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SLOW SYNAPTIC RESPONSES IN AUTONOMIC GANGLIA AND THE PURSUIT OF A PEPTIDERGIC TRANSMITTER

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SUMMARY

This account deals with studies of slow synaptic potentials, a new peptidergic transmitter, and integrative mechanisms at synapses in vertebrate autonomic ganglia.

In neurones of the cardiac parasympathetic ganglia of the mudpuppy (*Necturus maculosus*) both rapid excitatory and slow inhibitory synaptic potentials interact. The same transmitter, acetylcholine (ACh), causes in individual neurones a fast e.p.s.p. lasting up to 50 ms and a slow i.p.s.p. of about 2 s. The sequence of processes leading to these two synaptic potentials differs in important respects. Molecules of ACh released by terminals of the vagus combine with nicotinic receptors and within a fraction of 1 ms initiate the rapid e.p.s.p. which, as a rule, leads to a conducted impulse in ganglion cells. The e.p.s.p. resembles in its ionic mechanisms the rapid excitatory synaptic events seen at most neuromuscular and neuronal synapses. ACh also combines with muscarinic receptors whose activation is followed by an increased flow of K^+ ions and the generation of a slow i.p.s.p. There occurs, however, an apparent delay of over 100 ms between the time ACh reaches the muscarinic receptors and the detectable activation of the inhibitory conductance. During the delay the nicotinic e.p.s.p. has declined and ACh has disappeared from the synaptic cleft. It is suggested that at least three distinct processes are involved in the activation of the inhibitory conductances.

The second part of this paper describes synaptic events in sympathetic ganglia of the frog where release of ACh initiates three different synaptic potentials: (i) a standard fast nicotinic e.p.s.p. (about 30–50 ms duration); (ii) a slow muscarinic e.p.s.p. (30–60 s); (iii) a slow i.p.s.p. (about 2 s). The fourth synaptic signal, the 'late slow e.p.s.p.', lasts 5–10 min and is not caused by ACh.

We have evidence that a peptide, resembling luteinizing hormone releasing hormone (LHRH), is secreted by specific axons within ganglia where it initiates the late slow e.p.s.p.s. The evidence for such a peptidergic transmitter is as follows: (1) In nerves whose stimulation leads to the late slow e.p.s.p.s one detects by radioimmunoassay a peptide with a molecular weight of about 1000 daltons, resembling LHRH. (2) The peptide is released in isotonic KCl if Ca^{2+} is present in the high K^+ solution. (3) Five days after cutting the appropriate presynaptic nerves, about 95% of the peptide disappears from the ganglia. At the same time the content of peptide central to the cut is increased, suggesting that it is concentrated in axons and transported to the periphery from the spinal cord. (4) Application of synthetic LHRH mimics the action of the nerve-released transmitter in a specific

manner. Both substances cause similar changes in the postsynaptic membrane conductance and in the excitability of neurones, and the depolarizing effect of both agents changes in parallel when neurones are hyperpolarized. (5) An analogue of LHRH which in mammals blocks the release of gonadotropins also blocks the depolarizing effect of nerve-released transmitter and of applied LHRH in ganglion cells. Similar parallel actions occur after the application of other analogues of LHRH, some of which are more potent agonists, and others which are not effective. It is suggested that the natural transmitter and LHRH and its analogues act on the same receptors.

The role of slow synaptic potentials and the way in which they influence the effectiveness of cholinergic stimulation are discussed. Since the various synaptic potentials interact in individual cells, these neurones are suitable for a study of integrative mechanisms.

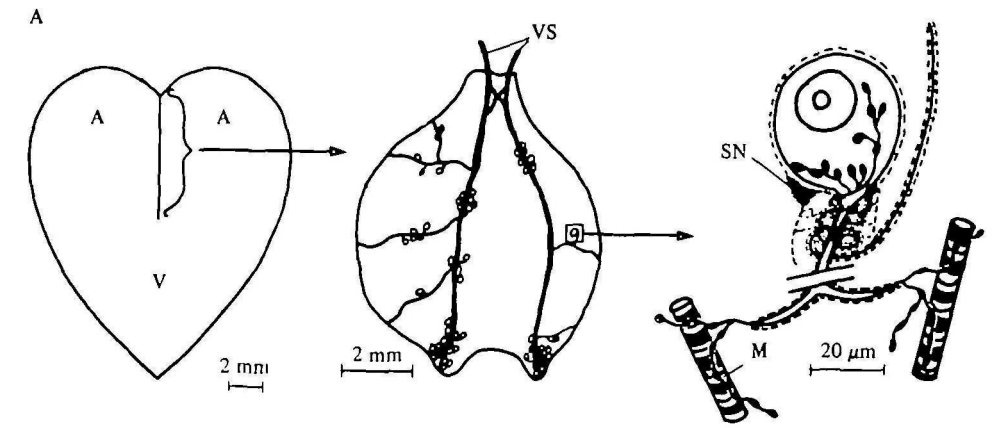
INTRODUCTION

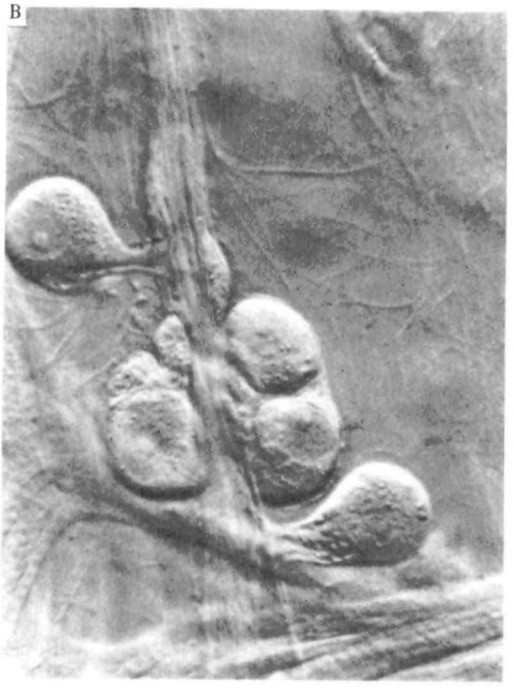
The dedication of a volume on neurotransmission by a group of distinguished scientists, most of whom are also good friends, is a pleasantly embarrassing event. No less embarrassing, and possibly a little out of order on the part of Ed Kravitz and John Treherne, was the invitation to contribute a paper under such unusual circumstances. My initial reaction was to write about various aspects of synaptic transmission in which I had been engaged for much of my career. Such an account could be made almost 'safe', because the most glaring mistakes of the past are likely to have been discovered and one need not reveal transgressions on which the statute of limitations has expired and the score has been settled. Having been given freedom by the editors, I decided to give a somewhat informal personal account about several current interests which are still in a stage of transition and whose future course is not certain. After all, it is ongoing work which makes one go to the laboratory with a feeling of suspense and cautious expectation. Although success is rare, we continue in the spirit expressed by Robert Louis Stevenson, that to travel hopefully is better than to arrive.

I shall focus on two topics which surfaced in the past few years: first, a slow inhibitory synaptic signal generated by acetylcholine in parasympathetic neurones (Hartzell *et al.* 1977); second, evidence for the hypothesis that in sympathetic ganglia a peptidergic transmitter is responsible for the slow excitatory synaptic potentials which last for 5 min or longer. This last topic in particular is still in its formative stage (Jan, Jan & Kuffler, 1979, 1980).

Some background and aims

Most detailed knowledge of chemical synaptic transmission is derived from the study of peripheral synapses. Skeletal neuromuscular junctions have been of greatest use because there the analysis has been extended to many components of the pre- and postsynaptic transmission process, including the operation of individual ionic channels in the synaptic membrane (Katz & Miledi, 1972). In crustacean nerve-muscle preparations fine details of synaptic inhibition as well continue to be analysed (Onodera & Takeuchi, 1979). The signals at all these nerve-muscle synapses are relatively rapid, in the range of fractions of a second. Analogous fast synaptic potentials with similar ionic mechanisms have been found in all of the various central synapses of all verte-





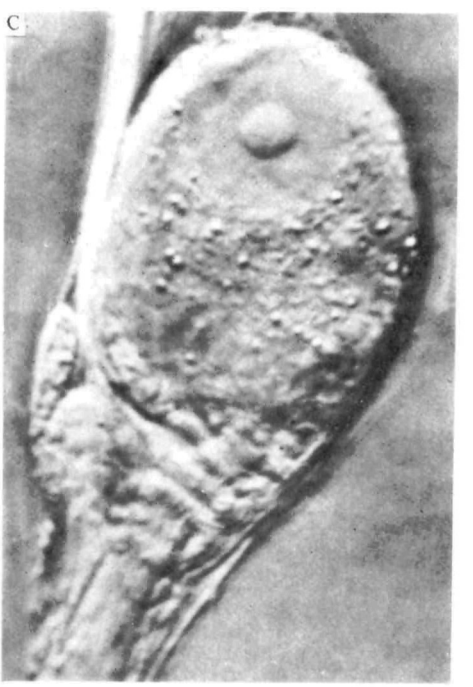


Fig. 1. Parasympathetic neurones in the heart of the frog. (A) Schematic drawing of the heart on left. The atria (A) are divided by a transparent septum which can be removed and examined in detail with differential interference contrast optics (middle). Vagosympathetic nerves (VS) run through the septum. The parasympathetic ganglion cells are seen scattered around the nerves and their branches. To the right is a sketch of a ganglion cell which receives a vagal presynaptic axon that coils around the axon hillock before depositing about a dozen synaptic boutons on the cell body. Schwann cell processes cover all neuronal structures (SN is Schwann cell nucleus). The ganglion cell innervates the heart muscle (M). (B) Cluster of live ganglion cells along a small nerve, several muscle fibres in the lower right. (C) Optical cross-section through a living ganglion cell. One sees the nucleus, nucleolus and various organelles within the cytoplasm. Coil of preganglionic axon and Schwann cell nucleus visible as indicated in sketch (for boutons see Fig. 3A). Neurones are 25–30 μm in diameter (from McMahan & Kuffler, 1971).

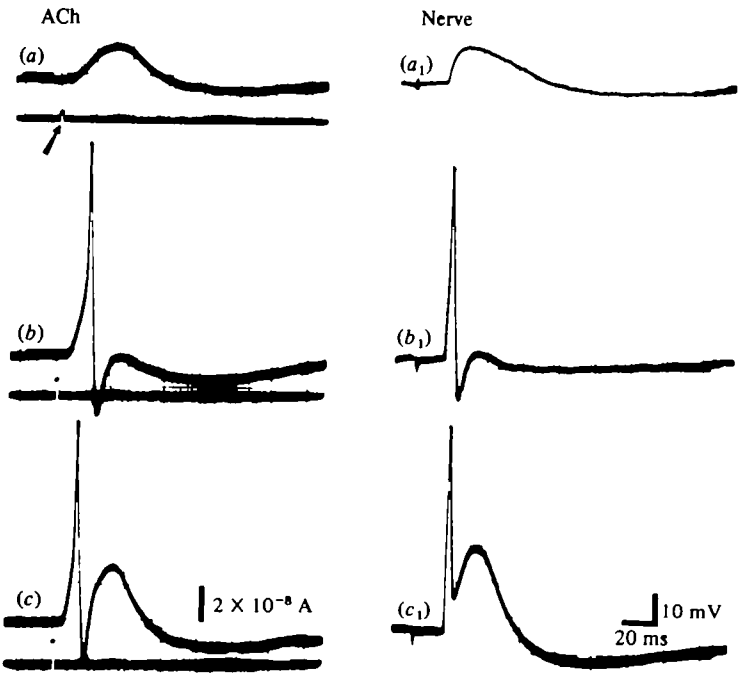


Fig. 2. Mimicking synaptic responses with iontophoretically applied acetylcholine (ACh) to a cluster of synaptic boutons on a cardiac parasympathetic ganglion cell. Left: Progressively increasing current pulses of 1 ms duration through an ACh-filled pipette that touches a synaptic bouton: (*a*) current pulse (arrow) delivers a small amount of ACh and causes subthreshold depolarization; (*b*) more ACh is ejected so that threshold is reached and the impulse is followed by a small residual depolarization; (*c*) suprathreshold response followed by a larger secondary depolarization which is due to ACh surviving after the nerve impulse. These responses are matched to the right by synaptically released ACh: (c_1) initial response after a single nerve stimulus when nerve-released ACh outlasts the action potential; (b_1) a single response during a sustained train of stimuli when less ACh is released and (a_1) when ACh initiates a subthreshold e.p.s.p. only. Full transmission sequence can be repeated after several seconds of rest (see Fig. 4 for more precise mimicking) (from Dennis, Harris & Kuffler, 1971).

brates that have been examined (Eccles, 1964; Katz, 1966; Kuno, 1971; Bennett, Model & Highstein, 1976; Llinas & Nicholson, 1976; Takeuchi, 1977).

About 10 years ago Jack McMahan and I set out to study nerve-to-nerve synapses of vertebrates in a preparation which we hoped would lend itself to as rigorous an analysis as had neuromuscular junctions. After a considerable search we came upon a suitable preparation in the interatrial septum of the frog (McMahan & Kuffler, 1971) (Fig. 1). We soon found out that our discovery had been anticipated by anatomists more than 100 years earlier; in fact, the preparation had been known and studied by Russian physiologists in the 1930s (Lavrentjev & Fedorov, 1934). In any event, in the parasympathetic cardiac neurones of the frog our attention became focused on postsynaptic processes in the vicinity of synaptic boutons which were visible on the cell surface in living neurones (Fig. 3A). As a result, we were able to map the distribution of chemoreceptors in the synaptic and extrasynaptic areas in normal and denervated neurones. Further, we could mimic with reasonable accuracy

the sequence of postsynaptic changes by iontophoresis. An early experiment of this type is shown in Fig. 2 (Dennis, Harris & Kuffler, 1971). This method aimed at obtaining an 'artificial synapse' in which the nerve terminal could be replaced by a transmitter-filled pipette. With such an arrangement one could control quantity and timing of transmitter reaching the subsynaptic membrane without having to rely on the vagaries of the nerve impulse in the terminals.

Eventually, with Doju Yoshikami we further improved the methodology and did more accurate analyses of synaptic events in skeletal neuromuscular junctions of the snake in which the motor axons at the endplate form terminals which in shape and fine structure closely resemble boutons on neurones. In Fig. 3 we can compare neuronal and neuromuscular boutons. In Fig. 3A the optics are focused on a rather prominent neuronal bouton, while 3B shows a cross-section through such a bouton, with all the features of a chemical synapse. Figure 3C gives a face-on view of an endplate in which one sees the bouton-like terminals embedded in the muscle surface in a ball-and-socket-like arrangement. A section through one of these boutons (Fig. 3D) again displays the characteristics of a chemical neuronal synapse, such as mitochondria and synaptic vesicles facing a synaptic cleft. The inset (in Fig. 3D) shows a micrograph of a pipette whose tip opening was about 500 Å. The tips of such pipettes can be placed accurately at the edge of a synaptic cleft for the release of ACh onto the subsynaptic membrane. Such pipette-release can rather faithfully reproduce in size and time course (left, Fig. 4) a miniature synaptic potential caused by a spontaneous nerve-released quantum of transmitter (right, Fig. 4). The number of ACh molecules that mimicked the action of a quantum was determined with an accuracy of several thousand. This showed that on the average fewer than 10000 molecules of ACh produced quantal effects. Since the number of quanta causing an e.p.s.p. could be measured, the method provided quantitative details about the amount of transmitter released by nerve impulses (Kuffler & Yoshikami, 1975*a*, *b*). The endplate of the snake also gave clues about the kinetics of transmitter-receptor activation (Hartzell, Kuffler & Yoshikami, 1975).

The experiments on the cardiac ganglia of the frog and the nerve-muscle junctions in the snake were quite satisfactory, in particular for bringing into line some essential elements in neuromuscular and neuronal synaptic transmission. They affirmed once more the general validity of the earlier studies on fast e.p.s.p.s in skeletal muscles, particularly those by Katz, Fatt, del Castillo, Miledi and their colleagues. Yet the approach fell short of some of our objectives, which included inhibitory transmission and slow processes at synapses. The study of such events in vertebrates previously had been confined mainly to sympathetic ganglia, as we shall see. Much of our basic information, however, derives from molluscan neurones, particularly in *Aplysia*, which have continued to reveal many novel features of neuronal mechanisms that are then frequently found in vertebrates (Tauc, 1967; Parnas & Strumwasser, 1974; Ascher & Kehoe, 1975; Gerschenfeld, 1977).

Around the time when we were searching for a more suitable preparation to explore various slow synaptic signals, McMahan & Purves (1976) made a fortunate discovery in the parasympathetic cardiac ganglion of the mudpuppy (*Necturus maculosus*), another amphibian. Unexpectedly, the organization of this parasympathetic ganglion differed from that in the interatrial septum of the frog, because not only did it contain the anticipated relatively large regular cholinergic ganglion cells (principal cells),

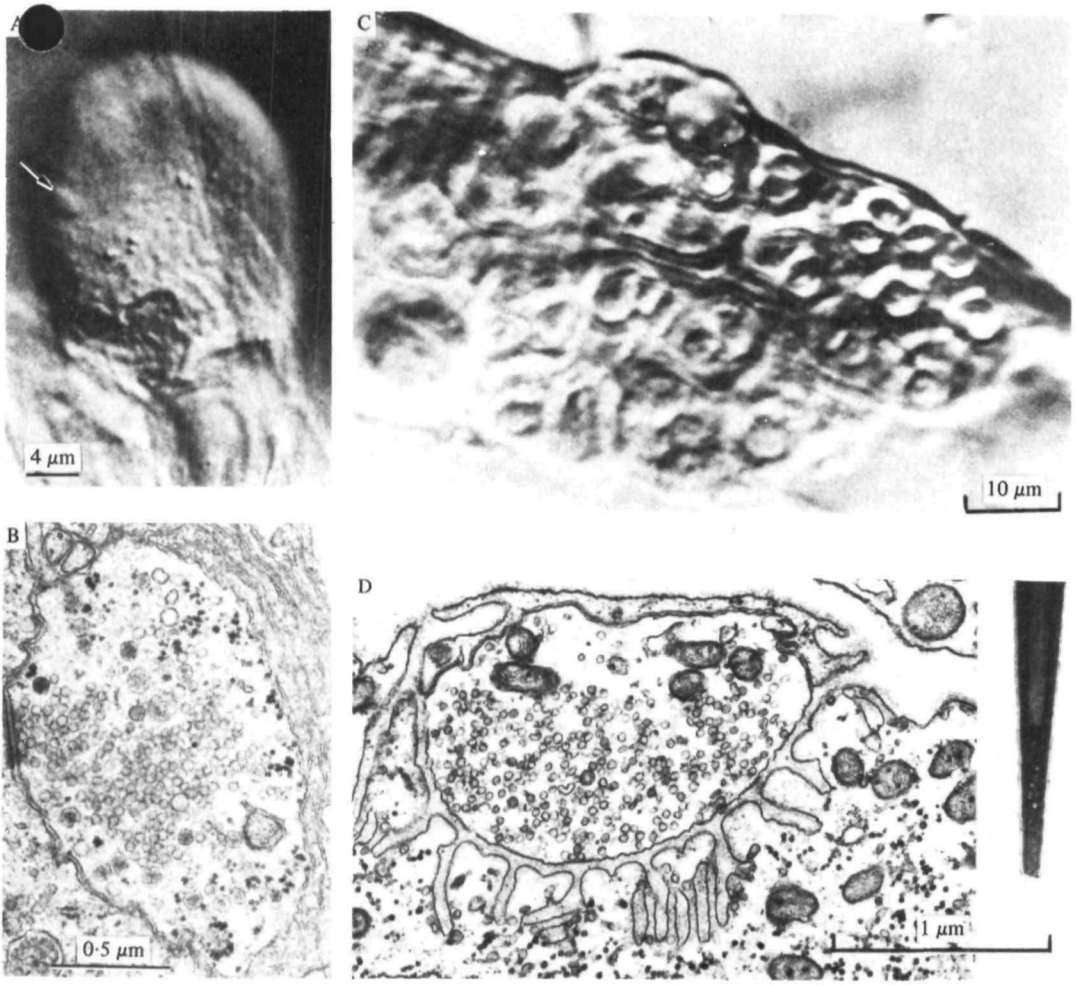


Fig. 3. Synaptic boutons on a living cardiac ganglion cell in the frog and on the surface of a skeletal muscle fibre in the snake. (A) Optics are focused on a prominent synaptic bouton (white arrow upper left) on a ganglion cell. Other boutons are revealed at different focal depth. (B) Cross-section through a synaptic bouton shows characteristic vesicles, mitochondria, pre- and postsynaptic specialization to the left. (C) Endplate region in a living muscle of snake. Individual synaptic boutons rest in craters sunk into the surface of muscle fibre, clearly resolved in upper right portion. (D) Cross-section through one of the boutons with characteristic elements of a chemical synapse. Subsynaptic folds radiate from the wall of the crater. Inset: electron micrograph of a pipette used for application of ACh (same magnification) ([A] and [B] from McMahan & Kuffler, 1971; [C] and [D] from Kuffler & Yoshikami, 1975*a*).

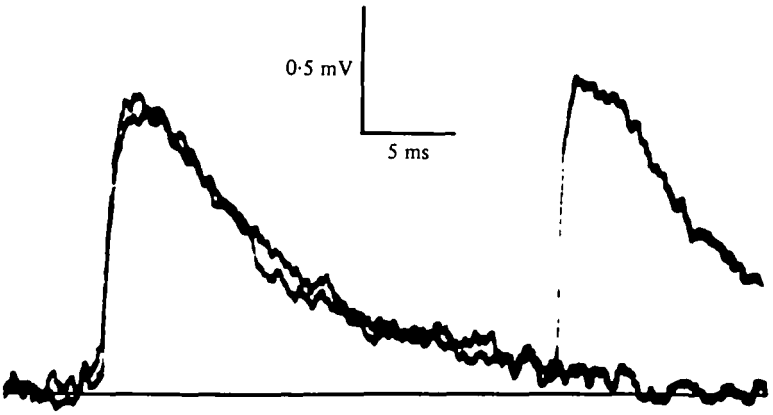


Fig. 4. Matching a miniature synaptic potential produced by a quantum of transmitter with applied ACh at a neuromuscular synapse in the snake. Two 1 ms pulses were passed in succession through an ACh pipette whose tip was accurately placed at the edge of a synaptic bouton (see Fig. 3 D) where the cleft opens (traces superimposed). During one trace a quantum was released spontaneously from a bouton, causing a similar depolarization. Close to 10000 molecules of ACh were released by the pipette to mimic the effect of such a nerve-released quantum (from Kuffler & Yoshikami, 1975*b*).

shown in Fig. 1, but in addition it had small catecholamine-containing neurones (see later, Fig. 5). These morphological findings by McMahan & Purves raised the interesting prospect of finding a second transmitter and a second type of synaptic response in these parasympathetic ganglia.

As we shall see, we did not detect the anticipated adrenergic transmitter, but found that ACh had a dual role in the neurones. Its release caused the usual fast excitation and in addition had an unexpected slow inhibitory action on the same neurones.

A second attempt to find a new transmitter, this time in sympathetic ganglia, proved to be more encouraging and led to the current evidence for peptidergic transmission. Despite such encouragements one is left with feelings of wonderment and even of ambivalence, because the new developments that emerged were rather fortuitous and not the result of forethought and well laid plans. As for myself, the main requirements seemed to be good luck and excellent young colleagues.

Section I

Slow inhibitory responses in a parasympathetic ganglion

Two types of synaptic responses in ganglion cells

The existence in parasympathetic ganglia of two types of cells with different chemistries suggested not only two transmitters but also two functional roles. An analogous cell diversity has been well known for many years in sympathetic ganglia where the catecholamine-containing neurones are called SIF (small intensely fluorescent) cells because they fluoresce strongly when treated by the method of Falck and Hillarp (Falck & Owman, 1965) or with 1% glyoxylic acid (Lindvall & Björklund, 1974) (for a comprehensive series of reviews see Eränkö, 1976). A schematic drawing Fig. 5 by McMahan & Purves (1976) gives the relationship between the two neuronal types in the parasympathetic ganglion of *Necturus*.

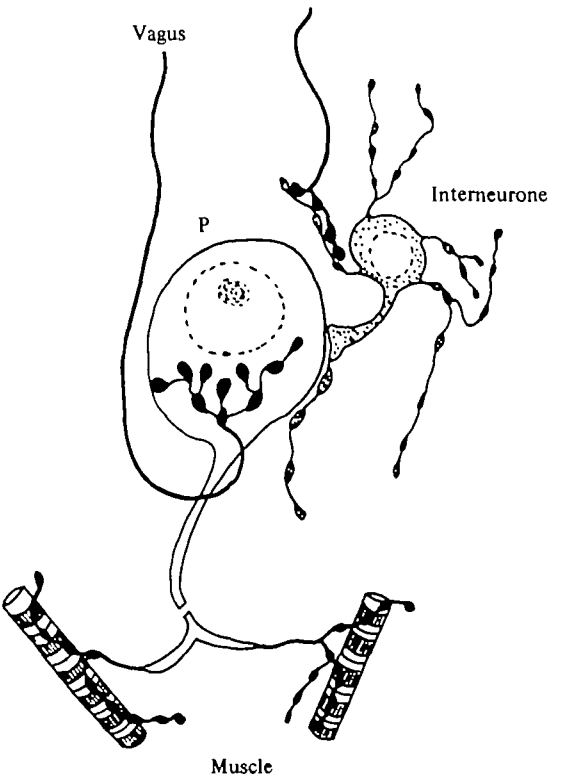


Fig. 5. Sketch of two types of neurones in a parasympathetic cardiac ganglion of the mud-puppy. Principal cell (P) and a catecholamine-containing interneurone (SIF cell). Both receive cholinergic input from the vagus nerve. P cell processes release ACh onto the heart muscle. SIF cells make occasional synapses with P cells (from McMahan & Purves, 1976).

In view of structural similarities with SIF cells (interneurones) in sympathetic ganglia, where a good deal of evidence assigns to them an inhibitory role (Libet & Kobayashi, 1974), we started with the hypothesis that our interneurones were inhibitory. However, we could not find the expected inhibitory effect of the interneurones on principal cells. Individual stimuli to the vagus caused no obvious inhibition (i.p.s.p.s); nor were we successful in evoking i.p.s.p.s after stimulation of interneurones with intracellular electrodes.

A second synaptic potential was eventually obtained from trials with trains of stimuli to the vagus nerve while recording from principal neurones. We had consistently overlooked the fact that the hyperpolarization following vagal stimulation is greater, compared to direct electrical stimulation of the same neurones, as shown in Fig. 6. There apparently existed a nerve-evoked slow hyperpolarizing component. The first task was to see whether this hyperpolarization could be separated from the after-effect of the regenerative impulses.

The demonstration of a synaptic contribution to the hyperpolarization after conducted impulses in Fig. 6 turned out to be straightforward. All one needed was the application of antagonists which differentiate between the nicotinic and muscarinic

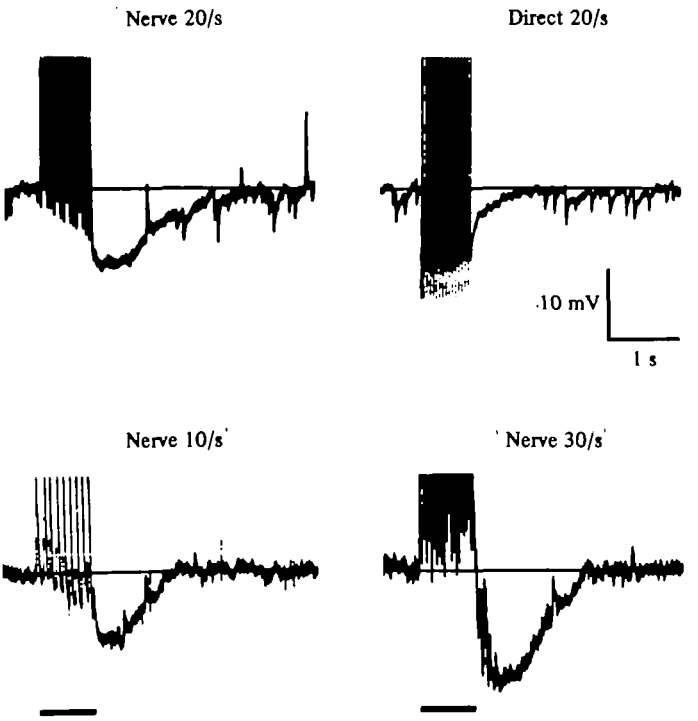


Fig. 6. Synaptically contributed hyperpolarizing component during vagal stimulation of a principal neurone in a parasympathetic cardiac ganglion in the mudpuppy. Trains of stimuli to the vagus nerve at different frequencies cause impulses (upward peak deflexions cut off) followed by hyperpolarization which is smaller if the neurone is stimulated directly through the intracellular electrode (20/s upper right). Large portion of the hyperpolarizations are due to nerve-evoked i.p.s.p.s (see Fig. 7) (from Hartzell *et al.* 1977).

actions of ACh. As expected, the rapid excitatory effect of vagus-released ACh was reduced by the curarizing agent dihydro-β-erythroidine (Roper, 1976*a*), which selectively blocks nicotinic receptors in relatively low concentrations (5×10^{-7} M). This procedure unmasked the underlying i.p.s.p. which had a total duration of about 2 s (Fig. 7A). Conversely, 5×10^{-9} M atropine, which acts on muscarinic receptors, blocked the i.p.s.p.s, leaving the excitatory action of ACh intact (Fig. 7B). This experiment, by separating excitation and inhibition, establishes the involvement of two kinds of receptors – nicotinic ones which initiate e.p.s.p.s and postsynaptic impulses, and muscarinic ones for postsynaptic inhibition.

The transmitter causing inhibition

Additional experiments showed that the muscarinic receptors that activated the ionic channels leading to a generation of slow i.p.s.p.s were localized on principal cells, like those initiating fast e.p.s.p.s. This is indicated by the effect of ACh which was iontophoretically released from a micropipette directly onto the surface of a principal cell (Fig. 8). Such application mimics the responses following a train of vagal stimuli, shown above in Figs. 6 and 7. Pharmacologically, the situation also was

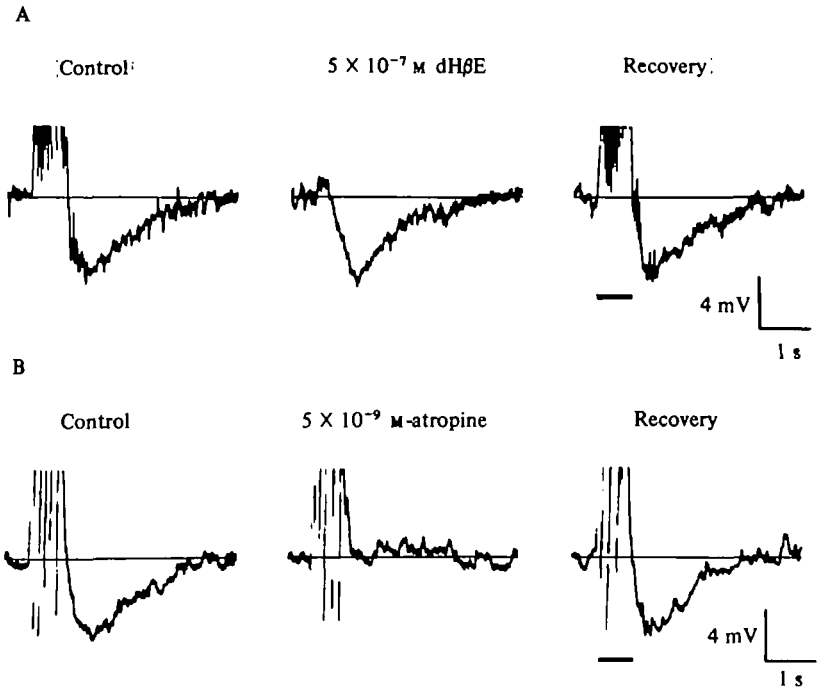


Fig. 7. Separation of inhibitory and excitatory components by cholinergic blockers in neurones of a parasympathetic cardiac ganglion. Stimulation in each record by 15 stimuli to the vagus nerve at 30 s (A). Addition of the nicotinic blocker dihydro-β-erythroidine (d$H\beta$E) selectively abolishes excitation, leaving an i.p.s.p. (B). Atropine, a muscarinic blocker, when applied to the same neurone, eliminates inhibition, leaving excitation intact. Effects are reversible (from Hartzell *et al.* 1977).

similar, because one could block nicotinic receptors by adding dihydro-β- erythroidine to the bath, so that applied ACh caused no excitation but only inhibition (Fig. 8B). Once more, the converse effect was obtained by blocking muscarinic receptors with atropine which did not interfere with excitation of principal cells (Fig. 8B). These results, therefore, are strictly analogous to those after nerve stimulation. The case for a direct inhibitory action of ACh on principal cells was further strengthened by iontophoresing onto principal cells the muscarinic agonist carbamyl-β-methylcholine (bethanechol), an analogue of ACh. This produced a hyperpolarization alone, without excitation.

Although unlikely, inhibition could still have resulted from the muscarinic action of ACh or of bethanechol on nerve terminals of interneurones which release an inhibitory transmitter onto principal cells. This possibility was excluded because inhibition was produced by applied ACh even when transmitter release from all terminals was blocked by reducing the Ca^{2+} concentration in the bath to 0·1 mM and increasing Mg^{2+} to 5 mM or more, or when release was blocked by 10 mM of cobalt (Hartzell *et al.* 1977).

We therefore concluded that nerve-released ACh has a dual action. ACh acts on principal neurones (i) by activating nicotinic receptors, which produce a rapid powerful e.p.s.p. (duration tens of ms) and set up nerve impulses, and (ii) by combining

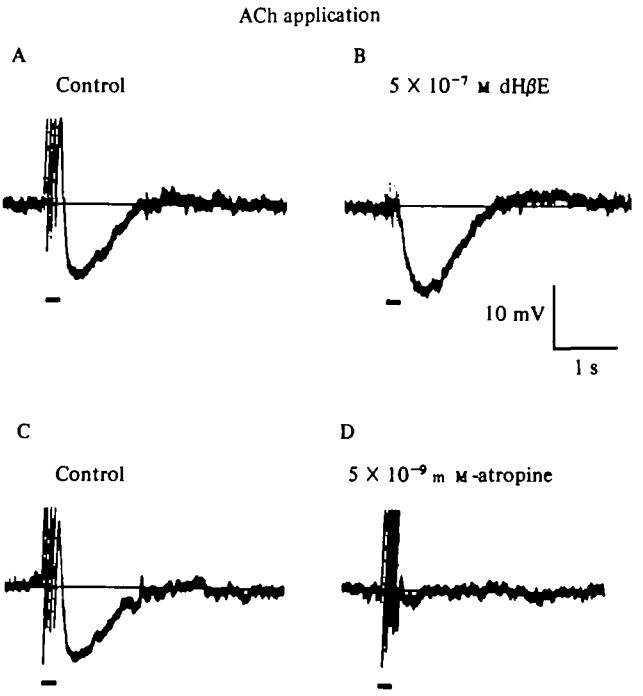


Fig. 8. Separation of excitation and inhibition in a neurone following application of ACh to the cell surface. Applied ACh mimics nerve stimulation (see Figs. 6 and 7). (A) and (C) Six current pulses at 30/s released ACh from a micropipette and set up nerve impulses (peaks cut off). The inhibitory synaptic component becomes evident in (B) after blocking nicotinic excitation by the addition of dihydro-β-erythroidine (dHβE), leaving the slow i.p.s.p. intact. (D) Atropine blocks inhibition, leaving excitation unchanged (from Hartzell *et al.* 1977).

with the muscarinic receptors to produce a much slower inhibition (total duration about 2 s).

Ionic conductances

It was less surprising to find that the ionic mechanism that causes the slow i.p.s.p. is principally the result of an increase in K^+ conductance. The i.p.s.p.s reverse near the equilibrium potential (E_K) for K^+ which is close to -100 mV. The ACh-evoked conductance, however, is voltage sensitive when the membrane potential is shifted between about -40 and -60 mV. At higher values the size of the i.p.s.p.s becomes smaller in proportion to the decrease in driving force on K^+ (Hartzell *et al.* 1977).

The long delay in the activation of i.p.s.p.s

There is a striking difference between the activation times of the conductances that initiate fast e.p.s.p.s and slow i.p.s.p.s. The nicotinic e.p.s.p. in parasympathetic neurones essentially resembles that at skeletal neuromuscular junctions where the ionic channels are activated in a fraction of 1 ms after release of the transmitter (Katz & Miledi, 1965). In the cardiac ganglion of the frog nicotinic responses appear within 1 ms after application of ACh or its release by nerve impulses (Dennis *et al.* 1971), and the situation is similar in the mudpuppy (Roper, 1976*a*, *b*). Depending on the

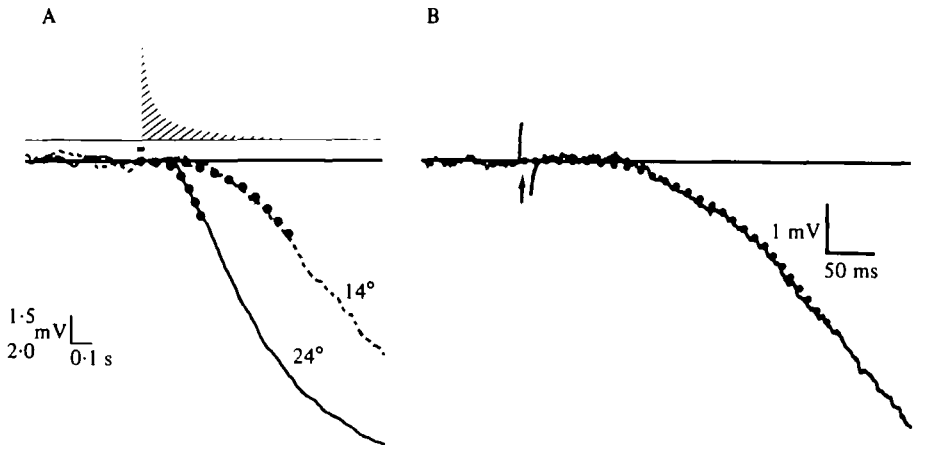


Fig. 9. Slow activation of responses in a parasympathetic neurone and in cardiac muscle of *Necturus*. (A) Delayed onset of muscarinic hyperpolarization after iontophoretic application of the muscarinic agonist bethanechol to the neuronal surface at 24 and 14 °C. Records were taken at high amplification and fast time base for an analysis of the initial portion only of the inhibitory activation. Peak amplitude was 20 mV at 20 °C and 15 mV at 14 °C. Bethanechol application represented by the bar. Calibration 1·5 mV at 14 °C and 2·0 mV at 20 °C. Filled circles indicate theoretical curves assuming that responses are proportional to t^3. The shaded area on top gives the estimated time course of the average bethanechol concentration at the cell surface, calculated from diffusion equations. (B) Similar experiment on the heart muscle of *Necturus*. Response produced by a 5 ms iontophoretic pulse of ACh activating muscarinic receptors (the muscle has no nicotinic receptors). Only the initial phase of a 30 mV peak response is shown. Potential rises after a latent period of about 100 ms. Early phase is proportional to t^3 as in (A). Temperature was 23 °C (from Hartzell *et al.* 1977).

time constant of the membrane, the total duration of e.p.s.p.s is less than 50 ms. Therefore, the nicotinic e.p.s.p. provides a good bioassay for the presence of ACh in the synaptic cleft. This is also borne out by the observation that if the survival of ACh in the cleft is prolonged by an anticholinesterase, the duration of the e.p.s.p. is also prolonged.

In the cardiac ganglia of *Necturus* the relationship between ACh in the cleft and the initiation of e.p.s.p.s and i.p.s.p.s is clearly seen after nerve stimulation of a curarized ganglion in which no conducted impulses are set up. The fast subthreshold e.p.s.p. has actually declined before the i.p.s.p. is detected after a latent period of more than 100 ms. Thus, continued presence of ACh in the synaptic cleft is not needed for the i.p.s.p. to develop.

The initiation of i.p.s.p.s was most conveniently analysed if the muscarinic receptors were activated exclusively by iontophoretically released bethanechol (BCh), without any preceding excitatory nicotinic response. Close examination of the initial portion of the BCh response in Fig. 9 (note high amplification and fast sweep speed) shows a long 'latent period'; i.e. no perceptible hyperpolarization occurs for over 100 ms after release of BCh within a few micra of the cell surface. The estimated decline of the BCh concentration at the cell surface, derived from equations for simple diffusion, is given by the shaded curve (top). The peak concentration of BCh has fallen to about 20% before the i.p.s.p. just begins its slow rise. The initial phase of activation can be described accurately by:

$$\text{response} = kt^3,$$

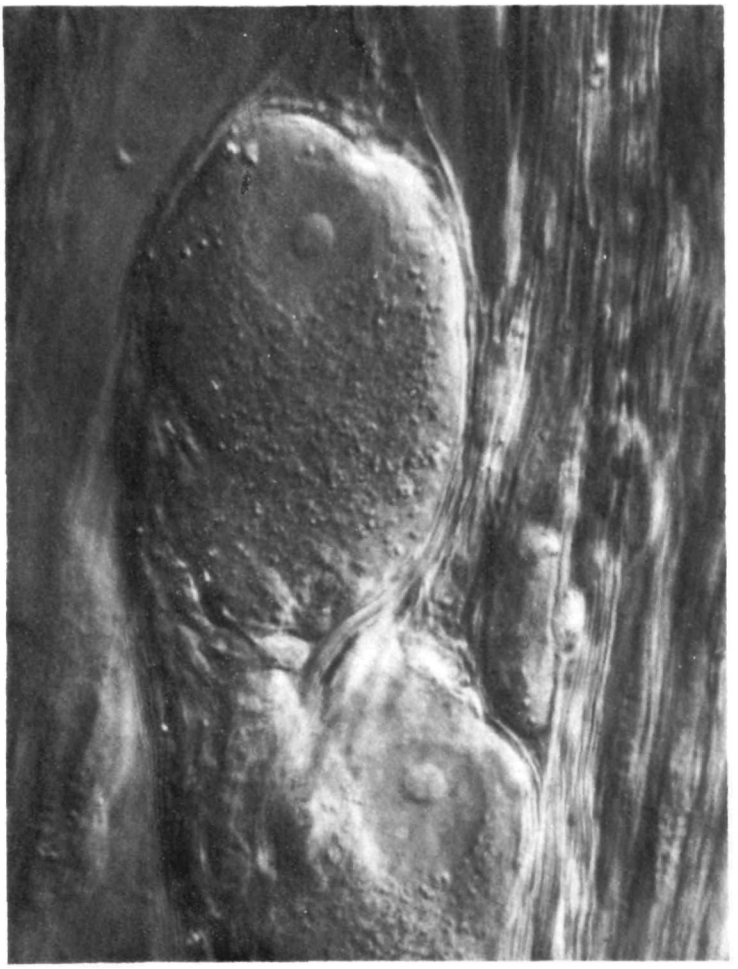


Fig. 10. Live B neurone in a lumbar paravertebral sympathetic ganglion in the bullfrog. Nucleus, nucleolus and assorted organelles are clearly seen with Nomarski optics, and so is a preganglionic axon spiralling around the axon hillock. In the lower right is a smaller C neurone. Diameter of B cell is about 30 μm. (Photograph kindly provided by Larry Marshall.)

where t is time and k is the apparent rate constant for activation of the inhibitory conductance increase. The full circles show the theoretical curves, assuming that responses are proportional to t^3. The value for k is eleven times greater at 24 °C than at 14 °C. Together with evidence that released ACh reaches the nicotinic receptors within a ms, the third order dependence on time indicates that diffusion plays no significant role in the long 'latent period' or activation of the i.p.s.p. Moving the BCh-releasing pipette by 10 μm or so increases the distance of diffusion to the cell surface but makes only a small expected difference in these times, further strengthening this conclusion. We therefore suggested that a series of three or more reactions underlie the activation of the inhibitory conductance increase (for details see Hartzell *et al.* 1977).

It was of considerable interest that the initial phase of the inhibitory activation of cardiac muscle fibres by ACh in the vicinity of the parasympathetic ganglion cells of *Necturus* also fitted the same cubic equation (response = kt^3). The response in muscle is simpler, since there are no nicotinic receptors on the surface of muscle fibres. Inhibition alone can be produced by brief pulses (several ms) of ACh. In Fig. 9B, which shows only a small early phase of the rising portion of the hyperpolarization, a peak of 30 mV is eventually reached. The early phase of the response is again proportional to t^3 and therefore the kinetics of the events leading to the activation of conductances via muscarinic receptors may be similar to those in neurones. Long latencies and slow activation have been observed also in smooth muscles (Purves, 1974; Hill-Smith & Purves, 1978; Bolton, 1979), and similar kinetics of activation were recently seen by Pott (1979) in guinea-pig atria in which the early phase of the hyperpolarization after vagal stimulation was proportional to t^3 and had a Q_{10} of about 12.

Section II

Slow synaptic responses in sympathetic ganglia

The unexpected finding of slow inhibitory synaptic responses in addition to the usual fast e.p.s.p.s in individual neurones of the cardiac ganglion of the mudpuppy raised our curiosity about the other branch of the autonomic system, the sympathetic ganglia. These have been known for their complexity for several decades. The likelihood of synaptic inhibition has already been indicated by studies in ganglia of the turtle by Laporte & Lorente de Nó (1950). Our current investigations have as their background the pioneering work from many laboratories, particularly those of Libet & R. M. Eccles, Nishi & Koketsu, Weight, and others (for reviews see Skok, 1973; Nishi, 1974; Volle, 1975; Ginsborg, 1976; Kuba & Koketsu, 1978; Libet, 1978).

For us, the greatest attraction of sympathetic ganglia in the frog was that these ganglia produced four distinct types of signals. Three of these are generated by mechanisms of which we have only a fragmentary understanding. The ganglia are not simple relays for excitation: several types of signals are generated in individual neurones and the manner of their integration is of considerable interest. Further, the transmitter for the slowest signal is not ACh.

We chose to work on sympathetic ganglia of the bullfrog because the preparations survive well in isolation, are accessible, and are relatively simple. In addition, much

background knowledge was available. Individual neurones in the thinner portions of ganglia, or at their edges, can be readily impaled with microelectrodes as one views them with Nomarski interference contrast optics, as was possible in cardiac ganglia (Figs. 1 and 3, and McMahan & Kuffler, 1971). An example of a neurone, photographed by Larry Marshall, is shown in Fig. 10.

Innervation patterns and variety of synaptic responses

Much interest is added to sympathetic ganglia by combined structural and physiological observations on their innervation patterns. These have revealed specific functional central projections to the ganglia, as indicated in Fig. 11 A which shows the last four paravertebral ganglia. The neurones in the ganglia, called principal cells, fall into two groupings, according to size (Fig. 11 B). The larger B cells in bullfrogs, with an average diameter near 50 μm, have fast conducting axons; and the smaller C neurones, about 20–30 μm in size, give rise to much more slowly conducting smaller fibres. Some of the central organization, linked with the innervation of ganglion cells, seems related to cell size and also to the chemistry of preganglionic axons. For example, all cells in ganglia 9 and 10 receive a cholinergic innervation, but the preganglionic fibres for the large B cells leave the spinal cord through roots above the 7th ganglion. The cholinergic innervation to the smaller C cells in the same two ganglia, however, emerges from the spinal cord almost exclusively through roots 7 and 8 (Nishi, Soeda & Koketsu, 1965; Libet, Chichibu & Tosaka, 1968). The latter roots contain in addition small non-cholinergic axons which supply all neurones in ganglia 9 and 10 (see later). For our experiments it was particularly important that B cells can be excited separately from two converging sources: first, by stimulating ACh-releasing axons in the ganglionic chain above ganglion 7; and second, by stimulating another set of smaller fibres leaving through roots 7 and 8 which do not release ACh onto B neurons. The cholinergic axons of roots 7 and 8 are predominantly destined for C cells in ganglia. The scheme in Fig. 11, which is likely to need modification in the future, omits the SIF cells because their connexions and exact relationship to the ganglion cells have not yet been worked out. In the frog, at least, their functional role remains obscure.

The four types of distinct synaptic responses in ganglion cells following stimulation of preganglionic fibres are seen in Fig. 12. Each has a different time course and there are also distinctive pharmacological differences: (1) there is the universal type of rapid excitatory response lasting tens of ms, the fast e.p.s.p. which results from the release of ACh and initiates impulses in all ganglion cells. It resembles in its time course and ionic properties the fast e.p.s.p.s elsewhere (Nishi & Koketsu, 1960). Neurones are usually supplied by more than one axon. The left record in Fig. 12 A shows first a subthreshold fast e.p.s.p. with a single nerve stimulus. If the stimulus strength is increased, bringing in another axon, a second e.p.s.p. is added, initiating an impulse. The e.p.s.p.s result from activation of nicotinic receptors which are blocked by dihydro-β-erythroidine, as we have seen with the fast e.p.s.p.s in neurones of the mudpuppy (Figs. 7 and 8). (2) The slow inhibitory synaptic potential recorded from C cells lasts about 2 s. Its time course resembles the i.p.s.p.s in the ganglia of the mudpuppy and is evoked, directly or indirectly, by ACh, because it is blocked by muscarinic antagonists such as atropine. The i.p.s.p. in Fig. 12 B was set up by

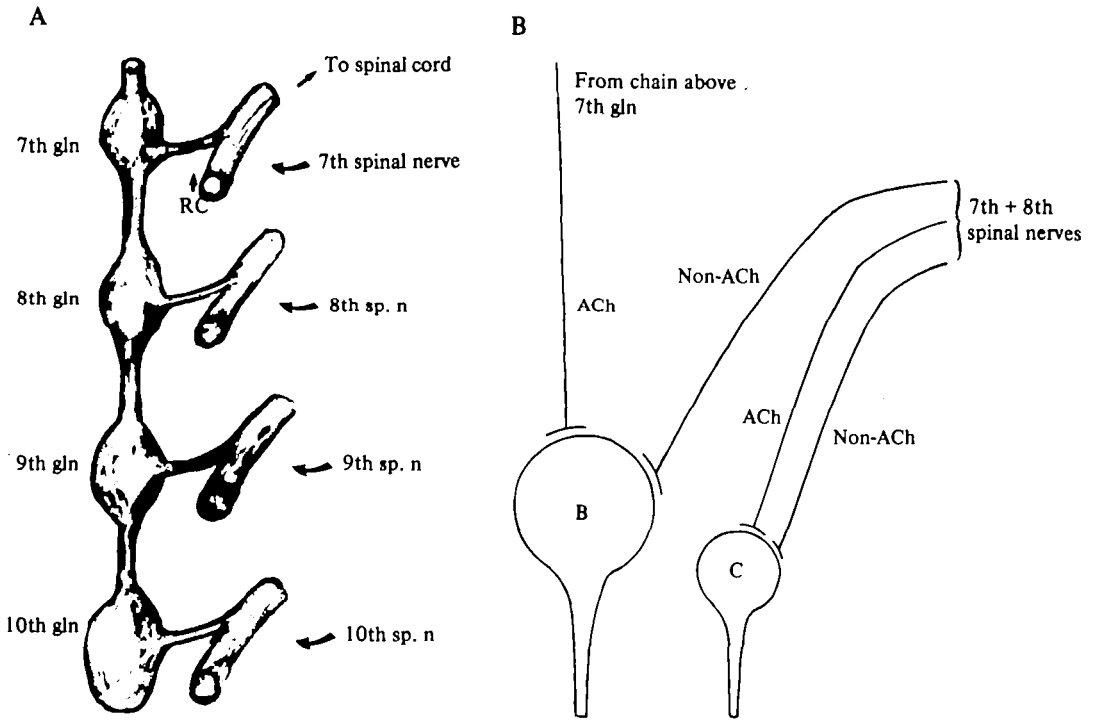


Fig. 11. (A) Sketch of the last four paravertebral lumbar sympathetic ganglia in the bullfrog. Preganglionic nerve supply leaves through the spinal roots into the spinal nerves and enters the ganglia through the rami communicantes (RC). (B) Innervation pattern of the larger B and smaller C neurones in the 9th and 10th ganglia. The catecholamine-containing interneurones are omitted. Note segregation of innervation. Outflow through roots rostral of ganglion 7 supplies predominantly cholinergic axons for B neurones in ganglia 9 and 10, while the non-cholinergic (peptidergic) axons come from the 7th and 8th nerves which supply cholinergic innervation for all the C neurones. The cholinergic and peptidergic axons for B cells can, therefore, be stimulated through separate nerves (from Jan *et al.* 1979).

13 stimuli at 20/s to the central portions of the 7th and 8th spinal nerves. The fast e.p.s.p.s and resulting impulses are not seen because they were almost completely blocked by dihydro-β-erythroidine. (3) Record C shows a slow e.p.s.p. of 30 s duration after four stimuli at 50/s. It is preceded by rapid deflexions which are four conducted impulses. It also is initiated by ACh, acting on muscarinic receptors on ganglion cells. (4) The late slow e.p.s.p. of Nishi & Koketsu (1968) in Fig. 12D lasted about 5 min after a train of 50 stimuli at 10/s. These are the only synaptic potentials in the ganglia of the frog that are known to be produced by a non-cholinergic transmitter.

Apart from the great spread in duration, by factors of 10000–20000, the most conspicuous feature of the three slow signals is their relatively small size. When the membrane potentials are 'at rest', near -50 to -60 mV, their peak amplitudes rarely exceed 10 mV. Another essential point is that all neurones can generate three types of synaptic potentials. Thus, we have regularly recorded, as have others, all three types of the excitatory e.p.s.p.s in B neurones. In C neurones we have registered the fast cholinergic e.p.s.p.s and slow i.p.s.p.s as well as the non-cholinergic late slow e.p.s.p.s. Such experiments show that ganglionic neurones offer many advantages for an

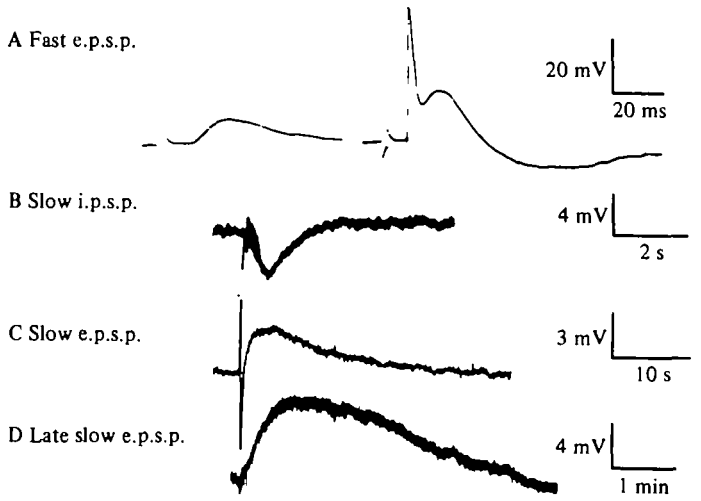


Fig. 12. The four types of synaptic responses recorded in neurones of the 10th sympathetic ganglion with intracellular electrodes. (A) Single preganglionic stimulus produces a fast subthreshold e.p.s.p. (left); stronger stimulus brings a second axon, producing a larger e.p.s.p. and an impulse. (B) Slow i.p.s.p. in a C neurone (lasting 2 s) on stimulation of the central portion of the 7th and 8th spinal nerves with 13 stimuli at 20/s. Fast e.p.s.p.s were blocked with dihydro-β-erythroidine. (C) Slow cholinergic e.p.s.p. lasting 30 s results from four stimuli at 50/s to synaptic chain above ganglion 7. The initial rapid deflexions are four large nerve impulses (cut off). (D) The late slow e.p.s.p. (peptidergic) lasts about 300 s on stimulation of the central portions of spinal nerves 7 and 8 (50 stimuli at 10/s). No drugs used except in (B) (from Jan *et al.* 1979).

analysis of integrative mechanisms, particularly if one understands their mode of generation and the action of the transmitters that are involved (see Discussion).

Evidence for a peptidergic transmitter

After Nishi & Koketsu (1968) discovered the late slow e.p.s.p.s they obtained evidence that these potentials were not significantly affected by nicotinic and muscarinic blocking agents, nor by cholinergic agonists. They concluded that ACh was not the transmitter producing these slow synaptic potentials. Since ACh had been the only known transmitter in autonomic ganglia for close to five decades (Feldberg & Gaddum, 1934), the possibility of finding a new one seemed attractive (see Burnstock, 1979). When Lily and Yuh Nung Jan joined the laboratory in 1977, we decided to attempt a systematic chemical approach which we knew might be highly uncertain in its outcome.

Before embarking seriously on chemical tests on the sympathetic ganglia, we confirmed Nishi & Koketsu's findings and then surveyed the effects of a series of known transmitter candidates. For this survey we recorded the various synaptic potentials within ganglia by extracellular suction electrodes, leading off from a post-ganglionic branch close to where it emerges from the ganglion. The suspected transmitters were then added to the bathing fluid to see whether they produced a depolarization resembling the late slow synaptic potential, or whether they modified nerve-evoked potentials. Among the agents tested were: gamma aminobutyric acid (up to 10 mM); D, L-octopamine (0·02 mM); ATP (1 mM); serotonin (0·01 mM); and also serotoni

blockers (0·05 mM methylsergide maleate and liserdol) and α- and β-adrenergic blockers (0·5 mM phentolamine and propranolol). These agents gave no striking positive results with respect to their action on the cell membranes or on nerve-evoked responses.

In the hope of avoiding laborious chemical analyses we took a chance on another short-cut by testing various peptides, partly because substance P, an undecapeptide, has been for some time a transmitter candidate in the spinal cord (Otsuka & Takahashi, 1977),* in the gut (Katayama, North & Williams, 1979), and possibly in the central nervous system (Krnjević 1977). Substance P has also been found immunohistochemically in sympathetic ganglia in the chicken (Hökfelt *et al.* 1977). We had been given many of our peptides by Drs Vale and Rivier in the Guillemin laboratory at the Salk Institute, shortly after they had originally been synthesized. After having kept them in the freezer for several years, we tested Vale and Rivier's peptides as well as some commercially available ones, including substance P, neurotensin, bombesin, somatostatin, thyrotropin releasing hormone, and angiotensin I and II. Up to concentrations of 0·1 mM none of these mimicked the action of the nerve-evoked late slow e.p.s.p.s. Only one peptide, the decapeptide luteinizing hormone releasing hormone (LHRH), had some effect in threshold concentrations of 1 μM.

A sample of a test is given in Fig. 13 which first shows a nerve-evoked late slow e.p.s.p. (recorded extracellularly) resulting from 25 stimuli at 5/s to the central portion of spinal nerves 7 and 8. In this test the cholinergic fast e.p.s.p.s (and the conducted impulses) had been blocked by 1 μM dihydro-β-erythroidine. Then a high concentration of 5×10^{-5} of LHRH was added to the bath, causing a slow depolarization during which the effect of nerve stimulation was reduced, compared to the control. This test did not seem particularly promising, because we thought that the required concentrations were too large for a transmitter or hormone.

Fortunately, at that critical stage Lily and Yuh Nung Jan recalled that in mammals several analogues of LHRH were 10–100 times more potent in releasing gonadotropins from the anterior pituitary (Rivier *et al.* 1976). A test of two of these, [D-Ala^6]-LHRH and ethylamide [D-Trp^6,Pro^9]-LHRH, showed that, as in mammals, they were about 100-fold more potent than LHRH, acting in concentrations of about 10^{-8} M. A test with bath application of one of the analogues is given in Fig. 14A. At a 100 × threshold concentration a ceiling depolarization is reached in less than 1 min. If nerve stimulation was repeated during the depolarization by the analogue (second full triangle), its effect was completely suppressed. The suppression was not due to a generalized blocking action on nerve terminals, because at that time ACh was still released and fast e.p.s.p.s appeared on stimulation of the larger rapidly conducting preganglionic axons. To decide whether the slow depolarization was due, at least in part, to a postsynaptic action we removed Ca^{2+} from the Ringer solution and added 4 mM Mg^{2+} to block release of a transmitter. Under such conditions all nerve stimulation became ineffective, while direct application of the analogue still depolarized (Fig. 14B). The finding does not exclude a presynaptic action but assures one that much of the LHRH effect is postsynaptic.

* The case for substance P has been greatly strengthened recently by Konishi, Tsunoo & Otsuka (1979) who found the substance in sympathetic ganglia of the guinea pig where it fulfils several of the requirements for a transmitter. See also Otsuka and Yonagisawa, *this volume*.

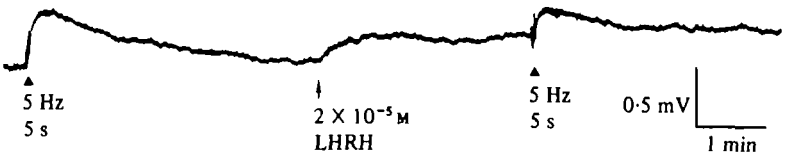


Fig. 13. Effect of luteinizing hormone releasing hormone (LHRH) on a ganglion, recorded with suction electrodes close to the 10th ganglion where a nerve branch emerges. Preganglionic stimuli (15 at 5/s) to the central portion of spinal nerves 7 and 8 produce a slow e.p.s.p. of several minutes (left). Addition of LHRH to the bath also causes a long lasting depolarization (middle) during which repetition of the nerve stimulation is reduced. The nicotinic synaptic potentials and impulses were blocked with 10^{-6} M dihydro-β-erythroidine (from Jan *et al.* 1979).

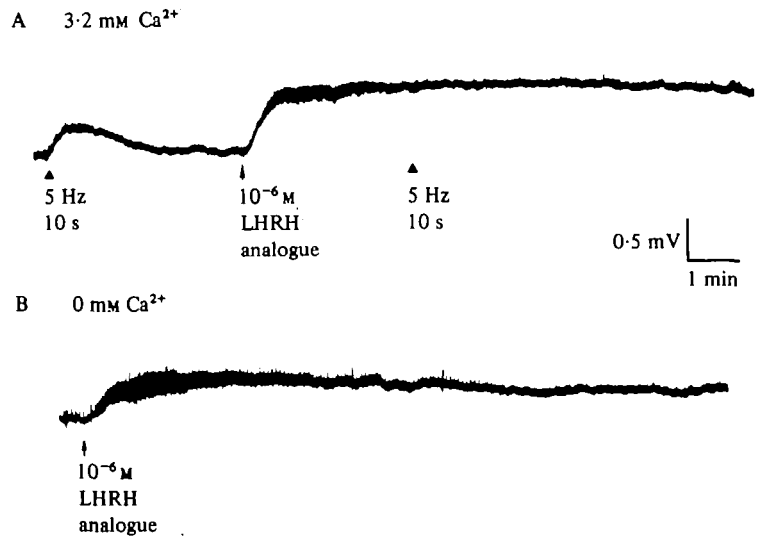


Fig. 14. An LHRH analogue acts postsynaptically on sympathetic ganglion cells. (A) Nerve-evoked slow response followed by bath application of ethylamide-[D-Tryp^6 Pro^9]-LHRH (about 100 × threshold concentration) causes long lasting depolarization during which repetition of nerve stimulation is ineffective. ACh is still released on stimulation of cholinergic axons. (B) Release of transmitters was blocked by reducing Ca^{2+} and adding Mg^{2+}. Analogue still depolarizes ganglion cells. Extracellular recording (from Jan *et al.* 1979).

At this stage the experiments merely showed that LHRH and some of its analogues did have a depolarizing effect on the membranes of ganglion cells. To consider seriously a hypothesis for a new transmitter the peptide had to be present in the tissue. We therefore used radioimmunoassays (RIA), the most sensitive available tests for LHRH. The assays turned out to be positive, as shown in Fig. 15. The standard curve (Fig. 15A) was done with iodinated antiserum for LHRH to which unlabelled LHRH was added (Nett *et al.* 1973). This showed that it noticeably displaced ^{125}I-LHRH from sites on the antibody in quantities of several pg. (The LHRH specific antiserum was kindly provided by Dr T. M. Nett of Denver.) Addition of some of the analogues also produced an inhibition curve (open squares) but, compared with LHRH, much more material was needed for an equivalent displacement of ^{125}I-LHRH from the antibody. For example, [D-Ala^6]-LHRH, which in the bioassay was 100 times mor

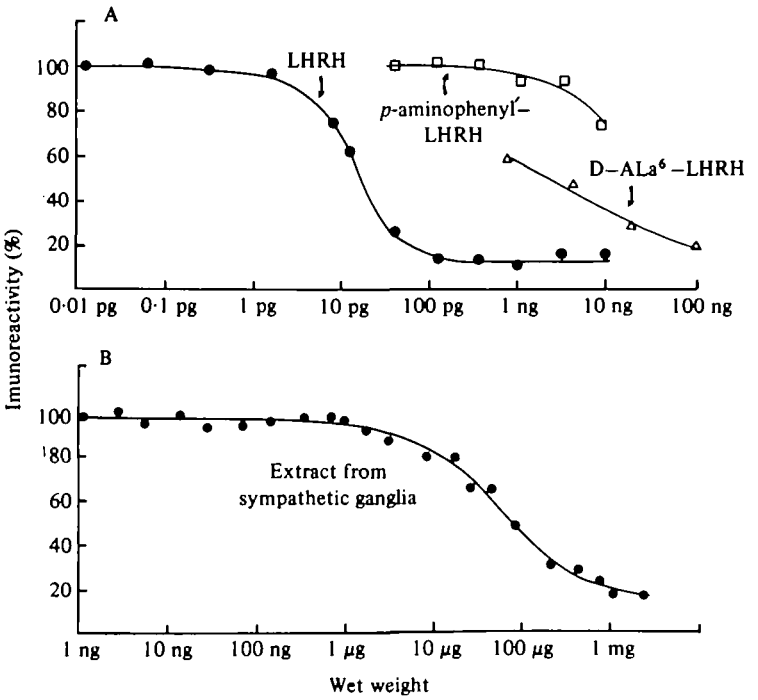


Fig. 15. Radioimmunoassay (RIA) for LHRH-like material in ganglia. (A) Standard curve shows displacement of ^{125}I-LHRH from antibody by unlabelled LHRH (full circles). Sensitivity is several pg. Also shown is the much lower immunoreactivity (by a factor of 100 and 1000) of two analogues of LHRH. Several other analogues gave no detectable reactivity. (B) Competition for binding sites on antibody by an extract from sympathetic ganglia. Immunoreactivity of ganglia indicated that between 100 and 800 pg of LHRH-like material per mg of wet weight is contained in ganglionic tissue (from Jan *et al.* 1979).

active (10^{-8} M) than LHRH, showed about 100 times less immunoreactivity; while ethylamide-[D-Trp^6,Pro^9]-LHRH, equally active biologically, was about 6000 times less effective in competing for sites on the antibody. A series of other peptides, including those initially tried in our bioassay, showed no immunoreactivity, or less than one part in 60000 compared with LHRH.

If we replaced unlabelled LHRH with extracts from sympathetic ganglia, the inhibition curve of Fig. 15B was seen. One hundred per cent in both curves expresses the amounts of ^{125}I-LHRH without an addition of extracts of unlabelled LHRH. By comparing the midpoints of the two curves of Fig. 15 one can estimate the total immunoreactivity of the ganglionic extracts, which in this example was 260 pg of LHRH-like material for 1 mg of wet tissue. The activity of sympathetic chains, consisting of the last four paravertebral ganglia, fluctuated between 100 and 800 pg/mg of wet tissue. (The average chain weighed about 1 mg.)

The presence of material resembling LHRH led us to a more serious examination of the hypothesis of peptidergic transmission.

LHRH-like material in preganglionic axons

The first aim was to find out whether the distribution of the immunoreactive material coincided with the distribution of nerves whose stimulation caused the late slow e.p.s.p.s. To obtain these in ganglion 9 and 10 we routinely stimulated the central segments of spinal nerves 7 and 8. The 9th spinal nerve may on occasion make a small contribution, while nerve 10 has never evoked late slow e.p.s.p.s. Radioimmunoassays on extracts of segments between the spinal cord and the rami communicantes, where axons pass into the ganglia (Fig. 11), reflected the physiological findings. The central segment containing the outflow from the 10th roots had no detectable peptide, while the 9th spinal nerve segment only occasionally contained LHRH-like material of 0·2 pg/mg wet weight. In contrast, in central segments of the 7th and 8th nerves we found 3–5 pg/mg wet weight.

The above findings did indicate a peptide distribution in 'appropriate' nerves but did not exclude the possibility of the peptide being confined to adventitious cells, such as Schwann cells or cells in connective tissue or blood vessels. This appeared unlikely because tests on peripheral nerves or other tissues, like skeletal muscles, heart or liver, showed no significant activity suggesting the presence of LHRH-like material. Nevertheless, to be more specific, various denervation experiments were made.

Denervation

It had previously been shown by Jan & Jan (unpublished) that cutting of preganglionic sympathetic nerves was followed by failure of transmission and noticeable degeneration of axon terminals within 5 days. In two frogs the peptide-containing spinal nerves were cut on one side, and so was the ganglionic chain above ganglion 7. After 5 days the LHRH-like material in the denervated ganglia was reduced to 7 pg/mg wet weight, compared with a value of 120 pg/mg on the control sides. The remaining 6% of peptide in denervated ganglia was probably contributed by communicating branches from the contralateral side, as established in several preparations by physiological tests. In five other frogs the 7th and 8th root outflow alone was cut on one side where it enters the rami communicantes (Fig. 11). After 5 days the content of LHRH-like material was increased threefold in the central segment, between the spinal cord and the cut, compared with the normal control side. This accumulation makes it likely that peptide is transported down the axons from the spinal cord, although proliferation of peptidergic fibres central to the cut cannot be excluded. An analogous accumulation of substance P was observed by Takahashi & Otsuka (1975) in ligated dorsal roots of the cat.

The degeneration experiments indicate that the peptide is localized in preganglionic axons and not in other tissues, but they still do not identify the actual axons which contain and release the material (see later).

Some chemical properties of extracts

A few relatively simple tests were also made to gain greater confidence that the LHRH-like material in the extracts was indeed a peptide. Like many other small peptides the material in our extracts was resistant to boiling, while its immunoreactivity was destroyed by proteolytic enzymes such as α-chymotrypsin. In oth

trials, extracts as well as synthetic LHRH were passed through a sephadex column and the eluted fractions were tested by RIAs. The extracts and LHRH both ran closely together, indicating that the LHRH-like material in the extract had a molecular weight near 1000. These tests, then, support our hypothesis that we are dealing with a small peptide, but the experiments do not identify the active component in the extract.

Ca-dependent release of LHRH-like material

One standard test for transmitter release consists in placing tissues in a high K^+ concentration. After ganglia were depolarized by immersion for 20–30 min in isotonic KCl containing Ca^{2+}, about 0·6% of the total LHRH-like material in the ganglia was released into the bathing medium, as detected by RIA. If, however, Ca^{2+} was absent, the depolarizing action of isotonic KCl was not effective in releasing the peptide. No peptide was detected in the bath, just as if the ganglionic chain had been bathed in normal Ringer solution. In preliminary tests Jan & Jan (unpublished) have also detected the release of a LHRH-like peptide after nerve stimulation.

Similarity of physiological action of LHRH and the nerve-released transmitter

Conductance changes

For a substance to qualify as a transmitter it should, on application, produce conductance changes in the postsynaptic membrane identical to those produced by the nerve-released transmitter. For such tests we impaled large nerve cells in the 9th and 10th ganglion, filled a capillary pipette with an opening of about 3–5 μm with 10^{-4} or 5×10^{-4} M LHRH, and placed the tip of the pipette within about 10–20 μm of the target cell. We then applied a pressure pulse of 3–10 s to eject the LHRH. The magnitude, time course and latent period of responses evoked by LHRH depended greatly on the ease of access to the neurone and the duration of pressure application; e.g. the changes were smaller and longer when connective tissue layers covered the cells. Interestingly, for well exposed cells the LHRH responses were often briefer than the nerve-evoked synaptic potentials in the same neurones. The concentrations of LHRH at the cell surfaces are not known, but they must be considerably lower than in the pipette because of dilution in the bathing medium and the continued flow of perfusion fluid over the cell toward the tip of the pipette. In spite of the variations of nerve-evoked and LHRH-evoked responses in different cells, some of the parallel features of conductances seem significant.

A comparison between a nerve-evoked peptidergic e.p.s.p. and an LHRH potential in a large B neurone is shown in Fig. 16. In this example, cholinergic innervation to the B neurone was also stimulated, causing a muscarinic e.p.s.p. which contributed to the first half of the rising phase of the response. Except for the initial part the general features of the potentials are similar.

In earlier experiments synaptic currents during the slow responses were obtained by the use of a manual voltage clamp (Jan *et al.* 1980). Manual clamping, however, does not follow changes in potential more rapid than a few seconds. Subsequently, in collaboration with Terry Sejnowski, conductance changes were measured with an electronic voltage clamp which followed faster changes. A single electrode was used to record the membrane potential and to pass currents into the cell through a bridge

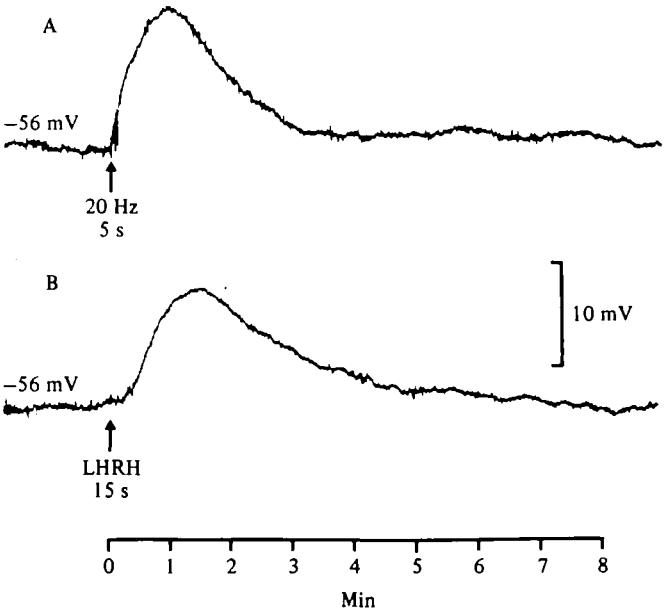


Fig. 16. Responses produced by the nerve-released transmitter and pressure applied LHRH recorded with an intracellular electrode from a sympathetic ganglion cell. (A) Stimulation of the central portion of spinal nerves 7 and 8 by a train of stimuli. In this example a muscarinic e.p.s.p. contributes to the early portion of the rising phase of the response which reaches 13 mV. (B) LHRH pressure application to the same neurone (Sejnowski & Kuffler, unpublished).

circuit. A sudden step in the command voltage produced a jump in the current, which was followed by a slower relaxing component (Brown & Adams, 1980). Because the responses were relatively slow, it was possible to step between two voltages every 5 s, thus allowing the current to reach a steady state over 2·5 s periods before each step. The envelope of the current steps in Figs 17 and 18, therefore, simultaneously provides the slow membrane currents during the responses at two different clamped potentials. The difference between the two currents is directly proportional to the conductance, as illustrated in Fig. 17.

In Fig. 17A the synaptic current is shown following nerve stimulation while stepping between −40 mV and −50 mV. The peak of the slow synaptic inward current during the response is 0·30 nA at −40 mV and 0·21 nA at −50 mV. During the first minute after stimulation the conductance decreased by about 20% and gradually returned to normal over the next 5 min. This means that the input resistance of the neurone that had been 27 MΩ before nerve stimulation increased to 32 MΩ under the influence of the action of the transmitter. The repolarizing jumps in voltage during the initial 1·5 min of the response were followed by impulses which were not present in the controls before stimulation. This indicates that a change in excitability occurred during the response, even though the membrane voltages were not altered (see later). In Fig. 17B the current response is shown in the same cell clamped at the same membrane potentials following pressure application of 5×10^{-4} M LHRH for 7 s. At its maximum, the current response to LHRH was similar to the nerve-evoked synapti

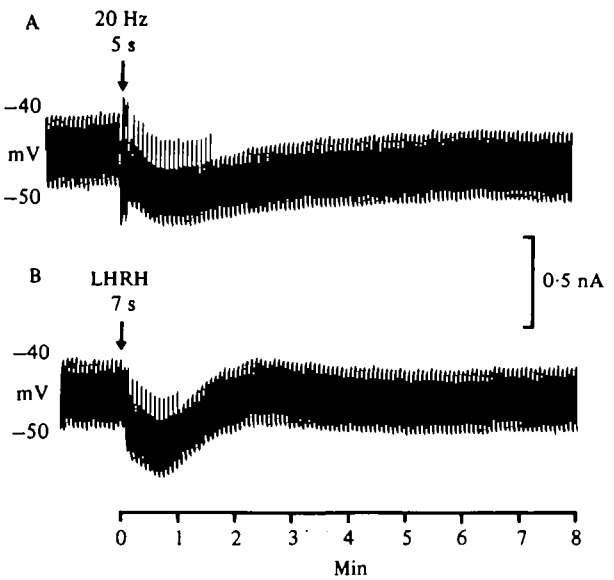


Fig. 17. Current responses in a voltage-clamped sympathetic neurone following a train of nerve stimuli and pressure application of LHRH to a sympathetic ganglion cell. The membrane potential was alternately clamped every 5 s, between −40 and −50 mV. The envelopes of the current steps provide the synaptic inward currents at the two membrane potentials. (A) Current responses following a 5 s train of nerve stimuli at 20/s. Although voltages are clamped, impulses arise during the first 1½ min of the synaptic current. An initial decrease in conductance of about 20 % gradually returns to the control value over about 7 min. (B) LHRH application to the same neurone produces similar membrane currents and impulses during the first minute and a similar decrease in conductance as after nerve stimulation. The time course of the inward currents is much shorter than in A. (Sejnowski & Kuffler, unpublished).

current, including a conductance decrease of 22 % and impulses during the first minute. The time course, however, was much briefer (see also Fig. 18B).

An increased input resistance during late slow e.p.s.p.s has been reported by Schulman & Weight (1976) who suggested that inactivation of the resting conductance for K^+ caused both the late slow and the muscarinic e.p.s.p.s (see also Weight & Votava, 1970). We therefore determined the equilibrium potential for K^+ (E_K) and hyperpolarized the neurones to E_K or beyond. In the majority of tests the size of the late slow e.p.s.p.s increased upon hyperpolarization, while in some it decreased and occasionally reversed. Any response caused by an exclusive conductance change for K^+ should disappear when the membrane potential is at E_K. Although we assume that an inactivation of K^+ conductances does occur, it can only be a portion of the ionic mechanism during these slow responses. In addition to finding conductance decreases during the peptidergic responses, we have measured conductance increases in some cells at hyperpolarized potentials, as mentioned by Kuba & Koketsu (1978). For example, in Fig. 18A the peak synaptic current at −75 mV is 0·5 nA and at −95 mV the peak current is 0·65 nA. At the same time the conductance at the peak of the response increased by 28%, a result that is paralleled by a similar 28% conductance increase following pressure application of LHRH for 3 s, as shown

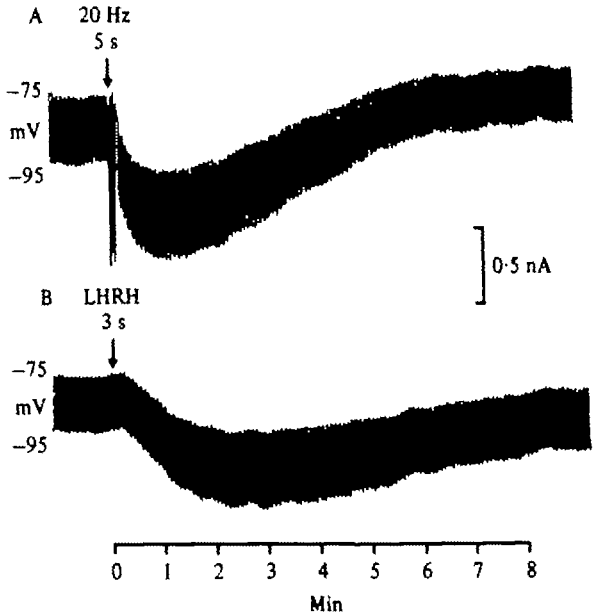


Fig. 18. An increase in conductance at membrane potentials alternately clamped at −75 and −95 mV. (A) A train of nerve stimuli produces a peak synaptic current of 0·5 nA at −75 mV; the conductance increased by almost 30%. (B) Application of LHRH produces similar increases in conductance and an inward current of longer duration in the same neurone (Sejnowski & Kuffler, unpublished).

in Fig. 18B. However, this time the duration of the LHRH response lasted over 8 min compared with about 6 min for the response after nerve stimulation.

The rules and mechanisms governing the conductances underlying the non-cholinergic late slow e.p.s.p.s remain obscure. Nevertheless, the sample results presented in Figs. 17 and 18 seem significant in the present context, because in spite of the variability between responses in different cells, nerve stimulation and peptide application produce parallel changes in individual neurones. This suggests that similar mechanisms are involved (see also later).

An antagonist to nerve-evoked late slow e.p.s.p.s and applied LHRH

In many studies of synaptic transmission, particularly at cholinergic synapses, the availability of specific blockers of nicotinic and muscarinic receptors (see Section I) was indispensable. It was therefore most helpful when Vale & Rivier kindly gave us some of their synthesized analogues of LHRH which blocked the release of gonadotropins (LH and FSH) in cultured pituitary gland cells in the rat and also prevented ovulation (Rivier & Vale, 1978).

A test of one of these LHRH antagonists, [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-LHRH, on sympathetic neurones is shown in Fig. 19 which illustrates nerve-evoked and LHRH-induced depolarizations (Fig. 19A). Addition of the antagonist to the bathing solution (2×10^{-5} M) abolished both responses. The cholinergic responses, the fast nicotinic and slow muscarinic e.p.s.p.s, however, were not significantly changed. This shows that conduction in cholinergic terminals remained intact. Further, the antagonist di

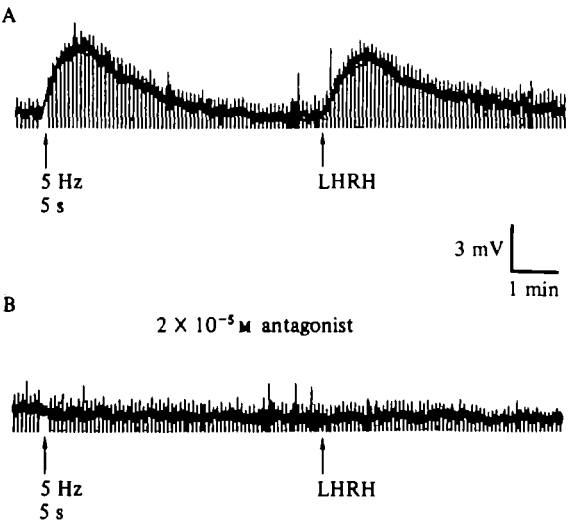


Fig. 19. An antagonist of LHRH which inhibits release of gonadotropins in the rat also inhibits nerve evoked peptidergic synaptic responses and LHRH induced depolarizations when introduced into the bathing solution. The vertical lines are test pulses whose peaks are not shown in the records (from Jan *et al.* 1980).

not appreciably alter the resting conductance of the membrane. The blocking effect, therefore, was confined to the peptidergic action of nerve stimulation.

The site of action of antagonists needs further analysis, because a whole series of reactions is involved in the production of late slow e.p.s.p.s. Interference with any of these intervening steps could lead to a blockage. Since we are dealing with an analogue, however, we suspect that the blocking action is, at least in part, at the postsynaptic receptors for the nerve-released transmitter.

Other analogues

Of the many other available analogues of LHRH (perhaps hundreds), we tested three that had a more potent action in releasing gonadotropins in cultured anterior pituitary cells of the rat and two that were ineffective (Rivier *et al.* 1976). The agonists were: [D-Ala^6]-LHRH; des-Gly^{10}-[D-Trp^6-Pro^9-ethylamide]-LHRH and des-Gly^{10}-[D-Ala^6-N$^\alpha$ Me Leu^7, Pro^9-ethylamide]-LHRH. The ineffective analogues were: des-Tyr^5-LHRH and des-Arg^8-LHRH. Again, there was a corresponding action in our ganglia; that is, the agonists were more effective at depolarizing the neurones at the same concentration than was pressure-applied LHRH. The ineffective analogues did not depolarize the neurones, even at 100 times higher concentrations than was required for LHRH (Jan *et al.* 1980).

Excitability changes and interaction between the peptidergic and cholinergic e.p.s.p.s

One would expect peptidergic e.p.s.p.s to increase the excitability of a cell. First, the depolarization should bring the membrane closer to its firing level. Second, the increased membrane resistance should enhance the effectiveness of currents that

change the membrane potentials. Finally, the actual threshold at which the post-synaptic membrane initiates impulses may be changed.

To measure increased electric excitability subthreshold depolarizing currents were passed through an intracellular electrode. During the e.p.s.p.s and during the applied LHRH depolarizations, such currents usually become effective in starting impulses in neurones. That the increased excitability was not due solely to the depolarizing action of e.p.s.p.s was demonstrated when the experiments were repeated with the peptidergic e.p.s.p.s or LHRH responses manually clamped at the resting potential. The same currents caused larger depolarizations during the clamped responses, as anticipated from the observations on increased cell resistance (Fig. 17).

Of direct physiological relevance are interactions with fast e.p.s.p.s, which represent the standard form of excitatory transmission. Several years ago Schulman & Weight (1976) showed that a subthreshold curarized e.p.s.p. may initiate impulses during late slow e.p.s.p.s. We have confirmed these observations and, as in the case of electric currents, such otherwise ineffective e.p.s.p.s became suprathreshold during the late slow e.p.s.p.

In view of the observed synergistic action with fast e.p.s.p.s it was surprising that cholinergic slow e.p.s.p.s (see Fig. 12C) were greatly depressed if they were evoked during a peptidergic e.p.s.p. The decrease was still seen if the peptidergic response was clamped at the resting potential. The same observation was made if LHRH was applied. This adds one more example of a parallel effect between applied LHRH and peptidergic nerve stimulation.

The basis of the depression of the slow muscarinic e.p.s.p. is not clear. On pharmacological grounds the interaction does not occur at the receptor level, since each e.p.s.p. can be blocked independently of the other, the cholinergic one by atropine and the peptidergic one by an analogue of LHRH. The two types of e.p.s.p.s may share ionic channels or have common processes leading to the activation of conductances (see Discussion).

DISCUSSION

The experiments discussed in this paper are part of our continued attempts to find suitable simple models for complex higher central nervous systems. The promise of the applicability of such models is supported by the realization that the nervous system appears to have maintained, at times with only small modifications, many features of neuronal mechanisms over the course of evolution. Good examples are the squid axon and impulse conduction, synaptic transmission, and the simple features of integration at various neuromuscular junctions or in ganglia of crustacea or molluscs (Kandel, 1976; Kuffler & Nicholls, 1976).

Although there exists in higher centres of vertebrates abundant evidence for a variety of slow potentials and for changes in excitability of seconds and longer, no transmitter-generated synaptic signals with a similar slow time course seem to have been analysed.

The slow parasympathetic i.p.s.p.

Multiple action of one transmitter

In the parasympathetic ganglion of *Necturus* the same transmitter, ACh, has a dual action on individual neurones, by causing excitation as well as inhibition. A different dual mechanism is known in sympathetic ganglia where ACh generates fast nicotinic and slow muscarinic e.p.s.p.s in B cells (Fig. 12), and a dual antagonistic action in C cells has been suggested by Weight & Padjen (1973). Our results are an extension of the well-established finding that the same transmitter may act differently on different cells, such as an excitatory effect of ACh on skeletal nerve-muscle synapses and an inhibitory effect on the heart muscle. One of the means of producing diverse and opposite effects is the provision in different tissues of different receptors in the post-synaptic membrane where receptors control conductances for different species of ions. In the case of our parasympathetic ganglia the nicotinic and muscarinic receptors are distributed on the same cell. In that respect and in their ability to give slow synaptic responses, autonomic neurones resemble some molluscan ganglia (Kandel, 1976). For example, in ganglia of *Aplysia* three types of cholinergic receptors have been activated (Kehoe, 1972).

The slow activation of conductances

A striking feature of the slow i.p.s.p.s is the long time it takes to activate the ionic channels, compared with fast e.p.s.p.s. On the assumption that both nicotinic and muscarinic receptors are subsynaptic, ACh should reach the two types of receptors simultaneously. The nicotinic receptors cause an opening of channels for Na^+ and K^+ ions within a ms of release of ACh, as measured by the rise of the fast e.p.s.p. The fast e.p.s.p. provides a good bioassay for the presence of free ACh in the synaptic cleft. Even if traces of ACh did persist for the entire duration of fast e.p.s.p.s, which is unlikely, all of the transmitter should have disappeared within 50 ms; yet a noticeable activation of the muscarinically controlled K^+ channels does not start for over 100 ms. The kinetics of the activation process are therefore different. Our experiments suggest at least three intermediary reactions between the time ACh reaches muscarinic receptors and the opening of K^+ channels. We still do not know the nature of the intermediary processes or the basis for the long duration of the i.p.s.p.s. In this respect the similarity in time course between the activation of muscarinic inhibitory channels in cardiac muscles and in parasympathetic neurones of *Necturus* is of interest, since it suggests a similarity in underlying mechanisms. Analogous long times of activation of K-dependent conductances have been reported in molluscan neurones (Ger, Katchman & Zeimal, 1979); in atria of the guinea-pig (Pott, 1979); and in smooth muscles (Purves, 1974; Bolton, 1976). For a review on comparative aspects of slow synaptic responses see Kehoe & Marty (1980).

Another unsolved question is the role of the catecholamine-containing interneurones which are likely to correspond to the SIF cells of sympathetic ganglia (Eränkö, 1976). In mammalian sympathetic ganglia there is evidence that the SIF cells have an inhibitory role (Libet & Kobayashi, 1974), a view not shared by Weight and his colleagues (Weight & Padjen, 1973) whose evidence, however, refers to frogs. The fact that our inhibitory potentials are not a result of interneuronal activity does not

decide this more general issue. It makes the parasympathetic ganglia of *Necturus* more interesting, however, because one can expect to find an additional transmitter, presumably with a different role. Roper (1976*a*) has already shown that SIF cells can be excited by vagal stimulation, and Hartzell, Kuffler & Yoshikami (unpublished) showed that they were depolarized by ACh.

The case for a peptidergic transmitter

The hypothesis that a peptide resembling LHRH is released onto sympathetic ganglion cells in the frog rests largely on the following chemical and physiological evidence by Jan *et al.* (1979, 1980):

(1) An LHRH-like peptide with a molecular weight around 1000 daltons is contained in ganglia and is concentrated in those preganglionic nerves whose stimulation initiates the late slow e.p.s.p.s. (2) The peptide is released in isotonic K^+ solutions which depolarize the ganglion cells, but no release occurs in the absence of Ca^{2+} from the high K^+ solution. (3) The peptide appears confined to preganglionic axons because within five days after cutting preganglionic axons, about 95 % of the LHRH-like peptide disappears from the ganglia, while its concentration triples in the portion central to the cut in those nerves whose stimulation causes the late slow e.p.s.p.s. Therefore, it is likely that the peptide is transported from spinal neurones to the periphery. (4) Application of synthetic LHRH mimics the action of the nerve-released transmitter on the postsynaptic membrane in some specific detail. Both nerve stimulation and applied LHRH change the conductances in the postsynaptic membrane in a parallel manner, at rest or when the neurones are hyperpolarized. (5) An analogue of LHRH which in mammals blocks the release of gonadotropins from cells of the anterior pituitary also blocks the nerve-evoked late slow e.p.s.p. and the action of applied LHRH. Conversely, two analogues which are more potent in the release of gonadotropins in the rat are also more potent in depolarizing ganglion cells. The same parallel holds for two analogues which are not effective in the rat (Rivier *et al.* 1976). These findings suggest that synthetic LHRH and its analogues act on the same receptors as does the nerve-released transmitter.

The results so far, then, give strong support for peptidergic transmission. Our peptide fulfils many of the requirements that one usually demands of a transmitter, such as synthesis, release, and mimicry of the nerve-evoked transmitter action in a specific manner on the postsynaptic membrane. The different time course of responses following nerve stimulation and applied LHRH may result from differences in delivery and access.

Gaps in information

One of the potential weak points in our case for peptidergic transmission is that the identification of the peptide relies so heavily on the specificity of the antiserum for mammalian LHRH (Nett *et al.* 1973). The actual structure of the peptide in our extracts has yet to be established; it need not be identical with LHRH but is likely to be close. While the many parallel effects of LHRH and of the natural transmitter are striking, the methods of mimicking the physiological effects are not yet sufficiently refined, compared with methods on skeletal neuromuscular synapses (Fig. 4). Another gap is that, although we know which nerves contain the peptides for the 9th and 10th

ganglion (Fig. 11), we still lack an identification of the specific axons and the relationship of their terminals to the neurones in the ganglia. Further, we do not know the groups of cell bodies in the central nervous system that give rise to the peptidergic axons. Immunohistochemical and other structural evidence will be essential. An LHRH-like peptide has been localized immunohistochemically in various parts of the brain of the frog (Alpert *et al.* 1976; Doerr-Schott & Dubois, 1976). Another difficulty is our limited understanding of the postsynaptic conductance changes caused by the peptidergic transmitter and of the steps leading up to them.

The physiological role of the slow synaptic signals

In the parasympathetic ganglia of *Necturus* and in sympathetic ganglia of the frog, integrative activity does occur at the cellular level, since diverse synaptic signals initiated by converging axons interact in individual neurones. Each signal influences the process of transmission because it alters properties of the postsynaptic membrane.

Inhibition in the mudpuppy is the simpler case because only two signals interact, a fast excitatory and a slow inhibitory one. One should note that the slow inhibitory potential differs significantly from i.p.s.p.s in the mammalian CNS where they tend to match more closely the duration of opposing e.p.s.p.s and where their inhibitory potency usually is stronger (Eccles, 1964). On first consideration it may not seem reasonable that the release of one transmitter should have a dual and opposite role on an individual neurone. The built-in delay in activation of inhibition makes sense because the i.p.s.p. does not develop effectively until the postsynaptic impulses have propagated away from the cell body. The long duration of i.p.s.p.s suggests a role of providing a prolonged inhibitory bias on neurones, without requiring high frequency activity in the presynaptic axons. The i.p.s.p.s are therefore suited to reduce the through-traffic in ganglia: in that way they may protect the heart from excessive slowing, should the vagus discharge at high frequencies.

In the sympathetic ganglia of the frog the slow peptidergic signals, e.g. in Figs. 16 to 19, increase for many minutes the background excitability of neurones and thereby the power of fast nicotinic e.p.s.p.s to transmit impulses. Unexpectedly, however, during the same period the slow muscarinic e.p.s.p. became less effective. The interaction with slow muscarinic i.p.s.p.s has not yet been studied. Since we are dealing altogether with four types of signals (Fig. 12), sympathetic neurones should offer a manageable ensemble of interacting elements for a study of integrating mechanisms at the cellular level, particularly since we can separately activate some of the various pathways converging on individual neurones. For example, the peptidergic innervation to the large B neurones in ganglia 9 and 10 can be stimulated most of the time exclusively through root outflow 7 and 8, while the cholinergic innervation to the same cells can be stimulated more rostrally through the roots above 7 (Fig. 11).

In conclusion, it seems that the sympathetic ganglia with their relative simplicity provide good models for a rigorous analysis of peptide action and of integrative mechanisms in neurones. Other models are likely to emerge from advances made recently in explorations of the cellular physiology of autonomic plexuses in the gut and various smooth muscles. In these systems activity is likely to be initiated by a variety of substances, in addition to the cholinergic and adrenergic transmitters (for recent survey consult Bülbring & Bolton, 1979).

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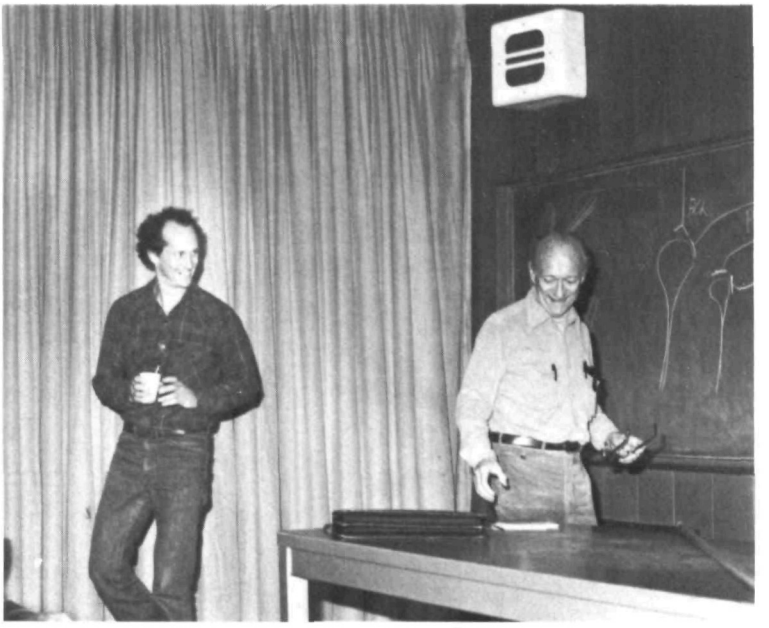
Photographs taken at the
Discussion Meeting held at Woods Hole,
8–11 April 1980



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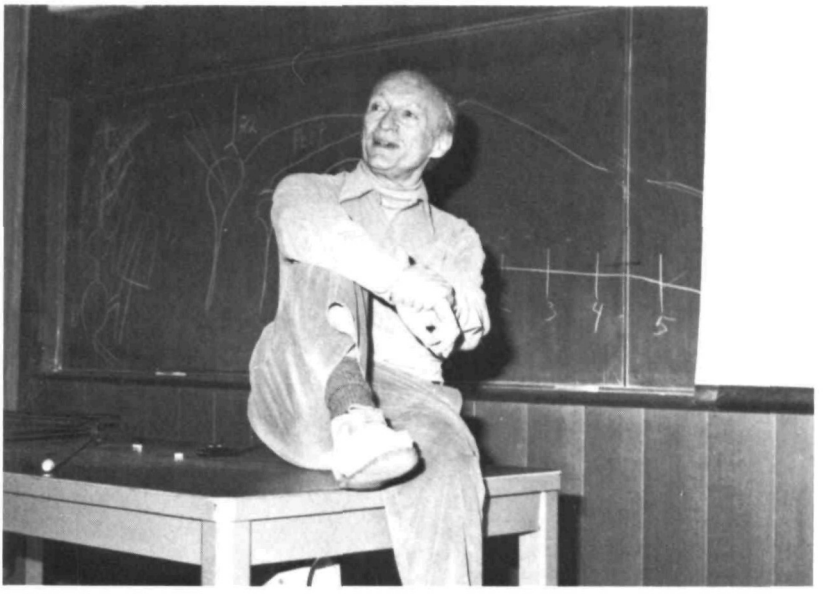

Steve Kuffler

Masanori Otsuka and Steve Kuffler

Susumu Hagiwara, Steve Kuffler and Otto Lowenstein

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