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A NOVEL MEMBRANE SODIUM CURRENT INDUCED BY INJECTION OF CYCLIC NUCLEOTIDES INTO GASTROPOD NEURONES

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SUMMARY

1. Injection of cyclic AMP (cAMP) or cyclic GMP into identifiable neurones from several different gastropod species immediately depolarized the cell membranes in a dose-dependent manner. Doses were monitored photometrically and evidence is presented for depolarizing effects following nucleotide injections of as little as 30–35 μmol.

2. The depolarizing effect was reversible and was demonstrated under voltage clamp to be primarily the result of a nucleotide-induced, transient increase in a membrane Na current, I_{Na} (cAMP). The charge-carrying species was identified by using ion-substituted salines, reversal potential in low-Na saline, and intracellular ion-sensitive electrode measurements. The current was resistant to tetrodotoxin, ouabain and amiloride. Substituting Trisma, tetramethylammonium or bis-tris propane for Na prevented the induced current, whereas Li substitution did not.

3. Duration of the induced current was greatly prolonged in neurones bathed in the phosphodiesterase inhibitor isobutylmethylxanthine, or following injection of any of several cAMP analogues, indicating that the reversible nature of the current stems primarily from *in situ* hydrolysis of the injected dose and not current inactivation.

4. Amplitude of the induced current either remained constant or decreased over the voltage range where it could be easily measured, i.e. $-30 > V_m > -100$ mV, reflecting a voltage as well as a chemical sensitivity of I_{Na} (cAMP).

INTRODUCTION

In spite of abundant information regarding the conditions for synthesis of cyclic nucleotides in nerve cells (Daly, 1977), there is only a relatively small amount of direct evidence concerning the intracellular functions of these nucleotides. Among the possible sites of action, changes in membrane resting potential and voltage-dependent conductances have received the greatest attention. Initially investigators applied non-hydrolysable analogues or phosphodiesterase (PDE) inhibitors extracellularly onto intact ganglia while recording from nerve cells (for review see Nathanson, 1977). However, a strictly cellular interpretation of these results has been difficult due to the likely non-specific and multicellular effects of these drugs. A more straightforward

approach has been to inject the nucleotide directly into cells while simultaneously recording membrane parameters (Tsien, 1973). Results from such experiments have demonstrated that cyclic nucleotides can affect resting potential and several ionic conductances in both vertebrate (Krnjevic & Van Meter, 1976; Gallagher & Shinnick-Gallagher, 1977; Miller & Nicol, 1979; Madison & Nicoll, 1982) and invertebrate neurones (Liberman, Minina & Golubtsov, 1975, 1977; Klein & Kandel, 1978; Kononenko & Mironov, 1981; Deterre, Paupardin-Tritsch, Bockaert & Gerschenfeld, 1981; Pellmar, 1981; Hockberger & Connor, 1983*a*). Similar techniques have been used to identify cyclic nucleotide effects upon synaptic transmission (Brunelli, Castellucci & Kandel, 1976; Shimahara & Tauc, 1977), evoked discharges (Kaczmarek & Strumwasser, 1981), and command functions in neurones (Gillette, Gillette & Davis, 1982).

In this report we use intracellular injections to characterize an unusual nucleotide-activated Na current in identified neurones found in a variety of gastropod (molluscan) species. In the following paper (Connor & Hockberger, 1984) we describe a cyclic nucleotide effect upon cellular metabolism, measured as a change in intracellular pH. Preliminary reports of these results have appeared elsewhere (Hockberger & Connor, 1981, 1982, 1983*a*, *b*, 1984; Connor & Hockberger, 1982).

METHODS

Several species of marine gastropod molluscs were obtained from either C. Eaton, Friday Harbor, WA, Pacific Bio-marine Supply Co., Venice, CA, or Marine Specimens Unlimited, Pacific Palisades, CA. Animals were kept in the laboratory for 1–5 weeks in artificial sea water (Instant Ocean Systems, Wycliff, OH) maintained at 12 °C. No noticeable differences were found between newly arrived animals and those contained up to 5 weeks after arrival as long as the apparent physical condition of the animals was good.

Throughout the course of these experiments only identifiable nerve cells were used. The majority of the data presented were obtained using the nudibranch *Archidoris montereyensis* which contains many large, identified nerve cells (see Blackshaw, 1976; Partrige & Stevens, 1976; Connor, 1979; Aldrich, Getting & Thompson, 1979). For several key experiments, we used identified neurones of *Aplysia californica* classified according to Frazier, Kandel, Kupfermann, Waziri & Coggeshall (1967). Neurones from several other species were also examined and identified using a classification system which was similar to the one employed by Frazier *et al.*, i.e. right *vs.* left hemiganglion, ganglion subtype (e.g. pedal), and numbered according to location within a ganglion.

Dissection procedures have been described previously (Connor, 1979). Briefly, the circum-oesophageal ganglia, containing many large (300–500 μm diameter) cell bodies, were removed from the specimen, and trypsin (Sigma, Type IX, 10 mg/ml) digestion at room temperature was used to facilitate removal of the endoneurium surrounding the cells. The length of exposure to the enzyme varied from a few minutes (dorids) up to 50 min (*Aplysia*). However, frequent dissections without enzyme treatment were done and showed no discernible differences in the parameters studied.

Isolated ganglia from most specimens remained viable in physiological saline for 2–3 days at 9 °C. Temperature in the experimental chamber was routinely controlled at $12 \pm 0{\cdot}5$ °C, unless otherwise stated, using a Peltier effect cooling system. Several different superfusion solutions (Table 1) were used in the experiments, and the following compounds were occasionally added to normal saline: isobutylmethylxanthine (IBMX, Calbiochem, La Jolla, CA), tetrodotoxin (Calbiochem), amiloride (Merck, West Point, PA), forskolin (Calbiochem) and ouabain (Sigma, St. Louis, MO). NaCl was replaced in Na-free saline by substituting one of the following ingredients in equimolar concentration: LiCl, tetramethylammonium chloride, Trizma base or bis-tris propane(1,3-bis[tris (hydroxy-methyl) methylamino] propane). During ion-substitution experiments membrane potential was monitored differentially with respect to a reference micro-electrode in the bath.

Recording electrodes were filled with 3 M-KCl and had resistances of 1–5 MΩ. Standard two-

electrode voltage-clamping techniques were employed as described elsewhere (Connor & Stevens, 1971). Data were displayed on either two-channel (Brush model 280) or four-channel (Gould model 2400S) chart recorders with selected signal filtering. Fast clamp records were first stored on a Gould digital storage oscilloscope (model OS4103) and then plotted on a chart recorder at an expanded time base.

TABLE 1. Ingredients used in the superfusion solutions (mM)

Ingredients	Normal saline	TEA saline	Na-free saline	High-K saline	High-Mg saline	Ca-free saline	Intern. saline
NaCl	490	450	0	460	340	490	50
KCl	8	8	8	40	8	8	350
$CaCl_2$	10	10	10	10	0	0	—
$MgCl_2$	20	20	20	20	60	30	3
$MgSO_4$	30	30	30	30	90	30	—
MOPS (H buffer)	10	10	10	10	10	10	50
Glucose	5	5	5	5	5	5	—
TEA Cl	—	40	—	—	—	—	—
TMA Cl or Trisma (base)	—	—	490	—	—	—	—
pH	7·6	7·6	7·6	7·6	7·6	7·6	7·2

Several different nucleotides were injected intracellularly either by pressure or ionophoresis using micro-electrodes identical in size to the recording electrodes. Ionophoretic injections were administered under voltage clamp using 100–200 nA of negative current for 0·8 s repeated at 1 Hz. Ionophoresis electrodes were filled with a 0·2 M solution of the Na salt (grade I, Sigma) of one of the following nucleotides: cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), cyclic uridine monophosphate (cUMP), cyclic cytosine monophosphate (cCMP), 8-azido-cAMP, 8-benzylamino-cAMP, 8-bromo cAMP, dibutyryl-cAMP, monobutyryl-cAMP, 5′-AMP, 5′-GMP, adenosine triphosphate (ATP), guanosine triphosphate (GTP) and 5′-guanylylimidodiphosphate (G_{pp}-NH_p). Ionophoretic doses (d) were estimated using the expression $d = Ni/zF$, where N represents the transport number for the injected species, i the injection current per pulse, z the charge of the species, and F Faraday's constant (Curtis, 1964). The transport number for our low-resistance electrodes filled with 0·2 M-cAMP was assumed to be 0·05 (Shoemaker, Balentine, Siggins, Hoffer, Henricksen & Bloom, 1975).

Pressure injections were made using a system which enabled intracellular recording and pressure injection through the same electrode. Electrodes were filled with 0·1 M-nucleotide, 5 mM-Arsenazo III (grade I, sodium salt, Sigma), and 1 mM-3-(*N*-morpholino)propanesulphonic acid (MOPS; free acid, Sigma) buffered to pH = 7·2 – 7·4. The Arsenazo dye was used to quantify the injected doses by measuring transcellular absorbance changes at the dye isobestic wave-length, 580 nm. Absorbance measurements were made using a rotating wheel spectrophotometer similar in design to one described by Brinley & Scarpa (1975) (also see Hockberger & Connor, 1983*b*). This enabled dual-wave-length absorbance measurements to be made which involved subtracting a reference signal (700 nm) from the signal of interest. This technique results in minimization of distortion and scattering artifacts which occur during injections. The amount of nucleotide injected was calculated 5–10 min post-injection using the net change in absorbance base line at the isobestic wave-length and the differential molar extinction coefficient of Arsenazo III (3×10^{-4} M^{-1} cm^{-1}). The injected mixture was assumed to have the same proportions as that in the electrode and the nucleotide dose was estimated accordingly. Ionophoretic and pressure-injected nucleotide doses are expressed as injected amount divided by cell volume.

In several experiments the volume of solution pressure-injected was quantified. These measurements were made by recording the distance that an air bubble in the electrode tip moved following an injection. The amount of solution in the tip between the bubble and electrode orifice was calculated using the equation expressing the volume of a right circular cone ($V = \frac{1}{3}\pi r^2 h$), where h represents the height of the solution and r the radius at its base (adjacent to the bubble). The volume of injected solution was then determined from the difference in the amount of solution between the bubble and the tip before and after an injection.

Na-selective micro-electrodes were constructed as described by Steiner, Oehme, Ammann & Simon (1979) using a synthetic, neutral ionophore (Fluka, ETH227). The micro-electrodes had tip diameters of approximately 1 μm and 90 % response times of approximately 1 min. The slopes of the micro-electrodes ranged from 50 to 58 mV for a change in Na concentration of 50–500 mM at pH = 7·4. The internal Na ion concentration, [Na_i], was calculated using the slope and by

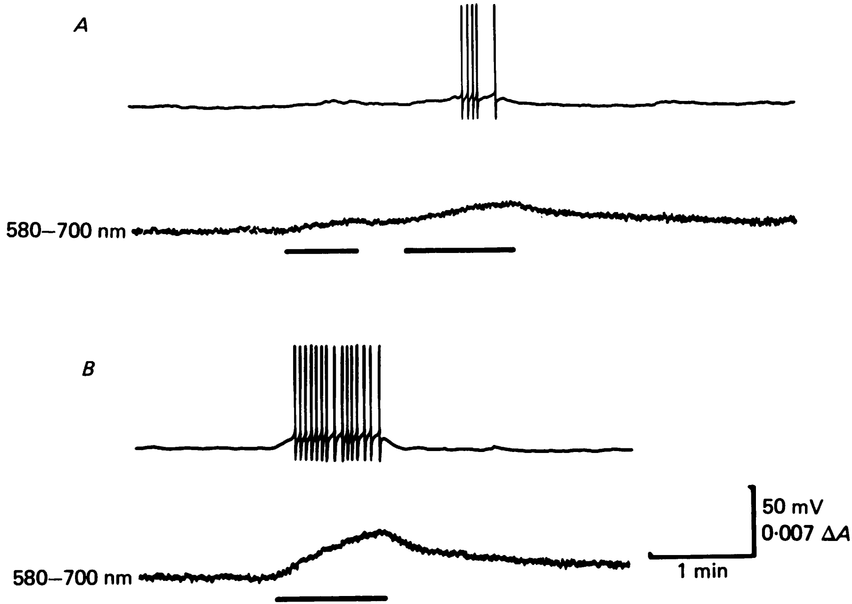

Fig. 1. Small pressure injections of cAMP–Arsenazo III mixture into *Archidoris* cell RPl 4 evoked action potential firing (upper traces) which increased with increasing dose. Dosages were monitored by measuring transcellular absorbance (580–700 nm) at the injection site (lower traces). This wave-length pair is insensitive to Ca, Mg and pH changes. Positive pressure on the injection pipette is denoted by horizontal bars. Post-injection absorbance decrease reflects dye diffusion from beneath the optic fibre.

measuring the drop in voltage upon moving the Na-sensitive electrode from the bath (490 mM-Na) into the cell. The output of the Na-selective micro-electrode was fed into a varactor bridge pre-amplifier and then into the non-inverting input of a differential amplifier. The inverting input was attached to the intracellular voltage-recording micro-electrode which served as the reference electrode. The Na ion electrode was considered to be inside the cell when a negative voltage step of 20 mV across the cell membrane produced a 2 mV or less change in the differential amplifier output.

RESULTS

Cyclic nucleotide effects on membrane potential

Injection of cAMP into any one of the fourteen identified *Archidoris* neurones (Hockberger & Connor, 1984) routinely induced a dose-dependent loss of membrane potential which typically resulted in spike activity. Fig. 1 shows records of membrane voltage and transcellular absorbance during slow pressure injections of nucleotide plus dye into one such neurone. With two small injections of nearly identical size delivered consecutively we found that doses resulting in barely detectable absorbance increases could depolarize the resting membrane (Fig. 1*A*). This effect was due

presumably to local saturation of PDE activity with the first injection. Following relatively small doses such as these the membrane potential recovered almost immediately upon removal of the applied pressure.

TABLE 2. List containing identifiable nerve cells from several species of gastropod mollusc (classified according to Beeman & Williams (1980)) which exhibited loss of resting potential following intracellular injection of cAMP. All cells listed also displayed a nucleotide-induced inward current at holding potentials of −40 mV, or below

Species	No. cells depolarized/ no. cells examined	Identified cells exhibiting depolarizations
Archidoris montereyensis	372/376	RCe 1, LCe 1, RPlG, RPl 2–4, LPl 1–4, RPe 1 and 2, LPe 1 and 2
Aplysia californica	67/76	R2, LP1, R15, L2–26
Archidoris odhneri	9/9	LPl 4, LPe 1
Diaulula sandiegensis	3/3	LPl 4, LPl 1
Armina californica	3/3	LPl 2, LPl 3
Triopha catalinea	1/3	LPe 1
Dendronotus albus	2/2	LPl 1, RPl 1

The dose delivered in Fig. 1*B* was estimated to be 0·1 ± 0·02 mM-cAMP (see Methods), the smallest dose which resulted in a net change in transcellular absorbance base line. The second injection in Fig. 1*A* yielded only a transient absorbance change, but nevertheless was sufficient to induce one-third as many action potentials as did the injection in Fig. 1*B*. Since the peak absorbance change was roughly one-third of that in Fig. 1*B*, we would suggest that the cAMP dose administered in the second injection was approximately 30–35 μM. Doses of similar magnitude were found to be effective in all fourteen cell types examined in *Archidoris*.

Larger doses of cAMP estimated to be 0·1–10 mM ($n = 45$) induced repetitive firing in these neurones which persisted in a dose-dependent fashion for several minutes beyond termination of the injection. Ionophoretic injections of cAMP under voltage clamp also evoked repetitive firing out of clamp for durations which were dose dependent. We have calculated that ionophoretic doses as low as 15 μM-cAMP (see Methods) resulted in membrane depolarizations. Both of these injection procedures for estimating the minimal effective dose ignored the regional localization of the injection by averaging over the entire cell volume. Although this results in under-estimates of the actual local concentration, we might assume that endogenous PDE activity will, on the other hand, significantly reduce the available nucleotide concentration. Injections made in the presence of PDE inhibitors have confirmed this latter assumption (see below).

A variety of control solutions were also pressure injected into these neurones and found to be without noticeable effects on membrane potential. Relatively large doses of 5′ AMP plus Arsenazo III (mean 5·7 mM) did not affect the resting potential in fifteen out of seventeen cells. Injections of 5′ AMP or internal saline were without effect so long as the cell volume increase was less than 5 %. Injections greater than 10 % by volume resulted in depolarizations which lasted a few seconds no matter what was injected. By contrast, cAMP injections which induced several minutes of

membrane depolarization were achieved with as little as a 0·2% increase in cell volume.

Identifiable neurones from several additional marine gastropod species were also injected with cAMP and examined in a similar fashion. Table 2 lists the species and the corresponding neurones which exhibited loss of resting potential with doses similar to those described above. Treistman & Levitan (1976) reported that they observed no effect from injecting cAMP into cell R_{15} (*Aplysia*). It is unclear to us at the present time just how to compare our results with theirs. However, we have noticed that *Aplysia* cells R15 and LP1 required substantially larger injections than the other neurones in order to obtain the observed results. Although each of the neurones listed in Table 2 exhibited depolarizing effects, there were other cells examined whose resting potentials were unaffected even by millimolar doses. We have not tested for other nucleotide effects (see Pellmar, 1981; Deterre *et al.* 1981; Connor & Hockberger, 1983) in this latter population of cells.

Nucleotide-induced changes examined under voltage clamp

Injection of cAMP into any of the neurones listed in Table 2 when held under voltage clamp at −40 mV or below (i.e. more negative) resulted in a dose-dependent, reversible increase in membrane holding current. Fig. 2*A* shows four increasingly larger pressure injections into an *Archidoris* cell voltage clamped at its resting potential, −40 mV. Each injection induced a correspondingly larger and longer-lasting current response. Injections larger than the fourth did not significantly increase the current amplitude, although they did prolong the period of current flow.

In no instance did we record a delay in the initiation of the current response, a result which might have occurred if the response were induced at a distance far from the injection site (e.g. neuropilar region). In fact the initial response was equally fast no matter where the injection electrode was placed around the soma. For these reasons we believe the current responses were generated initially by nucleotide diffusion to internal membrane receptors adjacent to the injection site, and that increased amplitude and duration of the responses reflected recruitment of receptors as the nucleotide diffused to more distant sites. The diffusion coefficient (D) for cAMP in water at 20 °C is $4{\cdot}4 \times 10^{-6}$ cm^2 s^{-1} (Dworkin & Keller, 1977). The value *in situ* might be considerably lower, though, due to the high viscosity of cytoplasm, cooler temperature ($\sim$ 12 °C) and diffusion barriers created by numerous organelles. Using $4{\cdot}4 \times 10^{-6}$ as a conservative estimate for D would enable diffusion of cAMP only 160 μm in 10 s ($x^2 = 6Dt$), far short of the neuropil in most instances.

Addition of the PDE inhibitor IBMX (10^{-3} M) to the bathing solution, as in Fig. 2*B*, substantially lowered the dose of cyclic nucleotide needed to invoke an inward current or to reach saturation (also see Liberman, Minina & Shklovskii-Kordi, 1981) and prolonged its duration, supporting the notion that endogenous PDE activity plays a significant role in the recovery of the inward current. Bath-applied IBMX (10^{-5}–10^{-3} M) also increased the base-line holding current of voltage clamped cells (Fig. 2*B*) and induced repetitive firing in unclamped neurones (see also Deterre *et al.* 1981). Similar results were obtained when this PDE inhibitor was applied in high-Mg (150 mM). Ca-free saline indicating that the base-line response could not have been entirely synaptically mediated, and making it probable that at least part of the effect of IBMX was to increase the endogenous cellular concentration of cAMP.

Ionic mechanism underlying induced current response

The current-carrying species underlying the inward current was examined in each of the *Archidoris* and *Aplysia* neurones listed in Table 2 using ion-substituted salines. Gradually replacing Na in the superfusion solution with either TMA, Trizma base,

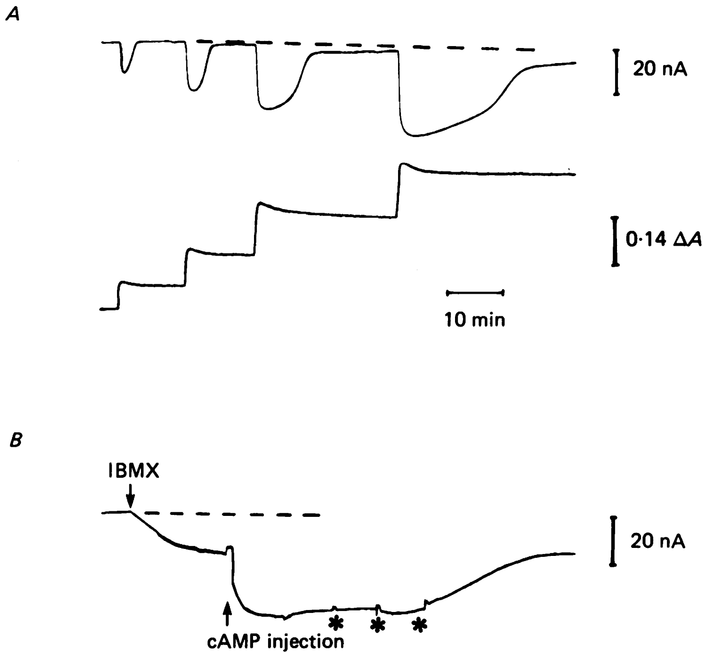

Fig. 2. *A*, records showing inward current under voltage clamp (upper trace) induced by the injection of cAMP–Arsenazo III mixture ($V_h = -40$ mV). Absorbance record (lower trace) monitors Arsenazo III concentration (proportional to injected dose) at a site slightly away from the injection pipette tip. The record is continuous for the four injections shown. Doses were computed to be 1·7, 2, 3·7 and 8·5 mM-cAMP. *B*, membrane current ($V_h = -40$ mV) induced in the same neurone after incubation in IBMX (10^{-3} M). Simultaneous absorbance record is not shown but was used to compute an injected dose of 4 mM-cAMP. *Archidoris* neurone LPl 1. Asterisks denote normal saline washes.

or bis-tris propane reversibly abolished the current response. Fig. 3 shows results from an experiment using TMA-substituted saline in which the current was no longer induced even though the injected dose was larger in the substituted saline. Since partial substitution of Na with any of these agents reduced the current response monotonically, it is unlikely that this was due to channel blockage by the substitutes. Bathing neurones in Ca-free or Cd-substituted (10 mM) saline, on the other hand, had no effect on normal current responses. *Archidoris* cells previously loaded with Arsenazo III (used as a Ca indicator dye) showed no elevation in internal Ca^{2+} levels during current flow (Hockberger & Connor, 1983*a*; Connor & Hockberger, 1984). These results, as well as the presence of the current at membrane potentials below -30 mV, indicated that the induced inward current reported here was unlike nucleotide-induced currents previously described (Pellmar, 1981; Deterre *et al.* 1981).

Gradual reduction of the extracellular Na concentration was invariably followed by a corresponding outward shift in the base-line holding current. Experiments using Arsenazo III for monitoring intracellular free Ca^{2+} levels showed no change in $[Ca^{2+}]_i$ during this shift (J. A. Connor & P. Hockberger, unpublished observations), nor was there an increase in membrane conductance as might be expected if intracellular Ca^{2+} increased as a result of blocking Na–Ca exchange. (For long exposures to Na-free

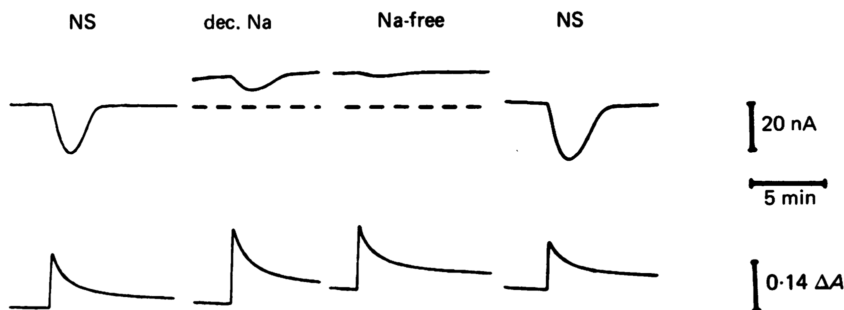

Fig. 3. Records showing the reversible reduction of cAMP-induced current (upper trace) in Na-free saline (NaCl replaced with TMA Cl). Record marked 'dec. Na' was taken before wash-out of the chamber was complete. Pressure-injected amounts were deliberately made larger during exposure to Na-replaced saline than for control (normal saline, NS) or recovery records (see absorbance monitor, lower trace). V_m during injections held at −40 mV. *Archidoris* neurone LPl 4.

solutions (> 45 min) we did note an approximately 2-fold increase in resting membrane conductance. These experiments were outside the range of our normal procedures.) The outward shift eventually plateaued with extended washing in Na-free saline as in Fig. 3, leading us to believe that the shift was due to the loss of a substantial, steady influx of Na^+ at the resting potential. If this shift represented the contribution of Na^+ to the resting leakage current, then it was equal in magnitude to oppositely flowing resting leakage current (K^+ and Cl^-). This prospect meant that the induced inward current may have been simply due to shutting down resting membrane conductance to K^+ or Cl^-. Three lines of evidence argued against this interpretation: (1) the size of the induced current could always be made much larger than the Na-free shift in holding current for a given cell; (2) the current was abolished in Na-substituted salines with normal $[Cl]_o$, as in Fig. 3; (3) the current size was not affected by changes in $[K]_o$.

Fig. 4 illustrates experiments in which $[K]_o$ was increased from 8 to 40 mM. Fig. 4*A* shows the effect of this on K ion tail current following brief voltage-clamp pulses to +20 mV in which the normally outward current, $E_K < -40$ mV, was converted to an inward current, $E_K > -40$ mV, by the change. In Fig. 4*B* matched ionophoretic doses of cAMP were injected into the same neurone before, during and after exposure to high K, with membrane voltage held at −40 mV. The sizes of the induced currents were not measurably different in five of six cells. Similar experiments in K-free saline or in 40 mM-TEA saline likewise showed no apparent effect of altered K conductance on the nucleotide-induced currents.

In order to find the reversal potential for the current response, injections were made

into *Archidoris* and *Aplysia* cells clamped at various holding potentials. Holding at steady potentials between −20 and −80 mV did not reverse the direction of the response (see below). Long-duration clamps at levels more positive than −20 mV were not practical in normal saline due to the large current required. However, such

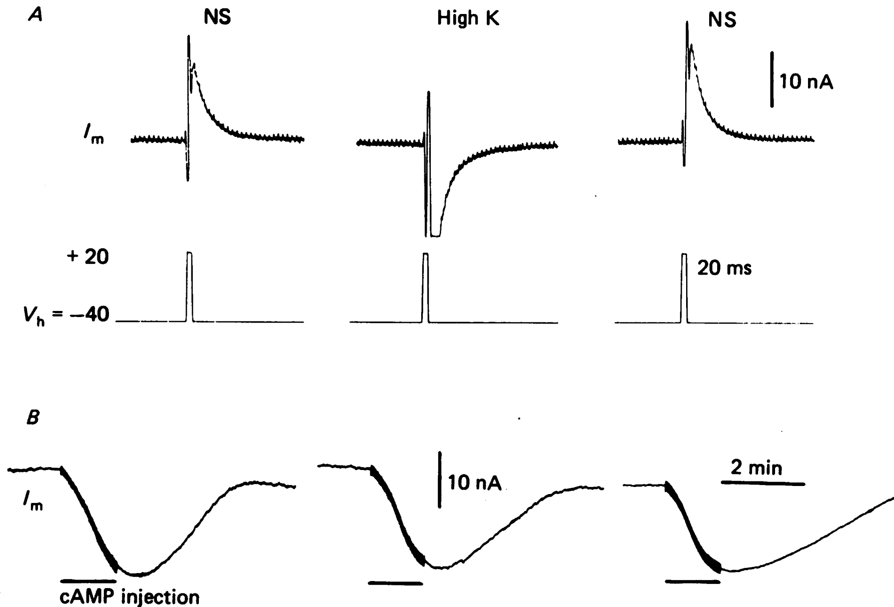

Fig. 4. Records showing that the cAMP-induced current is insensitive to reversing the driving force on K. *A*, membrane current (upper trace) was monitored after a 20 ms voltage pulse (lower trace) in normal, 40 mM-K, and normal salines (NS). The tail current, carried by K^+, clearly reverses in the high-K saline. *B*, current induced by ionophoretic injection of cAMP into the same cell bathed in the same salines (V_h = −40 mV). Each voltage pulse measurement of *A* was taken immediately before the corresponding cAMP injection of *B*. *Archidoris* neurone RPl 2.

measurements were more easily performed in salines where Na was partially replaced. In the experiment illustrated by Fig. 5 $[Na]_o$ was reduced to 20 mM by replacement with TMA, a low-affinity K-channel blocker. The current response obtained with ionophoretic injection of cAMP showed a clear reversal at around −10 mV. This is approximately the expected reversal potential for a response mediated exclusively by Na ions as predicted by the Nernst potential. Similar results were obtained with bis-tris propane substituted for Na.

An independent confirmation that the charge-carrying ion was Na was obtained using intracellular Na ion electrodes. A total of forty-six *Archidoris* and *Aplysia* nerve cells were impaled, displaying an average unbound $[Na]_i$ of 35·9 mM ± 15·5 (S.D. of an observation). This value is similar to those obtained by other investigators using gastropod neurones (e.g. Thomas, 1969; Eaton, Russell & Brown, 1975). Fig. 6 shows an example of the increase in $[Na]_i$ detected with a Na electrode following pressure injection of cAMP. For these experiments the free acid (crystalline) form of cAMP

was used and titrated to pH = 7·2 with KOH. This injection elevated the $[Na]_i$ by approximately 3·5 mM, a value close to an estimated increase of 3 mM based upon the integral of the current response with the assumption that the current was carried exclusively by Na ions which did not bind to cellular components after entering. Seven

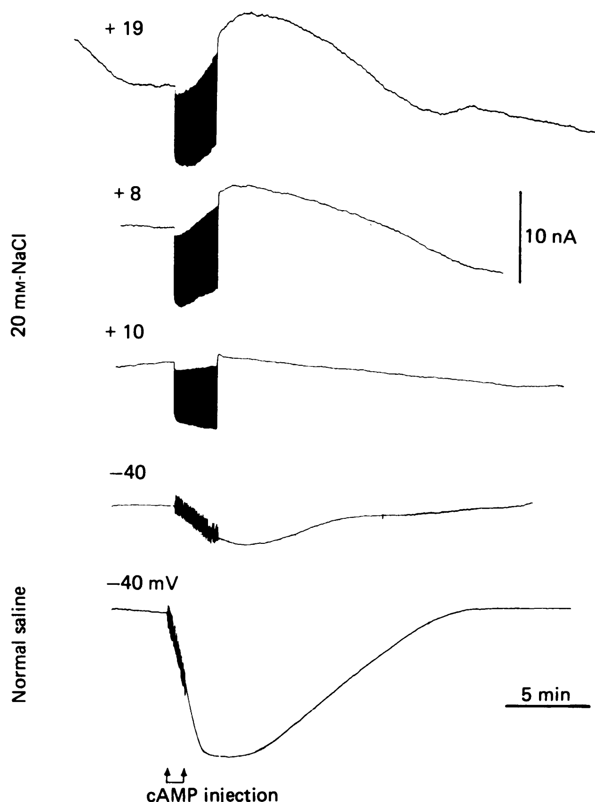

Fig. 5. Reversal potential measurement of the nucleotide-induced current response in *Archidoris* neurone LPl 1 made in 20 mM-NaCl saline. Identical ionophoretic injections were made at several holding potentials, and the current response reversed around −10 mV. Upon returning to normal saline (below) a larger inward current was obtained with a much shorter period of injection, consistent with the results in Fig. 3.

neurones (seventeen injections) were analysed in this manner, and the correlation coefficient between the integrated current and $\Delta[Na]_i$ was 0·92 (slope = 0·88), indicating that the rise in internal free Na concentration was primarily, if not entirely, due to Na ions entering through the cell membrane.

The above results clearly demonstrate that Na ions carry most of the cyclic nucleotide-induced current, termed I_{Na}(cAMP) in the following discussion, but leave the current-carrying mechanism undefined. We further examined the response in the presence of specific channel and pump blockers. Since some dorid neurones are insensitive to TTX (Connor, 1979), the following experiments were performed on cell R2 in *Aplysia californica* in which TTX and ouabain sensitivity have been established (Geduldig & Junge, 1968; Carpenter & Alving, 1968). Fig. 7 shows records taken

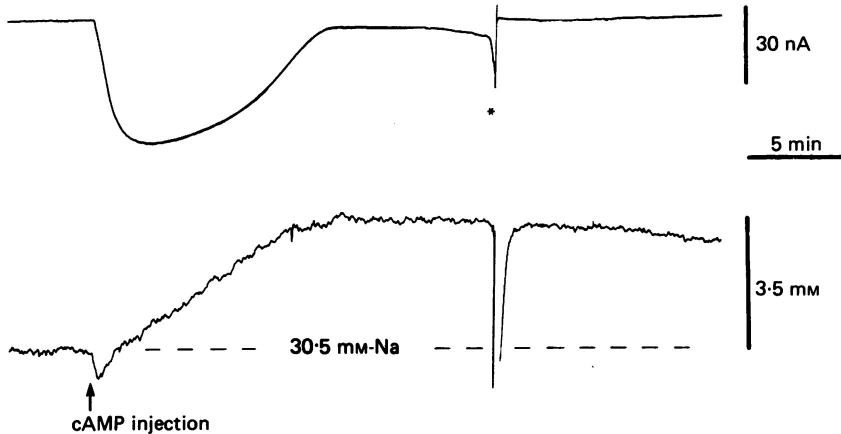

Fig. 6. Measurements of Δ $[Na]_i$ made using an intracellular Na-sensitive electrode (lower trace). Membrane current (upper trace) was induced by a pressure injection of the free acid form of cAMP titrated with KOH ($V_h = -40$ mV). An asterisk marks the spot where injection electrode was removed from the cell, causing a large transient in the Na-electrode trace. A small downward artifact in this trace occurred during the injection. *Archidoris* neurone LPl 1.

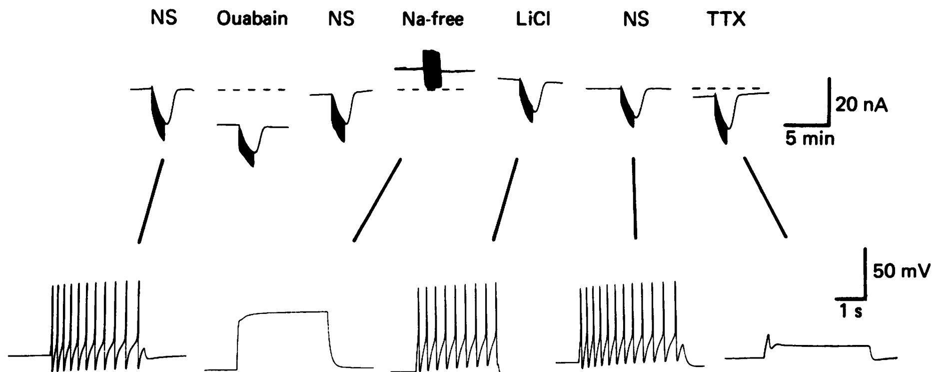

Fig. 7. Upper traces: currents induced in neurone R2 of *Aplysia* with matched ionophoretic injections of cAMP ($V_h = -40$ mV). Sequential records show the effects of ouabain, Na-free solution, Li substitution for Na, and tetrodotoxin (TTX). Lower traces: action potentials and electrotonic responses elicited in each saline. Where action potentials failed the stimulus current was increased to check for simple threshold changes.

during ionophoretic injections into cell R2 while it was bathed successively in several different salines. The results show that although 5×10^{-4} M-ouabain substantially increased the base-line holding current, it did not block the response. After washing off the ouabain in normal saline, Na-free (bis-tris propane substituted) saline abolished I_{Na}(cAMP) and prevented action potentials from being elicited with current stimulation. Replacing bis-tris propane with equimolar LiCl restored the action potentials and the induced current. Upon returning the cell to normal saline, 10^{-5} M-TTX was added to the bath. Although this concentration of TTX changed

the threshold for eliciting spikes (Fig. 7, bottom) it was ineffective at blocking the nucleotide-induced current.

We also examined several neurones bathed in 1·5 mM-amiloride, a compound which blocks Na transport across epithelial cells (Bentley, 1979). The nucleotide-induced response, examined at room temperature (since amiloride precipitated in the bath at 12 °C), was completely unaffected by amiloride treatment.

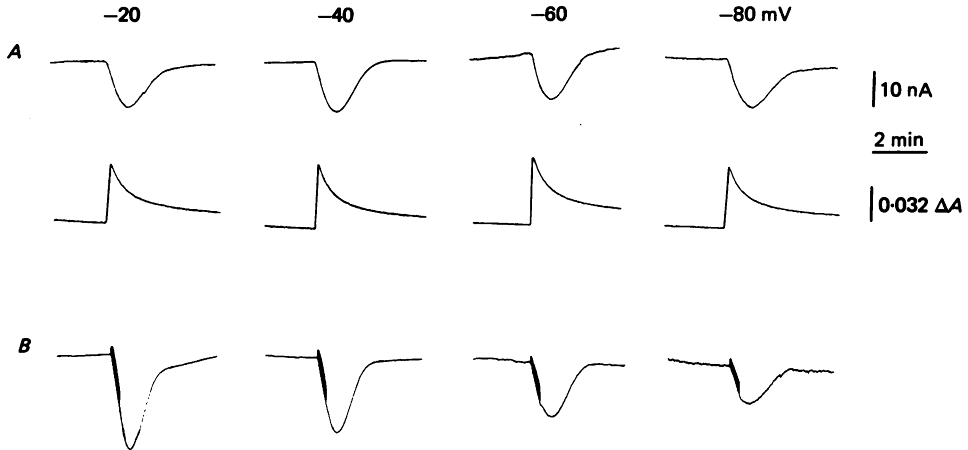

Fig. 8. *A*, records from *Archidoris* neurone RPlGC illustrating the negligible effect of membrane potential in the subthreshold range on cAMP-induced current (upper traces). Absorbance records (lower traces) show that the four pressure injections were nearly identical. *B*, records obtained from neurone R2 of *Aplysia* (probably the homologue of RPlGC), showing the strong decrementing effect of hyperpolarization on induced current. Matched ionophoretic pulses were used for this illustration.

Effects of holding potential and current–voltage relationships

In normal saline the magnitude of I_{Na}(cAMP) measured in *Archidoris* neurones was insensitive to membrane potential over the range from −20 to −110 mV. This finding is illustrated in Fig. 8*A* where pressure injections with dye tracking were employed to demonstrate the constancy of the injections. Ionophoretic injections gave the same result. In the *Aplysia* neurones tested (R2, LP1, the L2–L6 cluster) hyperpolarization *decreased* the induced current. This is shown in Fig. 8*B* where responses at the same holding potentials are compared, showing that the I_{Na}(cAMP) at −80 mV was approximately one-half of the value at −40 mV. In cell R2, the most frequently examined *Aplysia* neurone, steady hyperpolarization from −40 mV to −90 mV decreased I_{Na}(cAMP) by as little as 15 % to as much as 60 %. In neither *Aplysia* nor *Archidoris* neurones did the current increase in size with the increased driving force on Na. Voltage levels outside of this range were not examined due to practical difficulties, as mentioned above.

Conductance measurements before, during and after I_{Na}(cAMP) flow were made under voltage clamp in both *Aplysia* and *Archidoris* neurones using both positive and negative pulses from −40 mV. Sample records from such a series are shown in Fig. 9

for control (Arsenazo III alone) and cAMP (plus Arsenazo III) pressure injections. The absorbance records document that the two injections were nearly identical. Membrane conductance was monitored by applying 2 s pulses to −53, −67 and −80 mV. At the doses used the injection of Arsenazo III alone had no measurable effect on membrane conductance or holding current (Fig. 9, left-hand records). When

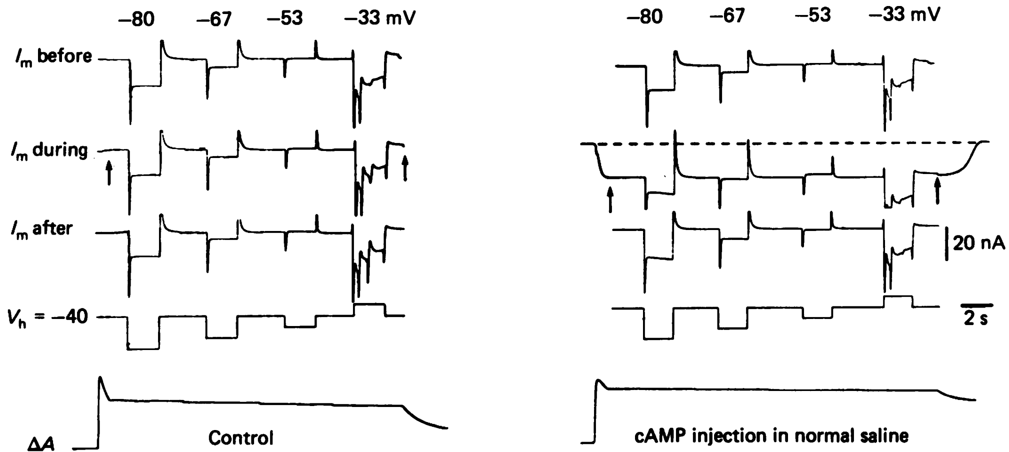

Fig. 9. Recordings illustrating the effect of cAMP injection on membrane conductance in *Archidoris* neurone LPl 1 measured by positive- and negative-going voltage pulses from −40 mV. Voltage during pulses is given at the head of each data column. Chart speed was 100 × slower prior to, and after regions marked by arrows (to show development and decay of induced inward current). Left panel shows the negligible effect on membrane conductance of injecting dye alone. For the right panel the cAMP–dye mixture was injected and negative-going steps demonstrated a small decrease in membrane chord conductance. Records labelled 'after' were taken 20 min after the injections. Capacitive transients were broadened by filter time constant (200 ms) and clipped by chart width. The A-current is activated in some instances making several asymmetries in positive and negative transients.

cAMP was injected along with the dye (Fig. 9, right-hand records), I_{Na}(cAMP) developed and the identical set of negative voltage pulses was accompanied by smaller currents. For small positive steps into the negative slope resistance region of the I–V curve (e.g. to −33 mV) there was typically an increased inward current as shown. Negative pulse current decreases similar to those shown were noted in approximately 75 % of the neurones studied (forty of fifty-four cells). In the remaining cells there was either no change in the current size (eleven cells) or a slight increase, less than 10 %, which persisted for periods up to an hour (three cells). The decreased pulse current was noted for both pressure and ionophoretic injections.

In *Aplysia* cells where the resting membrane conductance was small and the voltage dependency of I_{Na}(cAMP) was steep (see Fig. 8*B*), the direction of the pulse current actually reversed for the smaller voltage steps. That is, instead of a negative voltage pulse being accompanied by a negative-going current, the current response was positive-going. This effect was most dramatic in *Aplysia* cells L2–L6 as illustrated in Fig. 10 with data taken from neurone L3. It should be noted that *net*

current was never outward (due to the large induced inward current) and that the current pulse direction became negative with a sufficiently large voltage pulse. This behaviour also was noted in neurones from *Dendronotus* and cell R2 of *Aplysia*.

The *Aplysia* data of Fig. 10 can be interpreted in a straightforward manner based on static measurements of the type shown in Fig. 8. For the cell of Fig. 10, I_{Na}(cAMP) decremented by 19 nA when the holding voltage was moved to −60 mV from

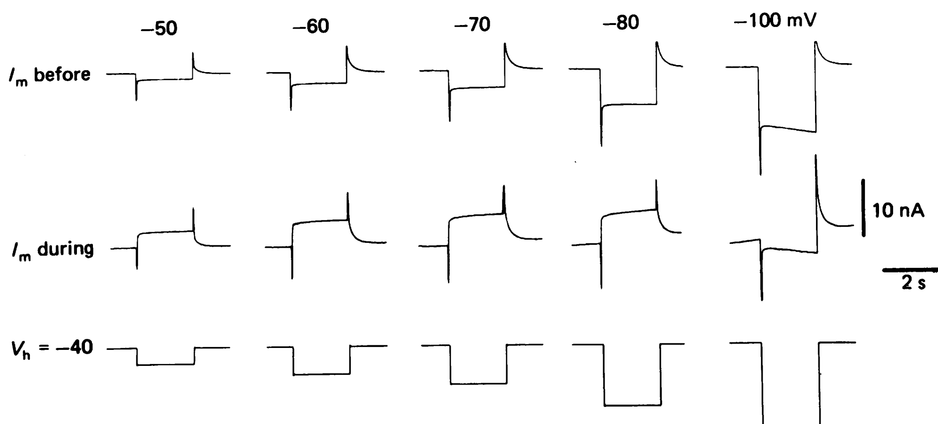

Fig. 10. Experiment similar to that of Fig. 9 performed on neurone L3 of *Aplysia*. Currents corresponding to the smaller voltage pulses are inverted during the period when I_{Na}(cAMP) was large (I_m during). Only for the largest pulse does the current retain normal negative-going polarity. The effect reversed as I_{Na}(cAMP) decayed.

−40 mV (static conditions). This amount is greater than the leakage current for a step pulse from −40 to −60 mV measured before the cAMP injection (−5 nA). This difference, assuming there are no other changes, would predict a positive step current deflexion of +14 nA during I_{Na}(cAMP), reasonably close to the value shown in the record.

It is more difficult to interpret the *Archidoris* data in terms of this scheme because there was no clear decrement in I_{Na}(cAMP) with hyperpolarization in the static measurements (see Fig. 8*A*), but there was a measurable suppression of the pulse currents in a large fraction of the cells (as in Fig. 9). At this point we believe that the current-carrying mechanisms are qualitatively the same in the two populations but that the voltage dependence of the transport mechanism is more shallow in *Archidoris* cells, causing the induced current to remain more nearly constant as driving force on Na is increased, rather than decreasing as in *Aplysia*. Pulse measurements were made in a number of neurones where I_{Na}(cAMP) had been virtually eliminated by superfusion with 20 mM-Na saline (470 mM-TMA or bis-tris propane). Under these conditions the size of the pulse currents after cAMP injections was nearly the same as the pre-injection value; i.e. much of the suppression was absent. The suppression returned when normal saline was restored. This finding suggests that there was a voltage dependency for the induced current even in *Archidoris* neurones. Using the analysis employed in connexion with Fig. 10, these pulse current differences could be accounted for by assuming that I_{Na}(cAMP) was

reduced by 10–15% when going from −40 to −80 mV. We tended to ignore such small differences in the static measurements (as in Fig. 8) since differences of this size fall into the range of variability between 'identical' doses. Large reductions, 40–50%, over the same voltage range in *Aplysia* were less ambiguous.

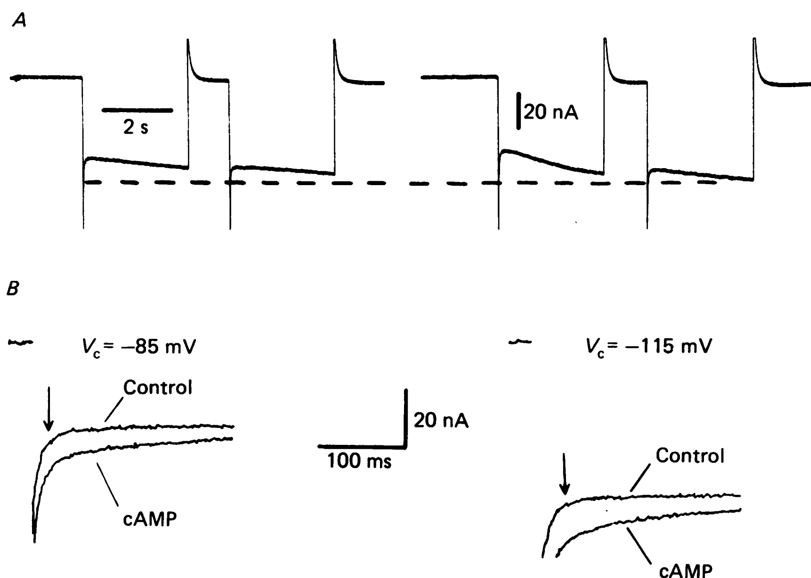

Fig. 11. *A*, current flow during twin, sequential voltage pulses (−40 to −100 mV) before (left-hand set) and immediately after injection of cAMP. Currents during the second pulses of the set are nearly identical. *Archidoris* neurone RPlGC. *B*, membrane current relaxations during hyperpolarizing voltage pulses (V_h = −40 mV). Currents recorded during the flow of I_{Na}(cAMP) are larger than control but relax to become equal to controls in several hundred milliseconds in this cell (off scale here). Pre-pulse currents have been shifted to overlay records. In other neurones the relaxation proceeded to a smaller value than control. *Archidoris* neurone RCe 1.

In some *Archidoris* cells where a decrease in the pulse current occurred there were two observations that suggested it was due in part to changes unrelated to the Na transport mechanism. The first was that suppression of the pulse current sometimes outlasted the period of I_{Na}(cAMP) flow in *Archidoris*. The pulse current suppression and reversal nearly paralleled the induced current flow in *Aplysia* neurones. To the other extreme, we often noted an increase in the pulse current after I_{Na}(cAMP) had decayed in both cell populations but have not analysed either effect. Secondly, in the *Archidoris* cells much of the decrease in pulse current could be transiently removed without affecting the magnitude of I_{Na}(cAMP). For example, Fig. 11*A* illustrates pairs of negative pulse records made before, during maximum flow of I_{Na}(cAMP), and after recovery. In the middle record, the pulse current was clearly suppressed during the initial part of the first pulse but relaxed toward the control value. Current for the second pulse was initially large and there was only a small further relaxation. As the spacing between pulses was increased, the current during the second pulse became identical to that during the first pulse. The current between the pulses was

approximately the same as the current before the first pulse, showing that the main current induced by the cAMP was not affected by changes in the pulse current. A similar but less pronounced relaxation was generally noted in control and recovery records.

Since cAMP is causing the activation of a Na transport mechanism with a voltage dependence, in principle one would expect to see a current relaxation in the pulse records corresponding to the decrease (or constancy) in I_{Na}(cAMP) with static hyperpolarization. If this mechanism is a channel or simple carrier, increasing the driving force on Na^+ should result in an instantaneous current increase. Then as channels close or other effects on the transport mechanism occur at the new voltage, the current should decrease. For example the pulse to -60 mV (during injection record of Fig. 10) should have initially caused a negative-going current that then relaxed to the more positive level. We have examined high-speed records in both *Aplysia* and *Archidoris* neurones but have been unable to demonstrate that such a relaxation actually occurs with consistency except in the two cerebral giant cells of *Archidoris*. Here the effect was clear, easily resolvable and repeatable. Fig. 11 *B* shows membrane currents in neurone RCe 1 for voltage pulses from a holding value of -40 mV to -115 mV and -85 mV. Superimposed currents were recorded during the flow of I_{Na}(cAMP) (23 nA) and after its decay to zero. Offsets have been adjusted so that pre-pulse currents were equal. The initial current jumps recorded during I_{Na}(cAMP) flow were larger than the initial jumps after recovery but the currents nearly converged toward the ends of the pulses (not shown in these records). In other examples the currents crossed over, the control current being larger at the end of the pulse. If we assume that I_{Na}(cAMP) is carried by an ohmic transport mechanism, the current induced at -40 mV in this example (23 nA) would require a conductance increase of 0·23 μS, taking the Na reversal potential as $+60$ mV. This conductance increase would allow an additional 10 and 17 nA to flow, over control values at -85 and -115 mV, at least until the transport mechanism changed, reducing current flow. The initial difference currents shown are 11 nA at -85 mV and 15 nA at -115 mV, very close to the values expected from the static measurement at -40 mV. Similar agreement was obtained in four of four cerebral giant neurones. The accuracy of these measurements is limited due to the fact that the injected cAMP is also affecting other membrane conductances which have an undetermined contribution to the current jumps. We limited our analysis to examples where these other effects were small.

Occasionally such current relaxations were seen in other identified neurones (both in *Aplysia* and *Archidoris*), but in general the initial current jumps were simply offset versions of each other. Rather than postulate a very different type of transport mechanism in these cases, we assume at present that the relaxation generally occurred too fast for us to measure and that the rate was modified by factors we have not yet identified. The capacitative transient in these neurones limited our time resolution for the voltage-clamp configuration from 2 to 4 ms; therefore, if the relaxation was faster than this we would not have been able to separate it out.

We have noted in several neurones from *Archidoris* and *Aplysia* a current similar to the M-current described by Adams, Brown & Constanti (1982). The current relaxations shown in Fig. 11 *B* were not due to a K-conductance relaxation such as the voltage-dependent M-current since they did not reverse direction around E_K.

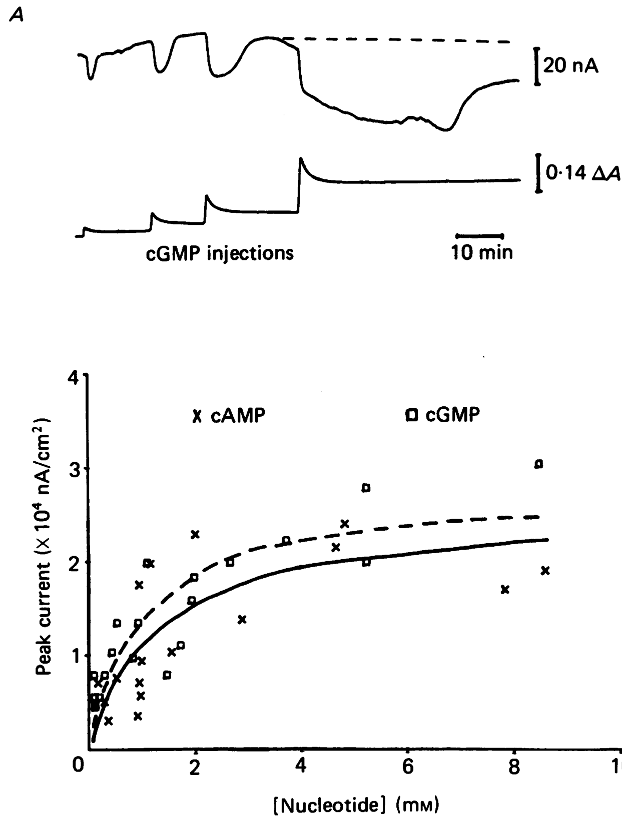

Fig. 12. *A*, records similar to those in Fig. 2*A* showing inward currents induced by the injection of cGMP-Arsenazo III mixture into *Archidoris* neurone RPl 2. *B*, dose–response plot of cAMP- and cGMP-induced currents in several different *Archidoris* neurones displaying inverse hyperbolic relationships between peak current (normalized for differences in soma sizes) and estimated concentration of injected dose.

Effects of other nucleotides

We have already pointed out that injections of 5′AMP caused no consistent changes in the resting potential of the *Archidoris* neurones studied. Similar injections of the following nucleotides into the same population of cells were also without effect: 5′GMP, ATP, GTP, cUMP and cCMP. However, injections of cGMP elicited dose-dependent depolarizations and inward currents (Fig. 12*A*) similar to the cAMP responses. Na-substitution experiments demonstrated Na ion dependence of the cGMP response. The current–voltage dependencies analysed in the preceding section for cAMP injections were identical to those obtained with cGMP injections. Fig. 12*B* shows a dose–response plot of the estimated amount of cAMP or cGMP injected into several different *Archidoris* neurones *vs.* the peak current response. Since current amplitudes following multiple injections were often erratic (see Fig. 12*A*), the data in Fig. 12*B* were taken only from initial injections (thus each point was a different cell) with stable base lines. The curves in the Figure were generated using a

least-squares fit computer program and illustrate that cGMP (dotted line) and cAMP (continuous line) evoked similar dose–response curves.

Injections of cAMP analogues into *Archidoris* neurones also induced inward currents, although they were quantitatively less effective than the non-substituted cyclic compounds. Several analogues were tried with the following order of potency

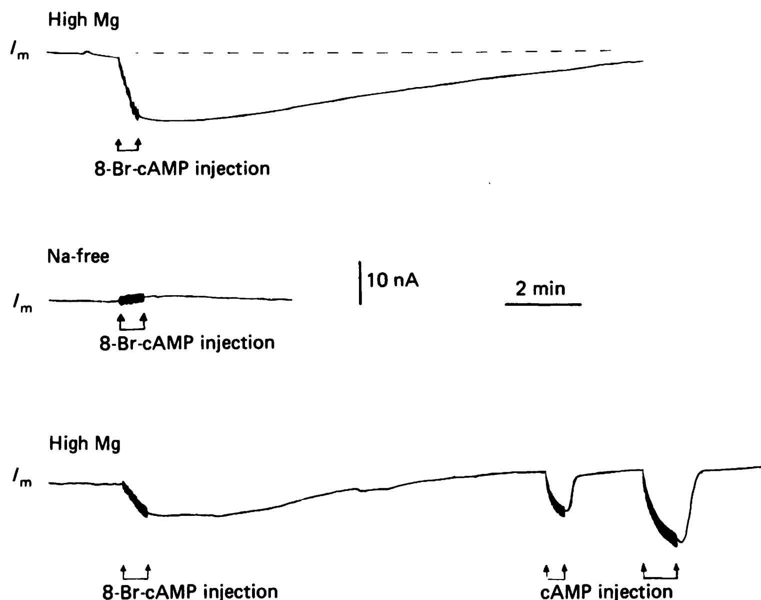

Fig. 13. Ionophoretic injection of 8-bromo-cAMP into *Archidoris* neurone RPlGC ($V_h = -40$ mV) induced an inward current which recovered much more slowly than currents evoked with cAMP (top and bottom traces). The injections were performed in high-Mg, Ca-free saline to prevent indirect effects caused by diffusion of analogue out of the injected cell into neighbouring cells. Removing Na from the bath blocked the analogue response (middle trace), which returned when Na was added back (bottom trace).

observed relative to cGMP: cGMP, cAMP > 8-bromo-cAMP, 8-azido-cAMP > 8-benzylamino-cAMP > dibutyryl-cAMP, monobutyryl-cAMP. However, even small injections of an analogue resulted in inward currents of long durations. Fig. 13 shows records from a neurone bathed in high-Mg, Ca-free saline and ionophoretically injected with 8-bromo-cAMP. The slow current recovery was expected for a compound which is only slowly hydrolysed by PDE. A subsequent injection in Na-substituted (bis-tris propane) saline produced no current response, although the induced current returned when Na was substituted back into the superfusion solution. For comparison a pair of cAMP injections were administered at the end of this experiment showing the faster rate of current recovery following injections of this nucleotide.

In contrast to the intracellular injection results, mono- and dibutyryl-cAMP were ineffective when applied in the bath at 10 mM. At the same concentration the 8-substituted analogues sometimes induced a small inward current in normal and in high-Mg saline. The changes were much smaller than the responses following injections, however, and they were slow to develop, indicating that these analogues did not easily penetrate the barriers which surround these cells.

Adenylate cyclase activators

As previously mentioned, intracellular injection of GTP did not affect cellular resting potential even though GTP levels are thought to underlie adenylate cyclase activation in a wide range of cell types (Rodbell, 1980). We also injected the non-hydrolysable analogue G_{pp}-NH_p into several of the *Archidoris* cells without observing any depolarization or inward current. Bath application of forskolin, a potent adenylate cyclase activator in mammalian cells (Seaman, Padgett & Daly, 1981), has been reported to induce inward currents in some molluscan neurones (Deterre, Paupardin-Tritsch, Bockaert & Gerschenfeld, 1982). However, we found no effect on holding current in *Archidoris* neurones clamped between -40 and -80 mV when exposed to 10^{-4} M-forskolin. Thus, at the present time we have not found a suitable method to activate adenylate cyclase in the molluscan neurones we examined.

DISCUSSION

The data presented in this report have characterized a Na-current mechanism, activated by either cAMP or cGMP, which does not exhibit a measurable steady-state conductance increase at voltages more negative than -40 mV. The results were found in identifiable neurones of several marine gastropods, and they are similar to observations made by Kononenko (1981) on neurone LP8 of the terrestrial gastropod *Helix pomatia*. The current we have described is not the same as the nucleotide-induced 'inward' currents described by Pellmar (1981) and Deterre *et al.* (1981, 1982). There are several prominent differences. Unlike the current we have described, their currents were induced only at holding potentials above -30 mV, and cyclic nucleotide injections had little or no effect upon cellular resting potential. The current described by Pellmar (1981) was blocked by 1–2 mM-Cd and persisted in Na-substituted (bis-tris propane) saline. These results are the reverse of the current we have described. Likewise the current of Deterre *et al.* (1981, 1982) was unaffected by two-thirds Na replacement (Tris), a procedure which monotonically decreased the current described here. We have also used the Arsenazo III technique in *Archidoris* neurones and found no direct relationship between the inward current and elevated internal Ca levels (Hockberger & Connor, 1983*a*). These data are not consistent with the interpretation that we were looking at the same currents as Pellmar (1981) and Deterre *et al.* (1981, 1982). More recently we have analysed several identified neurones in the land snail *Limax maximus* using Arsenazo III and cyclic nucleotide injections (Connor & Hockberger, 1983) and have found responses similar to those described by both Pellmar and Deterre *et al.* It appears to us that these different induced currents reflect real cellular differences.

The Na current characterized in this report demonstrated both pharmacological and voltage-sensitive properties which separate it from previously analysed Na currents. It was not like the fast inward current underlying the upstroke of the action potential which is TTX sensitive and exhibits activation and threshold around -30 mV. Neither did the nucleotide-induced Na current appear to be the result of Na–K pump inhibition since it persisted in either K-free or ouabain-containing saline. This current was also unlike the current underlying Na transport in epithelial cells which is amiloride sensitive. The nucleotide-induced current was activated in a

membrane potential range which overlaps the range where the inward current underlying the negative slope resistance region is found (Wilson & Wachtel, 1974; Partridge, Thompson, Smith & Connor, 1979). We consider it a possibility that the negative slope resistance current mechanism is somehow activated by cAMP, giving rise to the current measured here. However, it is not clear that the negative slope resistance inward current flows at membrane potentials more negative than -60 mV, as I_{Na}(cAMP) does. Nor is it understood whether or not injection of cAMP might somehow alter the ion selectivity of the negative slope resistance channels which ordinarily exclude Li ions (Smith, Barker & Gainer, 1975; J. A. Connor & P. Hockberger, unpublished observations), while permitting Na and Ca ions to pass through (Partridge *et al.* 1979). As mentioned above, we have found no evidence in dorid neurones to support the notion that Ca ions contribute to the nucleotide-induced current.

Another possible source of the Na current was a transient increase in Na–Ca exchange. Increased rate of Na–Ca exchange (Reuter, 1972) should cause a considerable reduction in internal Ca^{2+} since the Na fluxes measured were quite large. For example, a current of 10 nA flowing for 10 min would be sufficient to increase $[Na]_i$ by 1 mM in a 400 μm cell, in the absence of restoring mechanisms. Even with an exchange rate of 3 Na for 1 Ca (Blaustein & Russell, 1975), the obligatory Ca efflux would present an enormous drain on internal Ca stores and in all probability a decrease in free $[Ca]_i$. No such changes were detected using the Arsenazo III technique (Hockberger & Connor, 1983*a*), a detection system capable of tracking decreases in free [Ca] in these cells from normal resting levels brought about by EGTA injections (J. A. Connor & P. Hockberger, unpublished). A further possibility, Na–H exchange, was unlikely since data in the following paper (Connor & Hockberger, 1984) show that the H ion concentration was typically elevated in neurones following an injection.

It has been necessary to devote considerable attention to transient and static *I–V* measurements because the findings, superficially at least, are somewhat unorthodox. Most well described ligand-activated transport mechanisms have ohmic *I–V* characteristics; i.e. current increases nearly linearly with driving force for a wide voltage range, over 100 mV for end-plate current in striated muscle (Magleby & Stevens, 1972). In the present experiments the induced current either remained constant or decreased over the voltage range where we could measure it in normal saline, reflecting a voltage as well as chemical sensitivity of the Na transport system. In *Aplysia*, voltage dependence of the I_{Na}(cAMP) under static conditions was sufficient to account for most of the anomalous behaviour of current during voltage-clamp pulses (see Fig. 10). In *Archidoris* cells we were unable to demonstrate clearly a static voltage dependence of I_{Na}(cAMP) yet pulse currents were also suppressed (see Fig. 9). We believe it is most sensible to conclude that the mechanisms are basically the same across species, and involve ordinary ion channels, but that in *Archidoris* neurones the increase in electrochemical driving force which accompanies hyperpolarization is rather neatly balanced against a decrease in activatable channels.

In a few dorid neurones (e.g. Fig. 11*A*) an additional cAMP-related process may have contributed to the step conductance decrease. We have not investigated this mechanism in detail but it bears similarities to K conductance decrease following cAMP injection into other molluscan neurones (Klein & Kandel, 1980; Siegelbaum,

Camardo & Kandel, 1982; Deterre *et al.* 1982). Our ion-substitution experiments indicated that compared to I_{Na}(cAMP), there was relatively little current carried by this secondary process in the subthreshold voltage range; however, during depolarizations this process might become quite significant. We would note that a decreased conductance to K or Cl brought about by cAMP would tend to mask any static voltage dependency of the Na current and that this might be a contributing factor to the flatness of the I–V relationship in *Archidoris*. In all probability similar processes were initiated in the *Aplysia* neurones we studied but we could not separate them from the typically steep voltage dependency of I_{Na}(cAMP).

There are observations in two vertebrate systems which indicate a possible involvement of a cyclic nucleotide-activated inward current similar to the one characterized in this report. The muscarinic depolarizing response to acetylcholine in sympathetic neurones results from an inward current which occurs without an increase in membrane conductance (Kobayashi & Libet, 1968). Part of this muscarinic response is due to a suppression of an outward K current (Brown & Adams, 1980), but at least in mammalian cells part is mediated via an intracellular increase in cGMP where the electrogenic mechanism is still unknown (Hashiguichi, Kobayashi, Tosaka & Libet, 1982). Another example is the light-sensitive Na current in rod outer segments which is maintained by a high resting cGMP concentration (Miller & Nicol, 1981). This current is relatively voltage insensitive, unaffected by TTX and amiloride (Owen & Torre, 1981), and Li can substitute for Na (Yau, McNaughton & Hodgkin, 1981).

Although the amplitude of the Na current described here was shown to be dose dependent, the minimal effective dose could only be estimated due to limitations in the methods used. Our estimations of injected amounts also disregarded PDE activity as well as binding to non-catalytic sites (Yamazaki, Bartucca, Ting & Bitensky, 1982). Nevertheless we were able to obtain depolarizing effects with doses in the range of 30–35 μM (e.g. Fig. 1). Since the concentration of total cAMP in *Aplysia* neurones R2, R15 and LP1 has been determined to be approximately 5–10 μM (ignoring compartmentalization), and since certain neurotransmitters can elevate this amount at least 2–3-fold (Cedar & Schwartz, 1972; Levitan & Drummond, 1980; Bernier, Castellucci, Kandel & Schwartz, 1982; although none of the reports corrected for endogenous PDE activity which hydrolyses a significant amount of newly synthesized nucleotides), then our results are consistent with the idea that certain nuerotransmitters depolarize neurones by raising intracellular cyclic nucleotide levels. Until the temporal relationship between these events can be more rigorously tested, however, it is not clear if such elevation could underlie slow synaptic responses (Kehoe & Marty, 1980; Hartzell, 1981) or whether the role of cyclic nucleotides in synaptic transmission is merely modulatory (Ehrlich, Volavka, Davis & Brunngraber, 1979).

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Note added in proof. Since submission of this article two Reports discussing similar findings have appeared (Green & Gillette, 1983; Aldenhoff, Hofmeier, Lux & Swandulla, 1983).

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