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INTRACELLULAR pH CHANGES INDUCED BY INJECTION OF CYCLIC NUCLEOTIDES INTO GASTROPOD NEURONES

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SUMMARY

1. Injections of cyclic AMP (cAMP) or cyclic GMP (cGMP) into identifiable gastropod neurones under voltage clamp induced reversible cytoplasmic pH changes which were measured using the indicator dye Arsenazo III and pH micro-electrodes. Similar injections of 5′AMP or 5′GMP did not induce such effects.

2. In about one-half of the population of neurones examined, cAMP injections (0·1–10 mM) induced pH decreases with latencies of 1–5 min and maximum response times 12–25 min post-injection. Comparable injections of cGMP into these cells resulted in decreases with latencies less than 1 min and maximum response times 5–10 min. The amplitude and duration of these pH_i decreases were dose dependent.

3. In the other half of the population of neurones tested, cAMP injection induced an immediate alkalinization lasting 5–10 min followed by an acidification which displayed a maximum response time within the same range as those in the first group. cGMP injection into these cells induced only acidifications with faster peak responses than with cAMP. Since cGMP did not elicit alkalinizing responses, the slower response time of the first group of cells with cAMP may reflect an always present underlying alkalinization.

4. The nucleotide-induced acidification was potentiated in neurones bathed in the phosphodiesterase inhibitor IBMX. Injections of non-hydrolysable cAMP analogues also induced acidifications which were longer lasting than comparable doses of cyclic AMP. These results indicate that the elevated $[H^+]_i$ was not simply due to hydrolysis of the injected cyclic nucleotide.

5. The pH_i changes were invariably of much longer duration than a nucleotide-induced inward current (Connor & Hockberger, 1984) and persisted in Na-free salines which eliminated the current response. These results support the notion that cyclic nucleotide elevation can affect cellular metabolic processes distinct from effects on ion transport mechanisms.

INTRODUCTION

As transducers of membrane receptor signals, cyclic nucleotides exert a variety of actions upon the biochemical and physiological responses of cells. In the preceding paper (Connor & Hockberger, 1984), we have examined in detail a specific change

in membrane Na permeability and have briefly mentioned some of the membrane permeability changes reported by others. Though most of the neurophysiological studies reported to date have looked at induced changes in ion transport across the cell membrane, it is reasonable to expect cytoplasmic changes as well. We report here on changes in cytoplasmic pH of molluscan neurones induced by cyclic AMP (cAMP) and cyclic GMP (cGMP). Preliminary reports of these results have appeared elsewhere (Hockberger & Connor, 1982, 1983, 1984; Connor & Hockberger, 1982).

METHODS

Experiments were performed on identified neurones from *Archidoris montereyensis* (Hockberger & Connor, 1984) obtained from the waters around Friday Harbor, Washington. Basic experimental techniques and superfusing media have been described in the preceding article (Connor & Hockberger, 1984). Cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), 5'AMP, 5'GMP, dibutyryl-cAMP, and 8-benzylamino-cAMP (Nasalts, Sigma, St. Louis, MO) were injected using pressure pulses in all experiments, typically with the dye Arsenazo III to monitor dosage (see preceding paper). The pH of the injected solutions was buffered with 10 mM-MOPS (3-(*N*-morpholino)propanesulphonic acid) (MOPS, Sigma, free acid).

Recessed-tipped pH micro-electrodes were constructed according to the methods of Thomas (1978) and had tip diameters of 1–2 μm and response times (i.e. 90% completion following a unit change in pH) of 1–2 min. The pH micro-electrodes were routinely calibrated in an internal saline solution standard containing 350 mM-KCl, 50 mM-NaCl, and 50 mM-MOPS buffer adjusted to either pH 7·6 or 6·6. Selected electrodes were tested over a wider range (4–9). The slope of the electrodes was measured at room temperature in the buffered standards (see below) and only electrodes with slopes between 51 and 58 mV were used. Recording configuration was the same as that used for Na-sensitive electrodes in the preceding study. Penetration by the pH micro-electrode was considered satisfactory when a 20 mV voltage step across the cell membrane produced less than 1 mV change in the differential output. The intracellular pH, pH_i, was calculated using the slope of the electrode and by measuring the drop in voltage upon moving the pH electrode from the bath (pH = 7·6) into the cell.

pH standards were calibrated at room temperature using a high ionic strength calomel combination pH electrode (Corning, Medfield, MA) with a porous ceramic plug. The Corning pH electrode exhibited a 0·1 pH unit error when comparing 0·1 M with 1·0 M standard potassium phosphate buffers (Sigma) at pH = 7·5 and 7·2, respectively. This ionic strength-related error has been found to be inherent in all pH electrodes using porous ceramic plugs due to the difference in liquid–liquid junction potentials between standards of differing ionic strengths (Illingworth, 1981).

The indicator dye Arsenazo III (grade I, Sigma) was also used to detect changes in intracellular Ca and H ions as described elsewhere (Ahmed & Connor, 1979, 1980; Hockberger & Connor, 1983*c*). Changes in the internal concentration of these ions were detected by monitoring the absorbance of the dye at the differential wave-length pairs 610–700 nm and 660–700 nm with a rotating wheel spectrophotometer (Brinley & Scarpa, 1975) which minimized light scattering and distortion artifacts during injections. Since the dye absorbance is sensitive to both H and Ca ions (Ogan & Simons, 1979), simultaneous monitoring of pH_i using pH-sensitive micro-electrodes was also employed.

RESULTS

All of the experiments reported were performed using pH micro-electrodes to monitor changes in pH_i. Each of the fourteen identified neurones in *Archidoris* was examined at least twice in this manner ($n = 61$) and exhibited a range in resting pH_i between 7·05 and 7·75 (mean $7{\cdot}39 \pm 0{\cdot}16$, S.D. of an observation). This variability was found as often within cell types as between them, and the mean value is similar to that reported for other gastropod neurones (Thomas, 1974).

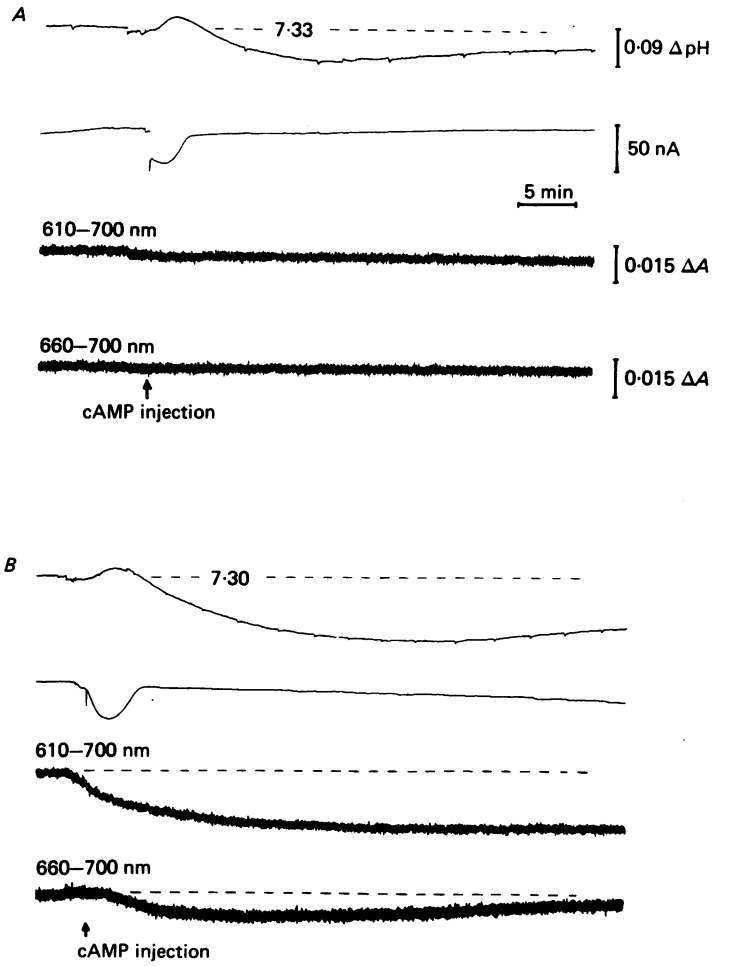

Fig. 1. Pressure injection of cAMP (pH = 7·3) into *Archidoris* neurone LPl 1 under voltage clamp ($V_H = -40$ mV) before and after loading the cell with the indicator dye Arsenazo III. *A*, without dye an injection induced a transient inward current and a pH_i decrease but did not affect the dual wave-length absorbance signals 610–700 nm and 660–700 nm. *B*, with dye in the cell an injection induced similar current and pH_i responses and decreased the 610–700 nm signal more than the 660–700 nm signal. The absorbance traces were monitored at the injection site whereas the pH micro-electrode was situated several hundred micrometres away. Absorbance signal time constant was 0·3s.

Injections of cAMP into neurones voltage clamped at resting potential induced large inward currents, described in the preceding paper (Connor & Hockberger, 1984), and pH_i changes. The latter portion of the pH response, an acidification, persisted for 10–20 min after the current response had recovered. During the initial portion (1–5 min) the pH remained either stable, or in some neurones increased. Fig. 1 shows an example of a pH response to pressure injection of cAMP tracked both by a pH micro-electrode and by Arsenazo III absorbance changes. The record of Fig. 1 *A* was obtained prior to loading the cell with Arsenazo III. The pH electrode

registered an initial alkalinization followed by a larger and longer duration acidification. The absorbance records demonstrate that the injection produced no measurable changes in intrinsic cell absorbance. After the cell was filled with Arsenazo III (0·3 mM) a second pressure injection of cAMP gave essentially the same current and pH electrode responses, as shown in Fig. 1*B*. However, dye absorbance decreased following the cAMP injection, with the magnitude of the 610–700 nm signal being

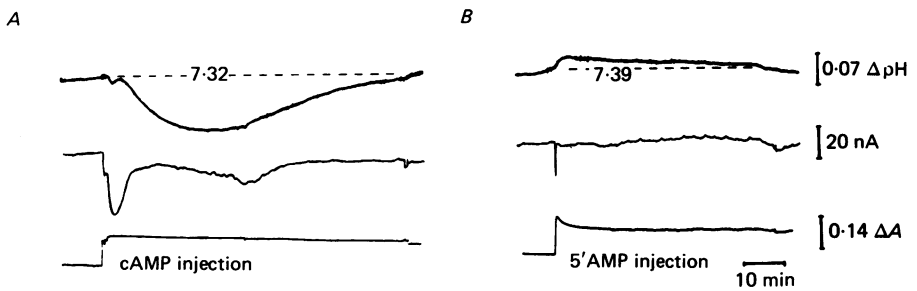

Fig. 2. *A*, simultaneous monitoring of changes in pH_i (upper trace) and membrane current (lower trace) brought about by cAMP injection. Inward current and decreasing pH are plotted downward. cAMP mixed with Arsenazo III (pH = 7·2) was injected under pressure. The absorbance record (lower trace) is a differential wave-length pair (580–700 nm) and is a monitor of injected quantity. Although both the current and pH_i responses were transient, the pH_i change lasted much longer. cAMP dose = 11·1 mM. *B*, a comparable dose of 5'AMP–Arsenazo III (pH = 7·5) did not evoke either the inward current or the acidification. 5'AMP dose = 10·0 mM. Calibrations the same for *A* and *B*.

greater than that of the 660–700 nm signal, indicative of a pH decrease (Ahmed & Connor, 1980; Hockberger & Connor, 1983). The initial dye absorbance increase, expected because of the trasient alkalinization, was apparently counterbalanced by dilution artifact. Dilution affects the 610–700 nm signal more severely than the 660–700 nm signal since the absorbance of Arsenazo III falls off sharply between 600 and 660 nm in a low-Ca milieu but very little between 660 and 700 nm. Thus, as reported previously, there is no indication that resting intracellular Ca concentration changes, either up or down, as a direct result of cAMP injection (Hockberger & Connor, 1983).

We have provisionally classified the cells studied into two groups based upon differences in the direction and time course of the pH response, although we will present evidence that supports the possibility that these differences may arise from subtle variations in a single response profile. In about half of the neurones injected with cAMP (fifteen out of twenty-nine), there was a simple latency of 1–5 min between injection and onset of an acidification. The amplitude and duration of the pH_i decrease were dose dependent, the amplitude maximum occurring 12–25 min post-injection. Fig. 2*A* shows an example of one such measurement in neurone RPe 2 in which the membrane current and dose (absorbance trace) were also monitored. The injected dose of cAMP was calculated to be 11 mM from the absorbance change (see Methods, in Connor & Hockberger, 1984). This estimate ignores hydrolysis by native phosphodiesterases. The corresponding pH_i decrease

lasted more than 1 h and reached its maximum response (approximately 0·14 units) about 20 min after the current recovered.

Fig. 2*B* shows recordings made during a similar injection of 5′AMP (plus Arsenazo III) into the same cell. This nucleotide did not evoke either the inward current response nor the acidification seen with cAMP injection. There was a small alkalinization which was probably due to the pH mismatch between cell resting pH and the

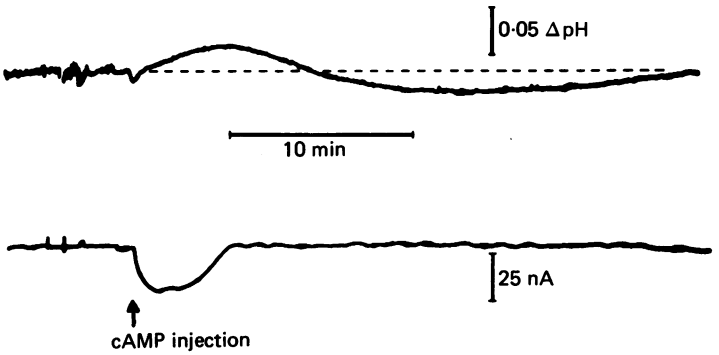

Fig. 3. Biphasic pH_i response was obtained following a cAMP injection (pH = 7·4) into neurone RPe 1. This type of response was elicited in about one-half of the neurones examined (also see Fig. 1). cAMP dose = 3·5 mM. $V_H = -40$ mV.

pH of the injected solution, since lowering the pH of the 5′AMP solution below the resting pH_i reversed the direction of this small response. When adjusted to the cell pH, neither 5′AMP, ATP nor internal saline injections changed the resting pH_i.

The second type of pH response following cAMP injections exhibited an initial, transient alkalinization ($n = 14$) followed by an acidification (Figs. 1 and 3). This type of response was often seen after injections into two white pigmented neurones (LPl 2 and LPl 3), although eleven of the twelve cell types showed this response at least once. The peak alkalinization occurred 5–10 min post-injection, depending upon the degree of acidification which followed. The subsequent acidification displayed a maximum response time similar to pH_i changes evoked in cells without an initial alkalinization. We believe it is possible that mechanisms underlying both types of pH_i responses were always present, and that half of the time the acidification was large enough to mask the initial alkalinizing response (see below).

The amplitude and duration of both the cAMP-induced inward current and acidifying pH_i responses were dramatically enhanced by bathing the cell in the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Fig. 4 shows this enhancement using neurone LPl 4 in 10^{-3} M-IBMX. Here a smaller dose of cAMP in the presence of IBMX produced greatly prolonged current and pH_i responses. Two additional observations were made on IBMX-treated cells. First, no change in pH_i was evident prior to a cAMP injection even though IBMX increased the holding current base line in most cells examined (Fig. 4*B*). If this change in holding current results from cAMP accumulation, then either the elevation resulting from IBMX exposure occurs at a site too distant to be detected by the pH micro-electrode, or else the elevation was localized at the cell membrane. A second, not infrequent,

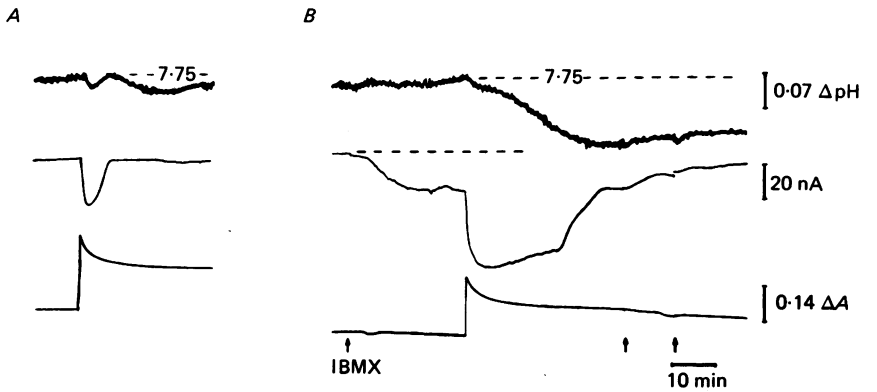

Fig. 4. *A*, small current and pH_i responses were induced in neurone LPl 4 with an injection of cAMP–Arsenazo III (pH = 7·5; cAMP dose = 4·7 mM). The initial transient decrease was probably an injection artifact (see text). *B*, much larger current and pH_i responses induced by a smaller dose of nucleotide (4·2 mM) following exposure to 10^{-3} M-IBMX. Addition of the IBMX saline increased the base-line inward current but did not change pH_i. The induced current response also began recovering before the IBMX was removed with normal saline washes (arrows). Calibrations the same for *A* and *B*. $V_H = -40$ mV.

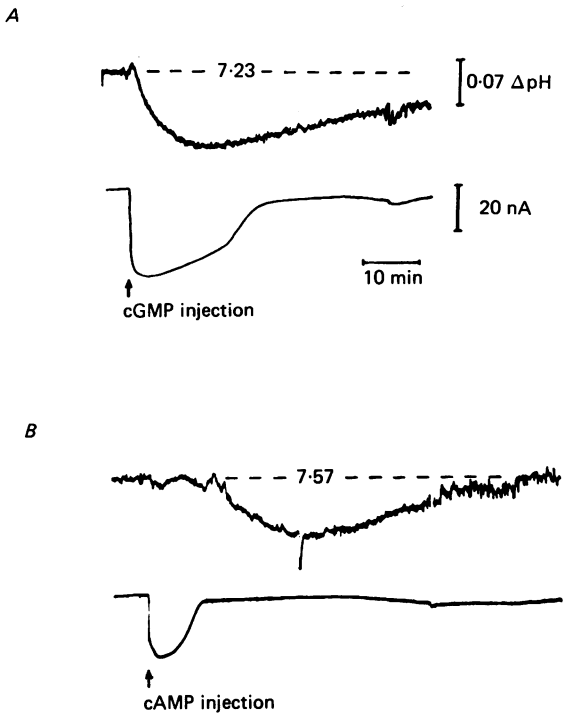

Fig. 5. *A*, injection of cGMP (pH = 7·4; dose = 8·5 mM) into neurone LPl 1 ($V_H = -40$ mV) induced a large inward current and showed virtually no pH_i response latency. *B*, injection of cAMP (pH = 7·5; dose = 4·1 mM) into neurone LPl 4 ($V_H = -40$ mV) induced a smaller inward current and the pH_i response exhibited a latency of almost 10 min. Calibrations the same for *A* and *B*.

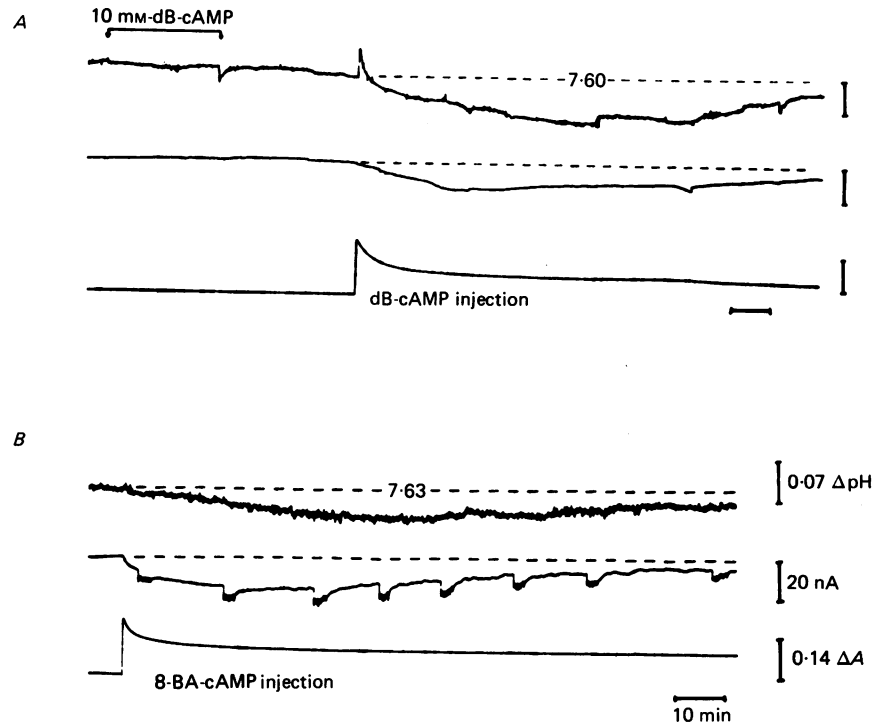

Fig. 6. *A*, initial part of record shows that bath application of dibutyryl-cAMP (dB-cAMP; 10 mM) did not appreciably change either the holding current ($V_H = -40$ mV) or the resting pH_i of this neurone (LPl 4). A subsequent injection of the analogue–Arsenazo III (pH = 7·4; dose = 1·6 mM) induced both a long-lasting inward current as well as a corresponding acidification. *B*, injection of 8-benzylamino-cAMP (8-BA-cAMP; pH = 7·5; dose = 3·8 mM) into the same cell ($V_H = -40$ mV) used to record Fig. 5*B* induced a long-lasting inward current response as well as a corresponding cellular acidification. Calibrations the same for *A* and *B*.

observation in IBMX-bathed neurones was that the current response following a cAMP injection inexplicably recovered even though its amplitude and duration were markedly enhanced (Fig. 4*B*).

Dose-dependent acidifications were also obtained with cGMP, but not 5′GMP, injections. The magnitude of the pH_i changes were similar to those evoked by cAMP, but the time courses differed. The latency of onset following cGMP injection was always less than 1 min and the maximum response time was 5–10 min. Fig. 5 shows records comparing the pH_i responses following injections of each nucleotide into different cells, although qualitatively similar results were obtained when using the same cell. The injection of cGMP induced an inward current and an internal acidification with virtually no latency. By comparison, cAMP induced a pH_i decrease with a latency of almost 10 min. The faster onset of the cGMP-induced acidification, coupled with the fact that cGMP has not been found to induce an alkalinization ($n = 7$ cells), provides additional support for the notion of a consistent underlying alkalinization induced with cAMP.

Injection of two different cAMP analogues (dibutyryl-cAMP and 8-benzylamino-cAMP) also resulted in intracellular acidifications with time courses far outlasting the cAMP and cGMP responses. Bath application of these analogues was less effective at inducing either a current (see also Connor & Hockberger, 1984) or pH_i response. Fig. 6*A* contains records from neurone LPl 4 showing that although bath-applied dibutyryl-cAMP (10 mM) was an ineffective stimulus, a subsequent injection of the analogue induced long-lasting responses in both pH_i and current measurements. The

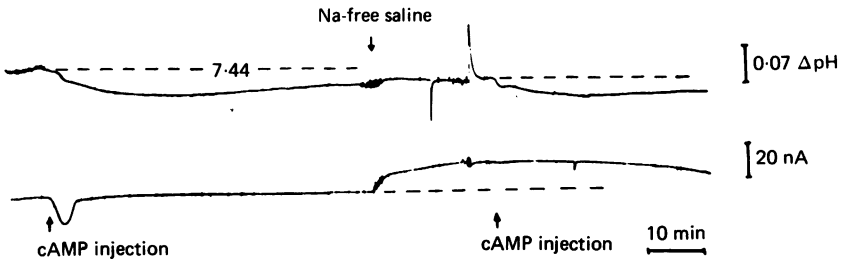

Fig. 7. Injection of cAMP (pH = 7·3; dose = 0·6 mM) into neurone RPl 4 ($V_H = -40$ mV) in normal saline exhibited a typical acidifying response. Bathing the cell in Na-free (tetramethylammonium-substituted) saline changed the base-line holding current and abolished the nucleotide-induced inward current response (dose = 0·8 mM) but did not affect the induced cellular acidification.

data presented in Fig. 6*B* show the effects of an injection of 8-benzylamino-cAMP into the identical cell used to record Fig. 5*B*. Comparison of the records shows that the time course of both the current and pH_i responses induced by the analogue were greatly prolonged. The data using poorly hydrolysed analogues in combination with the results obtained using the phosphodiesterase inhibitor IBMX, suggest that the normal current and pH_i recoveries following cyclic nucleotide injections were in large part due to hydrolysis of the nucleotide by phosphodiesterase. These results also indicate that the induced acidifications were not simply due to H ions liberated during this hydrolysis.

We examined the cAMP-induced acidification in Na-free saline, which eliminated the induced inward current, and found the pH_i response to be unaffected by this manipulation ($n = 9$). Fig. 7 shows an example of current and pH_i records during injections into neurone RPl 4 bathed first in normal saline followed by a comparable dose in Na-free saline. Each injection induced an acidification of about the same magnitude and time course, indicating that there was no obligatory coupling between the Na current and H ion concentration increase in the cytoplasm as would be expected from some form of co- or counter-transport mechanism (Thomas, 1977; Boron, McCormick & Roos, 1981).

DISCUSSION

The results presented here show that injections of cAMP (or cGMP) cause cytoplasmic pH changes that are relatively large, long-lasting, and reversible. Although studies on barnacle muscle have been published showing that pH_i recovery

mechanisms were enhanced by cAMP (Boron, Russell, Brodwick, Keifer & Roos, 1978), our results using neurones seem to be the first report of a direct effect of cAMP upon pH_i.

While we have not been able to determine the cellular processes which generate the pH_i changes, we have at least ruled out a number of possibilities which might have produced the dominant acidification. (1) Hydrolysis of the nucleotide load: induced pH_i changes were much larger with non-hydrolysable analogues or when phosphodiesterase activity was reduced. (2) Regulation of Ca load induced by the nucleotides: under voltage clamp at resting potential there was no significant Ca load as measured by Arsenazo III. (3) Increased Na–K transport and consequent ATP hydrolysis: the acidification persisted in low-Na saline where there was no appreciable load. (4) Modification of Na–H exchange: reversing the Na gradient had little effect on the pH_i change. The initial alkalinization sometimes observed possibly results from increased Na–H exchange but the effect has not proved robust enough in our experiments to study in detail. Also, such alkalinizations were not found with cGMP injections. It is our tentative conclusion that the underlying proton consuming or transporting processes are always accelerated by cAMP and that their effect is counterbalanced by other processes which generate protons. The net effect is generally the quasistable pH_i observed for the first 5–10 min following cAMP injection. Injection of cGMP apparently does not enhance the alkalinizing processes.

Since cyclic nucleotides, *in vitro*, are presumed to exert their effects by activating protein kinases (for reviews see Williams & Rodnight, 1977; Greengard, 1978; Walsh, 1978; Cohen, 1982), we may have demonstrated a consequence of this event under *in vivo* conditions; that is, H ions produced as a result of protein phosphorylation. Whether this pH change itself, or others of like magnitude, serves as a physiological signal is a question of some current interest (Roos & Boron, 1981; Nuccitelli & Deamer, 1982).

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