Volume 291, number 2, 341–344 FEBS 10283 October 1991

ADONIS 001457939100996E

Molecular mechanism of protein kinase C modulation of sodium channel α-subunits expressed in *Xenopus* oocytes

W. Schreibmayer[1], N. Dascal[2], I Lotan[2], M Wallner[1] and L. Weigl[1]

[1]*Institute of Medical Physics and Biophysics, Karl-Franzens University Graz, A-8010 Graz, Austria* and [2]*Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel*

Received 20 August 1991

The mechanism of modulation of sodium channel α-subunits (Type IIA) by a protein kinase C (PKC) activator was studied on single channel level It was found that (i) time constants for channel activation were prolonged, (ii) inactivation remained virtually unchanged, (iii) peak sodium inward current was reduced as evidenced by calculation of average sodium currents, and (iv) time constants for current activation and decay were prolonged (i), (iii) and (iv) were voltage dependent, being most prominent at threshold potentials The data show that a voltage dependent action on the activation gate can account for the observed reduction of peak inward sodium current and prolongation of current decay in macroscopic experiments

Sodium channel modulation, Protein kinase C (PKC), Phorbol ester, *Xenopus laevis*, Patch clamp, RNA expression

1 INTRODUCTION

Part of the pivotal and diverse cellular regulation by protein kinase C (PKC) [1] is accomplished via modulation of K^+-,Ca^{2+}- and Cl^--channels of the plasma membrane ([2–5]). The channel forming α-subunit of the voltage dependent sodium channel protein has been shown to be a target for PKC-mediated phosphorylation [6]. Regulation of functional sodium channels, expressed from vertebrate brain in *Xenopus* oocytes, is now well established [7–9]. Both an altered availability of the channels to open without affecting activation or inactivation [7] and interference with the channel's activation gate [8] have been suggested to be the mechanism of sodium channel modulation by PKC. In the present study this modulation was explored at the molecular level to gain detailed insight into the underlying mechanism

2 EXPERIMENTAL

2 1 *Chemicals and solutions*

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (Germany) All other chemicals used were of reagent grade ND96 contained (in mM) NaCl 96, KCl 2, $MgCl_2$ 2, HEPES/NaOH 5, pH 7 4 NA200 contained (in mM) NaCl 200, KCl 5 4, $MgCl_2$ 2, $CaCl_2$ 2, HEPES/NaOH 10, pH 7 4 PG180 contained (in mM) K^+/glutamate$^-$ 180, KCl 37 5, EGTA 2 5, $MgCl_2$ 0 5, $CaCl_2$ 0 25, glucose 10, HEPES/KOH 10, pH 7 4

2 2 *In vitro transcription and expression*

Capped cRNA was transcribed from *ClaI* linearized plasmid DNA containing the IIA sodium channel insert (pVA2580, Auld et al [10]) with the aid of T7 polymerase as described [8] Adult female *Xenopus laevis* were purchased from H Kähler, Germany, and kept at 18°C Defolliculated stage V and VI oocytes were prepared as described [8] After injection of 3–20 ng RNA oocytes were incubated at 21°C in ND96 plus 1 8 mM $CaCl_2$, 2 5 mM pyruvate/Na^+, 100 μg/ml streptomycin and 100 units/ml penicillin Two to four days after injection oocytes were tested with the double electrode voltage clamp technique [8] in ND96 plus 1 8 mM $CaCl_2$ and those with peak currents (I_p)>3 μA were taken for patch clamp recordings

2 3 *Patch clamp recordings*

Oocytes were placed for at least 5 min in PG180 and then the vitelline layer was removed Subsequently oocytes were transferred to plastic Petri dishes containing 3 ml fresh PG180 Patch clamp recordings were performed at 18°C according to Hamill et al [11] Sylgard-coated pipets were pulled (List L/M-3P-A puller, Germany) from standard haematocrit capillaries (Assistent No 564, Germany) and filled with NA200 Patch current was amplified with an Axopatch D (Axon Instr , USA) current amplifier and filtered at 5 kHz (4-pole Bessel filter) Sampling frequency was 33 kHz with the TL-125 interface (Axon Instr , USA) connected to an IBM SX386-compatible computer (Steiner, Austria) Voltage pulses were supplied by the same device. Data evaluation was performed with the PClamp 5 5 1 software (Axon Instr , USA) Holding potential was kept constant at −100 mV and every 2 s voltage pulses ranging from −90 mV to +50 mV and lasting for 50 ms were applied (range and increment were variable) Voltage jump sequences were run repetitively to allow observation of channel behaviour in time 5 nM PMA was introduced by the addition of 3 μl of a 5 μM PMA solution in PG180

3. RESULTS

Sodium channel currents were recorded from cell-attached membrane patches. Patches that did not display overlapping sodium channel opening events at depolarizations >+10 mV were considered single channel patches For calculation and evaluation of ensemble currents one membrane patch containing many func-

Correspondence address W Schreibmayer, Institute of Medical Physics and Biophysics, Karl-Franzens University Graz, A-8010 Graz, Austria Fax (43) 316 35566

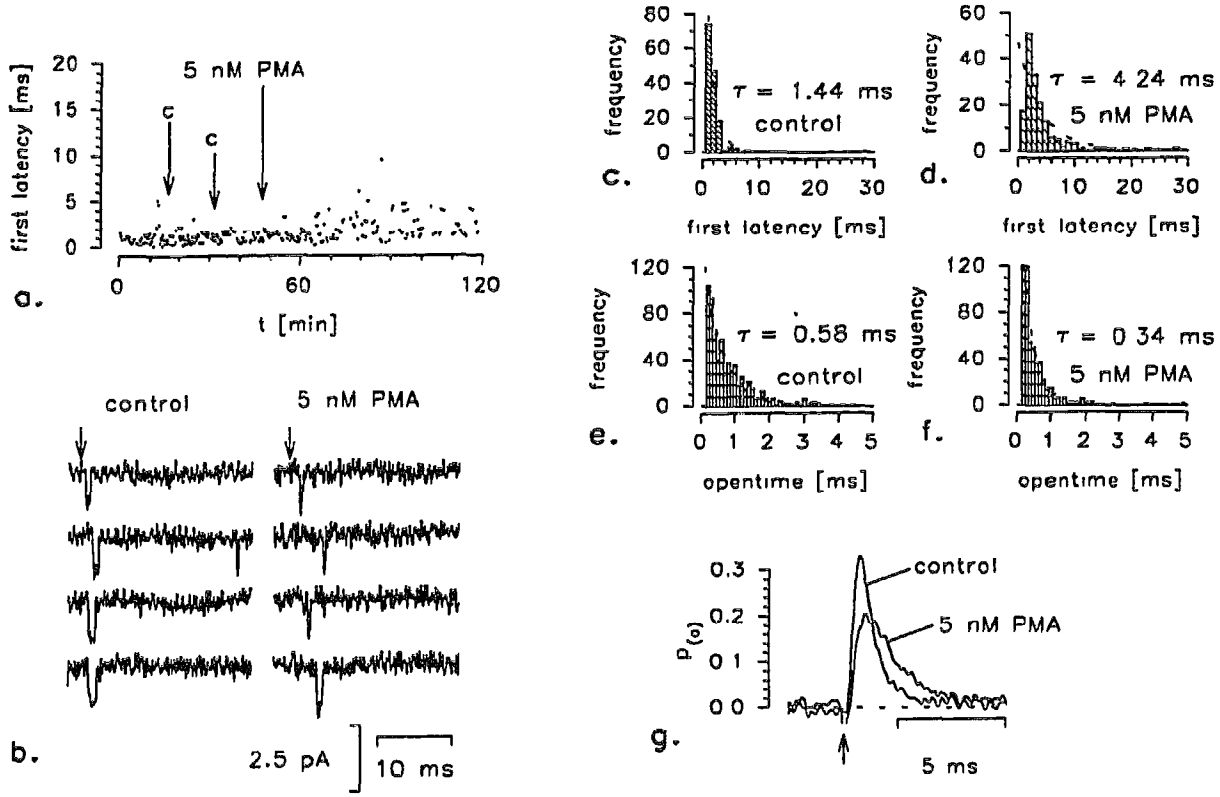

Fig 1 Effect of PMA at −30 mV (a) Development of the PMA effect on first latencies (b) Original single channel traces, filtered digitally at 2 0 kHz, starts of voltage jumps are marked by arrows (c–f) Histograms for first latencies and open times (g) Open probability vs time as calculated by average current/open channel current Digital filtering at 1 5 kHz

tional sodium channels ('macropatch') was used in addition

In 4 cell-attached patches, between 5 and 25 min after the addition of 5 nM PMA to the bathing solution (this time being variable from experiment to experiment), a marked effect on first latencies of sodium channel openings developed at −30 mV (Fig 1a, Table I) This effect could not be mimicked by control additions ('c' in Fig 1a) of bathing solution Selected single channel recordings before and after the development of this effect and corresponding histograms for first latencies are shown in Fig 1 At −50 mV first latencies were so much prolonged after addition of 5 nM PMA, that calculation of their time constant (t_f) became impossible for the given pulse length of 50 ms (Fig 2) In contrast, at −10 mV t_f's were virtually not influenced by the phorbol ester At depolarizations > −10 mV the resolution of sodium channel openings was hampered by single channel currents being small and fast, and microscopic analysis has not been performed. Mean values for the measured time constants are listed in Table I The patch-to-patch variation of t_f (and also of open time constant, t_o) was considerable (Table I), obscuring the effect of PMA Therefore, the % changes of time constants were calculated individually in each patch and the significance of the mean % change to be >0% was calculated according to [12] (P in Table I).

In contrast to t_f, the open time constant t_o was not markedly affected by the phorbol ester, albeit there was a tendency for a decrease at −50 mV (see Figs 1, 2 and Table I, respectively)

In additional three single channel patches, PMA had no effect on the channel This emphasizes the stochastic

Table I

Evaluated time constants in ms (mean ± SEM)

E_m (mV)	Control (ms)	5 nM PMA (ms)	% change (%)	n	P
Open times (t_o)					
−50	0 26±0 05	0 17±0 03	−31±18	3	<0 10
−30	0 38±0 07	0 33±0 04	−10±13	4	>0 10
−10	0 53±0 13	0 57±0 17	+4±11	3	>0 10
First latencies (t_f)					
−30	2 49±1 07	4 67±1 51	+113±29	4	<0 001
−10	0 95±0 39	0 91±0 30	+1±17	3	>0 10
Activation time constant					
−30	1 32±0 42	1 95±0 54	+55±16	5	<0 001
−10	0 74±0 29	0 81±0 35	+7±8	4	>0 10
+10	0 85±0 48	0 98±0 59	+7±8	4	>0 10
Decay time constant					
−30	3 45±1.16	5 33±1 52	+68±21	5	<0 001
−10	2 34±1 50	2 78±1 54	+34±31	4	>0 10
+10	2 23±1 40	2 52±1.61	+9± 5	4	>0 10

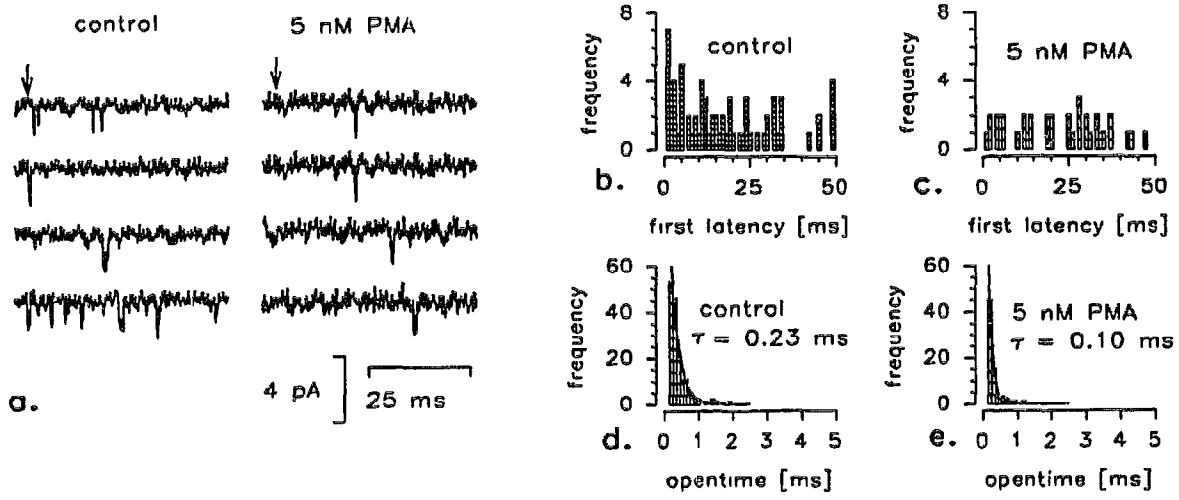

Fig 2 Effect of PMA at −50 mV (a) Original traces as in Fig 1a (b–e) Histograms for first latencies and open times

nature of the single channel behaviour and may be due to a high basal phosphorylation of those channels, or the absence of PKC molecules in these particular membrane patches In one particular patch (Fig 3) about 45 functional α-subunits were expressed (estimated as $I_p/P_{(o)}$, where I_p is the patch current, and $P_{(0)}$ the open probability) From the inspection of the macroscopic current traces and also from the I_p vs membrane potential (E) curve, it becomes apparent that the PMA effect is voltage-dependent, being perceptable only at voltages between −50 and −20 mV (Fig. 3) Reduction of I_p was also accompanied by a prolongation of both activation and decay of the current (Fig 3a) as also seen for $P_{(o)}$ of single channels (Fig 1g)

Average patch currents for all 5 patches were calculated before and after the addition of 5 nM PMA. I_p was measured and normalized to $I_{p\text{-}30(control)}$ (control I_p of a given experiment at −30 mV) Time constants were measured as the risetime to 63% I_p (activation time constant) and decay to 37% (decay time constant) and are summarized in Table I The % effect was calculated for every potential and experiment, and was found to be strongly voltage-dependent (Fig. 4)

4 DISCUSSION

Our single channel data show a marked decrease of the rate constant(s) for conformational transitions from the closed (c) to the active (a) channel state(s). This effect was most prominent at threshold potentials (−50 mV and −30 mV) and vanished at depolarizations >−10 mV, as would be expected for a modulation affecting the activation process. These findings are in agreement with those of Dascal and Lotan [8] who suggested, based on measurements of macroscopic I_p/E relations, that the activation process of sodium channels is shifted to positive potentials by PMA

As shown by the averaged patch current analysis in combination with microscopic assessment of channel activation and inactivation (e g. Fig 1c–g and Table I), the reduction of peak sodium inward current by PKC stimulation observed in this study (see also ([7,8,13]) can be explained by slowing down of the activation process. A parallel slowing of the macroscopic current decay was observed, as is directly predicted from kinetic theory [14] In accord with our findings, a slowing of the sodium current decay has been preliminarily reported for

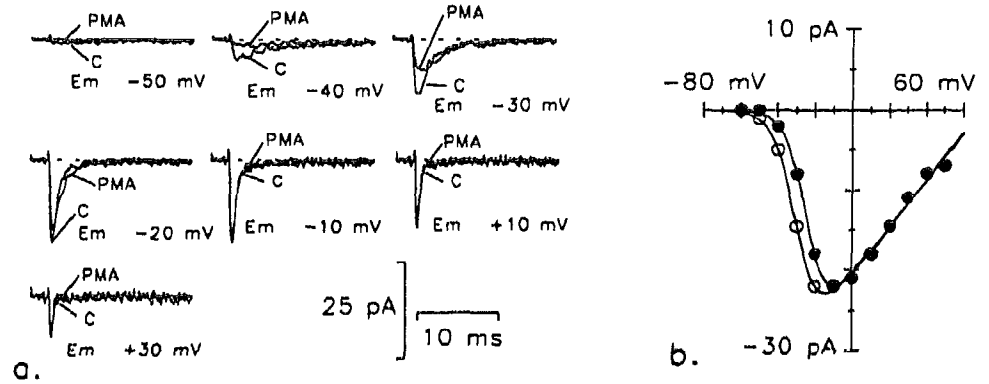

Fig 3 Macropatch currents (a) Original traces before and after the development of PMA effect at different voltages Average current from 10 consecutive sweeps, digital filtering at 3 0 kHz (b) I_p/E relation before (open circles) and after (filled circles) the development of PMA effect

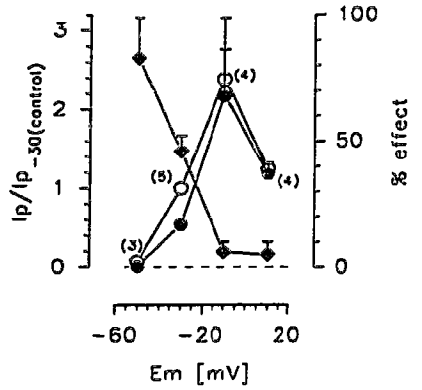

Fig 4 Voltage dependence of effect on I_p before (open circles) and after the development of PMA effect (filled circles) calculated from 5 different experiments % effect (diamonds) is plotted on separate scale Values are presented as mean±SEM

cultured embryonic rat brain neurons [13]. Differences to our findings (i.e. lack of voltage dependence, removal of inactivation) might be due to differences in α-subunit and/or PKC isoforms, higher phorbolester- and/or Na^+-concentrations in [13]

The inactivation process was little or not at all influenced by PMA Indeed, if the prolongation of the macroscopic Na^+ current were due to slower inactivation, then modulation affecting the *a->i* process (α-subunit inactivation) should be most prominent at higher membrane potentials and become of minor relevance near threshold potentials, were *c->a* and *a->c* transitions dominate the time course of the macroscopic current. However, microscopic inactivation (transitions to the inactivated channel state (*i*)), as assessed by measuring the dwell times in the open state (t_o, Table I), was little or not influenced in our experiments Especially at higher potentials (−30 mV and −10 mV), where the inactivation process dominates t_o [14], t_o's were practically identical

Our findings demonstrate that the effects of PKC on sodium channels expressed from mRNA (total rat brain) and of cRNA (VA200 and IIA α-subunits, respectively) are clearly different from those by the cAMP-dependent protein kinase, that do not affect the activation process [15], suggesting differential regulation by different sequence-specific kinases.

Detailed kinetic analysis of macropatch currents in combination with site-directed mutations will be necessary to distinguish whether (i) the channel's gating charge is reduced, (ii) the movement of gating particles, i e S4 segments [16] or (iii) conformational changes of the entire α-subunit, accompanying channel activation, are hindered allosterically by subunit phosphorylation As different α-subunit isoforms differ mostly in their intracellular regions (and hence are potentially targets for intracellular protein kinases), differential modulation of sodium channel isoforms might provide the evolutionary clue to the existence of at least 5 tissue specific isoforms in one organism [10,17–21]

Acknowledgements We thank Prof R Dunn (Montreal, Canada) for providing the pVA2580 and pVA200 constructs and Dr D Platzer (Graz, Austria) for valuable discussions about statistical evaluation of the data Skillful technical assistance and computer programs were provided by B Spreitzer and A Russ Financial support by the Austrian Research foundation (project S4505B), the Karl-Franzens University, Graz (fellowship to N D) and the USA–Israel BSF is gratefully acknowledged

REFERENCES

[1] Nishizuka, Y (1984) Nature 308, 693–698
[2] Strong, J A , Fox, A P , Tsien, R W and Kaczmarek L K (1987) Nature 325, 714–717
[3] Tohse, N , Kameyama, M , Sekiguchi, K , Shearman, M S and Kanno, M (1990) J Mol Cell Cardiol 22(6), 725–734
[4] McCann, J D and Welsh, M J (1990) Annu Rev Physiol 52, 115–135
[5] Li, M , McCann, J D , Anderson, M P , Clancy, J P , Liedtke, C M , Nairn, A C , Greengard, P and Welsh, M J (1989) Science 244(4910), 1353–1356
[6] Costa, M R C and Catterall, W A (1984) Cell Mol Neurobiol 4, 291–297
[7] Sigel, E and Baur R (1988) Proc Natl Acad Sci USA 85, 6192–6196
[8] Dascal, N and Lotan, I (1991) Neuron 6, 165–175
[9] Lotan, I , Dascal, N , Naor, Z and Boton, R (1990) FEBS Lett 267, 25–28
[10] Auld, V J , Goldin, A L , Krafte, D S , Catterall, W A , Lester, H A and Davidson, N (1990) Proc Natl Acad Sci USA 87, 323–327
[11] Hamill, O P , Marty, A , Neher, E , Sakman, B and Sigworth, F J (1981) Pflugers Arch 391, 85–100
[12] Kreyszig, E (1979) Statistische Methoden und ihre Anwendungen, Vandenhoeck & Ruprecht, Gottingen
[13] Numann, R , Scheuer, T and Catterall, W A (1991) Biophys J 59, 262a
[14] Yue, D T , Lawrence, J H and Marban, E (1989) Science 244, 349–352
[15] Gershon, E , Weigl, L , Lotan, I , Schreibmayer, W and Dascal, N (submitted)
[16] Stuehmer, W , Conti, F , Suzuki, H , Wang, X , Noda, M , Yahagi, N , Kubo, H and Numa, S (1989) Nature 339, 597–603
[17] Auld, V J , Goldin, A L , Krafte, D S , Marshall, J , Dunn, J M , Catterall, W A , Lester, H A , Davidson, N and Dunn, R J (1988) Neuron 1, 449–461
[18] Noda, M , Ikeda, T , Suzuki, H , Takeshima, H , Takahashi, T , Kuno, M and Numa, S (1986) Nature 322, 826–828
[19] Kayano, T , Noda, M , Flockerzi, V , Takahashi, H and Numa, S (1988) FEBS Lett 228, 187–194
[20] Trimmer, J S , Coopermann, S S , Tomiko, S A , Zhou, J , Crean, S M , Boyle, M B , Kallen, R G , Sheng, Z., Barchi, R L , Sigworth, F J , Goodmann, R H , Agnew, W S and Mandel, G. Neuron 3, 33–49
[21] Rogart, R B , Cribbs, L L , Muglia, L K , Kepharth, D D and Kaiser, M W (1989) Proc Natl Acad. Sci USA 86, 8170–8174