C are modulated similarly as the wild-type. The up-modulation was not disturbed by any single mutation of the putative phosphorylation sites. Although R26Q and R351Q were not well modulated by PMA under the protocol described above, this is probably due to the effects of removal of a positive charge (see below).

State dependent modulation of R26Q and R351Q

As described above, the R26Q and R351Q currents were not well modulated by PMA. These results were apparently not due to the inhibition of PKC-induced phosphorylation of Ser^{24} or Ser^{349} by the disruption of the consensus sequence because the S24A, S349P and S349C currents were modulated by PMA. Rather, the change of charge and/or conformation of AKv1.1a by the mutation may inhibit the

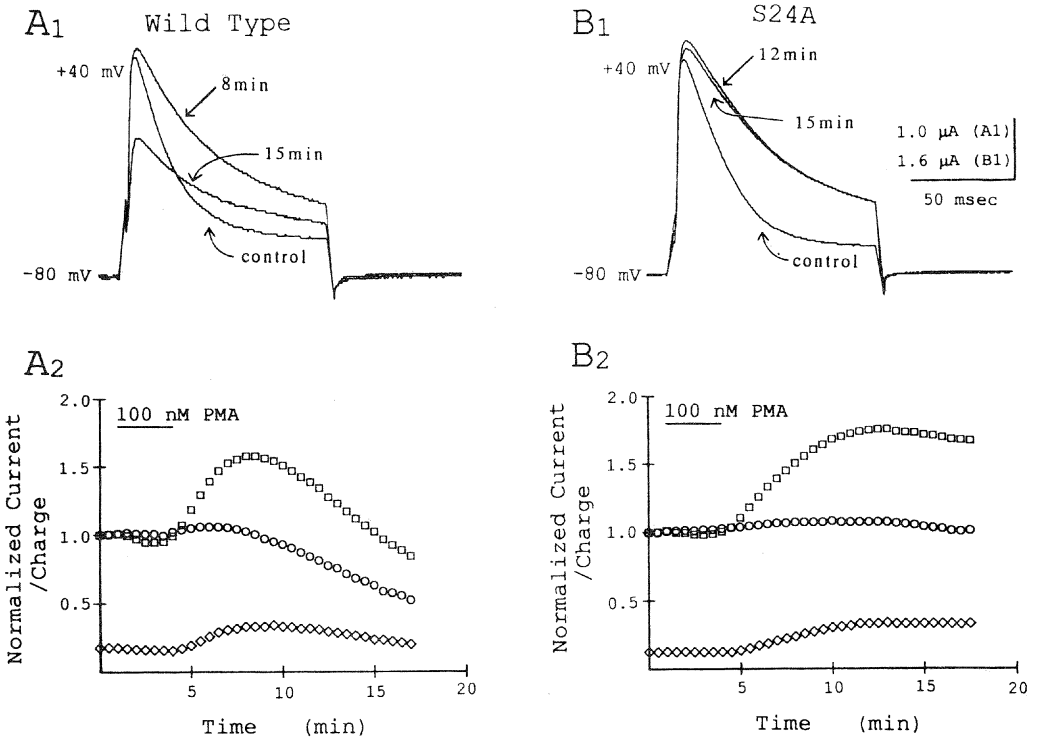

FIG. 5. Comparison of the PMA induced modulation between the wild type (A) and S24A (B). A1, B1: Membrane currents before and after the application of PMA. Membrane currents were elicited by depolarizing pulses to +40 mV every one minutes. 100 nM PMA was applied externally for 3 min. The time at which the current was measured after the PMA treatment is shown. A2, B2: Time dependent changes of membrane currents by the PMA treatment. The currents (peak, circle; at the end of 100 msec pulse, diamond), and the time integral of the current during a command pulse (charge, square) are plotted against time.

TABLE 2. Comparison of the up- and down-modulation by PMA among AKv1.1a and its mutants

	Maximum increase	N	20 min after	N
AKv1.1a	163.3±17.3	12	114.1±32.3	8
ΔN[1)]	89.6±12.8*	6	58.6±15.7*	6
S24A	176.5±25.3	15	170.0±25.1*	9
R26Q	89.9±8.0*	6	—	—
T345A	153.1±23.9	6	52.4±22.7*	6
S349C	150.4±14.5	4	84.1±14.9	3
S349P	161.1±15.8	5	84.4±19.7	4
R351Q	117.1±16.9*	3	—	—
S24A·T345A·R351Q	108.2±3.1*	5	86.4±10.5	4
R26Q[2)]	133.2±19.1	5	81.0±28.2	4
R351Q[2)]	150.5±21.9	3	85.4±20.0	3
S24A·T345A·R351Q[2)]	112.0±5.4*	5	79.7±16.9	5

A time integral of the current (charge) after the PMA treatment is shown as % of the charge before the treatment. All data are mean±SD for N oocytes. Significantly different values compared to AKv1.1a are indicated by asterisk. *; $P<0.01$.

1) For this mutant, a value under 'Maximum increase' was obtained 15 min after the PMA, and that under '20 min after' was obtained 30 min after the treatment.

2) The measurement was done using a hyperpolarizing protocol described in the text.

association of the activated PKC to the channels although there was no explicit kinetic difference between R26Q (or R351Q) and the wild-type (Table 1). Because these channels are voltage-dependent, their conformational states should be modified by changing the holding potential. The results of such experiments are shown in Figure 6. In an oocyte shown in Figure 6A, the R351Q current was modulated very slightly by the usual protocol (Fig. 6A). By contrast, in another oocyte from the same donor, a marked modulation was observed by using a more hyperpolarized holding potential during the PMA application (Fig. 6B). In Figure 6B, the R351Q current was recorded every 60 sec as

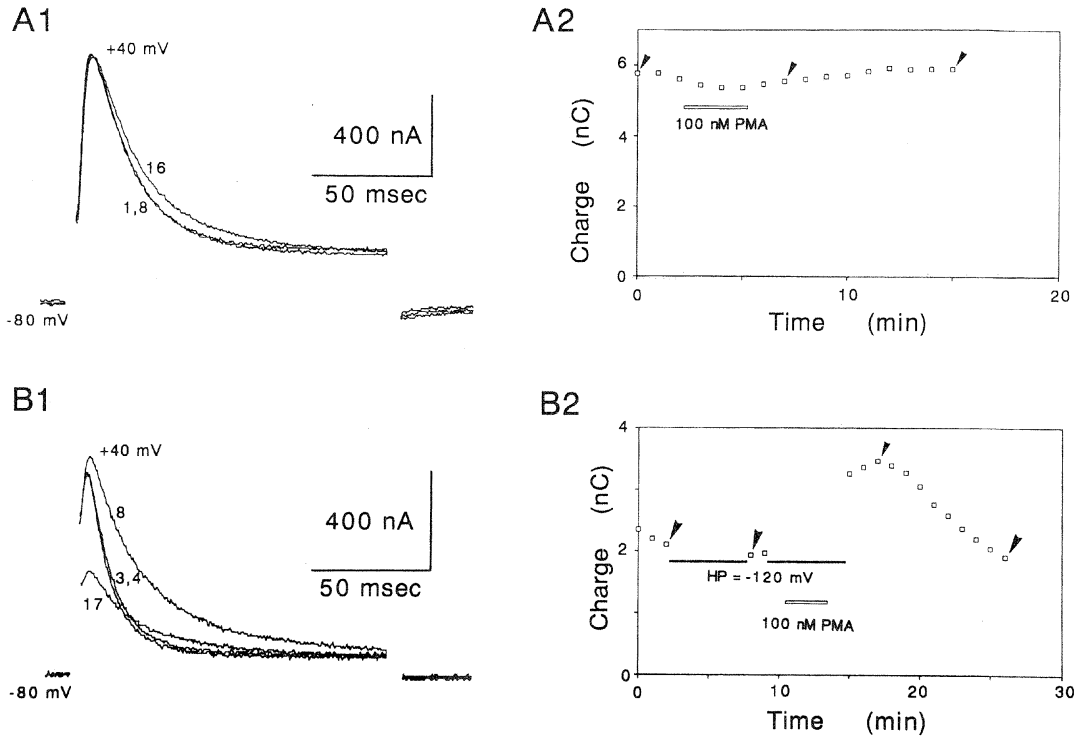

FIG. 6. Effects of PMA on the R351Q current. Membrane currents were elicited by depolarizing pulses to +40 mV from a holding potential of −80 mV every one minutes. In B, the pulse was not applied for two periods during which a holding potential was set to −120 mV (B2). A1,B1: Membrane currents at different time before and after the PMA treatment. Number near each trace shows sequential number of applied pulse. Corresponding time integrals of the currents are indicated by arrow-heads in A2 and B2. A2,B2: Time dependent changes of the time integral of current (charge) by the PMA treatment. In B2, the holding potential was changed to −120 mV during periods indicated.

described before except for two periods in which the oocyte was hyperpolarized to −120 mV without test pulses. Note a more hyperpolarized holding potential itself had no effect on the channel current. During a second hyperpolarization, 100 nM PMA was applied. After the treatment with PMA, there was a marked transient increase of the channel current. Similar results were obtained in R26Q. Mean charges at the maximum increase and about 20 min after the PMA treatment of R26Q and R351Q are shown in Table 2. Conditioning hyperpolarization such as shown in Figure 6 was preferable to observe a robust modulation but the modulation could be detected by just omitting test pulses during the application of PMA. These data suggest that the R26Q or R351Q currents are well modulated by PMA if the channels are in deep closed states during the application of PMA. The apparent discrepancy between the down-modulation of S24A and R26Q is currently unknown, but the removal of positive charge (Arg^{26}) may have additional effects.

Effects of PMA on the triple mutant

As described above, no single mutation of the putative PKC phosphorylation sites in the AKv1.1a resulted in the inhibition of the up-modulation. Thus, we next made triple mutants in which all the three putative sites were disturbed. One of them, S24A·T345A·R351Q, expressed functional channels as described above. When 100 nM PMA was applied by a usual protocol, the up-modulation was very slight. As shown in Table 2, the current was rarely increased by PMA. A hyperpolarizing conditioning pulse which was effective for R26Q and R351Q did not improve the up-modulation. By contrast, the down-modulation was still observed in this mutant.

DISCUSSION

In the present study, kinetic properties of a cloned *Aplysia* potassium channel, AKv1.1a, were found to be modulated by the activators of PKC. Because the effects were depressed by H7, phosphorylation of protein(s) by PKC is probably involved. In respect of the current amplitude, the modulation was both up- and down-regulation. Although phorbol esters activate most of known PKC isoforms exist in cells, physiological activation of PKC can be more selective [22]. Thus, some PKC isoforms may selectively induce the up-modulation, while other isoforms may cause the down-modulation. AKv1.1a is a homologue of the *Drosophila* Shaker K^+ channels [24]. A *Drosophila* Shaker K^+ channel is also modulated by PMA, but the effect is a reduction of the current without noticeable change in its kinetics [20]. Because two channels are quite similar both biophysically and structurally, it may be possible to locate amino acid residues which determine the type of PKC-induced modulation.

The modulatory effects of the PKC activators can be due

to either phosphorylation of AKv1.1a itself or phosphorylation of other protein(s) which exist in *Xenopus* oocytes and act as a regulator of AKv1.1a. Based on the knowledge of consensus sequence for phosphorylation sites by PKC [1, 32], we have examined the contributions of three putative phosphorylation sites (Ser^{24}, Thr^{345}, Ser^{349}) to the PMA-induced modulation by site-directed mutagenesis.

Although none of the examined mutants removed the PMA-induced modulation completely, we nevertheless observed substantial effects of the mutation: (1) a deletion of the amino-terminal domain removed the up-modulation by PMA as well as a fast inactivation of AKv1.1a, but not the down-modulation although the onset of the modulation became extremely slow. (2) the late decrease of the expressed current following the PMA treatment was not obvious in S24A, (3) T345A showed much larger down-modulation than others, (4) a state dependency for the expression of the PMA-induced modulation was observed in R26Q and R351Q, (5) the up-modulation was significantly inhibited in S24A·T345A·R351Q. These results suggest that the up-modulation by the PKC activators is due to the inhibition of the N-type inactivation, thereby increase the time the channels are conductive. It is difficult, however, to evaluate the contribution of three putative phosphorylation sites to the up-modulation because no single mutation of the sites affected the modulation. However, the results obtained by a triple mutant may suggest that phosphorylation of multiple sites induces a conformational change which is needed for the up-modulation. The apparent lack of the down-modulation in S24A as well as the larger down-modulation observed in T345A may imply the importance of the N-type inactivation for the down-modulation as well as for the up-modulation because the loci of both mutations are considered to be involved in the N-type inactivation [13, 15, 33]. At first sight, the results on ΔN may be considered to weaken such arguments because the down-modulation was observed in the channels without the N-type inactivation. However, because the time course for the development of the down-modulation of ΔN was very different from that of the wild-type AKv1.1a, we rather think that the down-modulation of AKv1.1a may be heterogeneous such that the faster down-modulation requires the intact amino-terminal domain and the slower one proceeds without the N-type inactivation. A further analysis of ΔN as well as S24A and T345A may be helpful to examine such possibility.

Because a substantial down-modulation by PMA was still observed even in a mutant which should lack putative PKC phosphorylation sites, additional mechanisms may also contribute to the observed modulation. A plausible mechanism is that PKC phosphorylates endogenous protein(s) in oocytes, and that a certain part of the observed modulation is due to the binding of such phosphorylated protein(s) to AKv1.1a. Indeed, a recent result in *Hermissenda* shows that the A-type K^+ currents in the photoreceptors are modulated by a phosphorylated small GTP-binding protein [21]. Otherwise, PKC-dependent phosphorylation may lead to the activation of other kinase(s) as suggested in the modulation of a potassium channel by the activation of m1 muscarinic acetylcholine receptor [14].

The present study clearly showed that the AKv1.1a current can be modulated by PKC-induced phosphorylation. The kinetic change of the AKv1.1a current by PKC activators resembles the modulation of I_{Kv} in *Aplysia* sensory neurons by 5-HT and PKC activators [3, 29], suggesting the operation of similar molecular mechanisms. However, recent experiments in *Aplysia* sensory neurons indicate that I_{Kv} is also modulated similarly by cAMP-dependent protein kinase (PKA) following the application of 5-HT [8, 11]. Although a membrane permeable cAMP analogue had no prominent effect on the AKv1.1a current expressed in *Xenopus* oocytes in a preliminary experiment [24], Drain *et al.* [5] have shown recently that the modulation of cloned *Drosophila* K^+ channels in *Xenopus* oocytes by PKA is observed only if PKA was applied following the treatment with phosphatase. Thus, the effect of PKA on the gating of AKv1.1a must be examined more thoroughly in future studies.

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