

Modulation of a *Shaker* potassium A-channel by protein kinase C activation

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Brain fast transient K^+ channel (A channel) is known to be modulated by PKC activation. We studied, by two-electrode voltage clamp, the molecular mechanism of modulation by PKC activation of A-channels expressed in *Xenopus* oocytes from the *Shaker* H4 clone. The modulation is inhibitory affecting primarily the maximal conductance of the channels. A secondary effect is a small change in the voltage-dependence of activation and inactivation of the channel.

Potassium channel; Modulation; Protein kinase C; Activation; Inactivation

1. INTRODUCTION

The fast transient outward K^+ current (I_A ; A-current) plays an important role in repetitive firing, spike regulation and in several aspects of synaptic transmission [1]. I_A can be physiologically modulated by neurotransmitters during learning, perhaps by mechanisms leading to channel phosphorylation by protein kinases [2,3]. The ubiquitous protein kinase C (PKC) appears to be an especially important modulator of ion channels [4] and has been reported to down-modulate I_A in invertebrate and vertebrate neurons [5–10]. We have shown that A-channels expressed in *Xenopus* oocytes from total brain RNA, are regulated by protein kinase C [11]. Now when A-channel clones have been isolated, it is possible to study the molecular mechanisms of their modulation. In the present study we show that A-channels, expressed in oocytes from the *Shaker* H4 clone isolated from *Drosophila* [12], are modulated by PKC activation. This clone is a member of the subfamily of K^+ channel genes that includes also several members in mammals [13–17]. The coding region of the K^+ channel genes corresponds to one of the four homologous internal repeats of the Na channel α -subunit; thus, by analogy, the functional K^+ channel is believed to be homo- or hetero-oligomers of subunits [18–20]. We show that the target of PKC modulation resides in a channel composed of only one type of subunit, the *Shaker* H4, and that modulation of A-channel by PKC activation is inhibitory, affecting the availability of channels to open and, to some extent, the voltage sensitivity of the channel.

2. EXPERIMENTAL

Frogs were maintained and dissected and oocytes prepared as described [21]. The A-channel RNA was generated from a recombinant Bluescript plasmid containing the H4 cDNA insert by linearization with *Hind*III and in vitro transcription of full-length capped transcript with T7 RNA polymerase, essentially as described [12], with a few modifications. During the first hour of the transcription reaction the GTP concentration was only 0.05 mM, and was raised to 0.5 mM for an additional 2 h of the reaction. The unincorporated nucleotides were removed by two ethanol precipitations from 1 M ammonium acetate aqueous solutions. The oocytes were injected with 1–5 ng of RNA and incubated at 22°C for 1–3 days in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5) supplemented with 1.8 mM $CaCl_2$, 2.5 mM sodium pyruvate, 100 μ g/ml streptomycin and 100 units/ml penicillin and then assayed electrophysiologically.

Recording of the currents, using a two-electrode voltage clamp technique, was done in ND96 solution supplemented with 1 mM $CaCl_2$, essentially as described [22]. The holding potential was set to -80 mV and stepped for 50 ms (prepulse) to -100 mV before depolarizing the membrane for 50 ms to different voltages. The net I_A was estimated by subtraction of current elicited by voltage step to the same depolarized voltage after a prepulse to -10 mV, where the current was completely inactivated [22]. By this procedure the background leak currents were eliminated. When the current/voltage relationship was studied, the protocol for estimation of net current at each test voltage included subtraction of sealed leak current elicited by voltage jump from -100 to -70 mV. For inactivation studies, currents were elicited by voltage steps to $+20$ mV from different prepulse potentials and the current elicited from -10 mV prepulse was subtracted. The peak of the current trace is referred to as I_A in Figs 2 and 3 and in Table I.

PMA and staurosporine were purchased from Sigma and α PMA from LC Services (Woburn, MA). They were dissolved in 0.1 mM dimethylsulfoxide and kept at -20°C . Final concentration was made in ND96 solution (PMA was kept light-protected). Statistical significance was calculated using the paired two-tailed *t*-test.

3. RESULTS

I_A was expressed in *Xenopus* oocytes injected with the *Shaker* H4 clone isolated from *Drosophila* and its

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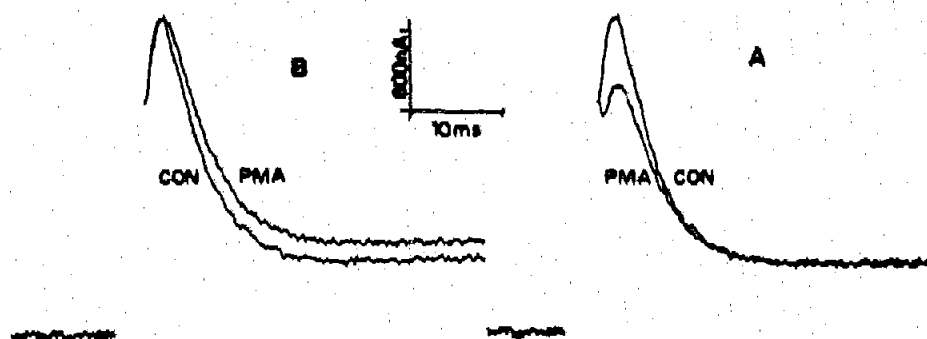


Fig. 1. Effect of 10 nM PMA on I_A . (A) I_A was evoked by a voltage step from -100 mV to $+20$ mV in the absence (control) and 15 min after the application of PMA. (B) The trace of the current recorded in the presence of PMA was scaled in order to compare kinetics.

kinetics and voltage dependence were found to be similar to those described [12].

The effect of the PKC activator PMA (β -phorbol 12-myristate 13-acetate) was evaluated by measuring the I_A amplitudes before and after the PMA application. Only cells in which the I_A amplitude was stable for at least 10 min were exposed to PMA. I_A was suppressed by PMA at doses as low as 0.5 nM. We routinely worked with 10 nM PMA (Fig. 1). The waveform of I_A was not consistently altered during the suppression as was verified by scaling up the amplitude of the current trace under PMA (Fig. 1B). The time course of the PMA effect is shown in Fig. 2. The amplitude of I_A started to decline about 5 min after PMA application and after 20 min reached $50.5 \pm 4.8\%$ (mean \pm SE; 15 oocytes) of its original value ($P < 0.0005$).

Several kinds of control experiments were carried out to verify that the effect of PMA was due to activation of PKC (Fig. 2). (i) The decrease in I_A was not due to an accompanying decrease in membrane surface area as was verified by monitoring the membrane capacitance alongside the development of the PMA effect on I_A . (ii) 100 nM α PMA, an isomer that does not activate PKC, had no effect. (iii) Staurosporine, a protein kinase inhibitor [23], attenuated the PMA effect. Staurosporine was introduced either by bath application of $1 \mu\text{M}$, 10 min before and during the application of PMA, or by microinjection into the oocyte of 50 pmol, 10 min to 3 h before the PMA application. Both procedures produced essentially the same results. We verified that these concentrations of the drug had no direct effect on I_A by recording the current for 10–20 min in the presence of staurosporine alone, before the exposure to PMA. I_A in oocytes pretreated with staurosporine was reduced after 20 min exposure to PMA only to $78.6 \pm 5\%$ (9 oocytes) of its value, a reduction which is significantly weaker ($P < 0.0001$) than that occurring in oocytes which were not exposed to staurosporine (see also Fig. 2).

Steady state activation curves were obtained by fitting the experimental current/voltage (I - V) relationship

data to a modified Boltzmann equation [24] (see Eqn 1 in legend to Fig. 3A) assuming two activation gates, as the assumption of one gate produced less satisfactory fits. The four free activation parameters in the fitting procedure are: half-activation voltage ($V_{1/2}$), slope factor (corresponds to a change in voltage (in mV) that produces an e -fold change in conductance; a_n), maximum conductance (G_{max}) and reversal potential (V_{rev}). All these parameters were allowed to change in very wide ranges.

The effects of PMA were assessed by comparing activation parameters before and after (15–30 min) PMA application and are summarized in Table 1A; effects in one oocyte are shown in Fig. 3A,B. PMA reduced G_{max} in a dose-dependent manner by: 23% at 0.5 nM (31% and 15%; 2 oocytes), $30 \pm 0.06\%$ at 1 nM (range 25–47%; 4 oocytes) and $52 \pm 0.03\%$ at 10 nM (8

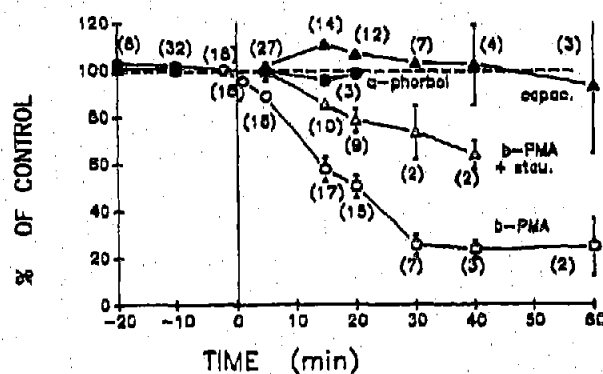


Fig. 2. Time course of the effects on I_A amplitude of 10 nM PMA alone (open circles), 10 nM PMA in the presence of staurosporine (open triangles) and 100 nM α PMA (closed circles). The effect of PMA on the oocyte's membrane capacitance (closed circles) was monitored in parallel to the effects on I_A . The capacitance of the oocyte membrane, measured as the area under the capacitive current elicited by a step from -80 to -70 mV, was 191.45 ± 7.5 nF (10 oocytes). I_A measurements as well as the protocols for the application of the drugs are explained in the text. At time point 0 min the PMA was introduced; staurosporine was introduced at least 10 min before PMA.

Table 1
Effects of 10 nM PMA on activation and inactivation parameters of I_A

(A) Activation					
	Control	PMA	Relative effect	<i>n</i>	<i>P</i> ^a
V_{rev} (mV)	-94.5 ± 0.4	-94.1 ± 0.5	0.5 ± 0.3*	8	>0.4
G_{max} (μS)	19.7 ± 2.7	9.4 ± 1.2	0.5 ± 0.0**	8	<0.001
V_h (mV)	-25.5 ± 0.9	-22.2 ± 2.0	-3.3 ± 1.3	8	<0.05
a_h (mV)	12.7 ± 0.8	13.8 ± 0.65	1.1 ± 0.8	8	<0.2
(B) Inactivation					
	Control	PMA	Difference	<i>n</i>	<i>P</i> ^a
V_h (mV)	-41.3 ± 1.2	-37.5 ± 0.8	3.9 ± 1.2	5	<0.05
a_h (mV)	4.2 ± 0.6	4.5 ± 0.4	0.4 ± 0.2	4	<0.1

The parameters describing the activation (A) and inactivation (B) curves were obtained in each oocyte by fitting the $I-V$ relation to eqn 1, or inactivation curve to eqn 2 (legend to Fig. 3). The values of each parameter were averaged across cells and are presented as mean ± SE; *n* is number of cells tested.

^a*P* was calculated using paired two-tailed *t*-test

*Difference (calculated individually in each cell by subtracting the control value from that in PMA)

**Ratio (G_{max} in 10 nM PMA divided by control G_{max}). Ratios were calculated instead of differences because of the wide variation of G_{max} among individual oocytes

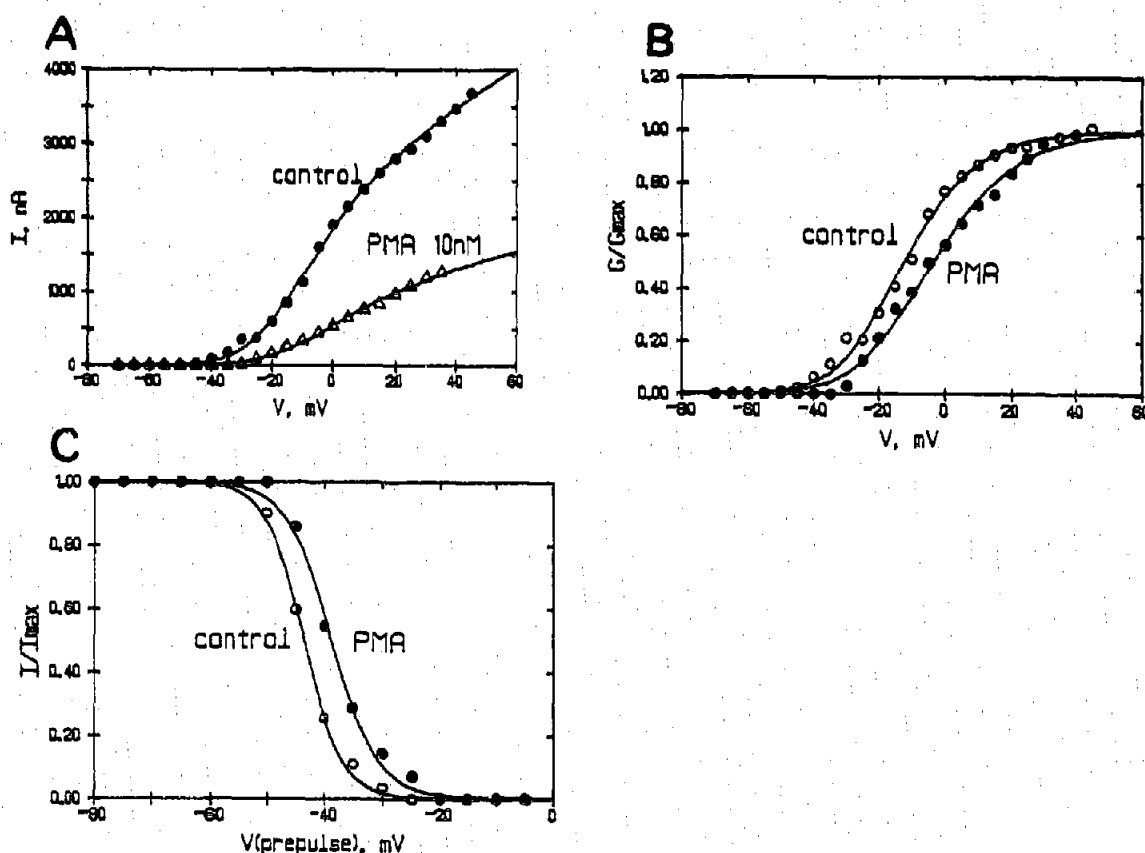


Fig. 3. Effect of PMA on the voltage dependence of I_A . Net I_A was determined as explained in section 2. (A) $I-V$ curves of I_A in the presence and absence (control) of PMA. The experimental results were fitted, using non-linear least square algorithm, to the equation:

$$G/G_{max} = 1/(1 + \exp(-(V_m - V_{rev})/a_n))^2 \quad (1)$$

where at each membrane voltage (V_m), $G = I_A/(V_m - V_{rev})$. (B) The $I-V$ relations shown in (A) presented as activation curve, i.e. G/G_{max} vs V_m . (C) Steady state inactivation curves of I_A in the presence and absence (control) of PMA. Experimental data were derived from voltage steps to +20 mV and were fitted to the equation:

$$I/I_{max} = 1/(1 + \exp((V_{prepulse} - V_h)/a_h)) \quad (2)$$

where I_{max} was the current obtained by the step from -100 mV to +20 mV. The parameters of V_h (half-inactivation voltage) and a_h (slope factor) were allowed to change in wide ranges.

oocytes, range 66–41%). 10 nM PMA shifted the activation curves to the right, toward more positive potentials, with an average change of V_A of 3.3 ± 1.3 mV (range 0.1–9 mV; $P < 0.05$). No significant changes in the slope factor or reversal potential were detected, the latter being -94.5 ± 0.4 mV (range -92 to -95), a value expected for a channel conducting K^+ ions in the oocyte membrane [25].

The effect of 10 nM PMA on steady state inactivation parameters derived from fitted steady state inactivation curves are summarized in Table IB; inactivation curves from one oocyte are presented in Fig. 3C. Half-inactivation voltage (V_H) was shifted to the right (to more positive potentials) by 3.9 mV ± 1.2 (range 0.2–7 mV; 5 oocytes). The slope factor a_H was not changed significantly.

4. DISCUSSION

In a previous work we have shown that the A-channel expressed from total brain RNA is subject to modulation by PKC activators and by purified subtypes of the enzyme itself [11]. In this work we extended the study in two aspects. Firstly, we studied the modulation by PKC of A-channel expressed from cloned gene (*Shaker H4*) and secondly, we analyzed the biophysical mechanism underlying the modulation.

The PMA effect which could be detected already at 0.5 nM is probably mediated via PKC since the analog α PMA, which does not activate PKC, had no effect at a 10-fold higher concentration, and staurosporine, a PKC inhibitor [23], markedly reduced the effect of PMA.

The fact that A-channel expressed from a single gene is modulated, indicates that the modulation ability resides in this single subunit of the channel. However, it is not yet known whether the channel protein is a substrate for PKC phosphorylation since the A-channel protein has not yet been purified. This study cannot resolve whether the modulation effect involves the phosphorylation of the channel protein itself or the phosphorylation of other regulatory proteins (existing in the oocyte), as was suggested to be the case in memory processes in *Hermissenda* photoreceptors where a phosphorylated G protein decreased I_A [26]. A study which correlates the biophysical modulation with phosphorylation analysis on SDS-PAGE of the injected oocyte proteins is necessary in order to discriminate between these possibilities.

Biophysical analysis revealed that the main effect of PMA is a reduction of the maximal conductance. This can be due either to a reduction in the number of channels, to a decrease in the open probability of the channels, or, though less probably, to altered single channel conductance.

A secondary effect was observed which was a change in voltage sensitivity of the channel (reflected in small

but statistically significant shifts to more positive potentials of the steady state activation and inactivation curves). This mode of modulation of I_A by PKC activation differs from that described for Na^+ channel [27], as PMA inhibits I_{Na} mainly by reducing the voltage dependence of activation of the channel without affecting markedly the availability of the channels to open.

The almost parallel shifts to the right of the activation and inactivation curves in the presence of PMA are quite similar in value so that they could result from alteration in the transmembrane surface voltage [28]. We would like to speculate that such a change could be the phosphorylation by PKC of a site in the cytoplasmic side of the channel protein.

In some individual oocytes the shifts both in activation and inactivation curves were more pronounced. This effect is being currently further investigated as we suspect it to depend on the level of expression of the channel in the oocyte. It has already been noticed that A-channel characteristics could be subject to the level of expression [29].

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