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MODULATION OF THE CALCIUM CURRENT OF MOLLUSCAN NEURONES BY NEUROTRANSMITTERS

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SUMMARY

In identified neurones of the snail *Helix aspersa*, the Ca^{2+} current can be modulated by biogenic amines and peptides in different ways.

1. A reversible increase of Ca^{2+} current is evoked by 1 μmol l^{-1} serotonin (5-HT) in a group of identified snail ventral neurones (D6, D7, E1). This 5-HT-induced enhancement of Ca^{2+} current is probably mediated by cyclic GMP. Neither cyclic AMP nor forskolin mimic the effect of 5-HT, but both the intracellular injection of cyclic GMP and the application of zaprinast (an inhibitor of a cyclic-GMP-dependent phosphodiesterase) induce a Ca^{2+} current increase. Moreover, when a maximal Ca^{2+} current increase is induced by cyclic GMP, 5-HT becomes ineffective and *vice versa*.

2. Decreases in the Ca^{2+} current can be generated by two mechanisms. (a) A reversible decrease of the Ca^{2+} current is evoked by both dopamine (1 μmol l^{-1}) and the neuropeptide FMRFamide (1 μmol l^{-1}) on different identified neurones. Intracellular injections of either cyclic AMP or cyclic GMP do not mimic the effect of dopamine or FMRFamide. Moreover, the intracellular injection of EGTA does not affect the Ca^{2+} current decrease induced by these transmitters. (b) An 'irreversible' decrease of the Ca^{2+} current of the D2 neurone is elicited by 5–20 μmol l^{-1} cholecystokinin octapeptide (CCK 8). Intracellular Ca^{2+} plays a key role in the intracellular mediation of this effect since the intracellular injection of EGTA prevents the CCK 8-induced decrease of the Ca^{2+} current.

INTRODUCTION

In recent years, a convincing body of knowledge has been accumulated indicating that neurotransmitters can modulate voltage-dependent channels such as those for Ca^{2+}.

The first observations supporting this idea were made in cardiac muscle fibres of both amphibians and mammals. In these, stimulation of beta-adrenergic receptors by adrenaline and its agonists caused an increase of the plateau component of the action potential of the cardiac muscle fibres, resulting from an increase of the Ca^{2+} current (Vassort *et al.* 1969; Reuter & Sholz, 1977; see reviews by Reuter, 1983; Reuter, Kokubun & Prod'hom, this volume; Tsien, 1983; McCleskey *et al.* this volume).

Key words: synapse, neurotransmission, serotonin, dopamine, FMRFamide, cholecystokinin.

This beta-adrenergic effect on the Ca^{2+} current was mimicked by the application of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) or its analogues (see Tsien, 1977; Reuter, Cachelin, De Peyer & Kobuku, 1983; Brum *et al.* 1983) and also by injection of the catalytic unit of a cyclic-AMP-dependent protein kinase (Osterrieder *et al.* 1982; Brum *et al.* 1983). More recently, patch-clamp studies of cardiac cells have shown that beta-adrenergic agents evoke an increase of the average number of functional Ca^{2+} channels per cell (Reuter *et al.* 1983; Bean, Nowycky & Tsien, 1984) and a slowing of the time course of both the activation and inactivation of these channels (Bean *et al.* 1984; Brum, Osterrieder & Trautwein, 1984; see the chapters by Reuter *et al.* and McCleskey *et al.* in this volume).

In other preparations, however, it was observed that the transmitter-induced increase of the Ca^{2+}-dependent spike duration did not involve a change of the Ca^{2+} current. For instance, serotonin (5-hydroxytryptamine, 5-HT) was found to increase the calcium spike duration of sensory neurones in *Aplysia californica*, and of some identified neurones in *Helix aspersa*, by evoking a decrease in a voltage-dependent K^+ conductance (Klein & Kandel, 1978, 1980; Paupardin-Tritsch, Deterre & Gerschenfeld, 1981). Similar effects were obtained by applying dopamine (DA) to identified snail neurones (Paupardin-Tritsch, Colombaioni, Deterre & Gerschenfeld, 1985*a*) or two small cardioexcitatory peptides (SCP_A and SCP_B) to *Aplysia* sensory neurones (Abrams *et al.* 1984). These effects were also mimicked eitherby the intracellular injection of cyclic AMP or by agents which increased the intracellular cyclic AMP concentration (Klein & Kandel, 1978, 1980; Deterre, Paupardin-Tritsch, Bockaert & Gerschenfeld, 1981, 1982). A similar increase in the duration of the action potential of *Aplysia* neurones could also be evoked by the intracellular injection of the catalytic unit of a cyclic-AMP-dependent protein kinase (Kaczmarek *et al.* 1980; Castellucci *et al.* 1982). The patch-clamp study of sensory *Aplysia* neurones revealed that both 5-HT and cyclic AMP elicit the closing of a population of 'background' K^+ channels (called the S-channels) which constitute the predominant open channel population when these cells are held at potentials near 0 mV (Siegelbaum, Camardo & Kandel, 1982; see Siegelbaum, Belardetti, Camardo & Shuster, this volume). The application of the catalytic unit of a cyclic-AMP-dependent protein kinase to inside-out membrane patches from the same neurones also closes the S-channels (Shuster, Camardo, Siegelbaum & Kandel, 1985; see Siegelbaum *et al.* this volume). In voltage-clamped *Aplysia* sensory neurones, the closing of the S-channels by 5-HT is reflected in the decrease of a specific outward current component, the S-current (Klein, Camardo & Kandel, 1982). 5-HT, DA and the neuropeptide FMRF amide were also able to decrease the S-current of identified snail neurones (Paupardin-Tritsch *et al.* 1985*a*, and unpublished results; Colombaioni, Paupardin-Tritsch, Vidal & Gerschenfeld, 1985).

In both cardiac muscle cells and peripheral neurones, neurotransmitters have also been reported to *decrease* the duration of the Ca^{2+} current. In cardiac muscle fibres, muscarinic agonists have been reported to decrease the plateau phase of their Ca^{2+}-dependent action potential (Giles & Noble, 1976; see Tsien & Siegelbaum, 1980). This effect appears to be associated with a decrease in Ca^{2+} conductance (Hino &

Ochi, 1980) and would involve cyclic 3′5′-guanosine monophosphate (cyclic GMP) as a second messenger (Trautwein, Taniguchi & Noma, 1982). In the case of peripheral neurones, noradrenaline has been observed to decrease the duration of the Ca^{2+}-dependent spike recorded in the soma of both rat sympathetic neurones (Horn & McAfee, 1980; Galvan & Adams, 1982) and chick embryo dorsal root sensory neurones in culture (Dunlap & Fischbach, 1980). The ionic mechanism involved in this effect of noradrenaline on both types of neurones is the same and consists of a decrease of the Ca^{2+} current resulting from a decrease in Ca^{2+} conductance, without apparent alteration of the Ca^{2+} current kinetics (Dunlap & Fischbach, 1980; Galvan & Adams, 1982).

GABA (γ-aminobutyric acid), serotonin, dopamine, enkephalin and somatostatin were also found to decrease the duration of the action potential of cultured dorsal root neurones from chick embryos (Dunlap & Fischbach, 1980; Canfield & Dunlap, 1984). In other vertebrate neurones, some neuropeptides were, in addition, found to decrease the Ca^{2+}-dependent spike. Thus, it was observed that met-enkephalin shortens the duration of the Ca^{2+}-dependent spike of amphibian Rohon–Beard neurones (Bixby & Spitzer, 1983) and leu-enkephalin, and other opioid peptides, decrease the duration of the somatic action potential of mouse sensory ganglion neurones in culture (Werz & Macdonald, 1982). In none of these cases have the ionic mechanisms of peptide action been analysed in detail.

In this article, we will review recent work from our own laboratory on the neurotransmitter-induced modulation of the Ca^{2+} current of identified neurones of the snail, *Helix aspersa*. In this preparation, biogenic amines and peptides were found to exert at least three different actions on the neuronal Ca^{2+} current: (1) 5-HT evokes, in a group of identified snail ventral neurones, a reversible increase of the Ca^{2+} current probably mediated by cyclic GMP (Paupardin-Tritsch, Hammond & Gerschenfeld, 1985*b*, 1986); (2) both dopamine and the neuropeptide FMRFamide elicit a reversible decrease of the Ca^{2+} current mediated by an unknown intracellular messenger which is not Ca^{2+}, cyclic AMP or cyclic GMP (Paupardin-Tritsch *et al.* 1985*a*; Colombaioni *et al.* 1985; see also Akopyan, Iljin & Chemeris, 1984); and (3) the cholecystokinin octapeptide (CCK 8) induces an irreversible decrease of the Ca^{2+} current of an identified neurone through an intracellular mechanism in which intracellular Ca^{2+} appears to play an important role (D. Paupardin-Tritsch, C. Hammond & H. M. Gerschenfeld, unpublished results).

ACTION POTENTIAL AND MEMBRANE CURRENTS IN SNAIL NEURONES

The electrophysiological experiments that will be reviewed in this article were all performed on *in vitro* preparations of isolated circumoesophageal ganglia of the snail *Helix aspersa*. Some large neurones of these ganglia could be recognized from one preparation to another by their position inside the ganglia (see Kerkut *et al.* 1975; Paupardin-Tritsch *et al.* 1986).

The action potential recorded in the cell body of these large molluscan neurones involves Na^+, Ca^{2+} and K^+ ions. Many of the transmembrane currents carried by

these ions can be specifically blocked by different pharmacological agents. Thus, the blockade of some membrane K^+-conductance components by the presence of 30 mmol l^{-1} tetraethylammonium (TEA) ions in snail saline (TEA-saline) prolongs the spike of these neurones by unmasking a Ca^{2+}-dependent plateau phase (see Fig. 1A).

To find which specific voltage-dependent membrane current of an identified snail neurone is modulated by the neurotransmitters, the neurones were voltage-clamped and the different current components were studied after isolating them pharmacologically. In our experiments, we first bathed the neurones in a saline containing 1 μmol l^{-1} tetrodotoxin (TTX) and 30 mmol l^{-1} TEA. Then, holding the membrane potential at -50 mV, the neurone was depolarized to between 0 and $+20$ mV using current pulses of 60 ms–1 s duration. In these conditions, a composite current was generally recorded: an initial inward component being followed by a large outward current. In the presence of TTX, the inward current of molluscan neurones is Ca^{2+}-dependent (see reviews by Kostyuk, 1980; Hagiwara & Byerly, 1981), their outward one corresponding to different K^+-current components, namely: I_{DR}, the K^+ current associated with delayed rectification: I_A, an early transient K^+ current; I_C, the Ca^{2+}-dependent K^+ current (see reviews by D. Adams, Smith & Thompson, 1980; P. Adams, 1982) and sometimes the S-current (Klein *et al.* 1982).

To study the effects of neurotransmitters on the Ca^{2+} current, it was essential to isolate the inward current from the different outward current components. Moreover, it was necessary to ensure that the action of the neurotransmitters was not exerted on those outward K^+-current components which do not depend on Ca^{2+}. For this purpose, both the inward current and the Ca^{2+}-dependent K^+ currents were blocked by bathing the snail neurones in a saline containing 1 μmol l^{-1} TTX and 30 mmol l^{-1} TEA and in which Ca^{2+} was replaced with Mg^{2+} (TTX/TEA/Mg^{2+}-saline). To study the remaining outward K^+ currents, the membrane potential was held at -50 mV and the cell depolarized by pulses of 1–1·2s to between $+10$ and $+20$ mV.

Once it was known that the neurotransmitter did not affect the Ca^{2+}-independent outward K^+ currents, its effects on the inward current through the Ca^{2+} channels were then studied. To separate this inward current from the other current components, and to facilitate it, the neurones were bathed in a saline containing both 1 μmol l^{-1} TTX and 30 mmol l^{-1} TEA and in which Ca^{2+} was replaced with Ba^{2+} (TTX/TEA/Ba^{2+}-saline). In these voltage-clamp experiments the neurones were generally held at -50 mV and depolarized to between 0 and $+20$ mV by pulses of 50–60 ms duration.

A SEROTONIN-INDUCED INCREASE OF THE CALCIUM CURRENT PROBABLY MEDIATED BY CYCLIC GMP

In a group of identified neurones (neurones D6, D7), located in the ventral face of the parietal ganglion of the snail, *Helix aspersa*, bathed in a TEA-saline, 5-HT (1 μmol l^{-1}) was found to evoke an increase of the duration of the Ca^{2+}-dependent

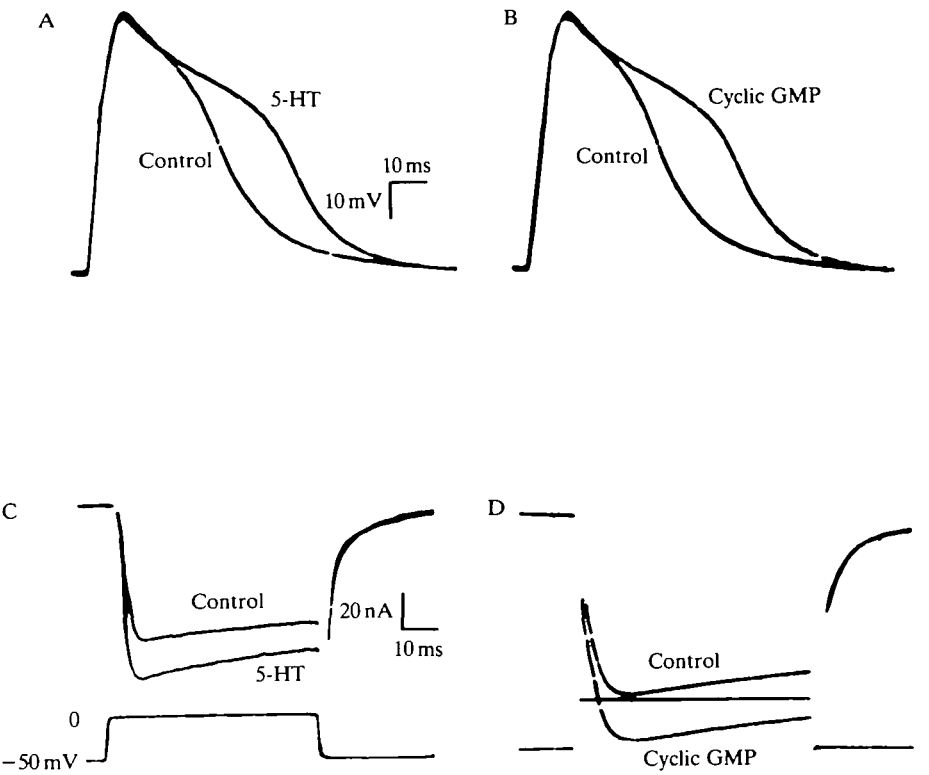

Fig. 1. Effects of serotonin (5-HT) and cyclic GMP on the action potential and inward current of identified snail ventral pallial neurones. (A) In a D6 neurone bathed in a TEA-saline, the somatic action potential shows a Ca^{2+}-dependent plateau phase (Control). Application of 1 μmol l^{-1} 5-HT increases the duration of the spike. (B) In the same snail D4 neurone bathed in a TEA-saline, intracellular injection of cyclic GMP evokes an increase of the duration of the Ca^{2+}-dependent spike similar to that induced by 5-HT in A. (C) Inward current recorded from a D6 neurone bathed in TTX/TEA/Ba^{2+}-saline (see text for details). The cell, held at −50 mV, is depolarized for 60 ms to 0 mV. Application of 1 μmol l^{-1} 5-HT evokes a 30% increase in the amplitude of the peak inward current. (D) Inward current recorded in a D6 neurone from another preparation. The cell, bathed in a TTX/TEA/Ba^{2+}-saline, was held at −50 mV and depolarized to +10 mV with a 60-ms pulse. Intracellular injection of cyclic GMP also evokes an increase of the inward current.

action potential (Fig. 1A). This effect showed a latency of 1–3 min and could be easily reversed by washing out the neurotransmitter for 5–10 min. In cases in which the 5-HT application was maintained for more than 15 min, no desensitization phenomena were observed.

The application of 1 μmol l^{-1} 5-HT to the same neurones did not affect any TEA-insensitive, Ca^{2+}-independent outward current components (not illustrated). In contrast, the application of 1 μmol l^{-1} 5-HT evoked a 30% increase in the amplitude of the inward current of the ventral parietal neurones bathed in a TTX/TEA/Ba^{2+}-saline (Fig. 1C).

To further confirm that 5-HT specifically increased the inward current through the Ca^{2+} channels, and to dismiss a possible effect of 5-HT on a hidden outward current component, the antibiotic nystatin was used to exchange completely the intracellular K^+ for Cs^+ (see Tillotson, 1979). In neurones loaded with Cs^+ and bathed in a Cs^+-containing Tris saline in which Ca^{2+} was replaced by Ba^{2+}, i.e. in conditions in which Cs^+ was the only intracellular monovalent cation and both K^+ and Na^+ were absent from the extracellular medium, 5-HT still markedly decreased the inward current.

Analysis of the I–V curves relating the inward current amplitudes to the membrane potential at which they were recorded in ventral parietal neurones bathed in a TTX/TEA/Ba^{2+}-saline also showed that 5-HT exclusively increased the inward current (Fig. 2A) throughout the membrane potential range explored. Moreover, the potential level at which the inward current became maximal either in the presence or in the absence of 5-HT in the bath was the same and at this potential level the inward current increase induced by 5-HT was maximal.

From these results it was concluded that 5-HT enhanced the inward current of the identified ventral parietal neurones by increasing the membrane Ca^{2+} conductance.

The possibility that an intracellular second messenger could be involved in the generation of the 5-HT-induced of Ca^{2+} current in the snail ventral parietal neurones was suggested by the relatively long latency of the effect of 5-HT on the Ca^{2+}-dependent action potential.

In the case of cardiac muscle cells, beta-adrenergic agonists were observed to enhance the Ca^{2+} current by increasing the intracellular concentration of cyclic AMP (see Introduction). It was found, however, that cyclic AMP did not intervene in the 5-HT-induced increase in Ca^{2+} current of the snail ventral neurones. Thus, intracellular injections of cyclic AMP modified neither the duration of the action potential of these neurones nor the outward or inward currents recorded in them. Moreover, neither the inward current nor the 5-HT-induced increase of this current were altered by forskolin, a potent adenylate cyclase stimulating agent (Seamon & Daly, 1981; see Deterre *et al.* 1982, for its effects on molluscan neurones).

The possibility of a participation of intracellular Ca^{2+} in the mechanism of the effect of 5-HT was also examined. It was found that the intracellular injection of EGTA in the identified ventral parietal neurones did not affect the 5-HT-induced increase of the inward current. To confirm that the cell loading with EGTA was effective, parallel experiments were carried out in which intracellular injections of EGTA were shown to block the Ca^{2+}-dependent afterhyperpolarization that follows a train of spikes. These findings thus suggested that the effect of 5-HT on the inward current is not related to changes in the intracellular Ca^{2+} concentration.

Since DeRiemer *et al.* (1985) had reported that either the intracellular injection of protein kinase C or the stimulation of this enzyme by phorbol ester TPA (12-*O*-tetradecanoyl-phorbol-13 acetate) evoked an increase of the inward current of neuroendocrine bag cells of *Aplysia*, the effect of phorbol ester TPA was also tested on the identified ventral parietal snail neurones. The application of $100\,nmol\,l^{-1}$ phorbol ester TPA to these neurones bathed in a TTX/TEA/Ba^{2+}-saline also

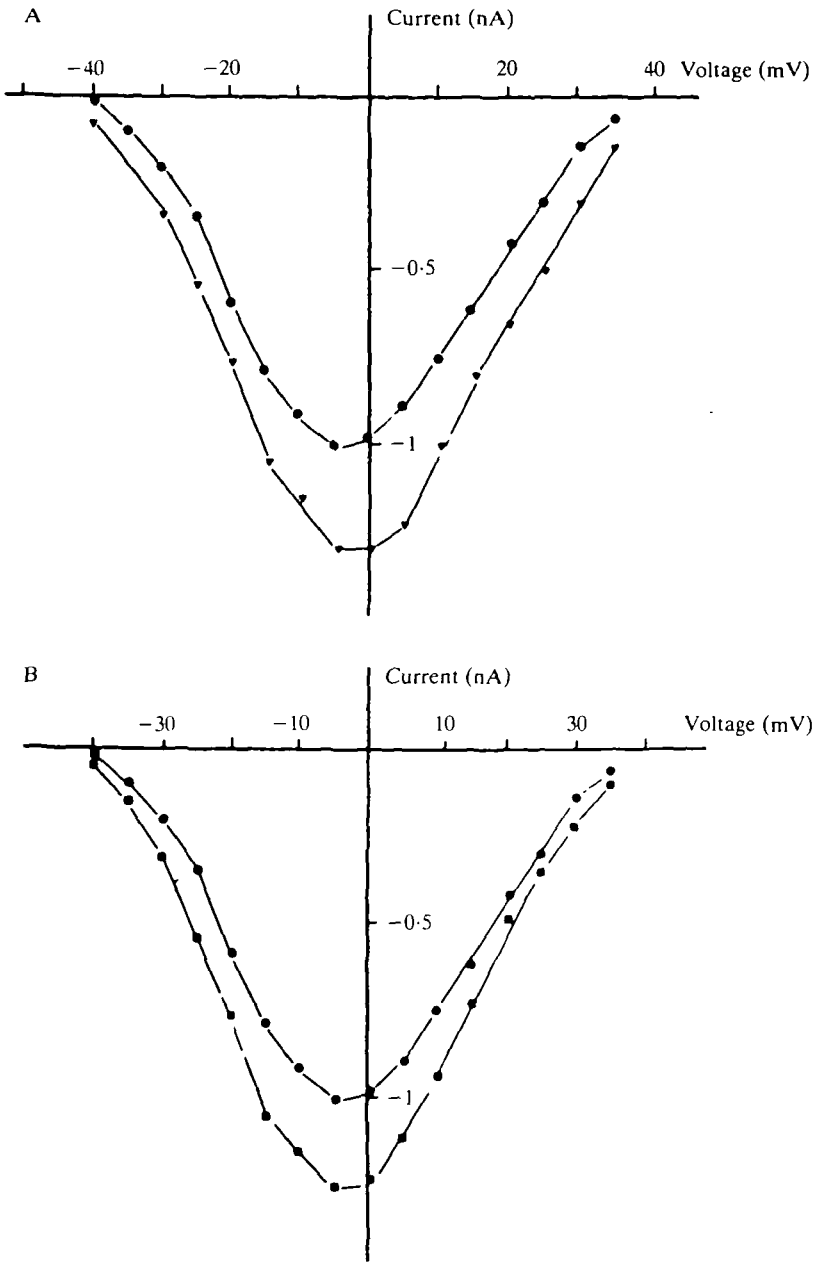

Fig. 2. (A) Effect of serotonin (5-HT) on the I–V curves obtained in a D6 neurone bathed in a TTX/TEA/Ba^{2+}-saline (see text for details). The normalized values of the peak inward currents obtained in the absence (circles) and in the presence (triangles) of 10 μmol l^{-1} 5-HT were plotted against the values at which the membrane potential of the neurone was stepped. Both curves were corrected for linear leakage conductance. (B) Effect of intracellular injection of cyclic GMP on the I–V curve obtained in the same D4 neurone bathed in a TTX/TEA/Ba^{2+}-saline. The neurone and the method of establishing the I–V curves were the same as in A. The control curve was obtained in the absence of all intracellular injection and the experimental curve was obtained after prolonged intracellular injection of cyclic GMP. Both curves were corrected for the linear leakage conductance.

evoked an increase in the inward current. In this case, the increase in the current was very small at the peak and much larger at the end of the pulse. Therefore, phorbol ester TPA apparently acted by reducing the inward current inactivation. Moreover, the application of 5-HT during the continued presence of phorbol ester was still able to induce an increase in inward current, even if the phorbol ester TPA-induced depression of the apparent inward current inactivation persisted.

The most interesting results were obtained with cyclic GMP which, unlike cyclic AMP, mimicked well the effects of 5-HT on the inward current of the ventral parietal neurones. The intracellular injection of cyclic GMP into these cells evoked an increase of the spike duration similar to that caused by 5-HT (Fig. 1B). Moreover, the intracellular injection of cyclic GMP in the ventral parietal neurones bathed in a TTX/TEA/Ba^{2+}-saline markedly increased their inward current (Fig. 1D). When a prolonged 5-HT application evoked a maximal increase in the inward current, the intracellular injection of cyclic GMP was unable to induce a further increase in the inward current. Conversely, when cyclic GMP induced a maximal increase in the inward current, the application of 5-HT became ineffective.

The effects of 5-HT and cyclic GMP on the I–V curves obtained from the same single ventral parietal neurones were also similar (Fig. 2). As with 5-HT, cyclic GMP was observed to induce an increase in the inward current at all the potentials examined (Fig. 2B). Moreover, the potential level at which the inward current became maximal both in control conditions and during the cyclic GMP injections was the same, the cyclic GMP effect being maximal at this potential level.

We examined next whether the inhibitors of phosphodiesterases which elicited an increase of the intracellular cyclic GMP concentration in other cells (for instance, vascular muscle cells, see Winquist *et al.* 1984) could also induce a similar enhancement of the inward current in the identified ventral parietal neurones. We failed to observe any overt action of isobutylmethylxanthine (IBMX) on the inward current. In contrast, zaprinast (M&B22948, see Winquist *et al.* 1984) was found to evoke an increase of the inward current similar to that evoked by 5-HT. However, zaprinast not only was unable to potentiate the effect of a low concentration of 5-HT on the Ca^{2+} current but it actually blocked the 5-HT-induced increase of the current.

In more recent experiments, it could be observed that the intracellular injection of a cyclic GMP-dependent protein kinase previously activated by cyclic GMP evoked a marked increase of the Ca^{2+} current of the identified snail ventral neurones. Moreover, the injection of this enzyme, in the absence of any previous activation of it by cyclic GMP, was found to potentiate the effect of very low concentrations of both 5-HT and zaprinast which, by themselves, were ineffective on the inward current (D. Paupardin-Tritsch, C. Hammond, H. M. Gerschenfeld, A. Nairn & P. Greengard, unpublished results).

Considered together, all these results on the identified snail ventral parietal neurones indicate that the 5-HT-induced increase in duration of their somatic Ca^{2+} action potential is due to an increase in membrane Ca^{2+} conductance. Moreover, they rule out the possibility that the 5-HT-induced increase of the Ca^{2+} current could be mediated by cyclic AMP, intracellular Ca^{2+} or diacylglycerol *via* the

stimulation of protein kinase C. In contrast, they are strongly in favour of a second messenger role of cyclic GMP in the generation of the 5-HT-induced increase of the Ca^{2+} current since they indicate that 5-HT and cyclic GMP probably act on the same Ca^{2+} channel population through the same chain of intracellular events leading to the inward current increase. However, the lack of effect of IBMX on the inward current and the block of the 5-HT responses by low concentrations of zaprinast still remain unexplained.

It has been recently proposed that the stimulation of cyclic GMP formation may involve the activation of receptors which trigger an increase of the hydrolysis of phosphoinositides (see Berridge, 1984; Nishizuka, 1984). These receptors would activate guanylate cyclase indirectly by releasing arachidonic acid which, converted to another metabolite, would stimulate the synthesis of cyclic GMP. Intracellular Ca^{2+} seems to be necessary to release the arachidonic acid (see Berridge, 1984). It is difficult to ascertain whether the arachidonic cascade intervenes in the formation of cyclic GMP in the snail ventral parietal neurones. However, the lack of effect of EGTA would rule out an intervention of the arachidonic acid cascade in the generation of the 5-HT-induced increase of Ca^{2+} current.

DOPAMINE AND THE NEUROPEPTIDE FMRFAMIDE DECREASE REVERSIBLY THE CALCIUM CURRENT

In contrast with the effect of 5-HT on the identified snail ventral parietal neurones, the application of dopamine (DA) (1–10 μmol l^{-1}) and FMRFamide (20 μmol l^{-1}), respectively, to some identified dorsal snail neurones (cells D2 and E2) bathed in a TEA-saline, evoked a decrease of the duration of the Ca^{2+}-dependent action potential recorded in these cells (Fig. 3A,B). A voltage-clamp analysis of the transmembrane currents of the same neurones revealed that the mechanism involved in these actions of both DA and FMRFamide was also different from that previously described in the ventral parietal neurones. Thus, when cells D2 or E2 were voltage clamped in a TTX/TEA/Mg^{2+}-saline, the application of 10 μmol l^{-1} DA (to cell D2) and of 50 μmol l^{-1} FMRFamide (to cell E2) did not alter any of the outward K^+ current components. In contrast, when the membrane currents of cell D2 and E2 were recorded in a TTX/TEA/Ba^{2+}-saline, the application of DA and FMRFamide evoked a 30–40% decrease of the inward current of neurones D2 and E2, respectively (Fig. 3C,D).

To confirm that DA and FMRFamide specifically decreased the Ca^{2+} current in the D2 and E2 neurones and that their effect did not involve any modification of a hidden outward current, nystatin was used to exchange completely the intracellular K^+ content of both cells with Cs^+ (see Tillotson, 1979). When the only monovalent cation inside the cells was Cs^+, the inward current of neurones D2 and E2 was still markedly decreased when DA was applied to neurone D2 and FMRFamide to neurone E2. Moreover, the I–V curves of neurones D2 and E2 (Fig. 4A,B) clearly showed that DA and FMRFamide, respectively, decreased the Ca^{2+} current over the whole range through which the membrane potential of these neurones was stepped.

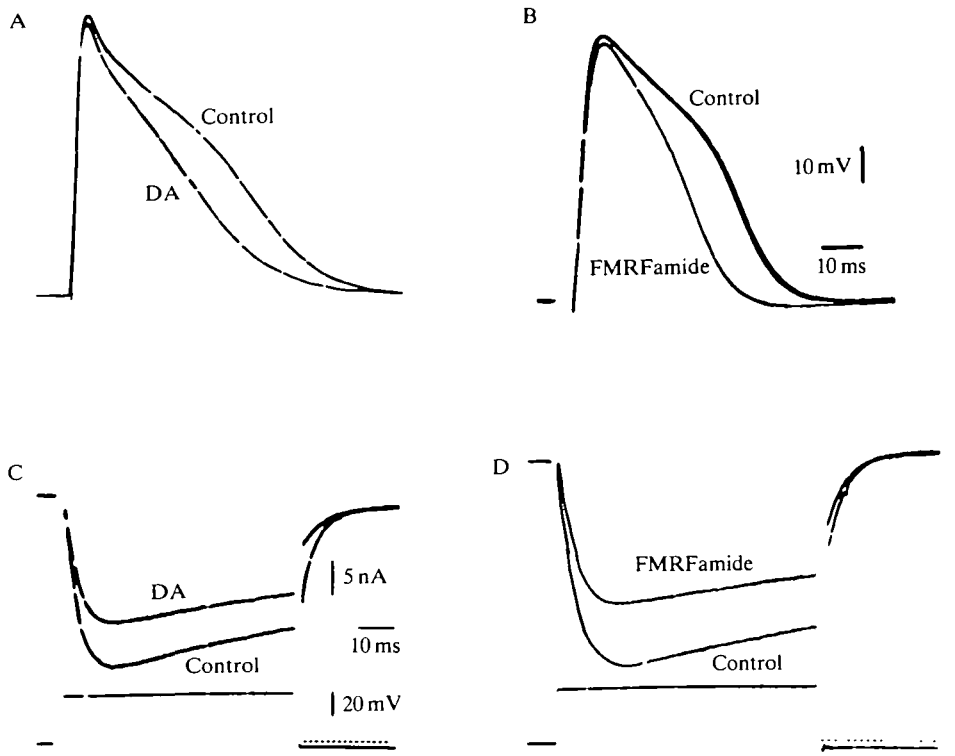

Fig. 3. Effect of 10 μmol l^{-1} dopamine (DA) and FMRFamide on the somatic action potential and the inward current of identified snail neurones. (A) The duration of the Ca^{2+}-dependent action potential of a D2 neurone bathed in a TEA-saline (Control) is decreased by the application of 10 μmol l^{-1} DA. (B) Bath application of 10 μmol l^{-1} FMRFamide to an E2 neurone bathed in a TEA-saline evokes a marked decrease in the duration of the Ca^{2+}-dependent spike. (C) Inward current recordings in a D2 neurone bathed in a TTX/TEA/Ba^{2+}-saline (see text for details). The neurone was held at −50 mV and depolarized for 60 ms to 0 mV and then repolarized to −75 mV. Bath application of 10 μmol l^{-1} DA decreases the inward current and the inward tail current. (D) Inward current recordings in an E2 neurone bathed in a TTX/TEA/Ba^{2+}-saline. The cell, initially held at −60 mV, was depolarized for 60 ms to +10 mV and then repolarized to −75 mV. Application of 20 μmol l^{-1} FMRFamide decreases the amplitude of both the inward current and the tail inward current.

The potential level at which the Ca^{2+} current became maximal either in the presence or in the absence of the transmitters in the bath was the same. Furthermore, the decrease of the inward current induced by DA and FMRFamide was maximal at this potential level. It can therefore be concluded that the main effect of DA on cell D2 and of FMRFamide on cell E2 was to decrease their Ca^{2+} conductance.

Experiments were then performed to investigate the possible intervention in the actions of DA or FMRFamide of the three second messengers already mentioned in the case of the 5-HT-induced increase in the Ca^{2+} current: cyclic AMP, cyclic GMP and Ca^{2+} ions. The intracellular injection of either cyclic AMP or cyclic GMP in D2 or E2 neurones affected neither the Ca^{2+}-dependent spike duration nor the inward current of these neurones. Biochemical single-cell assay of adenylate cyclase activity

on single D2 and E2 neurones showed that DA did not stimulate the enzyme activity in neurone D2 and that FMRFamide did not activate the enzyme in cell E2.

Finally, injection of EGTA into neurones D2 and E2 for periods of 30–60 min did not affect the Ca^{2+} current decreases induced by DA and FMRFamide, respectively, in these neurones.

Therefore, it can be concluded that DA and FMRFamide both induce a similar decrease in the duration of the Ca^{2+}-dependent action potential of neurones D2 and E2 by reversibly decreasing the Ca^{2+} current. We have not at present any definitive proof that the same mechanism underlies both actions. It is certain, however, that both actions probably involve an intracellular mechanism in which cyclic AMP, cyclic GMP or a decrease in the intracellular Ca^{2+} concentration do not seem to be involved.

A CCK 8-INDUCED IRREVERSIBLE DECREASE OF CALCIUM CURRENT

In addition to 5-HT, DA and FMRFamide, a CCK 8-like neuropeptide has been detected in molluscan nervous systems and has been localized by immunocytochemical methods in identified cells of the snail *Helix aspersa* (Osborne, Cuello & Dockeray, 1982).

It has been reported that CCK 8 exerts depolarizing excitatory effects on vertebrate central neurones (see e.g. Brooks & Kelly, 1985; Willetts, Urban, Murase & Randic, 1985). Moreover, in some dorsal horn motoneurones, CCK 8 was found to evoke a reversible decrease in duration of the Ca^{2+}-dependent action potential (Willetts *et al.* 1985). In some snail neurones, CCK 8 may evoke an inward current, but more interestingly, on neurone D2 (in which DA has been observed to evoke a reversible decrease of Ca^{2+} current, see above), CCK 8 was also found to induce a decrease of Ca^{2+} current. However, in contrast with DA, the effect of CCK 8 on the Ca^{2+} current was not reversed by washing out the peptide.

Moreover, CCK 8 acted on neurone D2 at much lower concentrations than DA (5–20 nmol l^{-1}). In a D2 neurone bathed in a TEA-saline, the application of 20 nmol l^{-1} CCK 8 caused, in 2 min, a marked decrease of the duration of the somatic Ca^{2+}-dependent action potential (Fig. 5A). This decrease progressed further with time (Fig. 5B). In our experimental procedure, CCK 8 was generally removed 9 min after the onset of its application, but it could be observed that the Ca^{2+}-dependent spike was still decreasing in duration during the first 2–3 min of washing out and then remained unchanged even if the washing was prolonged for as long as 45 min.

The transmembrane currents were then analysed in voltage-clamped D2 neurones of many preparations. No effect of CCK 8 was observed on the TEA-insensitive, Ca^{2+}-independent outward current. When a D2 neurone was bathed in a TTX/TEA/Ba^{2+}-saline in which NaCl had been replaced by Tris-Cl and to which 3 mmol l^{-1} 4-aminopyridine (4-AP) had been added, it could be observed that the application of 5 nmol l^{-1} CCK 8 also reduced the amplitude of its inward current. The time course of the action of the peptide at this concentration on the inward current was slower than that observed on the Ca^{2+} action potential. After 6–9 min of

application of 5 nmol l^{-1} CCK 8, the inward current began to decrease (Fig. 5C, trace 2) and when the peptide was removed after 9 min of application (Fig. 5C, trace 3), the inward current was reduced by 25 %. A further small decrease in the inward current was observed during the first minutes of washing out, the amplitude of the decreased inward current reaching a steady value equivalent to 30–40 % of the initial amplitude. No recovery was observed even if the washing with normal saline was prolonged for 30 min (Fig. 5C, trace 4).

The selectivity of the effect of CCK 8 on the inward current was confirmed by suppressing any possible hidden outward component by loading the D2 neurones with Cs^+ in the presence of nystatin (see above). In these experiments, in which all the intracellular K^+ was exchanged for Cs^+ and Tris-Cl replaced the extracellular NaCl, CCK 8 was still observed to induce a decrease of the inward current with a similar time course to that described above.

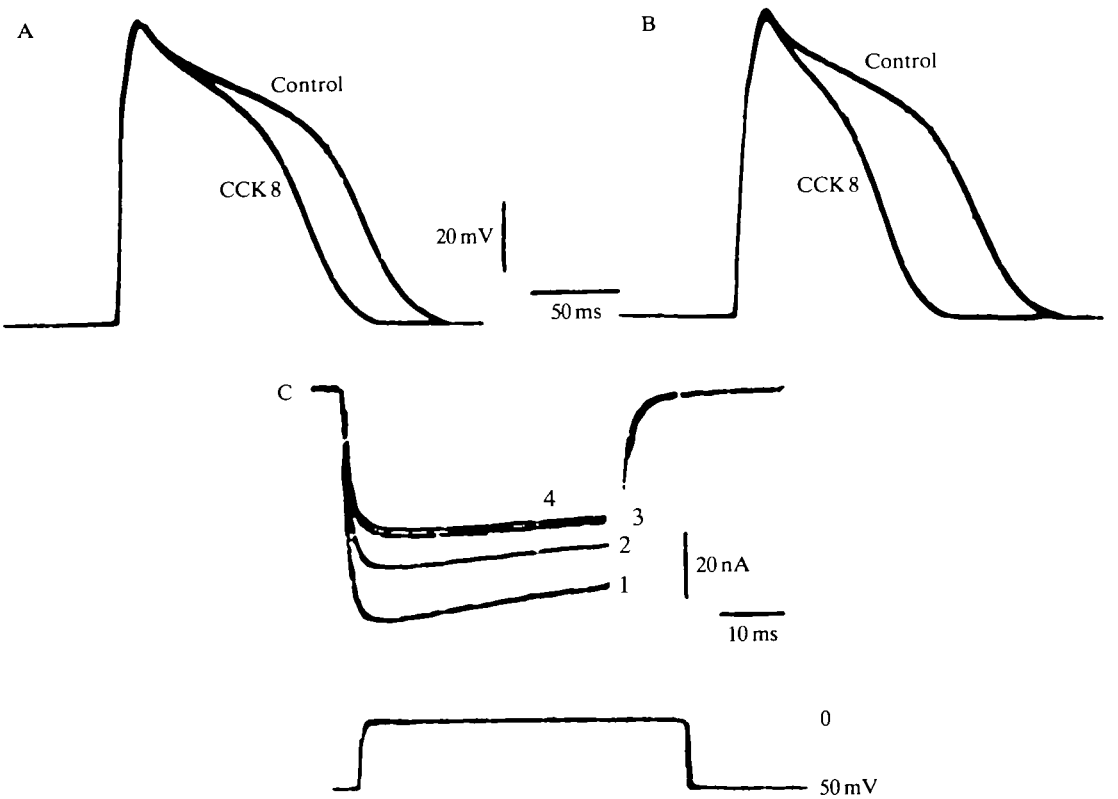

Fig. 5. Decrease in the duration of the Ca^{2+}-dependent action potential of neurone D2 by cholecystokinin octapeptide (CCK 8) (20 nmol l^{-1}). (A) Decrease in the spike after 2 min application of the peptide. (B) The spike duration is further decreased after 5 min of CCK 8 application. (C) Inward current recordings obtained in a D2 neurone bathed in a TTX/TEA/Ba^{2+}-saline (see text for details) to which 4-aminopyridine (4-AP) (3 mmol l^{-1}) was added. Trace 1, control recording. The inward current decreases after application of 5 nmol l^{-1} CCK 8. Traces 2 and 3 were recorded, respectively, 6 and 9 min after the peptide application and trace 4 was obtained after a 10-min washing out of CCK 8.

Analysis of the I–V curves relating the net currents evoked by CCK 8 to the membrane potentials at which they were recorded in D2 neurones bathed in TEA/TTX/Ba^{2+}-saline containing 3 mmol l^{-1} 4-AP confirmed that CCK 8 specifically decreased the inward current of the D2 neurone by decreasing its Ca^{2+} conductance.

The latency and the peculiar time course of the CCK 8-induced decrease of inward current suggested the intervention of a second messenger, as in the preceding examples of Ca^{2+} current modulation. As has already been shown during the study of the DA-induced decrease of Ca^{2+} current in the D2 neurone (see above), the intracellular injection of either cyclic AMP or cyclic GMP inside this cell was found not to affect the Ca^{2+} current. But, in contrast with what had been observed in the case of the DA-induced decrease in the Ca^{2+} current, the intracellular injection of EGTA prevented the Ca^{2+} current decrease evoked by CCK 8. Nevertheless, the intracellular injection of EGTA was not able to reverse the CCK 8 effect when it was performed once the Ca^{2+} current had already been decreased by the application of CCK 8.

It can therefore be concluded that CCK 8 evokes, in neurone D2, an irreversible decrease in the Ca^{2+} current and that an increase in the intracellular Ca^{2+} concentration appears to be a necessary initial event for the generation of the Ca^{2+} current modulation by the peptide.

DUALITIES OF THE NEUROTRANSMITTER-INDUCED MODULATIONS OF THE CALCIUM ACTION POTENTIAL OF SNAIL NEURONES

In the case of cardiac muscle cells (see Introduction), the target of the neurotransmitter-induced modulations of the Ca^{2+}-dependent action potential was the Ca^{2+} current. In the case of molluscan neurones, the situation is more complex because the increase in the duration of the Ca^{2+}-dependent action potential may result from a decrease of a specific K^+ current (the S-current), in the absence of any alteration to the Ca^{2+} current.

The duality of action of dopamine on snail neurones illustrates this complexity well. DA acts on identified snail neurones in two different ways: it may either prolong the duration of the Ca^{2+}-dependent action potential of some neurones by decreasing a cyclic-AMP-dependent K^+ current or it may decrease the Ca^{2+}-dependent spike duration of other neurones by decreasing the neuronal Ca^{2+} current (Paupardin-Tritsch *et al.* 1985*a*).

This duality of effects may even take place at the level of one single neurone. For instance, it has been observed that both DA and FMRFamide can induce, in identified snail neurones, a decrease of both the S-current and the Ca^{2+} current. These current modulations have been observed to exert opposite effects on the Ca^{2+}-dependent action potential. However, in these cells, in which DA or FMRFamide had a dual effect, these transmitters always elicited a decrease in the duration of the Ca^{2+}-dependent action potential (Paupardin-Tritsch *et al.* 1985*a*). The mechanism underlying the predominance of the decrease in the Ca^{2+} current on the action potential duration is unknown.

In other snail neurones, it was also observed that a neurotransmitter could evoke two different alterations of the membrane conductance having agonistic effects on the Ca^{2+}-dependent spike. For instance, in a group of identified *Aplysia* neurones, the RB cells, 5-HT probably increases the Ca^{2+} current and also evokes a slow inward current (Pellmar, 1984), probably due to a cyclic-AMP-dependent decrease in K^+ conductance.

MULTIPLICITY OF NEUROTRANSMITTERS AND SECOND MESSENGERS MODULATING THE CALCIUM CURRENT

It is important to emphasize that as for the neurotransmitters which modulate the Ca^{2+}-dependent spike duration, the second messengers mediating the modulation of the Ca^{2+} current can also display multiple actions. Thus, whereas in cardiac muscle cells cyclic AMP mediates the increase in Ca^{2+} current and cyclic GMP seems to intervene in the decrease of the Ca^{2+} current, in some molluscan neurones cyclic AMP mediates a decrease in K^+ conductance whereas cyclic GMP is probably involved in an increase in the Ca^{2+} current of other identified neurones.

A second intracellular messenger system also probably intervenes in the neurotransmitter-induced decrease in the Ca^{2+} current of vertebrate neurones. In sympathetic ganglion neurones, the noradrenaline-induced decrease in Ca^{2+} current appears to be modulated by alpha-adrenergic receptors (Horn & McAffee, 1980). Since activation of alpha-receptors has been reported to induce inhibition of adenylate cyclase activity (Jakobs, 1979), it has been postulated that the decrease in the Ca^{2+} current is linked to a fall in intracellular cyclic AMP concentration (McAffee *et al.* 1981). Moreover, more recently Holz, Rane & Dunlap (1986) have reported that GABA- and noradrenaline-induced decreases in Ca^{2+} current in embryonic spinal ganglion cells in culture were blocked both by pertussis toxin and by intracellular administration of GDP-B-S [guanosine 5′-*O*-(2-thiodiphosphate)], a non-hydrolysable analogue of GDP which competitively inhibits GTP binding. These results strongly suggest that the neurotransmitter-induced depression of the Ca^{2+} current of the spinal ganglion neurones involves the intervention of a GTP-binding protein.

In the case of an identified snail neurone, the D2 cell, two different transmitters were found to decrease the Ca^{2+} current, apparently using two different second messenger systems. The mechanism of the DA-induced reversible decrease in Ca^{2+} current is still unknown, but intracellular Ca^{2+} does not seem to be involved. The mechanism of the irreversible decrease in the Ca^{2+}-current elicited by CCK 8 probably involves the participation of intracellular Ca^{2+}.

PHYSIOLOGICAL IMPLICATIONS

The modulation of the Ca^{2+} current at neuronal synaptic endings plays an important role in the regulation of the transmitter release by them. Since it is not possible to measure directly the Ca^{2+} current in a large majority of presynaptic

endings, the cell bodies of neurones producing a Ca^{2+} spike and endowed with transmitter receptors can constitute rather useful models to analyse events which may be involved in the modulation of the transmitter release at the neuronal endings. Thus, Klein & Kandel (1978, 1980) have shown that the 5-HT-induced increase in duration of the soma action potential of *Aplysia* sensory neurones could account for the mechanism of the presynaptic facilitation evoked by 5-HT or for the stimulation of some interneurones which underlies the 'sensitization' of the gill withdrawal reflex. It is possible, following the same line of thought, that a 5-HT-induced increase in Ca^{2+} current at the endings of the snail ventral parietal neurones could also evoke a reversible presynaptic facilitation of their transmitter release.

In contrast, the decrease in Ca^{2+} conductance in chick embryo sensory ganglion neurones evoked by catecholamines, GABA and certain peptides (Dunlap & Fischbach, 1980) would account for the presynaptic inhibition at some of the dorsal root ganglion cell endings in the spinal cord (see Mudge, Leeman & Fischbach, 1979). A similar parallelism between decrease in the Ca^{2+} conductance of the soma membrane and presynaptic inhibition has been described in neurone L10 of *Aplysia* (Shapiro, Castellucci & Kandel, 1980). It can then be postulated that if DA is released near the endings of neurone D2 it could also be involved in presynaptic inhibition phenomena. Moreover, the CCK 8-induced decrease in Ca^{2+} current could also underlie a long-term presynaptic inhibition of neurones, such as snail neurone D2, showing CCK 8-induced responses.

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