cAMP-activated Na^+ Current of Molluscan Neurons Is Resistant to Kinase Inhibitors and Is Gated by cAMP in the Isolated Patch

Leland C. Sudlow, Rong-Chi Huang,[a] Daniel J. Green,[b] and Rhanor Gillette

Department of Physiology and Biophysics and the Neuroscience Program, University of Illinois, Urbana, Illinois 61801

The cAMP-dependent Na^+ current ($I_{Na,cAMP}$) modulates excitability in many molluscan neurons. Rapid activation of $I_{Na,cAMP}$ by cyclic nucleotide, its ion dependence, and its blockade by divalent cations resemble cyclic nucleotide–activated cation currents in vertebrate photoreceptors and olfactory receptors, where activation has been found to be independent of kinase activity. We tested the phosphorylation dependence of $I_{Na,cAMP}$ in neurons of the feeding and locomotory networks of the predatory marine snail *Pleurobranchaea*. Identified neurons of pedal and buccal ganglia were axotomized for recording the $I_{Na,cAMP}$ response to iontophoretic injection of cAMP under two-electrode voltage clamp. Intracellular injections of specific peptide inhibitor of protein kinase A had no blocking effects on activation of $I_{Na,cAMP}$ by iontophoretic injection of cAMP. Inward single-channel currents were activated in excised inside-out patches during exposure to cAMP in salines without added ATP. Sodium was the major current carrying ion. Two distinct types of $I_{Na,cAMP}$ channel activity were observed, where opening probability and open times differed, but conductance was similar, 36.7 pS. These observations suggest that $I_{Na,cAMP}$ activation occurs by direct binding of cAMP to a regulatory site at the channel, rather than by phosphorylation.

[Key words: cAMP, cAMP-gated ion channels, kinase inhibitors,* Pleurobranchaea*]

The cAMP-activated Na^+ current ($I_{Na,cAMP}$) of molluscan neurons is a prominent, second messenger–regulated ion current whose exact mechanism of activation has been uncertain. $I_{Na,cAMP}$ is widely distributed among central neurons of opisthobranch and pulmonate gastropods (Aldenhoff et al., 1983; Kononenko et al., 1983; Connor and Hockberger, 1984; Swandulla, 1987; McCrohan and Gillette, 1988a). The current participates in neuromodulatory responses (Kirk and Scheller, 1986; Huang and Gillette, 1987) and epileptogenic mechanisms (McCrohan and Gillette, 1988b), and it is coregulated in multiple ways by Ca^{2+} and intracellular pH (Gillette and Green, 1987; Green and Gillette, 1988; Kehoe, 1990).

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Correspondence should be addressed to Dr. Rhanor Gillette, Department of Physiology and Biophysics, 524 Burrill Hall, 407 South Goodwin Avenue, University of Illinois, Urbana, IL 61801.

[a]Present address: Department of Neuroscience, The Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205.

[b]Present address: The Eye and Ear Infirmary, 1855 West Taylor Street, University of Illinois at Chicago, Chicago, IL 60612.

There is a question as to whether this current is mediated by processes of cAMP-dependent phosphorylation, through the conventional A-type kinase ubiquitous to vertebrates and invertebrates. The alternative is that it may be activated in a more direct way independently of phosphorylation processes, like cyclic nucleotide–dependent cation currents of vertebrate photoreceptor, olfactory epithelium, cardiac muscle, and cochlear hair cells (Fesenko et al., 1985; Haynes and Yau, 1985; Nakamura and Gold, 1987; DiFrancesco and Tortora, 1991; Firestein et al., 1991; Kolesnikov et al., 1991; Zufall et al., 1991). Support for a nonphosphorylation mechanism, in neurons of *Helix* and *Aplysia*, comes from reports of resistance of the current to the actions of inhibitors of kinase A (Swandulla, 1987; Kehoe, 1990). In neurons of *Pleurobranchaea*, Huang and Gillette (1991) measured activation of $I_{Na,cAMP}$ with an estimated stoichiometry of 1.0, a value less than expected if a conventional kinase A, activated by two cAMP molecules (Beavo et al., 1974; Smith et al., 1981; Russell and Steinberg, 1987), were involved. On the other hand, cAMP-induced Na^+ currents in the snail *Achatina* are suppressed by isoquinalinesulfonamide kinase inhibitors and the specific peptide inhibitor of kinase A (Funase, 1990). We perceived a need for further testing the phosphorylation dependence of $I_{Na,cAMP}$ in molluscan neurons, and we wished specifically to test neurons of *Pleurobranchaea*.

We tested the phosphorylation dependence of $I_{Na,cAMP}$ in two ways. In the whole cell we assayed effects of specific and potent kinase inhibitors on activation of the current. In excised, inside-out membrane patches containing $I_{Na,cAMP}$ channels, we recorded the effects of cAMP added to the cytoplasmic side. The results of these experiments are evidence that mechanisms not involving phosphorylation mediate the activation and inactivation of the current.

Portions of these data were presented previously in abstract form (Huang and Gillette, 1989).

Materials and Methods

Specimens of the sea-slug *Pleurobranchaea californica* (20–600 gm) were obtained from Pacific BioMarine (Santa Monica, CA) and Sea-Life Supply (Sand City, CA) and kept in artificial seawater (Instant Ocean) at 15°C until use.

Testing kinase inhibitors under voltage clamp. The effects of kinase inhibitors were tested on the inward Na^+ current responses to iontophoretic injection of cyclic AMP in identified neurons. For voltage clamping, ganglia were removed and the somata of the identified ventral white cells of the buccal ganglion (Gillette et al., 1980; Green and Gillette, 1983) or members of a cluster of neurons on the dorsal surface of the pedal ganglia (Huang and Gillette, 1991; Sudlow and Gillette, 1991) were exposed by microdissection. The large size of the pedal neurons (200–400 μm) and their axon paths suggest function as peripheral effectors, probably foot motor neurons. Normal saline composition was (in mM) 420 NaCl, 10 KCl, 25 $MgCl_2$, 25 $MgSO_4$, 10 $CaCl_2$, and 10 MOPS buffer, adjusted to pH 7.5 with NaOH. Experiments were carried

Table 1. Effects of kinase inhibitors on $I_{Na,cAMP}$

	Amplitude (nA)		Duration (sec)	
	Before	After	Before	After
PKI protein injections (ventral white cells, $N = 3$)				
Mean	0.74	0.81	42.75	66.00
SEM	0.13	0.15	34.23	63.60
PKI fragment injections (ventral white cells, $N = 3$, and pedal cells, $N = 5$)				
Mean	0.97	1.09	24.71	30.14
SEM	0.19	0.21	3.11	4.58

All before/after comparisons were not significantly different; $p > 0.05$, paired t test.

out at 12–14°C. A voltage electrode and a double-barreled capillary electrode were inserted for voltage clamping. One of the double barrels was filled with 3 M KCl for the current electrode, and the second barrel was filled with 0.2 M cyclic AMP (Sigma) and 20 mM Tris buffer adjusted to pH 7.4 with KOH. For intracellular iontophoresis of cyclic AMP, negative current was passed through the cyclic AMP barrel during voltage clamp. Typically, iontophoretic currents were 20–50 nA, and injection duration was 5 sec. While the $I_{Na,cAMP}$ response is usually over 10 nA in amplitude at maximum, cyclic AMP injection current was adjusted to elicit ion currents near 1 nA, using a constant current source, to avoid saturation. Measurements of $I_{Na,cAMP}$ were recorded at −50 mV. For pressure injections a separate electrode was inserted into the neuron. Current and voltage traces from the voltage clamp were recorded on a chart recorder. Injections of cyclic AMP were made at regular 2–3 min intervals.

For intracellular pressure injection, protein kinase inhibitor (PKI) protein (bovine heart type II from Sigma) was suspended in 100 mM KCl and 10 mM HEPES at pH 7.3, dialyzed for 24 hr against the same buffer and diluted to a final concentration of 14.1 mg/ml. This concentration of the specific batch we used effectively inhibited cyclic AMP–dependent phosphorylation in *Pleurobranchaea* nervous system homogenates by more than 80% (Gillette et al., 1990). The synthetic, 20 amino acid active fragment of the PKI protein was obtained in >99% pure form (Bachem Ltd.). A 0.05% solution (225 μM) for intracellular injection was made up in 200 mM KCl and 10 mM MOPS buffer, pH 7.3. The K_i of this peptide fragment for inhibition of cyclic AMP–dependent protein kinase from rabbit skeletal muscle is 0.3 nM (Cheng et al., 1986). Prior to filling injection electrodes, solutions were briefly centrifuged to prevent clogging of the pipette. Pressure injection electrode tips were broken to 1–2 μm. Injections were made in repeated pulses under manual control over the course of the experiment. Final injection volumes in most cases were one to several volumes of the neuron somata, estimated from visible swelling of the somata. Thus, the final intracellular concentration for the inhibitor protein was well within an expected effective range for kinase inhibition, while the final intracellular concentration of the inhibitor fragment should have been in excess of 10^5 times the K_i. Successful injections were also indicated by transient changes in membrane current ascribed to viscoelastic effects of injection on the neuron membrane and ion conductance. Data are reported as the mean ± 1 standard error of the mean (SEM).

Patch-clamp procedures. Buccal and pedal ganglia were manually desheathed and digested in a proteolytic cocktail consisting of 13 U/ml dispase II (Boehringer-Mannheim) and 13 U/ml pronase E (Sigma) for 45 min at room temperature. The enzyme-cleaned neurons were then rinsed and patched in normal saline. The efficacy of enzyme cleaning was quite variable; it is our impression that the sources of variability appeared to proceed both from the batches of enzyme as well as from the animals themselves. Underdigestion failed to remove glia, while apparent overdigestion seemed to remove channels from the somata and often killed the cells. Electrodes were pulled from Corning 7052 glass, fire polished, coated with Sylgard, and filled with (in mM) 420 NaCl, 10 KCl, 50 $MgCl_2$, 12.5 MOPS, adjusted to pH 7.5 with NaOH; this saline did not have added Ca^{2+}. Electrodes had resistances of 2–8 MΩ and made patches with seals of 10–80 GΩ. Inside-out patches were excised and moved into one opening of a two-barrel gravity-feed perfusion system. From one barrel flowed an artificial intracellular saline

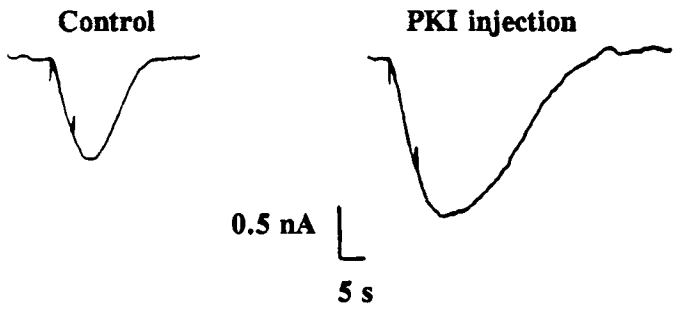

Figure 1. Lack of effect of kinase inhibitors. Intracellular injection of the kinase inhibitor protein into a ventral white cell failed to reduce the $I_{Na,cAMP}$ response to injected cAMP after 20 min (*right*). The actual increase over preinjection control record (*left*) is a nonspecific result of intracellular injection, and was not always observed (Table 1). Intervals of cAMP iontophoresis are marked in the records by small stimulus artifacts.

(in mM: 10 NaCl, 328 KCl, 10 KOH, 5 $MgCl_2$, 1 K_2EGTA, 140 L-arginine hydrochloride, and 12.5 MOPS at pH 7.5). Osmolarity of the cytoplasmic and electrode salines was verified on a vapor-pressure osmometer (Wescor), and final osmolarity was adjusted with mannose. In the second barrel the same saline contained 1 mM cyclic AMP. No nucleotide triphosphates were added. Excised patch longevity varied from a few minutes to over 1 hr. Single-channel currents were recorded with a patch-clamp amplifier (Axopatch-1D with integrating headstage IHS-1; Axon) and filtered at 2 kHz. Data were acquired at 10 kHz and analyzed with the pCLAMP software suite (version 5.5.1, Axon). Channel openings were attended by rapid, burst-like flickering; closures lasting less than 300 μsec were ignored in our analyses. Data are reported as the mean ± SEM.

Results

Effects of PKIs

Significant effects of kinase inhibitors were not observed, neither upon amplitude nor upon duration of the current. The results of experiments where kinase inhibitors were injected intracellularly are summarized in Table 1.

In each of three experiments where the PKI protein was injected into the ventral white cells, and in three further experiments where the active 20 amino acid fragment of the inhibitor protein was injected, no significant decrease in the amplitude of the cAMP-stimulated inward current was observed, even after multiple injections (Fig. 1). In fact, in the experiment shown in Figure 1 an increase in the current was actually observed; we attribute this change to a nonspecific effect occasionally caused by a spontaneous or induced decrease in phosphodiesterase activity (Mazzarella and Gillette, 1990). Similarly, in five further experiments intracellular injection of the PKI peptide fragment caused no measurable decrease in the cAMP response of the pedal neurons. Notwithstanding visible swelling of injected neuron somata, indicative of intracellular injection of appreciable volumes, the PKI protein and the inhibitor peptide fragment were without discernible effect on cAMP stimulation of the Na^+ current.

Patch-clamp recordings of $I_{Na,cAMP}$ channels

Active inward current channels could be identified in the intact neuron, consistent with the existence of a measurable basal level of $I_{Na,cAMP}$ in resting cells (Huang and Gillette, 1993). Such channels commonly ceased activity when excised and moved to artificial saline without added cAMP (Fig. 2*A,B*). However, in 43 patches the channels resumed activity when transferred to the saline barrel containing cAMP (Fig. 2*C*). The channel activity

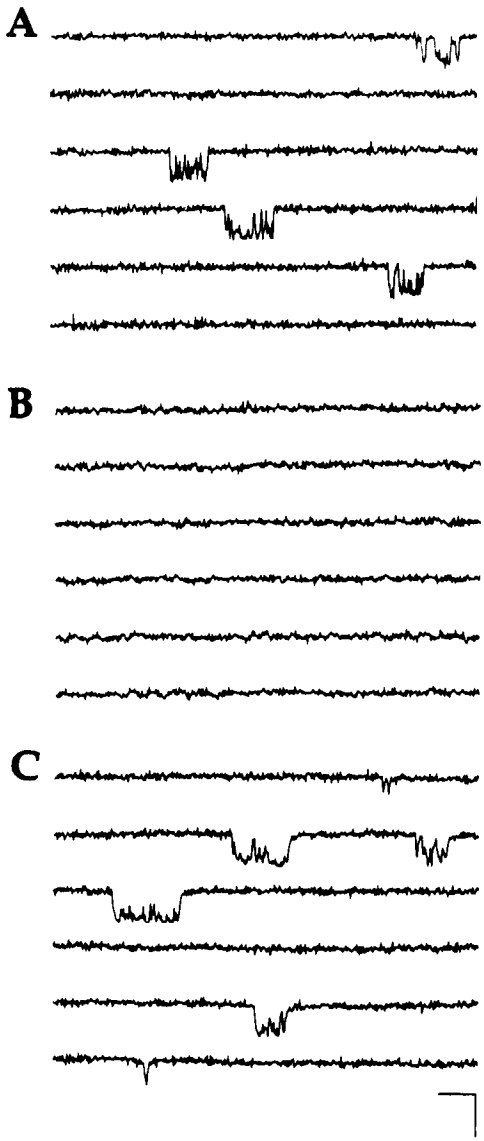

Figure 2. Single-channel recordings from the most frequently encountered type of $I_{Na,cAMP}$ channel. Excised patches were held at 0 mV. *A*, On-cell intact patch. *B*, The same patch after excision in the inside-out configuration, in 0 mM cAMP cytoplasmic saline. *C*, Activation of inward channels in the same patch by 1 mM cAMP in the cytoplasmic saline. Calibration: 9 msec, 3 pA.

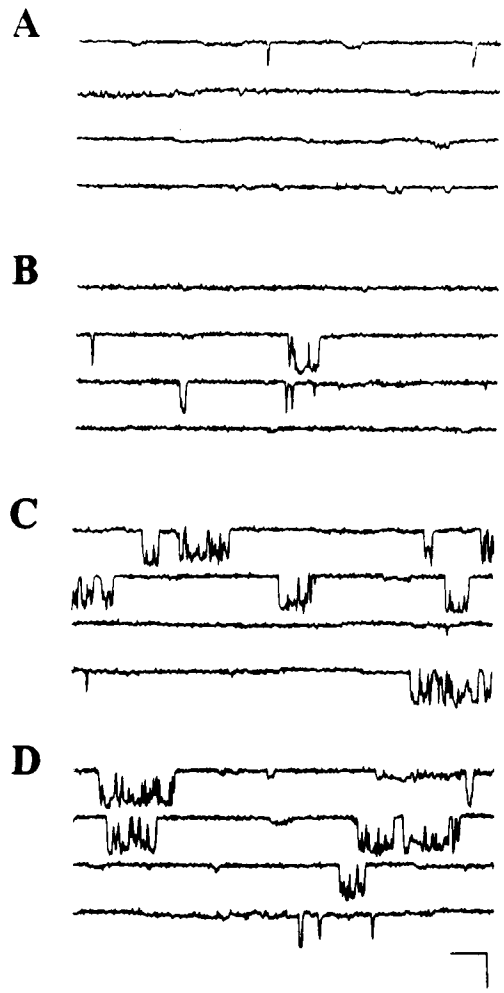

Figure 3. Single $I_{Na,cAMP}$ channel activity in a patch moved to increasing concentrations of cAMP. Excised patches were held at transmembrane potentials of 0 mV. *A*, saline alone, percentage open time = 0.1; *B*, 50 μM cAMP, percentage open time = 1.7; *C*, 100 μM cAMP, percentage open time = 5.0; *D*, 1 mM cAMP, percentage open time = 6.3. Calibration: 9 msec, 3 pA.

was constant over minutes-long exposures to cAMP. Channel activity appeared almost immediately after the pipette tip was moved to the 1 mM cAMP perfusion stream. Preliminary observations of dose–response relationships (Fig. 3) indicate that channel activity may saturate only at relatively high cAMP concentrations, probably above 100 μM, as predicted from studies of the macrocurrent (Connor and Hockberger, 1984; Huang and Gillette, 1991). The activating effects of cAMP on the channels were rapidly reversible upon moving the patch back into saline without cAMP (Fig. 4). Single openings indicative of unitary channel activity were observed in recordings from 14 excised patches of the 43 experiments. The average single-channel current calculated from such records was -2.08 ± 0.19 pA at 0 mV pipette potential. The current–voltage relationship for the channels exhibited a slope conductance of 37–40 pS in a saline with an E_{Na} of +94 mV and E_K of −88 mV (Fig. 5). A reversal potential was estimated at +50 to +55 mV in six separate experiments.

$I_{Na,cAMP}$ infrequently exhibited a second type of channel activity, distinguishable by the opening probabilities and mean open times. For one type, observed as single channels in nine patches (e.g., Figs. 2, 3, 5), the mean open time was 2.46 ± 0.46 msec with a percentage open time of 2.8 ± 0.4. The second type of observed channel activity showed markedly longer open times in response to exogenous cAMP (Figs. 4, 6). In three patches where the channels were observed singly, the averaged percentage time was $63\% \pm 1.92$ in 1 mM cAMP, and the mean open time durations (ignoring rapid flickering) averaged 47.04 ± 10.73 msec. The two types of channel activities did not switch between low and high percentage open time modes while under observation, nor were they detected in the same patch. The differing distributions of open times for the two channel activity types can be compared in the histograms of Figure 7.

Discussion

The results of these experiments are evidence against the involvement of a conventional type of cAMP-dependent kinase in mediating $I_{Na,cAMP}$ in molluscan neurons. The failures of the selective inhibitor protein of cAMP-dependent protein kinase, and of its more potent 20 amino acid fragment, to diminish the

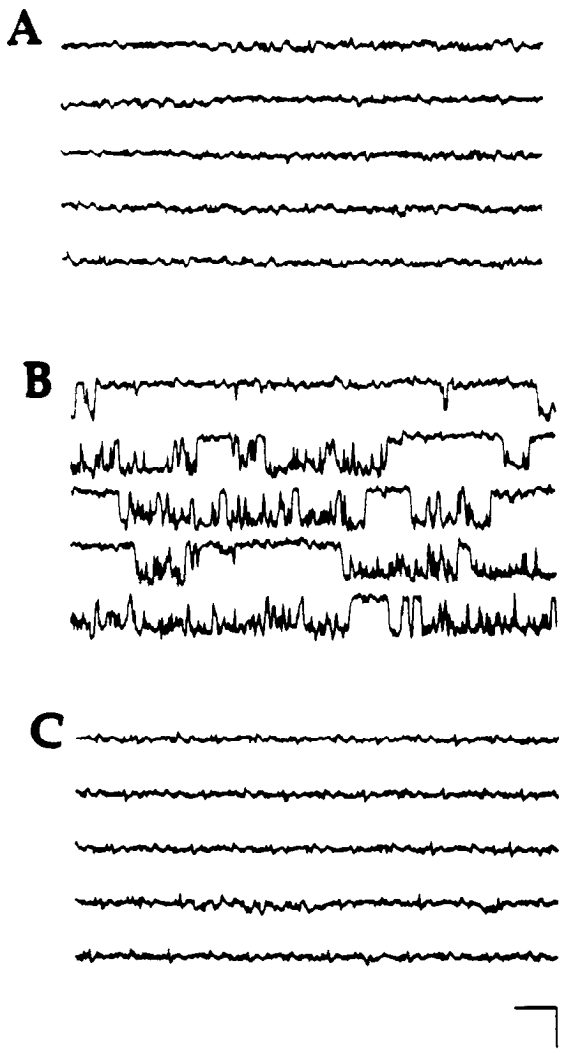

Figure 4. Reversibility of cAMP effects on single $I_{Na,cAMP}$ channels. Excised patches held at 0 mV. Representative traces were taken from the recordings of the same excised inside-out patch under the following conditions. *A*, Cytoplasmic saline alone. *B*, Saline plus 1 mM cAMP. *C*, Saline alone. The intervals of time elapsed between the records were approximately 12 sec. As the patch was moved manually between saline, changes in channel activity probably occurred much faster than was recorded. Calibration: 9 msec, 3 pA.

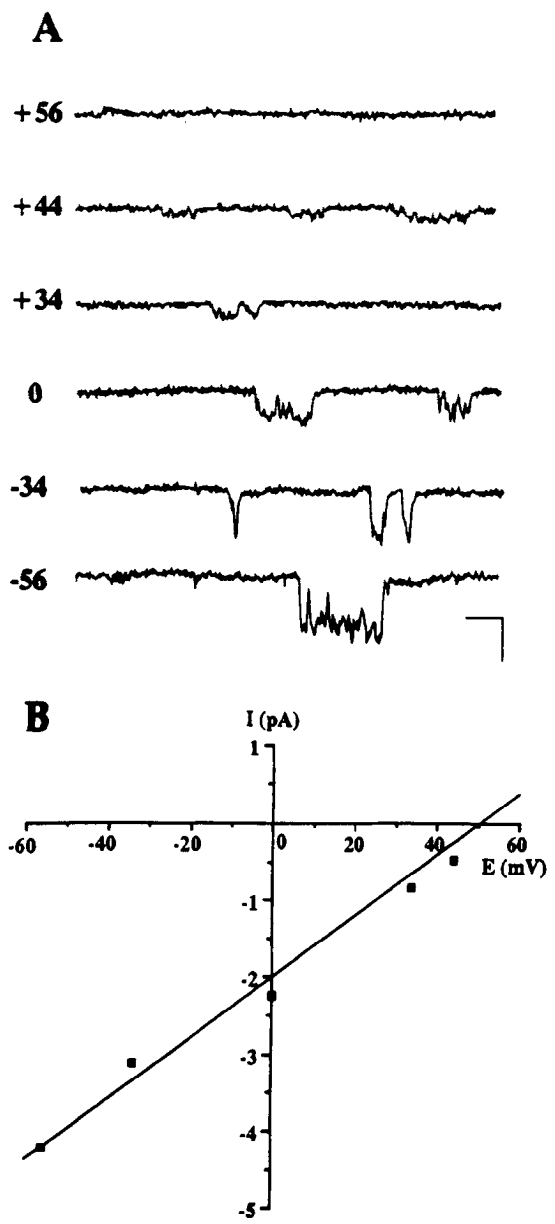

Figure 5. Current–voltage relationship for an $I_{Na,cAMP}$ channel. Cytoplasmic saline contained 1 mM cAMP. *A*, Clamp voltages of the pipette were stepped to the indicated potentials from the holding potential. *B*, Slope conductance was 39.7 pS with a reversal potential of +50.1 mV. Calibration: 9 msec, 3 pA.

$I_{Na,cAMP}$ response to injected cAMP indicate that an A-type protein kinase is not involved. The inhibitor protein has previously been shown to be effective in suppressing electrophysiological manifestations of cAMP-dependent phosphorylation in molluscan neurons (Castellucci et al., 1982; Siegelbaum et al., 1986).

The channels recorded here most likely participate in the macro-$I_{Na,cAMP}$ recorded in the whole cell, since they are largely permeant to Na^+ and they were excised from the identified neurons that exhibit $I_{Na,cAMP}$ under voltage clamp. Moreover, the sensitivity of the single channels to cAMP is in the high range expected, appearing to saturate at levels above 100 μM (Huang and Gillette, 1991). Sodium appears to be the major current-carrying ion associated with the $I_{Na,cAMP}$ channel; however, a component of the single-channel current may be associated with potassium ions, as the reversal potential for $I_{Na,cAMP}$ was estimated at about 40 mV lower than E_{Na} (+94 mV; also see Kehoe, 1990).

The patch-clamp results provide further evidence that the $I_{Na,cAMP}$ channels do not require exogenous ATP, and that a phosphorylation reaction is therefore unlikely. Activation of the $I_{Na,cAMP}$ channels by cAMP is rapid and the channels rapidly become quiescent during washout of cAMP.

The results also show that the $I_{Na,cAMP}$ channels can differ markedly in their responses to cAMP activation. This suggests that either there are at least two distinct populations of channels arising from distinct genes or posttranslational processing, or that a single channel type may manifest different states of activity. Both interpretations may be consistent with findings that addition of free Ca^{2+} to 0.2 μM in the cytoplasmic salines may cause a nearly fivefold increase in the percentage open time and mean open times over the zero-Ca^{2+} condition for channels from pedal ganglia neurons, but the $I_{Na,cAMP}$ channels from buccal ganglia cells have not yet been found to be Ca^{2+} sensitive (Sudlow and Gillette, 1992; unpublished observations).

The present results from pharmacological probes and single-channel recordings all argue that $I_{Na,cAMP}$ in these neurons of

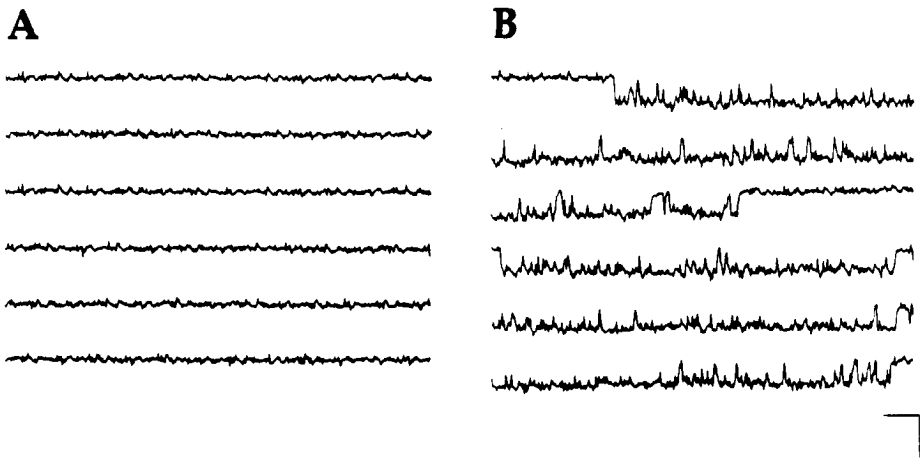

Figure 6. Records from a second type of $I_{Na,cAMP}$ channel. *A*, Excised inside-out patch in 0 mM cAMP cytoplasmic saline. *B*, The same patch in 1 mM cAMP in the cytoplasmic saline. Calibration: 9 msec, 3 pA.

Pleurobranchaea is not mediated by a conventional type of cAMP-dependent kinase. We conclude that activation of this current is probably mediated through direct binding of the cyclic nucleotide to the ion channel or to some closely associated regulatory macromolecule, as is the case for cation currents in vertebrate photoreceptors, olfactory epithelium, and cochlear hair cells (op. cit.).

Other data show that Ca^{2+} and H^+ modulate the effects of cAMP on $I_{Na,cAMP}$ in the intact cell (Gillette and Green, 1987; Green and Gillette, 1988; Huang and Gillette, 1993) and in single channels (Sudlow and Gillette, 1992); these observations add dimension to the function of the $I_{Na,cAMP}$ channel by suggesting allosteric regulation of $I_{Na,cAMP}$ by multiple ligands. Such combinatorial regulation may be particularly important in neurons of the CNS that generate endogenously patterned electrical activity. The general case of direct activation of ion channels by second messengers may be much more extensive than presently documented (cf. Hockberger and Swandulla, 1987). At present, such channels in vertebrates are only known in peripheral sense organs and tissues, while they constitute elements of major neuromodulatory mechanisms in the molluscan CNS. Withal, the class of cyclic nucleotide–activated cation currents typified by $I_{Na,cAMP}$ is widespread across phylogeny and broadly important in regulation of nervous function. The present demonstration of the independence of $I_{Na,cAMP}$ from phosphorylation is expected to facilitate future studies of its regulation by other second messengers, ions, and neurotransmitters.

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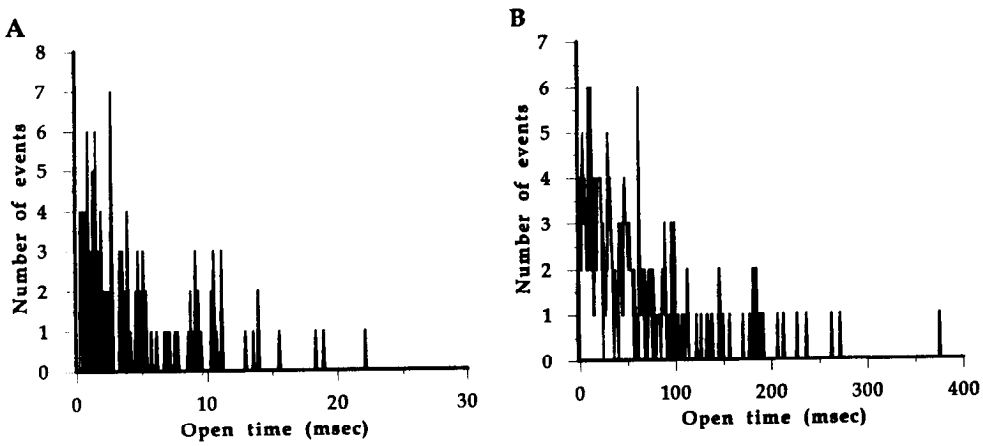

Figure 7. Distributions of open times induced by cAMP in the two types of $I_{Na,cAMP}$ channels. Data were fitted to single exponential distributions. *A*, The short-open-duration $I_{Na,cAMP}$ channel shown had a fitted time constant of 8.39 msec (111 channel openings). *B*, Channel openings from a long-open-duration $I_{Na,cAMP}$ channel displayed a time constant of 121.64 msec (233 channel openings).

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