Protein Kinase A Reduces Voltage-dependent Na⁺ Current in *Xenopus* Oocytes

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The voltage-dependent Na⁺ channel of the brain is a good substrate for phosphorylation by the cAMP-dependent protein kinase (protein kinase A, or PKA), but the physiological effects of PKA on Na⁺ channels are poorly documented. We studied modulation by PKA of voltage-dependent Na⁺ channels expressed in *Xenopus* oocytes injected with RNA coding for the α -subunit of the channel protein (rat brain type IIA and its variant VA200), using the two electrode voltage-clamp technique. Intracellularly injected cAMP or catalytic subunit of PKA, or extracellularly applied forskolin, inhibited the Na⁺ current by 20–30%. The effect of cAMP was attenuated by prior injection of PKA inhibitors. Injection of small doses of protein phosphatase 2A increased the Na⁺ current by 10%, whereas larger doses of protein phosphatase 1 and alkaline phosphatase were without effect. The inhibition by PKA showed little voltage dependence, being only slightly stronger at holding potentials at which the availability of the channels was reduced. The voltage dependence of activation and inactivation processes was not altered by cAMP. Similar effects were exerted by forskolin and cAMP on the Na⁺ channels expressed after the injection of heterologous (total) RNA from rat brain. Thus, PKA modulates the Na⁺ channel by a mechanism that does not involve major changes in the voltage dependency of the current and is exerted on the channel-forming α -subunit.

In recent years, it has been shown that, like the voltage-dependent Ca²⁺ and K⁺ channels, Na⁺ channels are also subject to neuromodulation [e.g., thyrotropic-releasing hormone and epinephrine reduce Na⁺ currents in neurons and heart cells, respectively (Schubert et al., 1989; Lopez-Barneo et al., 1990)], although the data on Na⁺ channel modulation are still scarce.

Brain Na⁺ channels consist of an α -subunit (260 kDa) and two smaller (30–40 kDa) β_1 - and β_2 -subunits. The α -subunit forms a functional channel when its RNA is injected into *Xenopus* oocytes (Noda et al., 1986b; Auld et al., 1988, 1990). The role of the smaller subunits is unknown; they may be involved in regulation of channel kinetics and incorporation into the

membrane (Schmidt and Catterall, 1986; Krafte et al., 1988). The predicted primary structure of the α -subunit suggests at least eight putative protein kinase A (PKA) phosphorylation sites, six of which are in the predicted cytoplasmic segment between the internal repeats 1 and 2 (see Barchi, 1988; Catterall, 1988). Like many other channels (Kaczmarek and Levitan, 1987), the voltage-dependent Na⁺ channel appears to be modulated by phosphorylation catalyzed by protein kinases. Thus, activation of protein kinase C (PKC) reduces the Na⁺ current (I_{Na}) in neurons and in neuroblastoma cells (Linden and Routtenberg, 1989; Numann et al., 1991) and in RNA-injected oocytes that express brain Na⁺ channels (Sigel and Baur, 1988; Lotan et al., 1990; Dascal and Lotan, 1991), and diminishes the action potential in sciatic nerve (Meiri and Gross, 1989). The physiological effects of another major protein kinase, the cAMP-dependent protein kinase (PKA) are less well characterized. Activation of PKA was shown to reduce Na⁺ fluxes through voltage-dependent Na⁺ channels reconstituted in synaptosomes (Costa and Catterall, 1984). Enhancement of inactivation of I_{Na} in cultured rat brain cells has been reported (Coombs et al., 1988). β -Adrenergic agonists reduce cardiac Na⁺ current and shift its steady-state inactivation to more negative potentials (Schubert et al., 1989). It has been suggested that this effect is mediated in part by a G-protein (G_s) directly coupled to the channels, and partly via activation of PKA (Schubert et al., 1989). The scarcity of data on modulation of the neuronal Na⁺ channel seems surprising, in view of the fact that phosphorylation of its main, pore-forming α -subunit by PKA is well established and thoroughly studied. Thus, it has been shown that this protein is a good substrate for *in vitro* phosphorylation by PKA, and the phosphorylated amino acid residues have been identified (Costa et al., 1982; Rossie and Catterall, 1987, 1989; Rossie et al., 1987; Gordon et al., 1988); that rat brain Na⁺ channels are substantially phosphorylated *in vivo* in their basal state; and that agents elevating cAMP cause additional phosphorylation of the channels (Rossie and Catterall, 1987). One of the reasons for the poor documentation of the PKA effect on the neuronal Na⁺ channels may be that the effect is relatively small and difficult to monitor, or that the modulatory effect is “running down” in perfused neurons (in whole-cell patch clamp) or perfused axons, due to washout of cellular components participating in the modulation. Therefore, we have chosen to study modulation of the rat brain TTX-sensitive Na⁺ channel expressed in *Xenopus* oocytes. This preparation allows extremely stable recordings (the Na⁺ current I_{Na} is recorded by the two-electrode voltage-clamp technique and does not run down) and intracellular injection of cAMP and PKA without the necessity to dialyze the cell's interior.

Received July 10, 1991; revised Mar. 30, 1992; accepted Apr. 15, 1992.

We are grateful to Prof. Philip Cohen for supplying PP1 and PP2A and for helpful suggestions, and to Drs. Y. Palti (The Technion, Haifa), D. Gordon (Hebrew University, Jerusalem), and K. Kral (Graz University) for stimulating discussion and useful comments. This work was supported in part by grants from U.S.–Israel Binational Science Foundation (N.D. and I.L.), The Israel Institute of Neurobiology (I.L.), and The Austrian Research Fund (W.S., Project S4505B).

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Here we report the inhibitory effect of cAMP and the catalytic subunit of PKA (PKA-CS) on Na^+ currents expressed in oocytes injected with RNA coding for the α -subunit of brain Na^+ channel (type IIA, $I_{\text{Na}}\text{-IIA}$; type VA200, $I_{\text{Na}}\text{-VA200}$), and on Na^+ channels expressed after the injection of total rat brain RNA. In all cases, the reduction in current amplitude was not accompanied by substantial changes in voltage dependence of activation and inactivation of the macroscopic Na^+ current. Thus, PKA applied directly or activated via cAMP appears to reduce the Na^+ current by acting on the α -subunit of the channel without appreciably changing the voltage dependence of the channel. Also, it is shown that injection of the protein phosphatase (PP) 2A increased I_{Na} . This finding may also imply that dephosphorylation could serve as a possible modulatory pathway of the sodium channel function.

Materials and Methods

Oocytes. *Xenopus laevis* females were purchased from the South African Snake Farm (Fish Hock, South African Republic), or Xenopus I Inc. (Ann Arbor, MI). The animals were maintained at a 13 hr light/11 hr dark cycle at 18–21°C and dissected under MS-222 anesthesia as described elsewhere (Dascal et al., 1984). Oocytes (stage 5 and 6; see Dumont, 1972) were defolliculated at 22°C by 3–4 hr treatment with collagenase (Sigma type 1A; 1.5–2 mg/ml in Ca-free ND96 solution: 96 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 5 mM HEPES, pH 7.5). After defolliculation, the oocytes were washed and injected as described previously (Dascal et al., 1986) with either 200–350 ng of total RNA extracted from 19-d-old rat brain, or 2–20 ng of Na^+ (VA200 or IIA) channel RNA transcribed *in vitro* from the corresponding cDNA (see below). The oocytes were then incubated for 2–5 d in ND96 solution with the addition of 1.8 mM CaCl_2 , 2.5 mM sodium pyruvate, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml potassium penicillin at 22°C and then used for the electrophysiological experiments.

RNA. Total RNA was extracted from whole brains of 19-d-old rats by LiCl-urea procedure (see Lotan et al., 1988, for details). Rat brain RNA was dissolved in water at 4–7 mg/ml and stored in 3–10 μl aliquots until injection into the oocytes. VA200 Na^+ channel cRNA was generated by linearizing pVA200 construct with *Clal* and *in vitro* transcription with SP6 RNA polymerase. IIA Na^+ channel cRNA was generated similarly from pVA2580 construct, linearized by *Clal* and transcribed *in vitro* with T7 RNA-polymerase. Both were prepared essentially as described previously (Auld et al., 1988, 1990), with few modifications: the transcription was done in the presence of 0.5 mM ATP, UTP, CTP, and 0.05 mM GTP for the first hour, and then GTP concentration was raised to 0.5 mM and transcription continued for an additional 2 hr. The unincorporated nucleotides were removed by two ethanol precipitations from 1 M ammonium acetate aqueous solutions.

Solutions and chemicals. Recording of the currents was done in the ND96 solution (see above) with 1 mM CaCl_2 .

cAMP and AMP were dissolved at 10 mM in water and stored at -70°C in 10 μl aliquots. Forskolin and dideoxyforskolin were dissolved at 20 or 50 mM in dimethyl sulfoxide, stored at -20°C , and brought to the final concentration in ND96 solution just before the experiment. PKA-CS (from bovine heart, purchased from Sigma, 250 phosphorylating units) was dissolved in 30 or 60 μl of 5 mM dithiothreitol (DTT) in water (Sigma), dialyzed against 5 mM DTT containing 5 mM phosphate buffer (pH 6.5), in order to remove traces of sucrose, and then microconcentrated to a final volume of 20 μl . The enzyme was stored in 4°C in 5 μl aliquots and was used only for the next 48 hr. The amount of injected enzyme was 10–30 nl per oocyte, which corresponds to 0.1–0.4 phosphorylating units (according to the manufacturer's specifications). One phosphorylating unit corresponded to about 0.028 μg of protein. To inactivate PKA-CS for control experiments, the enzyme was heated at 80°C for 1 hr. Since DTT could be degraded by this procedure, to provide a better control, cold DTT (from 50 mM stock) was added to a final concentration of 5 mM to the heat-inactivated PKA-CS before the injection into the oocyte. The denatured enzyme was injected into oocytes of the same donor in which the effects of active PKA-CS were tested.

Two protein kinase inhibitors were used: a synthetic 20-mer peptide and a protein inhibitor purified from rat muscle (both from Sigma). The

inhibitors were dissolved in water and stored at -70°C in 5 μl aliquots. Ten picomoles of the peptide inhibitor or 400 ng of the protein inhibitor were injected from freshly thawed aliquots 5 min before the oocyte was voltage clamped.

The catalytic subunits of protein phosphatases 1 and 2A (PP1 and PP2A) were obtained from Philip Cohen (University of Dundee, UK). PP1 and PP2A were dissolved at 15,000 and 3000 U/ml, respectively, in a buffer consisting of 50 mM Tris HCl (pH 7.0), 0.1% β -mercaptoethanol, 0.1 mM EGTA, and 50% (v/v) glycerol and stored at -20°C . About 4 nl of each phosphatase was injected into an oocyte without causing significant changes in holding current; appearance of large inward currents that interfered with I_{Na} records prevented the study of the effects of injection of larger volumes. Alkaline phosphatase was purchased as lyophilized powder (Sigma P8647, 60% protein) and dissolved at 50,000 U/ml immediately before the injection into the oocytes, in a buffer consisting of 50 mM Tris HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM dithiothreitol. Ten nanoliters of this phosphatase was injected into each oocyte with no significant effects on the holding current.

cAMP, protein kinase inhibitor (PKI), AMP, phosphatases, and PKA-CS were pressure injected using a microinjection needle as described before (Dascal et al., 1984). Except for PKIs, the substances were injected after the oocyte was already voltage clamped. Insertion of the microinjection needle into the oocyte usually caused a slight shift in the holding current, which usually recovered within 2–5 min to its previous level. The injection itself had no effects on the holding current.

Electrophysiological recordings. An oocyte was placed in a 1 ml bath, usually constantly perfused at a slow rate with ND96. When forskolin was applied, the solution was perfused at a higher rate (the “dead time” of the perfusion system was 10–20 sec). The change in rate of flow of solution either did not affect I_{Na} or sometimes caused a small decrease in the current amplitude (up to 5%) that reversed in a few minutes.

The electrophysiological recording system was described elsewhere (Dascal and Lotan, 1991). In brief, the cell was impaled with two conventional 3 M KCl electrodes having resistances of 0.5–2 M Ω . The bathing solution was connected to the ground through a 3 M KCl agar bridge. Two-electrode voltage clamp was performed using the Dagan 8500 amplifier (Dagan Corp., Minneapolis, MN). Stimulation and data acquisition were done with an IBM XT computer supplied with a Techmar Labmaster ADC/DAC board, using the pCLAMP software (Axon Instruments, Burlingame, CA; see Kegel et al., 1985). The membrane potential was set to different holding potentials (HPs) depending on the type of I_{Na} under study. In some cells, at HPs that were supposed to reduce the availability of the channels (-60 to -65 mV), I_{Na} continuously declined for many minutes and did not reach a steady level; such cells were excluded from the study. The currents evoked by the various experimental protocols were filtered at 5 kHz with an eight-pole Bessel filter and recorded by the computer (the sampling rate for recording the I_{Na} was either 5 or 10 kHz in different protocols). In parallel, the membrane voltage and current were monitored with a dual beam storage oscilloscope. When constructing I - V curves, the command voltage rather than the actually measured voltage was drawn at the abscissa. To avoid possible errors that could be introduced by this procedure, any cells in which, at the peak of I_{Na} , the measured values of voltage deviated by more than 2 mV from the command voltage were discarded. In most cells, the deviation of measured voltage from the command voltage was within 0.5 mV.

Statistical analysis. The mean values in the text and in the tables are presented with $\pm$ SEM. Unless otherwise stated, two-sided t test was used to calculate the statistical significance of the differences between samples or between a sample and a constant number (e.g., decreases caused by cAMP were compared with a 0% decrease considered as control).

Results

cAMP and PKA-CS reduce $I_{\text{Na}}\text{-IIA}$ and $I_{\text{Na}}\text{-VA200}$, while PP2A enhances $I_{\text{Na}}\text{-VA200}$

We first tested the effects of intracellularly injected cAMP on Na^+ currents expressed in the oocytes injected with “cloned” cRNA that coded for the α -subunit of a variant of the rat brain type IIA Na^+ channel, VA200, which differs from IIA in a single amino acid residue and has somewhat different activation and inactivation characteristics (Auld et al., 1988, 1990). It has been reported that cAMP-dependent phosphorylation may promote

Figure 1. The inhibitory effect of cAMP and PKA-CS injection on I_{Na} -VA200 or I_{Na} -IIA. *A*, Effect of cAMP injection (10 pmol) on I_{Na} -VA200. The HP was -80 mV. I_{Na} was evoked by a 100 msec depolarizing step to $+10$ mV after a 100 msec prepulse to -100 mV. Net I_{Na} was obtained by digital subtraction of the current obtained after a prepulse to -20 mV (which inactivated I_{Na} by about 90%; see Dascal and Lotan, 1991). *B*, Effect of the injection of 20 nl of PKA-CS on I_{Na} -IIA. I_{Na} was evoked from an HP potential of -100 mV. The same protocol as in *A* was used except that the IIA I_{Na} was evoked by a depolarizing step to 0 mV. *C*, Time course of the effect of cAMP injection on I_{Na} -IIA. *D*, Time course of the effect of PKA-CS injection on I_{Na} -IIA (same oocyte as in *B*). *E*, Time course of the effect of injection of 4 nl PP2A on I_{Na} -VA200.

the inactivation of the Na^+ channel, and that the effect of cAMP is stronger at holding potentials that partially inactivate the channel (Coombs et al., 1988; Schubert et al., 1989). Therefore, I_{Na} was evoked either from HP at which the channels were fully available (-80 mV) or from HPs at which the channels were inactivated by 30–50% (-60 to -65 mV). The stability of the current has been verified by continuous monitoring for at least 10 min before cAMP injection (e.g., Fig. 1*C,D*). Linear leakage current was accounted for by a simple subtraction procedure (see Fig. 1 caption).

After pressure injection of 10–20 pmol of cAMP, I_{Na} -VA200 amplitude began to decline over the next 15–20 min until it reached a steady level, 12–30% below the control (Fig. 1*A*). The decrease was similar with 10, 20, or 40–70 pmol cAMP (Table 1), and the data with 10 and 20 pmol/oocyte were pooled. Much of the delay might be due to the time of cAMP diffusion to the membrane, owing to the very large dimensions of the oocyte (diameter of 1.1–1.3 mm). The inhibitory effect was only slightly stronger at HPs that partially inactivated the channel (Table 1; $p < 0.05$, two-tailed t test). In control experiments, injection of 40 pmol of AMP (a noncyclic analog of cAMP that does not activate PKA) had no effect on the current (Table 1).

To verify that the effect of cAMP was mediated by activation of PKA, we injected two kinds of specific inhibitors (PKIs) of

this protein kinase before the injection of cAMP (Table 1): a synthetic 20-mer PKI peptide at 10 pmol/oocyte (Cheng et al., 1986) or a PKI protein purified from rabbit muscle, at 400 ng/oocyte (see Lotan et al., 1985). No PKI by itself altered I_{Na} -VA200 (three cells with each of the PKIs). The inhibitors were injected several minutes before the experiment. As seen in Table 1, cAMP still reduced the I_{Na} -VA200 in the presence of the PKIs, but the effect of cAMP was weakened by about 50% by the PKI peptide, and by 63% by the PKI protein, $p < 0.005$ for the PKI protein, $p < 0.05$ for the PKI peptide, one-tailed t test). Injection of higher amounts of PKI protein caused mechanical damage to the oocytes, and this prevented the possibility of fully blocking the cAMP effect.

I_{Na} -IIA was reduced by cAMP injection similarly to I_{Na} -VA200 (Fig. 1*C*, Table 2). We also tested the effect of the direct injection of PKA-CS on I_{Na} -IIA (Fig. 1*B*). The inhibitory effect of the enzyme developed somewhat slower than that of cAMP (Fig. 1*D*), possibly due to a slower diffusion in the cytoplasm. The inhibition of I_{Na} -IIA by the enzyme was similar at HPs of -100 mV (100% channels available) and -70 mV (about 30% channels inactivated) (Table 2). Injection of heat-inactivated PKA-CS had almost no effect on the current (Table 2).

The kinetics of decay of type IIA and VA200 I_{Na} were not appreciably changed by cAMP or PKA-CS injections (e.g., Fig.

Table 1. Effect of cAMP and PKI on I_{Na} -VA200

Injection	HP (mV)	% Decrease in I_{Na} ^a	n	p ^b
cAMP (10–20 pmol)	–65 or –70	28.8 ± 1.6	27	<0.001
cAMP (10–20 pmol)	–80	23.3 ± 2.2	15	<0.001
cAMP (40–70 pmol)	–80	24.6 ± 2.4	5	<0.001
AMP (40 pmol)	–80	2 ± 0.9	4	>0.1
cAMP (10 pmol) ^c	–80	18.6 ± 2.5	5	<0.001
cAMP + PKI peptide ^c	–80	9.7 ± 2.9	4	<0.02
cAMP (10 pmol) ^d	–80	28.7 ± 3.9	3	<0.01
cAMP + 400 ng PKI protein ^d	–80	10.7 ± 2.3	4	<0.01

Each cell served as its own control; thus, the percentage decrease was calculated in each cell. The amount of injected PKI peptide was 10 pmol/oocyte; the amount of injected PKI protein was 400 ng/oocyte.

^a Mean ± SEM.

^b Compared with a 0% decrease using two-tailed *t* test.

^c Tested in oocytes of one frog during a 2-d experiment.

^d Tested in oocytes of another frog during a 2-d experiment.

1). The kinetics of the rising phase of I_{Na} were not adequately resolved because of the large-capacity transient that lasted for 2–2.5 msec. Although subtraction of currents obtained after block of I_{Na} by TTX improved the time resolution to some extent, it was not employed in the present study in order to avoid subtraction artifacts (Dascal and Lotan, 1991).

To probe the possible effect of dephosphorylation on I_{Na} , we injected protein phosphatases PP1, PP2A, and alkaline phosphatase (see Cohen, 1989). Injection of PP2A (0.012 U/oocyte) resulted in a small but statistically significant increase of I_{Na} -VA200 in all eight cells tested (Fig. 1E). The increase varied between 5% and 15% with a mean ± SEM of 10.4 ± 1.2. In contrast, injection of PP1 (0.06 U/oocyte) reduced I_{Na} by 4% in two cells. Note that PP2A and PP1 were injected in the same buffer and at the same volume (4 nl/oocyte) and thus could serve as internal controls for each other. Injection of alkaline phosphatase (0.45 U/oocyte) reduced I_{Na} by 4 ± 0.4% in four oocytes.

Forskolin reduces I_{Na}

In two oocytes, the effect of extracellularly applied forskolin (an adenylate cyclase activator) was tested at HPs of –80 mV and –60 mV (Fig. 2A). I_{Na} -VA200 was evoked by steps to 10 mV from HP = –80 mV; then, the HP was changed to –60 mV, which reduced the availability of the channels by about 40%, and the current measured for several minutes. Then, the HP was set again at –80 mV, and the recovery of the current was verified. The same procedure was repeated in the presence of forskolin (25 μM). In additional experiments, the effects of for-

skolin were tested separately at different HPs in different oocytes. Altogether, forskolin reduced the current to a smaller extent at HP = –80 mV than at HP of –60 or –70 mV [see Fig. 2A; average decrease of I_{Na} at –60 to –70 mV was 23.7 ± 5.9% (*n* = 4), whereas at –80 mV the decrease was 9 ± 1.5% (*n* = 3)].

To test whether the inhibition by forskolin is due to elevation of cAMP levels or to a direct effect on the channel, similar experiments were performed with dideoxyforskolin, an analog of forskolin that does not activate adenylate cyclase (in this case, the IIA clone was used). At 25 μM, dideoxyforskolin did not affect I_{Na} at an HP of –100 mV in three cells (the current was 98.4 ± 1.2% of control), but reduced it by 14 ± 1.56% in other three cells in which the HP was –65 mV. In two additional oocytes, the effect of dideoxyforskolin was tested at two HPs, –100 mV and –55 mV (Fig. 2B). Holding the cell at –55 mV reduced I_{Na} by about 60%. Dideoxyforskolin (50 μM) did not affect I_{Na} at –100 mV in both cells but reduced the current at the inactivating HP by 24% and 30%. These results suggest a direct blocking effect of the Na⁺ channel by forskolin and its derivatives at inactivating HPs.

cAMP does not affect the voltage dependence of activation and inactivation of I_{Na}

Injection of cAMP had no appreciable effect on the current-voltage (*I*–*V*) relation of I_{Na} (both IIA and VA200), except for a general decrease in the amplitude at all voltages (Fig. 3). To assess the cAMP effect quantitatively, the *I*–*V* relations obtained before and after application of cAMP (in each cell) were fitted to a modified Boltzmann equation (Eq. 1 in Fig. 3 caption) as described previously (Dascal and Lotan, 1991), assuming the existence of three activation gates (Hodgkin and Huxley, 1952). In the fitting procedure, the reversal potential (*V*_r) was allowed to change within ±4 mV of the value obtained by simple extrapolation by eye of the *I*–*V* curve. The other three free parameters were *V*_a (the voltage at which the probability of a single gate opening is one-half), *a*_m (the slope factor, which corresponds to a change in voltage that produces an *e*-fold change in conductance), and *G*_{max} (the maximal Na⁺ conductance). These three parameters have been allowed to change over very wide ranges. Steady-state activation curves were drawn using the parameters obtained in these fits. In all cells, both the slope factor (*a*_m) and

Table 2. Effect of cAMP and PKA-CS on I_{Na} -IIA

Injection	HP (mV)	% Decrease in I_{Na} ± SEM	n	p
cAMP (10 pmol)	–80	26.42 ± 2.17	7	<0.001 ^a
PKA-CS (20 nl)	–100	23.16 ± 2.57	6	<0.001 ^b
PKA-CS (20 nl)	–70	23.0 ± 3.5	3	<0.02 ^b
Inactivated PKA-CS	–100	6.0 ± 1.8	3	>0.05 ^a

^a Compared with a 0% decrease using two-tailed *t* test.

^b *p* was calculated using paired two-tailed *t* test by comparing the decreases caused by active enzyme with that caused by heat-inactivated PKA-CS.

Figure 2. Effects of forskolin and dideoxyforskolin on I_{Na} . *A*, Time course of the inhibitory effect of 25 μ M forskolin on I_{Na} -VA200 (protocol as in Fig. 1*A*). I_{Na} was monitored at HP of -60 or -80 mV as indicated. *B*, Time course of the inhibitory effect of 50 μ M dideoxyforskolin on I_{Na} -IIA. I_{Na} was monitored at HP of -100 or -55 mV as indicated.

Figure 3. cAMP does not alter the voltage dependence of I_{Na} (IIA and VA200). *A*, The experimentally obtained current-voltage relations of I_{Na} -IIA in control (open circles) and in the presence of cAMP (solid circles). The data were fitted, using a nonlinear least square algorithm, to the equation

$$I_{Na} = G_{max}(V_m - V_r) / (1 + \exp(-(V_m - V_a)/a_m))^3, \quad (1)$$

where V_m is the membrane voltage, and the other parameters are defined in the text (see also Dascal and Lotan, 1991, for details). In the oocytes shown in this figure, the following parameters were obtained: controls, $V_r = 42.15$ mV, $G_{max} = 23.35$ μ S, $V_a = -17.32$ mV, $a_m = 7.37$ mV; cAMP, $V_r = 41.17$ mV, $G_{max} = 20.35$ μ S, $V_a = -14.03$ mV, $a_m = 7.7$ mV. *B*, The current-voltage relation shown in *A* presented as an activation curve, that is, G/G_{max} versus V_m . Open circles, control; solid circles, cAMP. *C*, Fast inactivation (of I_{Na} -VA200). The curve was obtained by holding the cell at HP = -80 mV and stepping the voltage to various voltages ($V_{prepulse}$) for 100 msec and then measuring I_{Na} by a step to 10 mV. Fractional current (I/I_{max}) was drawn as a function of $V_{prepulse}$, and the data 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 to 10 mV, and the parameters V_h (half-inactivation voltage) and a_h (slope factor) were allowed to change in wide ranges. In the experiment shown, the parameters (in mV) were as follows: control (open circles), $V_h = -37.28$, $a_h = 9.43$; cAMP (solid circles), $V_h = -39.1$, $a_h = 9.34$. *D*, Slow inactivation of I_{Na} -VA200. The curve was obtained using the same protocol as in *C* except that the duration of the prepulse was 2 sec. In the experiment shown, the parameters (in mV) were as follows: control (open circles), $V_h = -57.5$, $a_h = 6.02$; cAMP (solid circles), $V_h = -57.97$, $a_h = 6.00$.

Figure 4. The inhibitory effect of cAMP on the total rat brain I_{Na} . *A*, Effect of injection of 30 pmol of cAMP into the oocyte. The HP was -80 mV. I_{Na} was evoked by a 20 msec depolarizing step to -10 mV after a 40 msec prepulse to -100 mV. *B*, Activation curve of I_{Na} before (open circles) and after (solid circles) injection of 30 pmol of cAMP. The points represent mean $\pm$ SEM values of G/G_{max} obtained in five cells; no fits to Boltzmann equation were done. *C*, Steady-state inactivation of I_{Na} before (open circles) and after (solid circles) the injection of cAMP. The points represent mean $\pm$ SEM values of I/I_{max} obtained in five cells. No fits to Boltzmann equation have been performed.

the half-activation voltage (V_a) were not significantly affected by cAMP in both types of cloned channel (IIA, $n = 4$; VA200, $n = 4$; see Fig. 3). The reversal potential was slightly shifted to negative potentials (by about 4 mV at the average). This effect could reflect a genuine change in channel selectivity, but could also result from inaccuracies in the measurement of the small currents at positive potentials, which could be partially obscured by the capacity transient. The maximal conductance (G_{max}) was significantly reduced in both cases (average reduction of $20 \pm 2\%$ for I_{Na} -VA200, and average reduction of $21 \pm 8\%$ for I_{Na} -IIA; $n = 4$ in both cases).

As was shown by Krafte et al. (1990), two inactivation processes (slow and fast) can be distinguished in oocytes injected with RNA coding for the α -subunit of the type IIA and VA200 Na^+ channel. Two protocols were used to obtain the fast and slow steady-state inactivation curves of I_{Na} , which differed in the duration of the inactivating prepulse: 2 sec for the slow inactivation, 100 msec for the fast inactivation (see Fig. 3 caption). Figure 3, *C* and *D*, depicts the inactivation curves for I_{Na} -VA200. Both fast and slow steady-state inactivation properties of I_{Na} -VA200 (slow inactivation, $n = 3$; fast inactivation, $n = 5$) and I_{Na} -IIA (slow inactivation, $n = 8$) were not affected by the injection of cAMP (Fig. 3*C,D*).

To test whether the parameters of steady-state inactivation of I_{Na} -IIA are affected by HP, the effect of cAMP on the slow steady-state inactivation curve of I_{Na} -IIA was studied at HPs of -100 mV (all channels available) or -65 mV and -70 mV, at which the current is 30–40% inactivated (the protocol with 2 sec inactivating prepulses has been used). However, there was no difference in inactivation parameters of the current following cAMP injection at either HP (HP = -100 , $n = 3$; HP = -65 or -70 , $n = 5$).

I_{Na} in oocytes injected with heterologous rat brain RNA is modulated similarly to I_{Na} -IIA and I_{Na} -VA200

Heterologous (total) RNA from brain contains messages for brain-specific proteins that, in principle, could play a role in mediating the effect of PKA on the Na^+ channel. If this was the case, modulation by PKA of the Na^+ current expressed in oocytes injected with heterologous brain RNA could be different from that in oocytes that expressed only the α -subunit. Injection of 30 pmol of cAMP caused a small but consistent reduction of I_{Na} in oocytes injected with heterologous rat brain RNA (Fig. 4*A*) by $12.0 \pm 2.2\%$ (HP = -100 mV; $n = 6$; $p < 0.01$). The voltage dependence of I_{Na} activation and steady-state inactivation was virtually unaffected by cAMP, as can be seen in Figure 4, *B* and *C*. Both the fast inactivation and the activation were not significantly different in controls and cAMP-injected oocytes. The time course of I_{Na} decay was also unchanged by cAMP: the time constant of the decay (one-exponent fit) was 1.72 ± 0.29 msec ($n = 5$) in control, and 1.78 ± 0.25 msec ($n = 5$) after cAMP injection. Figure 5 illustrates the inhibitory effect of 50 μ M forskolin applied by perfusion. The oocytes were kept at HP = -65 mV (at this potential, only 5–10% of channels were inactivated in oocytes injected with rat brain RNA), and the current was monitored until it became stable. At 50 μ M, forskolin reduced I_{Na} by $17.2 \pm 4.8\%$ (Fig. 5*A*; $n = 4$), $p < 0.025$). The current was almost fully recovered after washout of the drug (Fig. 5*B*). As with the cAMP effect, forskolin did not have any significant effect on the voltage dependence of I_{Na} activation and inactivation (Fig. 5*C,D*).

Figure 5. The inhibitory effect of forskolin on the rat brain I_{Na} . *A*, Effect of 50 μ M forskolin on I_{Na} . The HP was -65 mV. Currents were evoked by a 20 msec depolarizing step to -10 mV. After the current in control solution reached a steady level (trace 1), forskolin was applied and the current was monitored until maximal reduction was achieved (trace 2). The current recovered after the washout of the drug (trace 3). *B*, Time course of the effect of forskolin application described in *A*. *C*, The activation curve of I_{Na} before (open circles) and after (solid circles) application of forskolin, drawn as in Figure 4*B* (four cells). *D*, Steady-state inactivation of I_{Na} before (open circles) and after (solid circles) application of forskolin, drawn as in Figure 4*C* (four cells).

Discussion

PKA reduces the Na^+ current by an effect on the α -subunit of the channel: roles of phosphorylation and dephosphorylation

The main finding of this study is that PKA and its activators cause a modest ($\sim 25\%$) but consistent reduction of the current flowing through the voltage-dependent brain Na^+ channel (type IIA). This has been demonstrated by direct intracellular injection of PKA-CS and of cAMP (the latter presumably acted via activation of the oocyte's endogenous PKA). Injection of AMP (the inactive, noncyclic analog of cAMP) did not affect I_{Na} , whereas the effect of cAMP was significantly (up to 63%) suppressed by PKA inhibitors, excluding the possibility of a non-specific effect. Extracellularly applied forskolin also inhibited I_{Na} , presumably via activation of adenylate cyclase leading to an increase in intracellular cAMP levels (although it might have a direct blocking effect at inactivating HPs; see below). These results strongly suggest that the observed inhibition of I_{Na} is a genuine result of PKA action.

Since 10, 20, and 70 pmol/oocyte cAMP and the employed doses of PKA-CS had similar inhibitory effects, it is likely that the observed 20–28% inhibition of I_{Na} was the maximum physiologically achievable effect. The basal cAMP level in the oocyte is 1–3 pmol/oocyte (see Dascal, 1987), and injection of 10 pmol already leads to a significant elevation. Further, assuming 50% free water in the oocyte (see Dascal, 1987), we calculated that

the injected amount of PKA-CS should give a concentration of 6–25 μ g/ml (0.15–0.6 μ M), whereas the physiological concentration of PKA in various tissues is 12–30 μ g/ml (Hofmann et al., 1977); thus, such injection would mimic activation of almost all intracellular PKA (i.e., an almost complete dissociation of the regulatory and the catalytic subunits). It is possible that the achieved inhibition is the result of a maximal phosphorylation of the channel polypeptide (four to five phosphates per molecule; see Catterall, 1988). However, it is more conceivable that, as in the brain, much of the channels are already partially phosphorylated in the basal state (Rossie and Catterall, 1987), and therefore each individual channel could be affected to a greater extent if it were completely dephosphorylated in the basal state. If this is correct, one would expect a great variability in the effects of PKA when tested on the single-channel level, since the basal phosphorylation state of each channel may be expected to vary quite substantially and in a “quantal” fashion.

The enhancement of I_{Na} by PP2A supports the idea that, as in the brain, the channel protein is phosphorylated in its basal state when it is expressed in the oocyte. The small extent of increase (10% on the average) of the basal current by PP2A was most probably the unfortunate consequence of the inability, due to technical difficulties (see Materials and Methods), to test higher concentrations of this phosphatase. The injected amount corresponded to a final concentration in the cytoplasm of about 24 U/ml assuming uniform distribution of the enzyme in the water

content of the oocyte and discounting the effects of inhibitory metabolites and macromolecules that are known to suppress PP2A activity strongly in concentrated tissue extracts (e.g., Ingebritsen et al., 1983). The potential concentration of PP2A and PP1 in the oocyte is about 50–100 U/ml based on activity measurements carried out in highly diluted extracts to minimize the effects of the above mentioned inhibitory substances (P. Cohen, personal communication). It is possible that a stronger effect of PP2A might have been obtained if higher concentrations could have been microinjected. Nevertheless, the fact that the injection of two other phosphatases at much higher concentrations (PP1 at 120 U/ml, alkaline phosphatase at 1000 U/ml) had no significant effect on I_{Na} suggests that the observed phosphatase action was genuine.

The present series of experiments was not intended to study in depth the effects of phosphatases on the Na^+ channel, and the results should at present be considered preliminary. Furthermore, we do not know at present dephosphorylation which sites of the channel caused the increase in I_{Na} ; those phosphorylated by PKA or maybe by PKC. Furthermore, calcineurin (PP2B), a phosphatase that is supposed to dephosphorylate specifically the PKA-phosphorylated sites of the Na^+ channel (Rossie and Catterall, 1987), was not available to us in reasonable amounts during this work and should be tested in the future. However, the present results already raise two important considerations. One is that, like phosphorylation, dephosphorylation may be a relevant physiological factor modulating the Na^+ channel activity (see also Rossie and Catterall, 1987). The second is that modulation by phosphatases of the Na^+ channel may be phosphatase specific, and thus separate signaling pathways mediated by different phosphatases may exist, similarly to those for protein kinases. Notably, PP1 downregulates a voltage-dependent Ca^{2+} channel, while PP2A is ineffective (Kameyama et al., 1986), while they show an opposite specificity toward the Na^+ channel (present results) and toward maxi- K^+ channels (Reinhart et al., 1991).

Most of the effect of PKA is exerted upon the main, pore-forming α -subunit of the channel, and does not depend on the presence of some brain-specific proteins that one might suspect to be involved in modulation. This is demonstrated by the fact that modulation by cAMP and forskolin of Na^+ channels directed by heterologous brain RNA is very similar to that of the channels directed by the cloned cRNA coding for the α -subunit alone. These findings strengthen the notion that the modulation of the Na^+ channel by PKA is due to phosphorylation of the main (α) subunit, as suggested by biochemical studies (Rossie and Catterall, 1987); this is also in agreement with what is believed to occur in a similar member of the ion channel family, the voltage-dependent L-type Ca^{2+} channel (Flockerzi et al., 1986; Kameyama et al., 1986; Mundina-Weilenmann et al., 1991), and what has been demonstrated for PKC modulation of the Na^+ channel (West et al., 1991). However, the possibility that the target for phosphorylation is an ubiquitous cellular protein associated with the Na^+ channel both in oocytes and in nerve cells cannot be excluded, and this problem will require further study.

The effect of PKA on brain Na^+ channel is not voltage dependent

In our experiments, the inhibitory effect of cAMP was largely voltage independent. The inhibition was only slightly stronger at holding potentials that partially inactivated the channel pop-

ulation (28% at inactivating HP vs. 23% at noninactivating HP); no shifts in inactivation curve have been observed at any HP, even with long (2 sec) inactivating prepulses that were sufficient to initiate the slow inactivation process. The effect of forskolin appeared to show a somewhat stronger dependence on HP than that of cAMP, inhibiting I_{Na} -VA200 by 9% at HP = -80 mV and by 24% at HP = -60 (or -65) mV. However, forskolin might partially inhibit the channel directly and voltage dependently (open channel block), rather than via cAMP, as has been shown for voltage-dependent K^+ (Garber et al., 1990, and references therein) and Ca^{2+} (Boutjdir et al., 1990) channels. Indeed, the inhibitory effect of the same dose (25 μ M) of dideoxyforskolin (14% at inactivating HP), an analog of forskolin that does not activate adenylate cyclase, fully supports this hypothesis. However, unlike forskolin, dideoxyforskolin was without any effect at HPs that render all channels available, suggesting that the mild inhibition of I_{Na} by forskolin at such potentials was due to its action via cAMP production.

The parameters of the voltage dependence of activation, as obtained by fitting the data to a Boltzmann equation using the classical Hodgkin-Huxley (1952) algorithm, were also unaltered by cAMP, and the only significant change that probably accounted for all of the cAMP effect was a reduction in the maximal Na^+ conductance, G_{max} . This might be the result of a decreased probability of channel opening, a decrease in the number of activatable channels, or a decrease in single-channel conductance. To discriminate between these possibilities, single-channel recordings will be necessary.

Although our results are in general agreement with the published data suggesting an inhibitory effect of PKA on Na^+ channels, there is one major difference between our results and the two reports in which the voltage dependence of PKA modulation has been mentioned or implied. In cultured embryonic rat brain cells, Coombs et al. (1988) observed a negative shift in the Na^+ current steady-state inactivation curve caused by intracellularly perfused cAMP. However, this shift occurred only in about 50% of the cells, and only 20–70% of the channel population appeared to be affected. One of the reasons for the discrepancy between our results and those of Coombs et al. might be a different modulation by PKA of Na^+ channel subtypes; there are at least four in the brain (Noda et al., 1986a; Auld et al., 1988, 1990; Kayano et al., 1988). Thus, type III channel is predominant in the embryonic nerve cells (Beckh et al., 1989), while we have been working with the IIA type or its variant, VA200, that might be modulated differently. A smaller effect of cAMP on the Na^+ current in oocytes injected with heterologous 19 d rat brain RNA (~12% inhibition as compared with 25% with the IIA or VA200 cRNA), which presumably gives rise to several subtypes of the Na^+ channel found in the postnatal brain (I, II, and IIA; see Beckh et al., 1989), seems to support the assumption that different channel subtypes may be modulated differently, and some of them may not be sensitive to PKA at all. Another reason for the discrepancy may be the different recording methods. Whole-cell patch-clamp recording used by Coombs et al. involves dialysis of the cell that by itself may cause shifts in inactivation curves (e.g., Schubert et al., 1989); cAMP might affect this process and thus complicate the interpretation of the results. In the two-electrode voltage-clamp recordings employed in the oocytes, the interior of the cell remained intact in the course of the experiment, and no spontaneous shifts in the activation and inactivation parameters could be detected over tens of minutes of recording.

In cardiac myocytes, isoproterenol (a β -adrenergic agonist) inhibits the voltage-dependent Na^+ current and shifts the steady-state inactivation curve to negative potentials; both effects are more pronounced at HPs that reduce the availability of the channels (Schubert et al., 1989). Schubert et al. have also shown that a major part of this effect is due to a direct coupling of G_i with the Na^+ channel, but another part of the inhibition appears to be mediated via the activation of PKA, since it is mimicked by 8-bromo-cAMP. However, the voltage dependence of the effect of 8-bromo-cAMP has not been reported (only values obtained at inactivating HP have been presented). Thus, it is difficult to compare our results with those of Schubert et al., and it is early to conclude that modulation of cardiac and nerve Na^+ channels by PKA differs in its voltage dependence.

Possible physiological significance

Recent data (see introductory remarks), including those presented here, strongly suggest that the voltage-dependent Na^+ channel is subject to modulation by neurotransmitters (hormones) and second messengers, despite what could be conceived owing to the all-or-none properties of the action potential. Indeed, a substantial inhibition of the Na^+ current by a neurotransmitter would abolish the action potential and interrupt intraneuronal communication. However, a mild reduction in I_{Na} , such as found in the present work, will not suppress the action potential, but it will decrease its amplitude and shorten the duration, increase the threshold of excitation, and delay the appearance of the next action potential (a 3–5% decrease in amplitude and up to several milliseconds delay were observed by using a simple simulation program, given a 25% decrease in G_{Na} ; not shown). As we have already proposed for the PKC modulation (Dascal and Lotan, 1991), the PKA modulation of the Na^+ channel may be of the “silent” kind; that is, the transmitter that elevates the cAMP level does not necessarily have to produce any change in the resting membrane potential or conductance in order to affect excitability. One pattern of modulation may thus be an increase in the threshold of excitation at the axonal hillock. In another scenario, by slightly reducing the amplitude of an action potential a neurotransmitter may modulate the extent of opening of voltage-dependent Ca^{2+} channels, thus regulating Ca^{2+} entry into nerve terminals and the amount of the released transmitter. Indeed, a partial block of Na^+ channels by low doses of TTX has been shown to diminish the transmitter release at the neuromuscular junction without inhibiting the propagating action potential (Dascal et al., 1981). Other patterns of neuromodulation through altered Na^+ channel function are conceivable, and it will be of interest to track them in the nervous system.

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