Modulation of vertebrate brain Na^+ and K^+ channels by subtypes of protein kinase C

Ilana Lotan[1], Nathan Dascal[1], Zvi Naor[2] and Rony Boton[1]

[1] *Department of Physiology and Pharmacology, Sackler Faculty of Medicine* and [2] *Department of Biochemistry, G.S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel*

Received 11 April 1990

Effects of purified subtypes I, II and III of protein kinase C (PKC) on voltage-dependent transient K^+ (A) and Na^+ channels were studied in *Xenopus* oocytes injected with chick brain RNA. The experiments were performed in the constant presence of 10 nM β-phorbol 12-myristate-13-acetate (PMA). Intracellular injection of subtype I (τ) reduced the A-current (I_A), with no effect on Na^+ current (I_{Na}). PKC subtype II ($\beta_1+\beta_2$) and III (α) reduced both currents. PKC did not affect the response to kainate. Inactivated (heated) or unactivated (injected in the absence of PMA) enzyme and vehicle alone had no effect. Our results strongly suggest that I_{Na} and I_A in vertebrate neurons are modulated by PKC, all PKC subtypes exert a similar effect on the A-channel while only subtypes II and III modulate the Na^+ channel.

Protein kinase C isoform; Sodium channel; A channel; RNA expression; Modulation; Oocyte; *Xenopus*

1. INTRODUCTION

Although voltage-activated Na^+ and A-type K^+ channels are among the most abundant and important ion channels in the brain, little is known about their modulation [1-5]. There are indications that, like many other ion channels [3], I_A and I_{Na} may be modulated by the ubiquitous protein kinase C (PKC), which is activated by a variety of neurotransmitters [6,7]. Thus, purified brain Na^+ channel is phosphorylated by PKC [2]; phorbol esters (PKC activators) inhibit I_{Na} expressed in *Xenopus* oocytes injected with chick brain RNA [8]; *cis*-fatty acids reduce I_{Na} in mouse neuroblastoma cells, apparently via PKC activation [9]. PKC activators reduce I_A in heart, glial cells and neurons [10-13]. Modulation of I_A by purified PKC has been studied only in molluscan neurones, where purified heterologous PKC reduces I_A [13]. Moreover, the roles of the recently discovered isoforms (subtypes) of PKC are unclear. It has been proposed that subspecies of PKC may (at least in part) account for the diversity of cellular effects of PKC, e.g. different modulations of ion channels [14,15]. However, differential effects of the PKC subspecies purified to date have been demonstrated only in one case: gonadotropin secretion in cultured pituitary cells [16]. Effects of PKC subspecies on ion channels have not been studied.

We have examined the effects of phorbol esters and of purified PKC subspecies on I_A and I_{Na} from chick brain expressed in RNA-injected *Xenopus* oocytes, which proved to be a good model system to study modulation of ion channels [8,17-22]. We have found that both PKC activators and the purified PKC reduce these currents, and there is a certain specificity of the different subtypes towards the Na, but not A, channel.

Correspondence address: I. Lotan, Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel

Abbreviations: PMA, phorbol 12-myristate-13-acetate; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; I_A, voltage-dependent potassium current of the A-type; I_{Na}, voltage-dependent sodium current

2. EXPERIMENTAL

Frogs were maintained and dissected and the oocytes prepared as described [23]. Total RNA was isolated from brains of 19-day chick embryos by LiCl/urea method [24]. The oocytes were injected with 50 nl of total RNA (6-10 mg/ml in water), and incubated for 2-5 days at 22°C in sterile ND96 solution [22] (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes/NaOH; pH 7.5) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 mM sodium pyruvate, with or without phorbol-12-myristate-13-acetate (PMA) or phorbol-12,13-dibutyrate (PDBu). The currents were recorded using the two-electrode voltage-clamp method [18,22] in ND96 solution with the addition of 2 mM 9-antracenecarboxylic acid (to block Cl^- channels [25]) and 20 or 30 mM tetraethylammonium chloride. The routine protocols to measure the currents were as follows. Net I_A was obtained by recording currents evoked by voltage steps from -100 mV (5 s prepulse) to 40 mV (test pulse) and subtraction of currents evoked by voltage steps from -50 mV to 40 mV [26]. Net I_{Na} was obtained by recording currents evoked by voltage steps from -100 to -10 mV, and subtraction of currents evoked by voltage steps from -20 to -10 mV [26]. Both currents were recorded in the same cell. The estimates of each current were not significantly affected by the presence of the other one because: (i) at 40 mV, at which I_A was measured, I_{Na} is negligible; (ii) at -10 mV, the peak of I_{Na} occurs while I_A only starts rising, and I_A is usually more than 4-fold smaller than I_{Na} [26].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Type I and III PKCs were purified to homogeneity from adult male rat brains as described [27,28]; type II was partially purified as described [29] and found to be free of activity of cAMP-and Ca/calmodulin-dependent protein kinases. The identity of the PKCs was confirmed with type-specific antibodies [28,29]. The PKCs were diluted at about 20 ng/μl in buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.01% Triton X-100, and 10% glycerol and stored in small aliquots at $-70°C$. 4.5 nl of the enzyme or vehicle (about 0.5% of the oocyte's volume) were pressure-injected into the oocytes as described [23] All PKCs had approximately the same specific activity; the activity of PKC introduced into an oocye by a single injection was approximately 8×10^{-15} mol/min (measured by incorporation of $^{32}P_i$ into H1 histone from [γ-^{32}P]ATP at 30°C) [16] The activity of the endogenous PKC in the oocytes was measured in homogenates of 100–200 oocytes.

3. RESULTS AND DISCUSSION

I_{Na}, I_A and the response to kainate ($I_{kainate}$) were characterized previously [26]. Currents were recorded with the two-electrode voltage-clamp technique. PMA and PDBu reduced I_{Na}, had no effect on $I_{kainate}$, in accord with [8], and also reduced I_A. 20–30 min after application, 10 nM PMA reduced I_{Na} and I_A to $47 \pm 9\%$ ($n = 5$) and $88 \pm 3\%$ ($n = 5$) of control before PMA addition, respectively (all data are mean ± SE). No significant changes in oocyte capacitance during this time have been observed, suggesting that the surface area was unchanged. Similar results were obtained with 50–100 nM PDBu. We also observed changes in voltage dependency of activation and inactivation of I_{Na} (to be reported elsewhere).

The effect of phorbol esters were assumed to be mediated by the oocytes' endogenous PKC (native or directed by the brain RNA). To avoid interference of the endogenous PKC when injecting the exogenous PKC, the routine protocol included 30–48 h incubations of the oocytes with 10 nM PMA or 50 nM PDBu (higher concentrations were lethal or reduced the oocytes' capacitance, in accord with [30]). I_A and I_{Na} were then recorded in the continuous presence of the same concentration of the phorbol ester. Under these conditions, the currents were steady for tens of minutes (before the

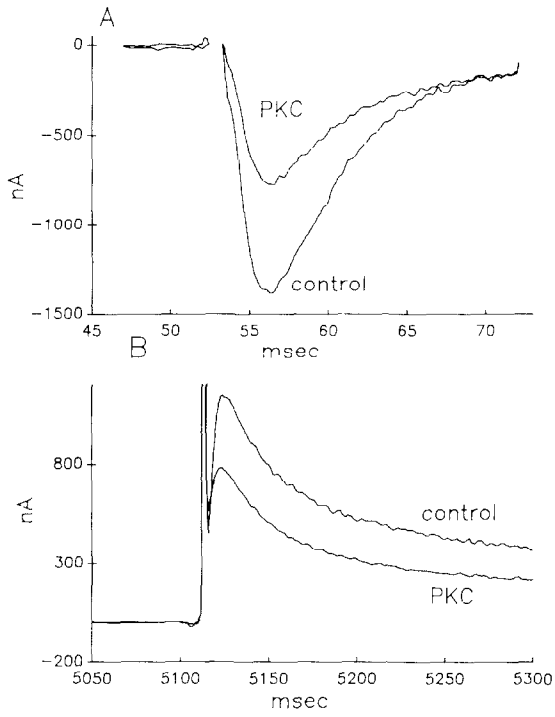

Fig. 1. The effects of injection of PKC (type III) on I_{Na} (A) and I_A (B) in the presence of 10 nM PMA, after a 48 h preincubation in PMA. The currents denoted by 'PKC' were recorded 20 min after the injection of PKC. Net I_{Na} was measured at −10 mV by subtraction of currents obtained in the presence of 100 nM tetrodotoxin; net I_A was measured at +40 mV as explained in section 2.

Table I

Effects of protein kinase C on I_A, I_{Na} and $I_{kainate}$

	Active enzymes				Controls		
	Type III	Type II	Type I	Mixture* I + II + III	Vehicle	Unactivated type III	Inactivated type III
I_A	67 ± 4 (11) $P<0.001$	63 ± 4 (8) $P<0.001$	72 ± 3 (11) $P<0.001$	73 (66,80) (2)	93 ± 2 (15)	91 ± 4 (4)	93 ± 2 (6)
I_{Na}	77 ± 6 (11) $P<0.01$	68 ± 8 (5) $P<0.02$	100 ± 6 (8) $P>0.2$	94 ± 10 (3)	94 ± 4 (8)	101 ± 2 (4)	103 ± 3 (5)
$I_{kainate}$	98 ± 4 (4)	-	100, 100 (2)	-			

*Stoichiometric mixture of the 3 subtypes; the concentration of each one was one-third of that used in injections of individual subtypes

Currents recorded 20–30 min after the injection were expressed in percent of the currents before the injection, in the same cell. The numbers are mean ± SE and number of cells is indicated in parentheses. The stability of the current before the injection was verified during at least 10 min. After the insertion of PKC-containing injection needle, the amplitude of I_A and I_{Na} sometimes changed; in these cases, stability of the currents' amplitude at the new level was again verified as above. If one of the tested currents was not stable within 5%, the results were discarded. The significance of the effects of protein kinase subtypes was calculated using two-tailed Student's t-test. The effects of types I and II were compared with those of the vehicle, whereas the effects of type III were compared with those of the inactivated and of the unactivated enzyme (identical probability levels were obtained in both cases).

injection of PKC). The endogenous PKC in these oocytes was down-regulated by 75 ± 15% ($n = 3$). These results suggest that, although full down-regulation (see [7]) of endogenous PKC did not always occur, a steady-state has been achieved in which the currents were not affected anymore by the presence of phorbol esters at the concentration employed.

Injection of PKC II and PKC III reduced both I_{Na} and I_A by 20–40% on the average after 20–40 min (Table I, Fig. 1A,B, and Fig. 2A). PKC I had a different effect: it reduced I_A, but produced no change in I_{Na} (Fig. 2B), although increases or decreases (range: 76–140% of control) were observed in individual oocytes. These results imply that PKC I may have a dual effect on I_{Na}, e.g. by phosphorylating two different sites on the channel molecule, but testing of this assumption requires further study. The responses to kainate in the same cells were not altered by PKC injection (Table I). We did not find evidence for synergism between PKC subtypes, since a stoichiometric mixture of all 3 subtypes reduced I_A to about the same extent as the individual subtypes, and did not reduce I_{Na} (Table I). The latter probably reflects the combined influence of all subtypes, supporting the possibility that PKC I may sometimes enhance I_{Na}.

The time course of the effects of PKCs is shown in Fig. 2A,B. In most cases, the main reduction in the currents occurred within a few minutes.

Injection of vehicle (the buffer in which PKC was dissolved) produced a small reduction of I_A (7%, $P < 0.001$) and I_{Na} (6%, $0.1 > P > 0.05$) (Fig. 2C). Therefore, the statistical analysis has been done by comparing the effects of all PKC subtypes with those of the vehicle, and for PKC III (which had marked effects on both currents), with additional controls. Injection of inactivated PKC III (heated to 65°C overnight and then stored for several days at room temperature) had the same effects as the vehicle (Fig. 2E). 'Unactivated' PKC III (enzyme injected into cells not pretreated with PMA, in the absence of any added PKC activator) had no effect on any of the currents (Fig. 2D). Table I summarizes the results of all injection experiments.

Our results demonstrate that the vertebrate neuronal voltage-dependent Na^+ and K^+ channels are modulated by PKC. This is the first direct demonstration of modulation of I_{Na} by purified PKC. Reduction

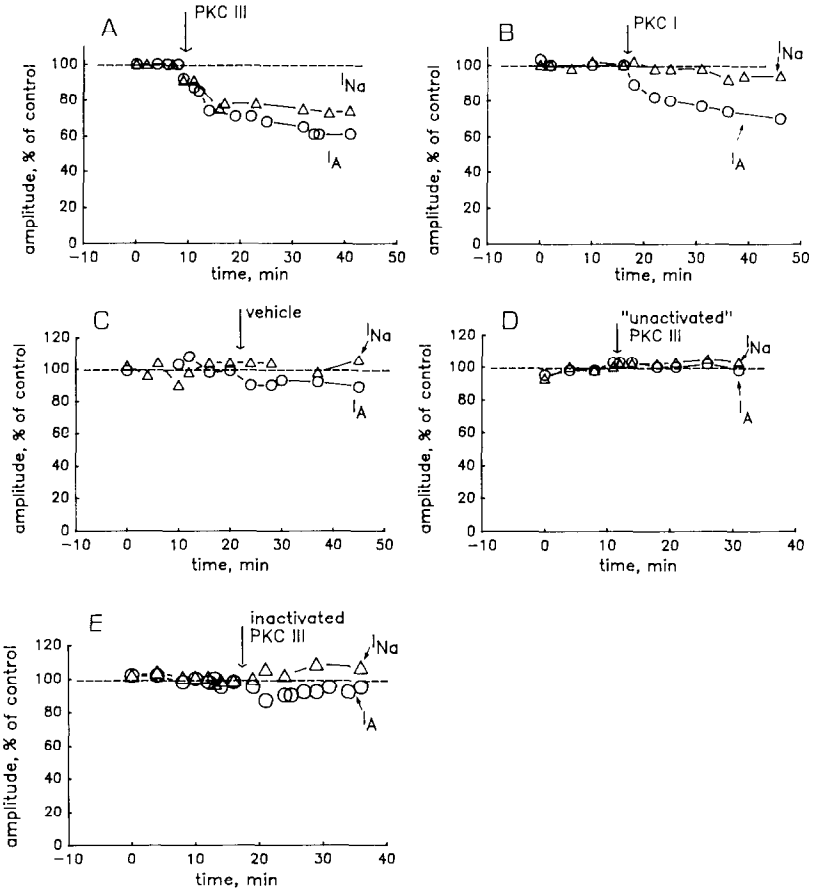

Fig. 2. The time course of the effects of injection of PKC and of control solutions. In all cases, except D, the oocytes were incubated in 10 nM PMA before and during the recording session, as described in the text. (A) PKC III. (B) PKC I. (C) vehicle alone. (D) 'unactivated' PKC (in the absence of PMA). (E) PKC III inactivated by heating (see text).

of vertebrate I_A by PKC is similar to that found in molluscan neurones [13], although we did not observe potentiation of I_A by PKC in the absence of activators. Another interesting finding is that PKC subtypes may differentially modulate the same cellular process (Na^+ channel), while in other cases, the heterogeneity of PKC subtypes may have no physiological meaning (A-channel). The differential distribution of PKC subtypes in brain neurones [14], together with the differential physiological activities of the subtypes, may play a role in the multitude of the effects by PKC-activating transmitters.

Acknowledgements: Supported by grants from the US-Israel Binational Science Foundation (BSF) and the Israel Academy of Sciences. We thank Dr Y Nishizuka for samples of purified PKC subspecies and type-specific antibodies used in some of the experiments.

REFERENCES

[1] Hille, B. (1984) Ionic Channels of Excitable Membranes, Sinauer, Massachusetts.
[2] Catterall, W.A. (1988) Science 242, 50-61.
[3] Jan, L.Y. and Jan, Y.N. (1989) Cell 56, 13-25.
[4] Kaczmarek, L.K. and Levitan, I.B. (1987) Neuromodulation, Oxford Univ. Press, Oxford.
[5] Rogawski, M.A. (1985) Trends Neurosci. 8, 214-219.
[6] Nishizuka, Y. (1984) Nature 308, 693-698.
[7] Nishizuka, Y. (1986) Science 233, 305-312
[8] Sigel, E. and Baur, R. (1988) Proc. Natl. Acad. Sci. USA 85, 6192-6196.
[9] Linden, D J. and Routtenberg, A. (1989) J. Physiol. (Lond.) 419, 95-119.
[10] Apkon, M. and Nerbonne, J. (1988) Proc. Natl. Acad. Sci. USA 85, 8756-8760.
[11] Soliven, B., Szuchet, S., Arnason, B.G.W and Nelson, D.J. (1988) J Membr. Biol. 105, 177-186.
[12] Werz, M.A. and Macdonald, R L. (1987) Neurosci. Lett. 78, 101-106.
[13] Alkon, D.L., Naito, S., Kubota, M , Chen, C , Bank, B., Smallwood, J., Gallant, P. and Pasmussen, H. (1988) J. Neurochem. 51, 903-917.
[14] Nishizuka, Y (1988) Nature 334, 661-665.
[15] Cussens, L., Parker, P.J., Rhee, L. Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U. and Ullrich, A. (1986) Science 233, 859-866
[16] Naor, Z., Dan-Cohen, H , Hermon, J. and Limor, R. (1989) Proc. Natl. Acad. Sci. USA 86, 4501-4504.
[17] Dascal, N (1987) CRC Crit. Rev. Biochem. 22, 317-387
[18] Cohen-Armon, M., Sokolovsky, M. and Dascal, N. (1989) Brain Res. 496, 197-203.
[19] Moran, O. and Dascal, N. (1989) Mol. Brain Res. 5, 193-202
[20] Leonard, J.P., Nargeot, T.P , Snutch, N., Davidson, N and Lester, H. (1987) J Neurosci 7, 875-881.
[21] Lotan, I., Dascal, N , Oron, Y., Cohen, S and Lass, Y. (1985) Mol Pharmacol. 28, 170-177.
[22] Dascal, N., Snutch, T.P., Lubbert, H., Davidson, N. and Lester, H.A (1986) Science 231, 1147-1150.
[23] Dascal, N , Landau, E.M. and Lass, Y. (1984) J Physiol. 352, 551-574.
[24] Dierks, P., Van Ooyen, A., Mantei, N. and Weissmann, C. (1981) Proc. Natl Acad. Sci. USA 78, 1411-1415.
[25] Boton, R., Dascal, N., Gillo, B. and Lass, Y. (1989) J. Physiol 408, 511-534.
[26] Lotan, I., Volterra, A., Dash, P., Siegelbaum, S.A. and Goelet, P. (1988) Neuron 1, 963-971
[27] Kikkawa, U., Ono, Y., Ogita, K , Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, Y., Igarishi, K and Nishizuka, Y. (1987) FEBS Lett. 217, 227-231
[28] Shearman, M.S., Naor, Z., Kikkawa, U. and Nishizuka, Y. (1987) Biochem. Biophys. Res. Commun. 147, 911-919.
[29] Naor, Z., Shearman, M.S., Kishimoto, A and Nishizuka, Y. (1988) Mol. Endocrinol. 2, 1043-1048.
[30] Vassilets, L., Madefessel, K., Schwarz, W. and Schmalzing, G. (1989) Naunyn-Schmiedeberg's Arch Pharmacol. 340 Suppl., R91 (abstr.).