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MODULATION OF CALCIUM-ACTIVATED NON-SPECIFIC CATION CURRENTS BY CYCLIC AMP-DEPENDENT PHOSPHORYLATION IN NEURONES OF *HELIX*

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SUMMARY

1. Currents through calcium-activated non-specific cation (CAN) channels were studied in the fast burster neurone of *Helix aspersa* and *Helix pomatia*. CAN currents were activated by reproducible intracellular injections of small quantities of Ca^{2+} utilizing a fast, quantitative pressure injection technique.

2. External application of forskolin (10–25 μM), an activator of adenylate cyclase, caused the endogenous bursting activity of the cells to be replaced by beating activity. These same concentrations of forskolin reduced CAN currents reversibly to about 50%.

3. External application of IBMX (3-isobutyl-1-methylxanthine, 100 μM), an inhibitor of phosphodiesterase, the enzyme which breaks down cyclic AMP, reduced CAN currents reversibly to about 40%.

4. External application of the membrane-permeable cyclic AMP analogues 8-bromo-cyclic AMP and dibutyryl-cyclic AMP (100 μM) caused almost complete block of the CAN current. A marked reduction in the CAN current was also observed following quantitative injections of cyclic AMP (internal concentrations up to 50 μM) directly into the cells from a second pressure injection pipette.

5. Similar results were obtained with quantitative injections of the catalytic subunit (C-subunit) of the cyclic AMP-dependent protein kinase (internal concentrations 10^{-4} units of enzyme) directly into the cells from a second pressure injection pipette.

6. Injection of the non-hydrolysable GTP analogue, GTP-γ-S (internal concentrations 100 μM), which stimulates G-proteins, produced a prolonged increase in CAN current amplitude by as much as 300%.

7. External application of serotonin (100–200 μM) caused a transition from bursting to beating activity of the neurones and mimicked cyclic AMP's effects on CAN currents. Two other neurotransmitters, dopamine and acetylcholine, were not significantly effective in reducing CAN currents.

8. Injection of a peptide inhibitor of cyclic AMP-dependent protein kinase suppressed serotonin's action on bursting and on CAN current.

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9. Our results indicate that CAN currents in *Helix* burster neurones are modulated by cyclic AMP-dependent membrane phosphorylation. They suggest that the physiological transmitter that induces this second messenger action is serotonin. The dual control of CAN channels by two second messengers, namely Ca^{2+} and cyclic AMP, has important functional implications. While Ca^{2+} activates these channels which generate the pacemaker current in these neurones, cyclic AMP-dependent phosphorylation down-regulates them, thereby resulting in modulation of neuronal bursting activity.

INTRODUCTION

Many different neurones, even when isolated from any form of phasic input, display bursting spike activity. Bursting activity itself, rather than the simple average firing frequency, plays an important role in the function of these neurones. Neurosecretion (Dutton & Dyball, 1979) and regulation of motor function (Prior & Gelperin, 1977) are two examples in which the effector action of a neurone is dependent upon the grouping of action potentials into bursts. Thus the ability to modulate the burst firing of a neurone can be an important factor in the determination of its function. Calcium-activated non-specific cation (CAN) channels (Partridge & Swandulla, 1987) have been shown to play an important role in the generation of burst firing in snail neurones (Swandulla & Lux, 1985). CAN channels appear to be present in many cell types (Partridge & Swandulla, 1988) and may represent a common feature in the ability to generate bursts.

Cyclic AMP-dependent mechanisms have been shown to modulate bursting activity in a number of neurones, most notably in *Aplysia* neurone R15 (Lotshaw, Levitan & Levitan, 1986). We studied modulation of bursting in the fast burster neurone of *Helix*. In this neurone the CAN current, which generates the depolarizing wave underlying the bursts, can be studied under conditions of controlled activation by quantitative injections of Ca^{2+} ions (Hofmeier & Lux, 1981; Swandulla & Lux, 1985; Müller, Swandulla & Lux, 1989). We have found that CAN currents are, in fact, modulated by a mechanism involving cyclic AMP.

In an effort to understand the mechanism of this modulation, we have utilized a number of means to alter intracellular cyclic AMP levels, and have directly increased the levels of intracellular cyclic AMP-dependent protein kinase. In addition we applied several neurotransmitters that are normally present in the snail ganglion and investigated their effects upon the CAN current that follows pressure injection of Ca^{2+} into these cells. Processes that would be expected to increase intracellular cyclic AMP levels depress CAN currents. These processes cause bursting spike activity to be replaced by more regular beating activity.

METHODS

Preparation and recording

The circumoesophageal ganglia of the snails *Helix pomatia* and *Helix aspersa* were utilized in these experiments. After removing the capsules from the ganglia, the remaining connective tissue sheaths were exposed to pronase (1 mg/ml in Ringer solution) for 3–5 min and then removed. The right parietal fast burster (F1 of Kerkut, Lambert, Gayton, Loker & Walker, 1975), or occasionally other large identified neurones such as E18 and E19, were used in these studies. Cells were voltage-clamped using a standard two-electrode clamp (for details see Hofmeier & Lux, 1981).

Intracellular injections

The cells were impaled with a pressure injection electrode for Ca^{2+} injection. Electrodes were filled with a solution containing 100 mM-$CaCl_2$ and 66 mM-KCl that was buffered with HEPES at pH 7·4, the internal pH of these neurones (Thomas, 1974). Calcium solutions were injected into the cells using a fast, quantitative method of pressure injection, which is described in detail by Hofmeier & Lux (1981) and Müller *et al.* (1989). Quantitative injections in the range of 1 % of the cell volume were delivered rapidly (10–100 ms) into the cell using this technique. Typical injection pulses utilized pressures of 10^{-6}–10^{-5} Pa. Injection electrodes were pulled in two steps. In the first step, the capillary was thinned over a length of 1·5 mm to provide an even, cylindrical chamber (inner diameter 30–50 μm) for the injection solution. In the second step, the tip (diameter, 1–2 μm) was formed. Potassium ion exchanger (477317; Corning Glass Works, Corning, New York, USA) was filled from the back into the capillary before the injection solution was sucked through the tip into the cylindrical chamber. Ion exchanger and injection solution formed a phase boundary which was easily visible under the microscope. The injected quantity was determined with a resolution of 10^{-11} l from the shift of the phase boundary measured with an ocular micrometer. A 10 nA negative current was passed constantly through the injection electrode to prevent Ca^{2+} leak and to indicate successful penetration of the electrode tip.

With individual Ca^{2+} injections to around 1 % of the cell volume, no cumulative depression of the CAN current was observed. In one control experiment, injections were made at 2 min intervals for more than 20 min with no systematic variation in the size of the resultant CAN current. Successive CAN currents in any experiment were compared only when the injected volumes of Ca^{2+} solution were equal. For several experiments, a second pressure injection electrode was inserted into the cell and the same quantitative techniques were used to introduce solutions which contained adenosine 3′,5′-cyclic monophosphate (cyclic AMP, 10 mM), the catalytic subunit of cyclic AMP-dependent protein kinase isolated from bovine heart (1×10^3 units/ml distilled water; one unit is capable of transferring 10^{-12} mol of phosphate ions/min), protein kinase inhibitor (rabbit sequence, 4×10^5 units/ml distilled water; one unit is capable of preventing the transfer of 10^{-12} mol of phosphate ions/min) or guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) into the cells. Injected volumes were 1–10 % of the cell volume. Effects of the catalytic subunit on CAN currents were unchanged when adding dithiothreitol (50 mg/ml, as recommended by Sigma) as a reducing agent to the injection solution.

Solutions

The composition of the normal Ringer bathing solution was as follows (mM): NaCl, 80; KCl, 4; $CaCl_2$, 10; $MgCl_2$, 5; glucose, 10; HEPES, 5 (pH 7·8). Ringer solutions containing in addition forskolin (10–25 μM), 8-bromo-cyclic AMP (100 μM), dibutyryl-cyclic AMP (100–500 μM), 3-isobutyl-1-methylxanthine (IBMX, 100 μM), serotonin (10–200 μM), acetylcholine (ACh, 100 μM) and dopamine (10–100 μM) were made to the appropriate concentration immediately before use and then either exchanged for the bathing solution or applied to the cell from a wide-bore perfusion pipette (tip diameter about 100 μm) that was placed about 500 μm from the cell under study. Forskolin was prepared as a stock solution of 10 mM in dimethylsulphoxide (DMSO). Controls with Ringer solution containing DMSO in the appropriate concentrations neither affected discharge activity of the cells nor modified currents under investigation.

Drugs were all from Sigma Chemical Co. All experiments were carried out at room temperature.

RESULTS

Forskolin modulates spontaneous activity

The diterpene, forskolin, has been shown to activate directly the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981, 1986). Thus adenylate cyclase activity can be conveniently stimulated by bath application of forskolin. The records in Fig. 1 indicate that burst activity in *Helix* burster neurones can be reversibly modulated by forskolin. Normal bursting activity of the fast burster neurone of *Helix pomatia* in normal Ringer solution is seen in Fig. 1*A*. Seven minutes after application of 25 μM-forskolin to the bath (Fig. 1*B*) bursting activity ceased and was replaced by

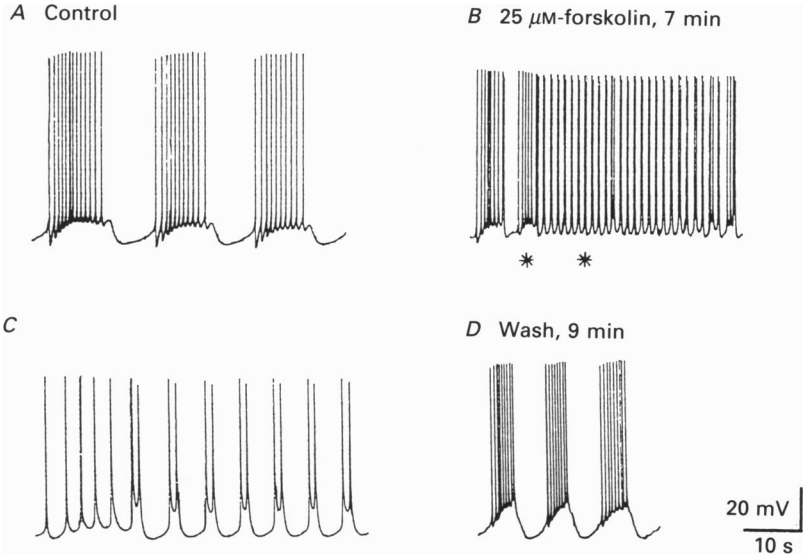

Fig. 1. Modulation of spontaneous activity by forskolin. *A*, spontaneous burst firing of *Helix pomatia* burster F1 in normal Ringer solution. *B*, beating and doublet firing observed 7 min after application of 25 μM-forskolin to the bath. *C* shows, on an expanded time scale, the portion of *B* marked with asterisks. *D*, 9 min after washing forskolin from the bath, the cell resumed burst firing.

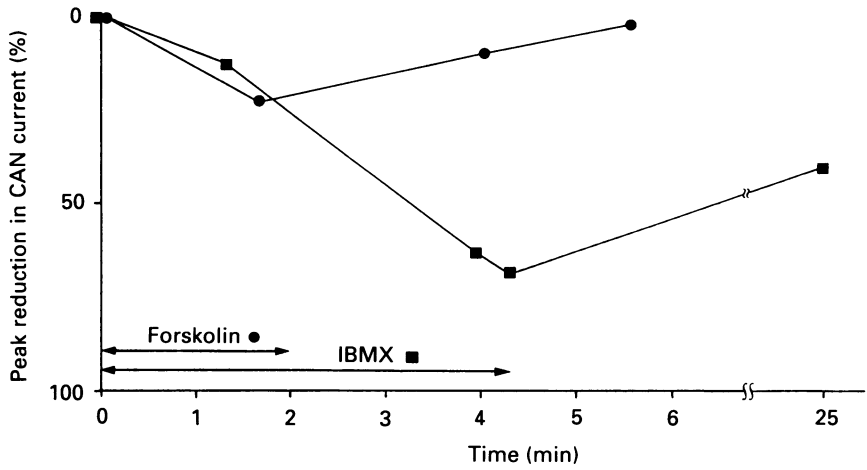

Fig. 2. Effect of external forskolin (10 μM) and IBMX (100 μM) application on CAN current. Forskolin and IBMX were perfused onto the tested neurone during the period marked on the abscissa. CAN currents in this and the subsequent figures were elicited in each cell by injecting equal amounts of $CaCl_2$ into the cells (see Methods). Typical current responses to Ca^{2+} injections are shown in the inset of Fig. 4. Following drug application the cells were rinsed several times with normal Ringer solution. Peak current amplitudes before drug application (control) were normalized for each cell in this figure and in Figs 3, 4 and 6 and the percentage reduction of peak current during and after drug application was plotted against time. Control current amplitudes were 2·2 nA (forskolin) and 5·8 nA (IBMX).

beating and doublet firing (see expanded time scale in Fig. 1*C*). The effect of forskolin was reversible, and, as shown in Fig. 1*D*, bursting activity resumed 9 min after wash-out.

Since the depolarizing wave which underlies bursts in these neurones is largely the result of CAN current activation (see Swandulla & Lux, 1985; Partridge & Swandulla, 1987), we investigated the effect of forskolin directly on CAN currents.

Forskolin and IBMX depress calcium-activated non-specific cation (CAN) currents

Pressure injection of Ca^{2+} into bursting neurones activates an inward current through CAN channels, which is insensitive to membrane potential, and a two-component outward potassium current (Müller *et al.* 1989). The more rapid component of the outward current may overlap somewhat with the inward CAN current. In order to minimize the contribution of potassium currents, we voltage-clamped the neurones to −70 mV, i.e. near the potassium equilibrium potential (Heyer & Lux, 1976), in the majority of our experiments. In addition, we ionophoresed TEA from the current-passing clamp electrode, which was filled with 1 M-TEA-Cl, into the cell soma. The CAN currents shown in Fig. 4 demonstrate the effectiveness of the above procedure in minimizing Ca^{2+}-activated outward currents.

We applied 10 μM-forskolin directly onto specific voltage-clamped neurones with a perfusion pipette and injected Ca^{2+} to activate the CAN current. Small, controlled pressure pulses were used for Ca^{2+} injections in these experiments, and the resultant CAN currents never exceeded a few nanoamperes. Figure 2 (●) shows one example of the reduction in CAN current following forskolin application. In this experiment, a full recovery from the forskolin-induced CAN current depression was observed within a few minutes. In a total of five experiments, forskolin caused an average reduction of CAN current of $53 \pm 32\%$ (mean ± S.D.), with an average recovery to $72 \pm 24\%$ (mean ± S.D.) of the current amplitude in the control after prolonged wash-out.

Since our results with forskolin suggested that activation of adenylate cyclase and subsequent increases in intracellular cyclic AMP might be responsible for the observed effects, we tried in three experiments to block the action of phosphodiesterase (PDE), which breaks down cyclic AMP, with the idea of possibly increasing intracellular cyclic AMP levels. Following application of the PDE inhibitor IBMX to the outside of the cell, we did indeed observe a gradual depression of CAN current amplitude by $62 \pm 17\%$ (mean ± S.D.) over the course of several minutes with some recovery in CAN current amplitude after a prolonged period of rinse (see Fig. 2).

While forskolin is a useful drug for activation of adenylate cyclase, it has been shown to have other direct effects on ion channels that do not depend on cyclic AMP (Lindner & Metzger, 1983; Coombs & Thompson, 1987; Hoshi, Garber & Aldrich, 1988; Joost, Habberfield, Simpson, Laurenza & Seamon, 1988; Krause, Lee & Deutsch, 1988; Wagoner & Pallotta, 1988; Zünkler, Trube & Ohno-Shosaku, 1988). We therefore sought to substantiate our results by studying the effects of directly elevating intracellular cyclic AMP.

Increasing internal cyclic AMP levels depresses calcium-activated non-specific cation (CAN) currents

Figure 3 shows the action upon CAN currents of internal cyclic AMP. Two different approaches were chosen to increase intracellular cyclic AMP levels. The

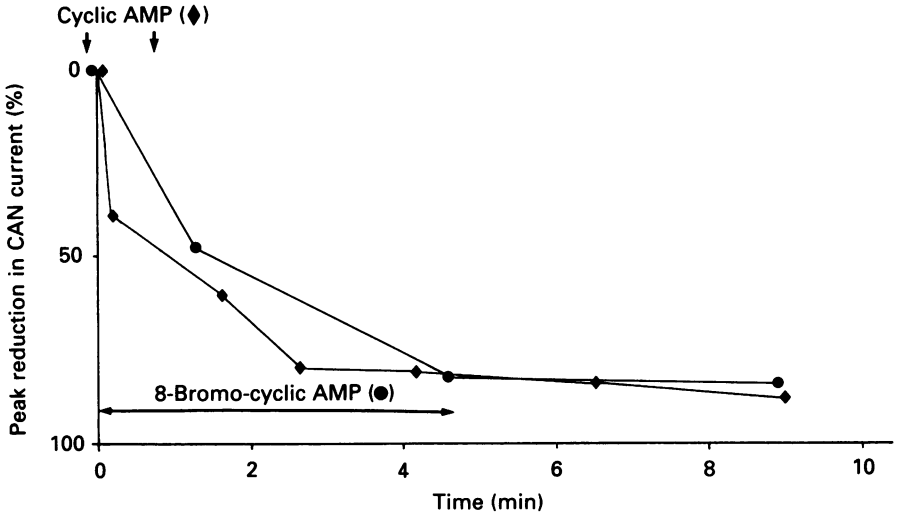

Fig. 3. Cyclic AMP action on CAN currents. The membrane-permeable cyclic AMP analogue 8-bromo-cyclic AMP (100 μM) was bath-applied and CAN current amplitudes were measured. 8-Bromo-cyclic AMP reduced CAN currents by approximately 85%. In another cell cyclic AMP was injected directly into the cell with brief pressure pulses from a second injection electrode (injections indicated by arrows), and CAN current was measured. The first cyclic AMP injection was about 30 s prior to the first test Ca^{2+} injection. Control current amplitudes were 12·5 nA (8-bromo-cyclic AMP) and 25 nA (cyclic AMP).

filled circles in Fig. 3 show the results of a 4·5 min external application of the membrane-permeable cyclic AMP analogue, 8-bromo-cyclic AMP. In this instance and three other cases, there was a marked depression of CAN current within minutes following the application of 100 μM of this drug that reached a maximum of 76 ± 11% (mean $\pm$ S.D.). In two of these cells we observed some recovery of the CAN current after several minutes of rinse. We also tested another membrane-permeable analogue, dibutyryl-cyclic AMP (100 and 500 μM) in five cells, and, again, in each instance the CAN current was depressed within minutes of the application of the drug (maximum reduction for 100 μM, 67 ± 24%; mean $\pm$ S.D.).

A controlled increase in internal cyclic AMP level is achieved by injecting cyclic AMP directly into the cells. For this purpose we introduced a second pressure injection electrode from which cyclic AMP was injected quantitatively into the neurone under investigation. Single injections quickly increased free internal cyclic AMP levels by 10–30 μM. These values compare well with those reached upon pharmacological stimulation of molluscan neurones, which elevated free cyclic AMP concentration from a basal level of 1–6 μM by more than 400% (Hockberger &

Yamane, 1987). We succeeded in obtaining controlled injections of both cyclic AMP and Ca^{2+} in one cell. The results of this experiment are shown with filled diamonds in Fig. 3. Injection of cyclic AMP itself activates an inward current in these neurones (Swandulla & Lux, 1984; see also Discussion), and each of the cyclic AMP injections into the cell shown in this figure activated a transient 2–3 nA inward current that lasted up to 30 s. Calcium injections were applied only after the cyclic AMP-induced current had completely decayed. Two Ca^{2+} injections preceded the first cyclic AMP injection as controls. Following the cyclic AMP injection, which should have increased the intracellular cyclic AMP concentration by about 20 μM, we were successful in activating a CAN current with six quantitative Ca^{2+} injections. The earliest of these Ca^{2+} injections induced a CAN current which was already markedly diminished compared to control. Following this current two more cyclic AMP injections were applied in rapid succession (marked as a single point in Fig. 3), amounting to a total increase in the internal cyclic AMP concentration of 50 μM. CAN current reduction continued steeply over the next few minutes and the current amplitude was reduced by about 90% by 9 min after the first cyclic AMP injection.

Calcium-activated non-specific cation (CAN) current depression involves cyclic AMP-dependent phosphorylation

A common cellular action of cyclic AMP in the modulation of cellular processes is through the phosphorylation of proteins by cyclic AMP-dependent protein kinase (PK) (see Huganir, 1987). The catalytic subunit of cyclic AMP-dependent PK without the cyclic AMP-sensitive regulatory subunit should have a similar effect that is independent of intracellular cyclic AMP levels. Injection of the catalytic subunit of cyclic AMP-dependent PK has been successfully exploited as a means of indicating the role of channel phosphorylation in neuromodulation (Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982; dePeyer, Cachelin, Levitan & Reuter, 1982). Figure 4 summarizes the results of four successful injections of the catalytic subunit of cyclic AMP-dependent PK into three cells. Quantitative injections of catalytic subunit were made as with cyclic AMP (see above) following three to four control injections of Ca^{2+}. We injected about 10^{-4} units into each cell. C-subunit injection, unlike cyclic AMP injection, did not produce any inward current. In each instance, however, there was a marked reduction of the CAN current within minutes (73 ± 16%; mean $\pm$ S.D.) following the injection of the catalytic subunit. CAN currents than started to recover and, in one cell, almost complete recovery was observed by about 20 min after the subunit injection.

Possible involvement of G-proteins in cation-activated non-specific cation (CAN) current modulation

G-proteins are one important intermediate allowing numerous membrane receptors to control internal cyclic AMP levels and by consequence cyclic AMP-dependent processes (Dunlap, Holz & Rane, 1987; Neer & Clapham, 1988). We hypothesized that the modulatory effect of cyclic AMP on the CAN current might be the final step in a process wherein adenylate cyclase is affected by a membrane receptor through the intermediary of a G-protein. We sought to increase the levels of the active subunits of G-proteins by injection of the non-hydrolysable GTP analogue, GTP-γ-S.

We accomplished these double injections in four cells. The total GTP-γ-S injection in each cell would produce an intracellular drug concentration of about 100 μM. At this concentration GTP-γ-S did not produce any changes in steady-state membrane conductance as measured with 20 mV de- or hyperpolarizing voltage steps from the

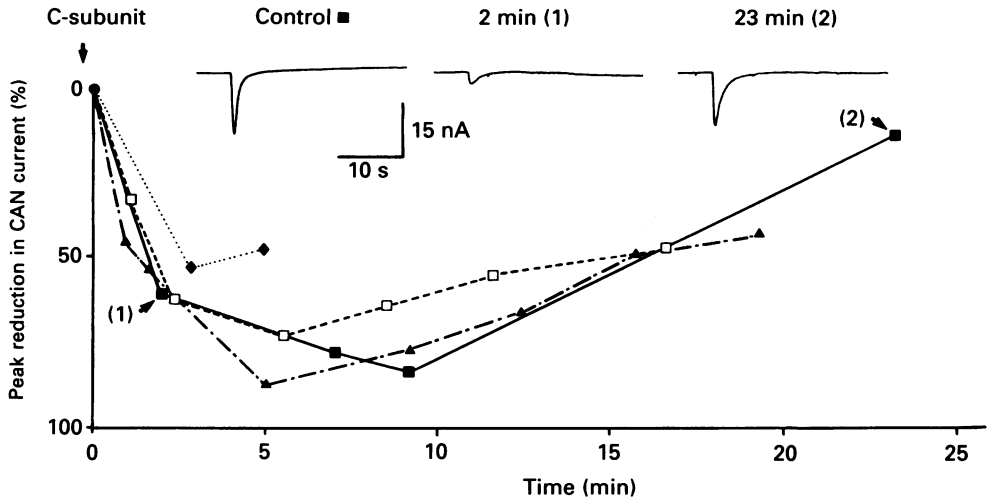

Fig. 4. Effect of intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase (PK) on CAN currents. The C-subunit of cyclic AMP-dependent PK was injected into three cells. The CAN current in one cell (■) recovered sufficiently to allow a second C-subunit injection (□). The blocking effect of C-subunit (injection indicated by arrow) on CAN current, and the recovery of current amplitude, are illustrated for one cell in the insets. One out of four control Ca^{2+} injections is shown. Control current amplitudes (indicated by filled circle) were 18 nA (■), 16·5 nA (□), 13 nA (◆) and 12 nA (▲).

holding potential of −70 mV. In marked contrast to our other experiments, however, we noted a drastic increase with time in the amplitude of the CAN current when the cell contained GTP-γ-S (Fig. 5).

Serotonin mimics cyclic AMP effects on calcium-activated non-specific cation (CAN) currents

Serotonin is one transmitter that is found in significant quantities in the ganglion of *Helix* (Cottrell & Osborne, 1970; see Gerschenfeld, 1973) and that has been shown to increase intracellular cyclic AMP levels. Levitan & Levitan (1988) have shown that serotonin via cyclic AMP can enhance both hyperpolarizing and depolarizing membrane conductances in molluscan neurones. One action of this transmitter is to depress bursting activity in these neurones (Kaczmarek, Jennings & Strumwasser, 1978; Levitan & Levitan, 1988). We investigated the effect of serotonin on bursting in *Helix* and the possibility that a serotonin receptor might be involved in the cyclic AMP-dependent modulation of CAN current. When we added 100–200 μM-serotonin to the bathing solution, the cells depolarized and switched to a beating activity. To investigate whether CAN current modulation contributes to this effect, we applied serotonin and studied its action on CAN currents. In the experiment shown in Fig. 6, 200 μM-serotonin was applied and within 2 min the CAN current was depressed

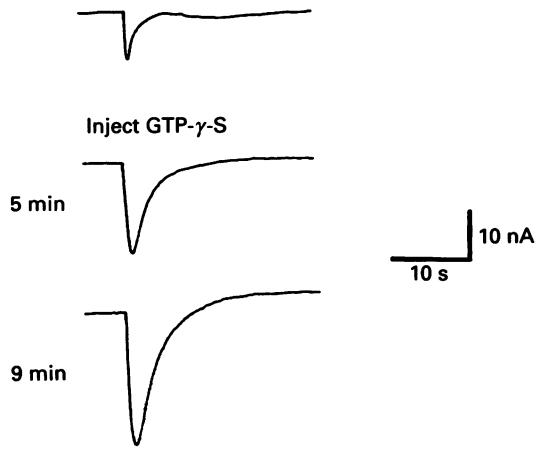

Fig. 5. Effect of intracellular injection of GTP-γ-S on CAN current. Upper trace shows a control. CAN currents were elicited following GTP-γ-S injection at the times indicated. The increase in CAN current amplitude induced by GTP-γ-S was not reversible in the course of the experiment.

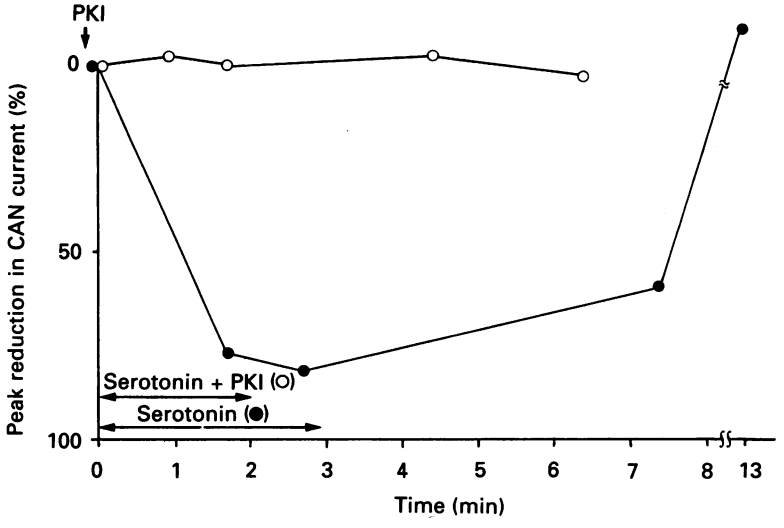

Fig. 6. Serotonin depresses CAN currents. Serotonin (200 μM) was added to the bathing solution and CAN currents were measured. Serotonin reduced the CAN current almost completely within a few minutes. After wash-out of serotonin the CAN current recovered fully. In another cell protein kinase inhibitor (PKI) was injected about 1 min before serotonin application (injection indicated by arrow). Control current amplitudes were 17 nA (●) and 6 nA (○).

from a control level of 17 nA to a minimum of 3 nA. The current gradually returned to its control level during a period of rinse. Five other cells showed similar depressions of CAN current (amplitude $10{\cdot}8 \pm 6{\cdot}3$ nA; mean ± S.D.) in the presence of serotonin (maximum reduction 72 ± 13 %; mean ± S.D.). The effects on CAN currents of two other transmitters of the snail ganglion, dopamine and ACh, were studied. In neither case was a consistent depression of CAN currents observed.

In order to ascertain that cyclic AMP-dependent membrane phosphorylation is indeed involved in serotonin's action on bursting as well as on CAN current, in four experiments a peptide inhibitor of cyclic AMP-dependent protein kinase (PKI) was injected into the cells from a second injection pipette about 1 min prior to serotonin application. Protein kinase inhibitor itself did not reduce CAN current (amplitude $9{\cdot}7 \pm 3{\cdot}9$ nA, mean $\pm$ S.D.). We injected 0·04–0·1 units of PKI into each cell. Under these conditions serotonin application failed to change bursting to beating, and its effect on CAN current was suppressed (Fig. 6).

DISCUSSION

The pattern of a neurone's firing is at least as important to neuronal functioning and information processing by the nervous system as is the average firing frequency. Bursting, in particular, has been associated with various forms of neuronal function, and agents that have the ability to modulate bursting activity should play a key role in the regulation of numerous effector functions of the nervous system. Of particular interest are the actions of intracellular second messengers on the ionic currents that affect bursting activity (see e.g. Benson & Levitan, 1983; Kramer, Levitan, Wilson & Levitan, 1988; Levitan & Levitan, 1988). We have investigated cyclic AMP-dependent modulation of bursting activity in *Helix* neurones and have focused on the effects of cyclic AMP on the CAN current, which has been shown to be essential for burst generation in these neurones (Swandulla & Lux, 1985).

While the membrane-permeable cyclic AMP analogues consistently depressed CAN currents, their action was only slightly reversible during prolonged wash-out. Although the intracellular concentration of these compounds would be expected to fall once they had been removed from the bathing solution, it is possible that a significant elevation of drug level remained for the duration of the experiments, especially since these agents resist break-down by PDE. To circumvent these difficulties, as well as to rule out possible direct effects of the membrane-permeable cyclic AMP analogues on the CAN channels, we attempted the straightforward, although more difficult, approach of injecting cyclic AMP through a second injection electrode. Increasing internal cyclic AMP levels using this fast and quantitative method affected CAN currents similarly to external application of cyclic AMP analogues.

Injection of cyclic AMP itself into these neurones induces a cationic current which is transient compared to the effect of cyclic AMP on CAN current (for details see Aldenhoff, Hofmeier, Lux & Swandulla, 1983; Swandulla & Lux, 1984). This raises the question whether cyclic AMP might transiently activate a fraction of CAN channels which, following activation, enter a non-conductive state where they cannot be activated by Ca^{2+} for a period of several minutes. Subsequent Ca^{2+} injections could then induce CAN currents which are reduced in amplitude.

Since it is possible to effectively suppress CAN currents by injecting the catalytic subunit of cyclic AMP-dependent PK, i.e. without increasing internal cyclic AMP levels, such a mechanism seems unlikely. Furthermore, injections of C-subunit, unlike cyclic AMP, failed to induce an inward current. These findings are in line with previous observations which suggest that the cyclic AMP-induced current is

activated by a direct action of cyclic AMP on membrane channels whose selectivity for cations differs from that of CAN channels (Swandulla & Lux, 1984, 1985; Hockberger & Swandulla, 1987; Swandulla, 1987; Swandulla & Partridge, 1990).

While the time course of the cyclic AMP-activated inward current (see above) is thought to reflect the intracellular concentrations of cyclic AMP (Connor & Hockberger, 1984; Swandulla & Lux, 1984), we observed a depression of CAN current which far outlasted the cyclic AMP-dependent current. It would appear, then, that the rate-limiting step in the recovery process from cyclic AMP-dependent modulation of CAN current is not cyclic AMP break-down by PDE, but perhaps dephosphorylation of the CAN channel by a phosphatase.

A direct demonstration of the role of cyclic AMP-dependent phosphorylation in the depression of CAN current was obtained by injecting the catalytic subunit of cyclic AMP-dependent PK into the neurones. We injected about 10^{-4} units into each cell. Since one unit is capable of transferring 10^{-12} mol of phosphate ions per minute (Sigma Assay), the injected amount of subunit should be able to transfer 5×10^7 phosphate ions per minute. At a density of one CAN channel per 6 μm^2 (Partridge & Swandulla, 1987), a typical *Helix* burster neurone would have about 4×10^4 CAN channels. Thus the injected amount should have been sufficient for the phosphorylation of the cell's CAN channels during the observed time. The results suggest that this process is reversible over tens of minutes and that there can be channel dephosphorylation. On the other hand, we believe that it is unlikely that Ca^{2+} ions activate CAN channels by means of channel dephosphorylation. First, the CAN current is activated rather rapidly after a Ca^{2+} injection. Second, CAN channels still operate in the presence of Ca^{2+} in inside-out patches (Partridge & Swandulla, 1987), a condition in which phosphatases are unlikely to be acting. To address these questions in more detail, we attempted to reverse the cyclic AMP effect on CAN currents by injecting non-specific phosphatases into the cells. We were unsuccessful in these attempts, most certainly because of various other actions these phosphatases exerted.

Cyclic AMP has numerous other effects in these neurones such as the activation of membrane channels permeable for monovalent cations, the depression of Ca^{2+}-activated K^+ channels and voltage-activated Ca^{2+} channels (Aldenhoff *et al.* 1983; Kononenko, Kostyuk & Shcherbatko, 1983; Swandulla & Lux, 1984). Its modulation of CAN channels must therefore be viewed in conjunction with those effects. It should be noted, however, that compared to other cyclic AMP-dependent processes observed in these cells, which occur within tens of seconds, the modulatory effect of cyclic AMP on CAN channels is long-lasting and comparable to that which has been described for cyclic AMP-dependent phosphorylation of K^+ channels in *Aplysia* neurones (Kramer *et al.* 1988). The time scale of this process appears suitable for modulation of the neurone's firing pattern regulating such effector functions as neurosecretion.

Neurotransmitter actions on burst activity have been extensively studied in the bursting neurone R15 of *Aplysia* (Parnas & Strumwasser, 1974; Boisson & Gola, 1976; Benson & Levitan, 1983). Both dopamine and serotonin have been found to hyperpolarize this cell and thereby to decrease or suppress bursting (Ascher, 1972; Drummond, Benson & Levitan, 1980). We have found that serotonin acts in a

complex manner to change burst firing into beating in *Helix* F1 burster cells. Our results suggest that at least part of the serotonin-induced action is modulation of CAN channels through activation of adenylate cyclase which leads to an increase in internal cyclic AMP and subsequent phosphorylation of CAN channels (Fig. 7).

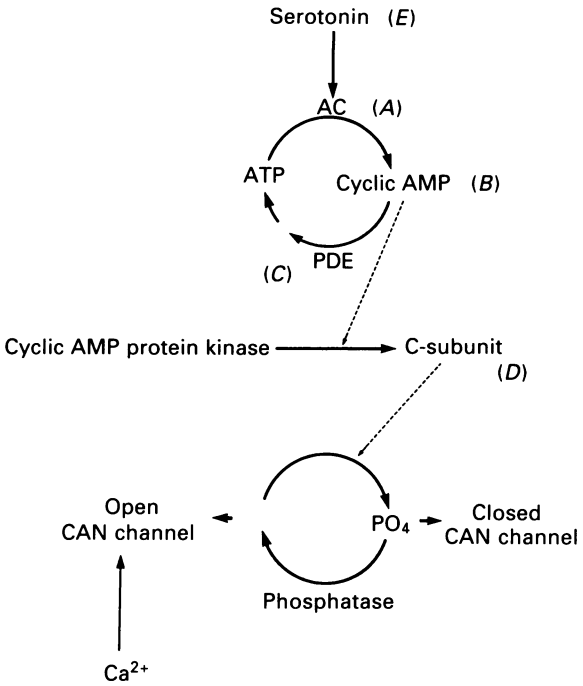

Fig. 7. A tentative model for the modulation of CAN current by cyclic AMP-dependent phosphorylation. The results from directly increasing intracellular cyclic AMP (*B*), activating adenylate cyclase (AC) with forskolin (*A*), and depressing PDE activity with IBMX (*C*) implicate a role of the level of intracellular cyclic AMP in the modulation of CAN currents. Because increased levels of the C-subunit of the cyclic AMP-dependent PK (*D*) has a similar effect to increasing the cyclic AMP level, phosphorylation of the CAN channel, or a regulatory protein closely associated with the channel, is implicated as a means by which the CAN current is depressed. Finally, one action of serotonin in changing the cell's activity from bursting to beating may be through the cyclic AMP level through activation of adenylate cyclase (*E*).

Our observation of increased CAN current following GTP-γ-S injection suggests a more complex involvement of G-proteins than the simple linking of a serotonin receptor to adenylate cyclase by means of a stimulatory α-subunit. One possibility is that GTP-γ-S has a dominant effect on CAN current other than through adenylate cyclase-controlled CAN channel phosphorylation. In support of this suggestion are preliminary experiments that show that the ability of serotonin to depress CAN currents seems to be unaffected when stimulating G-proteins by GTP-γ-S. A second possibility is that CAN currents are modulated by two independent second messenger systems in a manner similar to that found for the S-type K^+ channel of *Aplysia* (Volterra & Siegelbaum, 1988). One of these systems would involve the activation of

adenylate cyclase by serotonin through a G-protein. The second system would lead to an increase in CAN current, perhaps by G-protein-controlled action through a phosphatase.

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REFERENCES

ALDENHOFF, J. B., HOFMEIER, G., LUX, H. D. & SWANDULLA, D. (1983). Stimulation of a sodium influx by cAMP in Helix neurones. *Brain Research* **276**, 289–296.

ASCHER, P. (1972). Inhibitory and excitatory effects of dopamine on *Aplysia* neurones. *Journal of Physiology* **225**, 173–209.

BENSON, J. A. & LEVITAN, I. B. (1983). Serotonin increases an anomalously rectifying K^+ current in the Aplysia neuron R15. *Proceedings of the National Academy of Sciences of the USA* **80**, 3522–3525.

BOISSON, M. & GOLA, M. (1976). Current–voltage relations in ILD- or dopamine-stabilized bursting neurone in Aplysia. *Comparative Biochemistry and Physiology* **54C**, 109–113.

CONNOR, J. A. & HOCKBERGER, P. (1984). A novel membrane sodium current induced by injection of cyclic nucleotides into gastropod neurones. *Journal of Physiology* **354**, 139–162.

COOMBS, J. & THOMPSON, S. (1987). Forskolin's effect on transient K current in nudibranch neurons is not reproduced by cAMP. *Journal of Neuroscience* **7**, 443–452.

COTTRELL, G. A. & OSBORNE, N. N. (1970). Subcellular localization of serotonin in an identified serotonin-containing neurone. *Nature* **225**, 470–472.

DEPEYER, J. E., CACHELIN, A. B., LEVITAN, I. B. & REUTER, H. (1982). Ca^{++}-activated K^+ conductance in internally perfused snail neurons is enhanced by protein phosphorylation. *Proceedings of the National Academy of Sciences of the USA* **79**, 4207–4211.

DRUMMOND, A. H., BENSON, J. A. & LEVITAN, I. B. (1980). Serotonin-induced hyperpolarization of an identified Aplysia neuron is mediated by cyclic AMP. *Proceedings of the National Academy of Sciences of the USA* **77**, 5013–5017.

DUNLAP, K., HOLZ, G. G. & RANE, S. G. (1987). G proteins as regulators of ion channel function. *Trends in Neurosciences* **10**, 241–244.

DUTTON, A. & DYBALL, R. E. J. (1979). Phasic firing enhances vasopressin release from rat neurohypophysis. *Journal of Physiology* **290**, 433–440.

GERSCHENFELD, H. M. (1973). Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. *Physiological Reviews* **53**, 2–119.

HEYER, C. B. & LUX, H. D. (1976). Control of the delayed outward potassium currents in bursting pacemaker neurones of the snail *Helix pomatia*. *Journal of Physiology* **262**, 349–382.

HOCKBERGER, P. & SWANDULLA, D. (1987). Direct ion channel gating. A new function for intracellular messengers. *Cellular and Molecular Neurobiology* **7**, 229–236.

HOCKBERGER, P. & YAMANE, T. (1987). Compartmentalization of cAMP elevation in neurons of Aplysia. *Cellular and Molecular Neurobiology* **7**, 19–33.

HOFMEIER, G. & LUX, H. D. (1981). The time courses of intracellular calcium and related electrical effects after injection of $CaCl_2$ into neurons of the snail, *Helix pomatia*. *Pflügers Archiv* **391**, 242–251.

HOSHI, T., GARBER, S. S. & ALDRICH, R. W. (1988). Effect of forskolin on voltage-gated K^+ channels is independent of adenylate cyclase activation. *Science* **240**, 1652–1655.

HUGANIR, R. L. (1987). Biochemical mechanisms that regulate the properties of ion channels. In *Neuromodulation: the Biochemical Control of Neuronal Excitability*, ed. KACZMAREK, L. K. & LEVITAN, I. B., pp. 64–85. Oxford University Press, New York.

JOOST, H. G., HABBERFIELD, A. D., SIMPSON, I. A., LAURENZA, A. & SEAMON, K. B. (1988). Activation of adenylate cyclase and inhibition of glucose transport in rat adipocytes by forskolin analogues: structural determinants for distinct sites of action. *Molecular Pharmacology* **33**, 449–453.

KACZMAREK, L. K., JENNINGS, K. & STRUMWASSER, F. (1978). Neurotransmitter modulation, phosphodiesterase inhibitor effects, and cAMP correlates of afterdischarge in peptidergic neurites. *Proceedings of the National Academy of Sciences of the USA* **75**, 5200–5204.

KERKUT, G. A., LAMBERT, J. D. C., GAYTON, R. J., LOKER, J. E. & WALKER, R. J. (1975). Mapping of nerve cells in the suboesophageal ganglia of *Helix aspersa*. *Comparative Biochemistry and Physiology* **50A**, 1–25.

KONONENKO, N. I., KOSTYUK, P. G. & SHCHERBATKO, A. D. (1983). The effect of intracellular cAMP injections on stationary membrane conductance and voltage- and time-dependent ionic currents in identified snail neurons. *Brain Research* **268**, 321–338.

KRAMER, R. H., LEVITAN, E. S., WILSON, M. P. & LEVITAN, I. B. (1988). Mechanism of calcium-dependent inactivation of a potassium current in Aplysia of calcium-dependent inactivation of a potassium current in Aplysia neuron R15: interaction between calcium and cyclic AMP. *Journal of Neuroscience* **8**, 1804–1813.

KRAUSE, D., LEE, S. C. & DEUTSCH, C. (1988). Forskolin effects on the voltage-gated K^+ conductance of human T cells. *Pflügers Archiv* **412**, 133–140.

LEVITAN, E. S. & LEVITAN, I. B. (1988). Serotonin acting via cyclic AMP changes both the hyperpolarizing and depolarizing phases of bursting pacemaker activity in the Aplysia neuron R15. *Journal of Neuroscience* **8**, 1152–1161.

LINDNER, E. & METZGER, H. (1983). The actions of forskolin on muscle cells is modified by hormones, calcium ions and calcium antagonists. *Arzneimittel-Forschung* **33**, 1436–1441.

LOTSHAW, D. P., LEVITAN, E. S. & LEVITAN, I. B. (1986). Fine tuning of neuronal electrical activity: modulation of several ionic channels by intracellular messengers in a single identified nerve cell. *Journal of Experimental Biology* **124**, 307–322.

MÜLLER, T. H., SWANDULLA, D. & LUX, H. D. (1989). Activation of three types of membrane currents by various divalent cations in identified molluscan pacemaker neurons. *Journal of General Physiology* **94**, 997–1014.

NEER, E. J. & CLAPHAM, D. E. (1988). Roles of G protein subunits in transmembrane signalling. *Nature* **333**, 129–134.

OSTERRIEDER, W., BRUM, G., HESCHELER, J., TRAUTWEIN, W., FLOCKERZI, V. & HOFMANN, F. (1982). Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca^{++} current. *Nature* **298**, 576–578.

PARNAS, I. & STRUMWASSER, F. (1974). Mechanisms of longlasting inhibition of a bursting pacemaker neuron. *Journal of Neurophysiology* **37**, 609–620.

PARTRIDGE, L. D. & SWANDULLA, D. (1987). Single Ca-activated cation channels in bursting neurons of Helix. *Pflügers Archiv* **410**, 627–631.

PARTRIDGE, L. D. & SWANDULLA, D. (1988). Calcium-activated non-specific cation channels. *Trends in Neurosciences* **11**, 69–72.

PRIOR, D. J. & GELPERIN, A. (1977). Autoactive molluscan neuron: reflex function and synaptic modulation during feeding in the terrestrial slug Limax maximus. *Journal of Comparative Physiology* **114**, 217–232.

SEAMON, K. & DALY, J. W. (1981). Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. *Journal of Biological Chemistry* **256**, 9799–9801.

SEAMON, K. & DALY, J. W. (1986). Forskolin: its biological and chemical properties. *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* **20**, 1–150.

SWANDULLA, D. (1987). Cationic membrane conductances induced by intracellularly elevated cAMP and Ca^{++}: measurements with ion-selective microelectrodes. *Canadian Journal of Physiology and Pharmacology* **65**, 898–903.

SWANDULLA, D. & LUX, H. D. (1984). Changes in ionic conductances induced by cAMP in Helix neurons. *Brain Research* **305**, 115–122.

SWANDULLA, D. & LUX, H. D. (1985). Activation of a nonspecific cation conductance by intracellular Ca^{++} elevation in bursting pacemaker neurons of Helix pomatia. *Journal of Neurophysiology* **54**, 1430–1443.

SWANDULLA, D. & PARTRIDGE, L. D. (1990). Non-specific cation channels. In *Potassium Channels. Structure, Classification, Function and Therapeutical Potential*, ed. COOK, N. S., pp. 167–180. Ellis Horwood, Chichester.

THOMAS, R. C. (1974). Intracellular pH of snail neurones measured with a new pH-sensitive glass microelectrode. *Journal of Physiology* **238**, 159–180.

VOLTERRA, A. & SIEGELBAUM, S. A. (1988). Role of two different guanine nucleotide-binding proteins in the antagonistic modulation of the S-type K^+ channel by cAMP and arachidonic acid

metabolites in *Aplysia* sensory neurons. *Proceedings of the National Academy of Sciences of the USA* **85**, 7810–7814.

WAGONER, P. K. & PALLOTTA, B. S. (1988). Modulation of acetylcholine receptor desensitization by forskolin is independent of cAMP. *Science* **240**, 1655–1657.

ZÜNKLER, B. J., TRUBE, G. & OHNO-SHOSAKU, T. (1988). Forskolin-induced block of delayed rectifying K^+ channels in pancreatic β-cells is not modified by cAMP. *Pflügers Archiv* **411**, 613–619.