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SYNAPTIC BLOCK OF A CALCIUM-ACTIVATED POTASSIUM CONDUCTANCE IN *APLYSIA* NEURONES

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SUMMARY

1. In the preceding paper (Kehoe, 1985) it was shown that the firing of any one of three neurones (I, II, III) presynaptic to the medial cells of the pleural ganglion of *Aplysia californica* causes a diminution of the cholinergically controlled K conductance in those cells. Firing of the same three presynaptic neurones was shown here to cause a similar diminution in a depolarization-induced K-dependent conductance in the same post-synaptic cells.

2. The depolarization-induced K conductance was found to disappear when Ca ions were removed from the sea water bathing the ganglion or when the cell was injected with the Ca chelator ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetra-acetic acid (EGTA).

3. The diminution in this Ca-activated, K-dependent current occurred even when the presynaptic neurone was fired a few seconds after the end of the depolarizing voltage step to the post-synaptic neurone, showing that the diminution in K conductance was not an indirect effect of a transmitter-induced diminution in Ca influx during the depolarizing pulse.

4. The two K conductances affected by the 'blocking neurones' could be selectively eliminated. The cholinergic conductance could be blocked by receptor-specific cholinergic antagonists (e.g. 1 mM concentrations of phenyltrimethylammonium (PTMA), choline and tetraethylammonium (TEA)). Even at 10 mM concentrations, none of these compounds (including TEA, which is known to block certain Ca-activated K conductances) had an effect on the depolarization-induced, Ca-activated K conductance studied here. This latter conductance, on the other hand, was selectively blocked by an intracellular injection of EGTA. The three blocking neurones continued to diminish the K conductance (cholinergic or depolarization induced) that remained intact under these different experimental conditions.

5. The depolarization-induced influx of Ca was shown to block the cholinergically controlled K conductance, but Ca was excluded as the possible mediator of the diminution in K conductance caused by the three blocking neurones.

6. An intracellular injection of Ca ions into the medial cells was shown to activate a variety of changes in membrane conductance; in particular, two K-conductance increases: an early, TEA-sensitive one, and a slowly developing, TEA-insensitive one.

7. Both the permeant cyclic AMP analogue *p*-chlorophenylthioadenosine 3′,5′-monophosphate (CPT-cyclic AMP) and the phosphodiesterase inhibitors aminophylline and isobutyl-1-methylxanthine (IBMX) were shown to block the depolarization-induced K conductance, and to reduce, though not eliminate, the slowly developing K conductance activated by an intracellular injection of Ca. These agents had no effect on the early increase in K conductance activated by such an injection.

8. Although an intrasomatic injection of cyclic AMP failed to diminish the depolarization-induced K conductance (which presumably originates in the axon of the medial cell), cyclic AMP is proposed as a possible mediator of the synaptically activated diminution in the depolarization-induced K conductance, as it was for the similar action of the same presynaptic neurones on the cholinergically activated K conductance (see Kehoe, 1985).

INTRODUCTION

A K conductance that is gated by an increase in intracellular Ca (see Meech, 1972, 1974*a*, *b*) has been shown in the cells of the myenteric plexus of the guinea-pig small intestine to be reduced by synaptic stimulation (Grafe, Mayer & Wood, 1980; North & Tokimasa, 1982), by serotonin (5-HT) (Wood & Mayer, 1979; Grafe *et al.* 1980) and by muscarinic agonists (Morita, North & Tokimasa, 1982; North & Tokimasa, 1983, 1984). Likewise, noradrenaline (NA) (Madison & Nicoll, 1982), acetylcholine (ACh) (Bernardo & Prince, 1982; Cole & Nicoll, 1983), histamine (HA) (Haas & Konnerth, 1983), and corticotropin releasing factor (Aldenhoff, Gruol, Rivier, Vale & Siggins, 1983) have been shown to reduce adaptation by a similar mechanism in hippocampal pyramidal cells. Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) is thought to mediate the nonadrenergic blocking effect on these latter neurones (Madison & Nicoll, 1982). Finally, it appears that the K conductance activated by an intracellular injection of Ca in certain molluscan neurones can be diminished by the neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) (Cottrell, Davies & Green, 1984).

In this paper, it is shown that the three 'blocking neurones' that were shown in the preceding paper (Kehoe, 1985) to diminish a transmitter-induced K conductance in the medial cells can also block a K conductance triggered in the same cells by a depolarization-induced increase in intracellular Ca. This slowly developing K conductance, shown to be unaffected by tetraethylammonium (TEA) (10 mM) and thereby to resemble that described by Thompson (1977) (and most likely that observed by Meech (1972, 1974*a*, *b*) and by Hofmeier & Lux (1981), and most recently by Deitmer & Eckert (1985)), seems to differ from the TEA-sensitive conductance typically studied in molluscan neurones in response to intracellular injections of Ca (e.g. Hermann & Gorman, 1981; Hermann & Hartung, 1982).

The possibility that cyclic AMP is the mediator of the synaptically activated diminution in the depolarization-induced conductance studied here was evaluated.

METHODS

Most of the procedures used in the experiments reported here were the same as those described in the preceding paper (Kehoe, 1985). That paper should be consulted for a description of the experimental preparation, the composition of the external solutions, the drug suppliers, the particulars of the current- and voltage-clamping systems, etc.

Ca injections, not described in the preceding paper, were made using interbarrel injections from two independent electrodes pulled on the Narashige vertical puller. The electrode connected to the positive pole of the WPI ionophoresis unit contained 0·2 M-$CaCl_2$, whereas that connected to the negative pole contained 0·5 M-K_2SO_4. Some Cl was inevitably injected into the cell because of the braking current applied to the $CaCl_2$ electrode. Parameters of the injection currents are indicated in the appropriate Figure legends.

The response under study in this paper is a long-duration outward current elicited in voltage-clamped medial cells by a 1–2 s depolarization to +10 or +20 mV. In order to evaluate the changes caused in the post-depolarization currents by the firing of various presynaptic neurones, these currents were digitized, and the currents accompanied or not by synaptic inputs were subtracted one from the other. Owing to this type of processing of a 30–60 s response, the rapid elements of the synaptic response were not faithfully represented. However, since only slow changes in membrane currents were the object of the investigations described here, this defect posed no problem. Digitized records are presented in Figs. 2, 3, 5, 6 and 7. For a more accurate representation of the rapid elements of the responses to the five presynaptic neurones, Fig. 1 of the preceding paper (Kehoe, 1985) should be consulted.

The net outward current elicited by the depolarization is maximal only a few seconds after the cell has been returned to holding potential. This of course does not imply that the forces generating the underlying conductance change are maximal at that point, since many other conductance changes contribute to the net current over the initial 2 s post-depolarization period. However, this study has been limited to an evaluation of the current measured from about 2–4 s to about 40–60 s following the depolarization, at which stage only one of the many conductance changes activated by the voltage step appears to be present. Since this conductance increase orginates at least in part in the axonal membrane, and since the synaptic currents being studied here are purely axonal, it was necessary to work with the whole cell, rather than with an isolated soma preparation. Consequently it was impossible to clamp the entire membrane during the depolarizing pulse. However, the amplitude and time course of the depolarization-induced current remained remarkably constant over repeated pulses, suggesting that successive voltage steps elicited identical depolarizations. The response of the cell during the depolarizing voltage step itself was evaluated when a change occurred in the depolarization-induced current, and such control evaluations are discussed below when appropriate.

Drugs. The only drug used in the experiments reported here that was not used in the previously described experiments (Kehoe, 1985) was FMRFamide, which was obtained from Cambridge Research Biochemicals (U.K.).

RESULTS

Description of the depolarization-induced outward current

The effects of five identifiable presynaptic neurones (see Fig. 1, Kehoe, 1985) have been studied on a long-duration current (outward at resting potential) that is induced in the medial cells by a 1–2 s depolarization. An example of this current, activated by a 2 s depolarization to +10 mV and measured in a medial cell that was clamped at −35 mV before and after the voltage step, is given in the top left-hand record of Fig. 1. It can be seen that the current induced by the depolarization is a net outward current which peaks at about 2 s after the end of the depolarizing voltage step.

Although the time constants of the depolarization-induced outward current,

measured at −30 to −40 mV were found to be cell and ganglion dependent, they remained quite stable for a given cell in a given ganglion. The net outward current usually peaked 2–5 s after the voltage step, had a half-time decay on the order of 10–30 s, and returned to base line after 1–3 min. Since these time constants did not

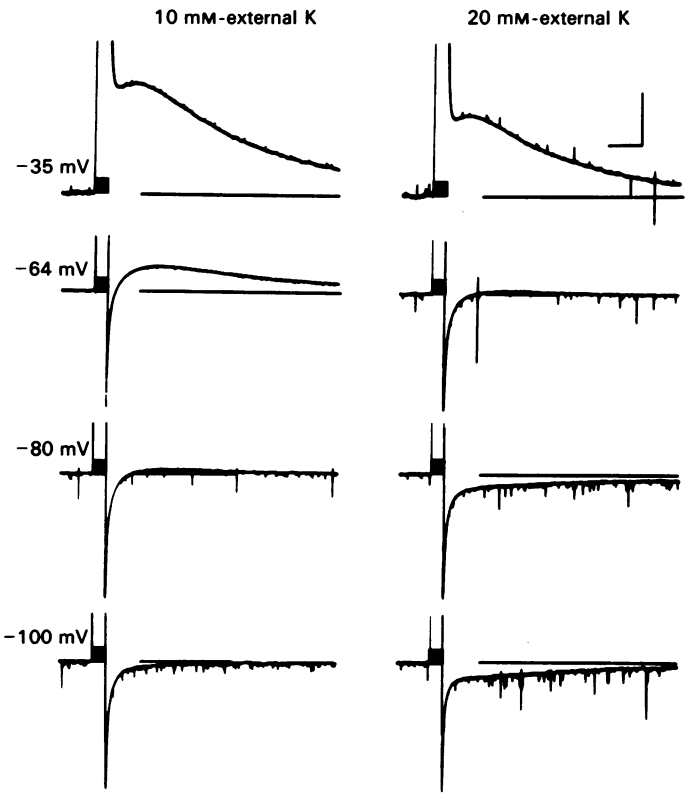


Fig. 1. Effect of varying external K concentration on the slowly developing depolarization-induced current. In each record, the current trace goes off scale during the 2 s voltage step to +10 mV (see filled square indicating the period during which the cell is stepped to +10 mV). The object of study here is the current recorded from about 2 s onwards following the return of the cell to the pre-set holding potential. The inversion potential of this depolarization-induced current is just beyond −80 mV in 10 mM-K, and just beyond −64 mV in 20 mM-K. Calibration: 2 nA, 5 s.

seem to be related to cell diameter or to membrane resistance, but rather seemed to be consistent from one medial cell to another for a given ganglion, it has been assumed that they reflect physiological rather than methodological differences (e.g. differential efficacy of the axon clamp). Furthermore, since none of the phenomena described in this paper appeared to vary with the temporal characteristics of the depolarization-induced conductance, these characteristics were not made an object of study.

The depolarization-induced outward current, found to be markedly reduced in the isolated soma preparation, has been assumed to be primarily an axonic event. Consequently, in all experiments shown here, the axons were left attached to the soma. It was of course impossible to clamp the voltage in the axon during the depolarization of the soma to +10 or +20 mV. In spite of this limitation, the voltage

step apparently remained constant as witnessed by the constancy, throughout a given experiment, of the post-depolarization membrane currents. The axon has been previously shown (see Kehoe, 1972*a*) to be isopotential with the soma at membrane potentials more negative than −30 mV.

Effect of doubling the external K concentration on the inversion potential of the depolarization-induced outward current. The change in the depolarization-induced current as a function of membrane potential and external K concentration is shown in the records presented in Fig. 1. The medial cell was depolarized to +10 mV for 2 s periods, with the voltage step being initiated from different holding potentials (−35, −64, −80 and −100 mV). The post-depolarization currents were measured first in 10 mM, then in 20 mM, K. It can be seen that in normal sea water (10 mM-K) the delayed, depolarization-induced current, evaluated from 2 s onwards following the voltage step, was outward at −35 and −64 mV, almost at its inversion potential at −80 mV (estimated inversion = −82 mV), and inward at −100 mV. When the K in the sea water was doubled, only the response measured at −35 mV remained outward. The inversion potential of the depolarization-induced current shifted to about −65 mV, and the responses measured at more negative holding potentials (−80 and −100 mV) were inward. This shift in inversion potential of the depolarization-induced outward current is equivalent to the shift expected in the equilibrium potential for K ions (E_K), thereby indicating that the depolarization-induced current, recorded from about 2 s onward following the voltage step, is a pure K-dependent current.

Synaptic diminution of the depolarization-induced K-dependent current

Differential ability of presynaptic neurones, I, II, III and V to diminish the depolarization-induced K current. The depolarization-induced K-dependent current was found to be markedly affected by firing of any one of three identifiable neurones presynaptic to the medial cells (presynaptic neurones I, II and III of Fig. 1, Kehoe, 1985). This finding is illustrated in Fig. 2. In the records labelled I, the curve labelled *a* shows the normal amplitude and time course of the K current activated by a 2 s depolarization (to +10 mV) of a medial cell clamped, before and after the voltage step, at −40 mV. This 'control' current decreases from a peak amplitude (reached about 2 s following the end of the 2 s voltage step) to half-amplitude in about 10 s. When, however, presynaptic neurone I was fired shortly after another identical depolarizing step, the depolarization-induced K current was considerably smaller (compare *b* with *a*). The diminution in the depolarization-induced K current can also be evaluated by comparing the synaptic response elicited in control conditions (*c*), i.e. in which no depolarization preceded the activation of the synapse, with that elicited following a depolarizing voltage step (*b*−*a*). The record *b*−*a* has been obtained by subtracting the normal depolarization-induced K current (*a*) from that seen when the synapse was activated just after the post-synaptic depolarization (*b*). The slow, long-lasting inward-going current seen in *b*−*a*, but not present in *c*, reflects the synaptic diminution in the depolarization-induced K conductance.

Records II, III and V are from similar experiments in which presynaptic neurones II, III and V, respectively, were used. It can be seen that the presynaptic neurones that are capable of diminishing the depolarization-induced K current (I, II and III) are the same neurones as those previously shown to diminish the cholinergically

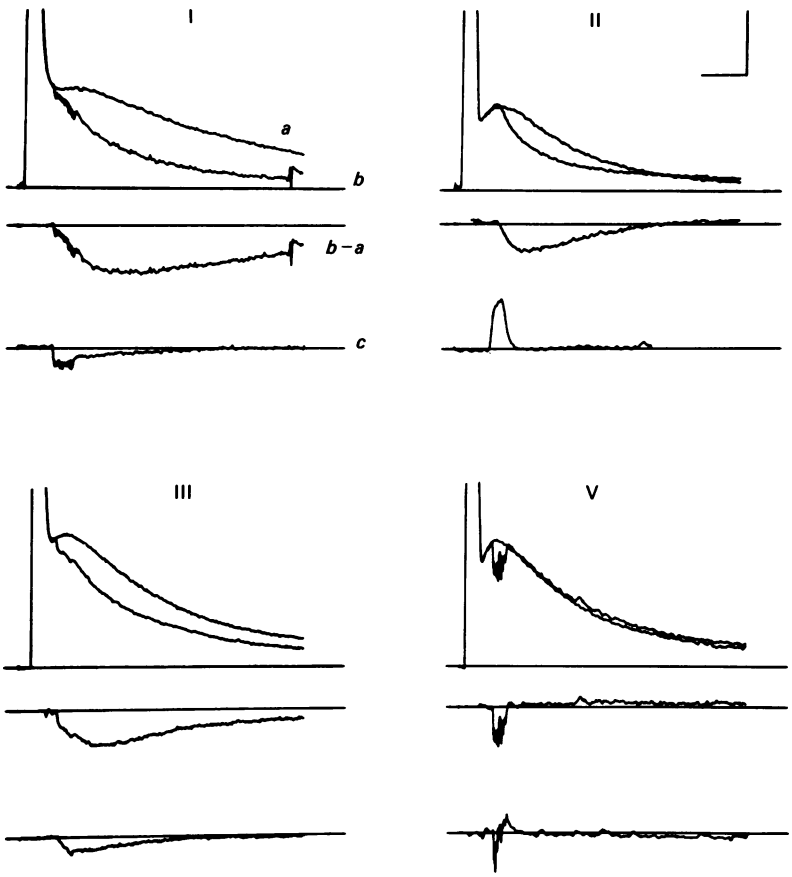


Fig. 2. Synaptic diminution in the depolarization-induced K current measured at −40 mV in voltage-clamped medial cells. Each of the four sets of records represents an experiment performed with one of four presynaptic neurones (I, II, III, V, respectively). In each set, the current recorded from the post-synaptic cell during the 2 s voltage step to +10 mV is off scale. Following the end of the voltage step and the return on scale of the records, two traces can be distinguished in the first row of each set of records. The upper trace of each pair (*a*) represents the depolarization-induced K current when no presynaptic neurone had been fired after the depolarizing voltage step to the medial cell. In the second of the two upper traces (*b*), one of the four presynaptic neurones was stimulated for a 2 s period within 1 s following the depolarization of the post-synaptic neurone. In the second row of each set of records (*b*−*a*) is the computer-calculated difference between the upper and lower traces of the first row. In the final row of each set of records (*c*) is the response to firing of the same presynaptic neurone at the same post-synaptic holding potential when not preceded by a depolarizing step in the post-synaptic cell. Whereas presynaptic neurones I, II and III caused a marked diminution in the outward, depolarization-induced current, presynaptic neurone V caused no alteration in that current. The transformation of the synaptic response to neurones I, II and III that occurs when elicited during the post-depolarization outward current can best be seen by comparing the traces of the middle (*b*−*a*) and last (*c*) rows of each set of records. The diminution in the outward synaptic current elicited by presynaptic neurone II that occurs when it is elicited during the post-depolarization period will be discussed later. Calibration: 2 nA, 5 s. Depolarizing voltage step: to +10 mV. For more faithful recordings of the rapid elements of the synaptic responses, see Fig. 1, Kehoe (1985).

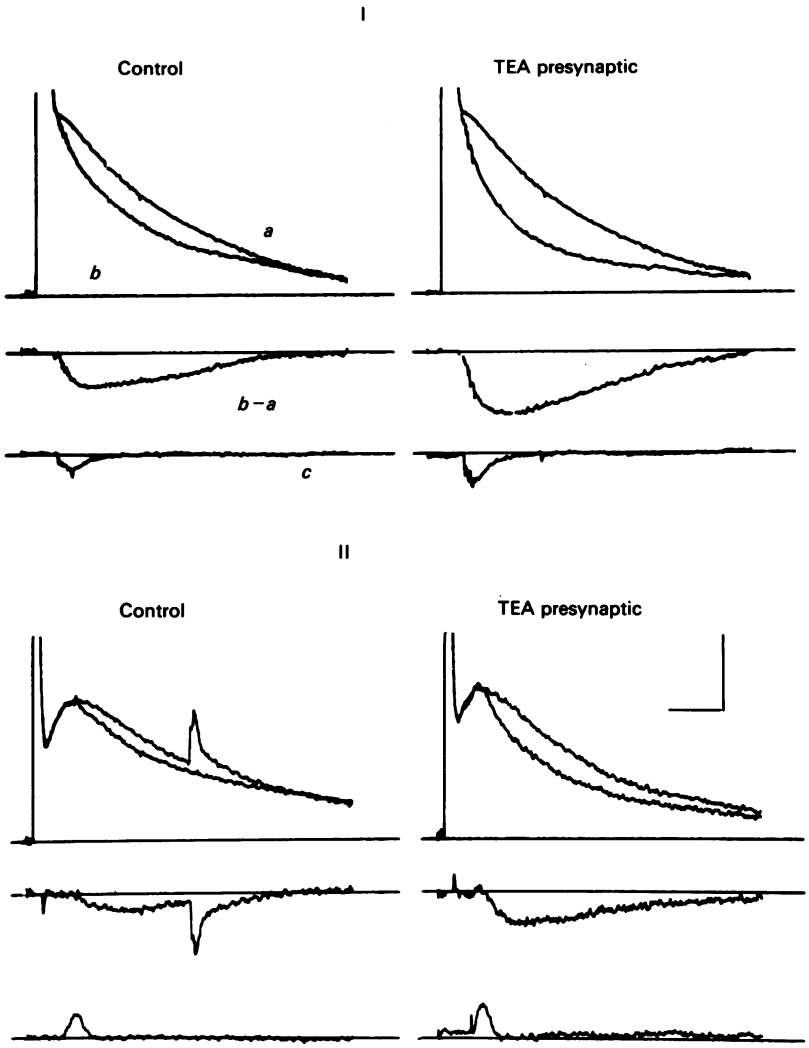


Fig. 3. Increase in the synaptic diminution of the depolarization-induced K conductance caused by an injection of TEA bromide in the presynaptic neurones I and II (see I and II, respectively). See Fig. 2 for the signification of traces *a*, *b*, *c* and *b*−*a*. In the left-hand records, no TEA had been injected in the presynaptic neurone. In the right-hand records, the synaptic responses were measured after an electrode filled with 0·5 M-TEA bromide had been inserted into the presynaptic neurone, and had been used for depolarizing the presynaptic cell during approximately ten 2 s firing periods. It can be seen that the diminution of the depolarization-induced K conductance by the presynaptic neurones I and II is increased following an injection of TEA into the presynaptic cell (compare records *b*−*a* in control and TEA-injected conditions). Furthermore, it can be seen that injection of TEA into either presynaptic neurone I or II also increased the amplitude of the other elements of the synaptic response seen in the 'resting' cell. However, these effects of an injection of TEA in the presynaptic cell are better evaluated in Fig. 3 of Kehoe (1985), where the early responses are more faithfully represented. Holding potential: −40 mV. Voltage step in post-synaptic cell: to +10 mV; 2 s duration, upper records; 1 s, lower records. Calibration: 2 nA, 5 s.

activated K conductance (see Kehoe, 1985). It is equally clear that the response to presynaptic neurone V (see Fig. 1), which was shown in the preceding paper to be identical whether fired in the presence or absence of cholinergic agonists (see Fig. 4, Kehoe, 1985), is also unchanged when it is superimposed upon the depolarization-induced K current.

The factor limiting the duration and amplitude of the slow inward-going synaptic current elicited by the blocking neurones is often the duration and amplitude of the depolarization-induced outward current, rather than those same parameters of the synaptic effect itself.

Accentuation of synaptically activated diminution in depolarization-induced K current with increase in transmitter release. As can be seen in Fig. 3, when the action potential of the presynaptic neurone was prolonged by an intracellular injection of TEA bromide, the response element representing a diminution in conductance increased, as did all other elements of the synaptic response. The TEA-induced increase in amplitude of the post-synaptic response can be rapidly produced. In the experiment presented in Fig. 3, for example, the TEA records were taken after about ten 2 s depolarizing pulses through the TEA electrode placed in the presynaptic neurone.

Ca dependence of the depolarization-induced K current

Elimination of the depolarization-induced K current in Ca-free sea water. Fig. 4 illustrates a typical experiment demonstrating the dependence of the depolarization-induced K current on the presence of extracellular Ca. When the Ca of the normal sea water was replaced by Mg, the depolarization-induced K current disappeared completely. In the lower row of the same Figure, it can be seen that synaptic transmission (between the cholinergic presynaptic neurone IV and the medial cell) was also blocked by the Ca deprivation. Many minutes are required (in this experiment, about 25) for the Ca-free sea water to block completely either the depolarization-induced K conductance or synaptic transmission. This delay probably represents the time required for a complete elimination of Ca from the intercellular spaces. When the Ca-free sea water was replaced with normal sea water (wash), both the depolarization-induced K conductance and synaptic transmission returned to normal.

Elimination of the depolarization-induced K conductance by an intracellular injection of ethyleneglycol-bis-(β-aminoethylether)N,N′-tetraacetic acid (EGTA). To reinforce the conclusion that an increase in intracellular Ca is indispensable for the activation of the depolarization-induced K conductance, the effects on that conductance of an intracellular injection of EGTA into the medial cell were evaluated. As can be seen in Fig. 5, by chelating the Ca entering during the depolarizing voltage step, EGTA impedes the development of the post-depolarization outward current. The EGTA-induced block shown here increased gradually with the duration of the 30 nA injection (see the inset records and the Figure legend for details). The depolarization-induced transformation of the synaptic response also disappeared, confirming that this transformation is indeed dependent upon the presence of the K conductance itself, and not simply another side-effect of the depolarizing voltage step that precedes it.

The middle record in the bottom of the Figure (*c′*) shows the response measured

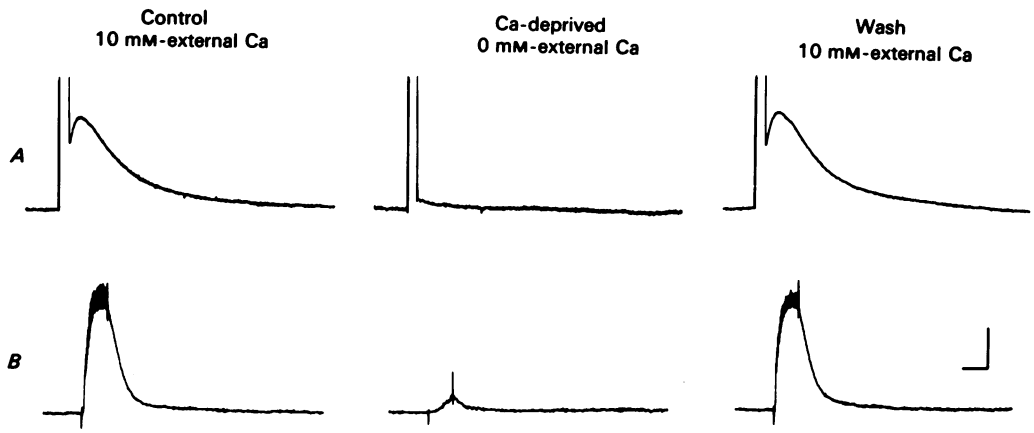


Fig. 4. Effect of Ca-free sea water on the depolarization-induced K conductance (*A*) and on transmitter liberation from the cholinergic presynaptic neurone IV (see *B*). Note the disappearance of the synaptic current (middle record of lower trace) and of the depolarization-induced K current (middle record of upper trace) when the 10 mM-$CaCl_2$ in the sea water bathing the ganglion was replaced by 10 mM-$MgCl_2$ (Ca deprived). Both of these currents returned to control values when the ganglion was once again bathed in normal sea water (wash). Calibration: 2 nA, 5 s. Holding potential: −40 mV. Depolarizing voltage step in *A*: 2 s to +10 mV; duration of presynaptic firing in *B*: 4 s.

in the EGTA-injected cell to firing of presynaptic neurone I after 20 μM-arecoline had been added to the bath. Even in the EGTA-injected cell, arecoline elicited a K-dependent current of normal amplitude (not shown), and this arecoline-induced current, as in non-EGTA-injected cells, was diminished by the firing of presynaptic neurone I (see record *c'*), as well as by the firing of either of the two other 'blocking' neurones (not shown).

Synaptic diminution in K conductance as a function of the temporal relationship between presynaptic firing and post-synaptic depolarization

The diminution in the K conductance occurred whether the presynaptic neurone was fired shortly before or shortly after the depolarizing voltage step. The fact that firing after the depolarization is as effective, if not more so, than firing prior to or during the depolarizing voltage step shows that the synaptic action is not due to an interference with the influx of Ca ions itself. Rather, it suggests that it be due to a perturbation either in the gating of the Ca-activated K conductance or in the conductance of that channel once opened.

Pharmacological comparison of the two K conductances (cholinergic and depolarization induced) that are diminished by the three blocking neurones, and analysis of their apparent interaction

Failure of the block of the cholinergic K conductance to affect either the depolarization-induced K conductance or its synaptic diminution. It was shown in the preceding paper (Kehoe, 1985) that the three presynaptic neurones shown here to be capable of blocking the depolarization-induced K conductance can also cause a diminution in

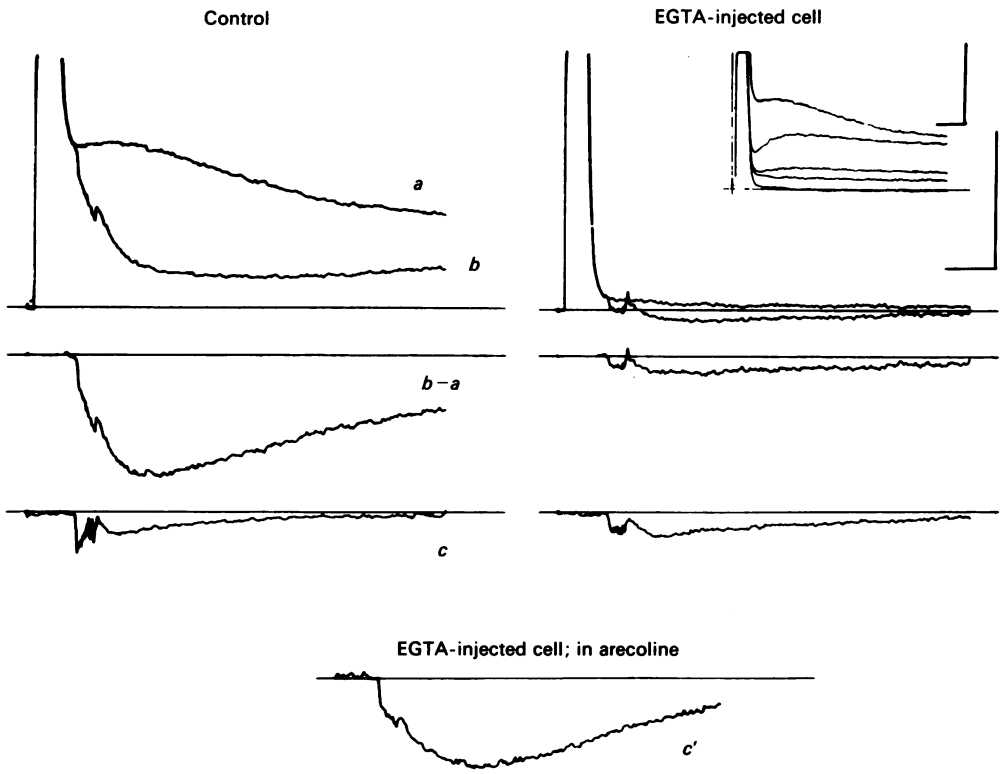


Fig. 5. Effect of intracellularly injected EGTA on the depolarization-induced K conductance and on the depolarization-induced transformation in the synaptic response to presynaptic neurone I. The post-synaptic cell was voltage clamped at −35 mV except during the 2 s voltage step which brought the cell to +20 mV. Following a 60 min 30 nA injection of EGTA into the medial cell, the depolarization was no longer followed by an outward current (see upper right-hand traces), and the response to the firing of the presynaptic neurone I was no longer transformed by the depolarizing voltage step (compare $b-a$ in control and EGTA conditions). The records in the inset (upper right) show the progressive diminution in the depolarization-induced K conductance over the period of the intracellular injection of EGTA (records taken at 0, 15, 35, 45 and 60 min). The single record in the last row (c') shows the response of the EGTA-injected medial cell to firing of the same presynaptic neurone after 20 μM-arecoline had been added to the bath. This cholinomimetic elicited a steady-state outward current in the voltage-clamped medial cell (held at −40 mV) of more than 5 nA. The slow inward-going current in response to presynaptic neurone I in the last row (c') represents the diminution in the arecoline-induced current, and should be compared with record (c) of the EGTA-injected cell prior to application of arecoline. Calibration: 3 nA, 5 s.

the K conductance activated by cholinergic agonists. To verify that the two synaptically diminished conductances are independent, experiments were performed in the presence of four different antagonists (TEA, phenyltrimethylammonium (PTMA), choline and methylxylocholine (β-TM 10)) which have been previously shown to block completely, at 0·5–1·0 mM concentrations, the cholinergic K conductance (Kehoe, 1972*b*; Gardner, Ruff & White, 1984). Fig. 6, which presents

records from one such experiment, shows that neither the depolarization-induced K conductance nor its diminution by the blocking neurones is affected by the cholinergic antagonist TEA (1 mM). This conclusion also holds true even for 10 mM concentrations of any of the four antagonists (not shown).

In the control records of Fig. 6 are shown the same type of recordings described in Figs. 2, 3 and 5. The trace labelled *a* shows the usual depolarization-induced

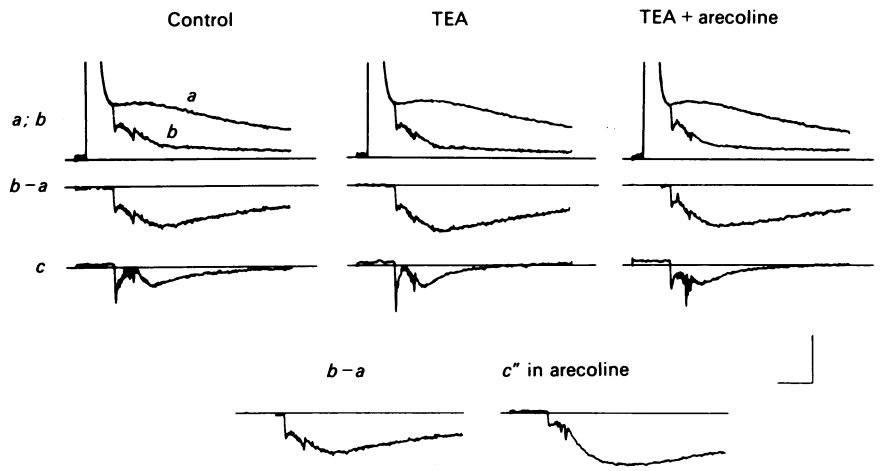


Fig. 6. Effect of the cholinergic antagonist TEA on the depolarization-induced K conductance and its diminution by the presynaptic blocking neurone I. The upper two traces (*a* and *b*) in each column represent the depolarization-induced K conductance with (*b*) and without (*a*) the firing of the presynaptic blocking neurone. The middle record ($b-a$) shows the computed difference between the upper two traces. The lowest trace of each column (*c*) shows the synaptic response when not preceded by a depolarization of the post-synaptic cell. It can be seen in these records that the depolarization-induced K conductance and its synaptic diminution persist in the presence of 1 mM-TEA (TEA) (compare control and TEA records). A comparison of the second and third column of records reveals the failure of 20 μM-arecoline, when accompanied by 1 mM-TEA (TEA + arecoline), to elicit either a change in steady-state current or a transformation in the synaptic response (*c*). The final two records of the Figure (last row) compare the synaptic diminution in the depolarization-induced K conductance ($b-a$) with the synaptic diminution in the steady-state arecoline current (c″ in arecoline) elicited in the same cell once the cholinergic antagonist TEA was washed out of the chamber. Calibration: 2 nA, 3 s. Holding potential: −35 mV; post-synaptic voltage step: 1 s to +5 mV; duration of presynaptic stimulation: 2 s.

outward K current. In trace *b*, the response of the medial cell to firing of presynaptic neurone I is elicited during the depolarization-induced K current, and the inhibition of this current can be evaluated by comparing $b-a$ with the 'resting' synaptic response *c*. The middle column of records show that none of these phenomena have been changed by adding 1 mM-TEA to the sea water bathing the ganglion. On the other hand, that this concentration of TEA completely blocks the response of the medial cell to arecoline can be seen by the persistence of a normal 'control' synaptic

response in the presence of arecoline, when accompanied by TEA (see the last column of data, labelled TEA + arecoline). When TEA is present, no arecoline-induced K conductance develops, and there is no development of an inward-going wave in the synaptic response (see *c*, TEA + arecoline). Neither was there any change in the depolarization-induced current or in its synaptic diminution. The response to the presynaptic neurone I measured at −40 mV in the presence of arecoline (after TEA had been washed out) is that seen in the right-hand record (*c″*) of the bottom row, where it is possible to compare (in the same cell) the transformation of the synaptic response that was caused by a preceding depolarization (*b* − *a*) with that caused by arecoline when unaccompanied by TEA (*c″*). The fact that the inward-going wave lasted longer in the presence of arecoline simply reflects the fact that the K conductance being reduced in that case was a steady-state current, whereas that which followed the depolarizing voltage step to the post-synaptic neurone decreased over time.

Similar experiments performed with the other two blocking neurones, and with the other three cholinergic antagonists (choline, see Gardner *et al.* 1984; PTMA and *β*-TM 10, see Kehoe, 1972*b*) yielded identical findings.

Effect of the depolarization of the medial cell on the cholinergic synaptic response. The data of Fig. 6 have demonstrated that blocking the cholinergic K conductance has no effect on the depolarization-induced K conductance. However, this separation of the two K conductances does not imply that depolarization of the post-synaptic cell has no effect on the cholinergic K conductance. In fact, if the cholinergic K conductance is triggered following a depolarization of the medial cell, the transmitter-induced current is markedly reduced. This interaction is analysed as a function of membrane potential in Fig. 7 (see also similar results of Ascher & Chesnoy-Marchais, 1982 and Chesnoy-Marchais & Ascher, 1983). The three pairs of traces shown in the upper row of this Figure are the computer-processed, depolarization-induced K currents measured when no synapse had been activated (*a*) and when the cholinergic neurone IV had been fired for 2 s just after the depolarizing voltage step to +10 mV (*b*). The percentage block of the cholinergic conductance by the depolarizing step (i.e. the ratio of *b* − *a* to *c*) was the same at all three holding potentials (−30, −50 and −60 mV). Further details are given in the legend of Fig. 7.

Ca-dependence of the depolarization-induced block of the cholinergic synaptic response by membrane depolarization

As was shown in Fig. 5, when EGTA is injected in the medial cell, there is a progressive decline and an eventual elimination of the depolarization-induced K conductance. As can be seen in Fig. 8, when the depolarization-induced K conductance is eliminated by chelation of the inflowing Ca, the block by depolarization of the cholinergic synaptic K conductance also disappears. The failure of depolarization to cause a diminution of the synaptically activated K conductance in an EGTA-injected cell reinforces the findings of Chesnoy-Marchais & Ascher (1983), who showed that an increase in intracellular Ca reduces the cholinergic K conductance activated by carbachol applied either in the bath solution or by ionophoresis.

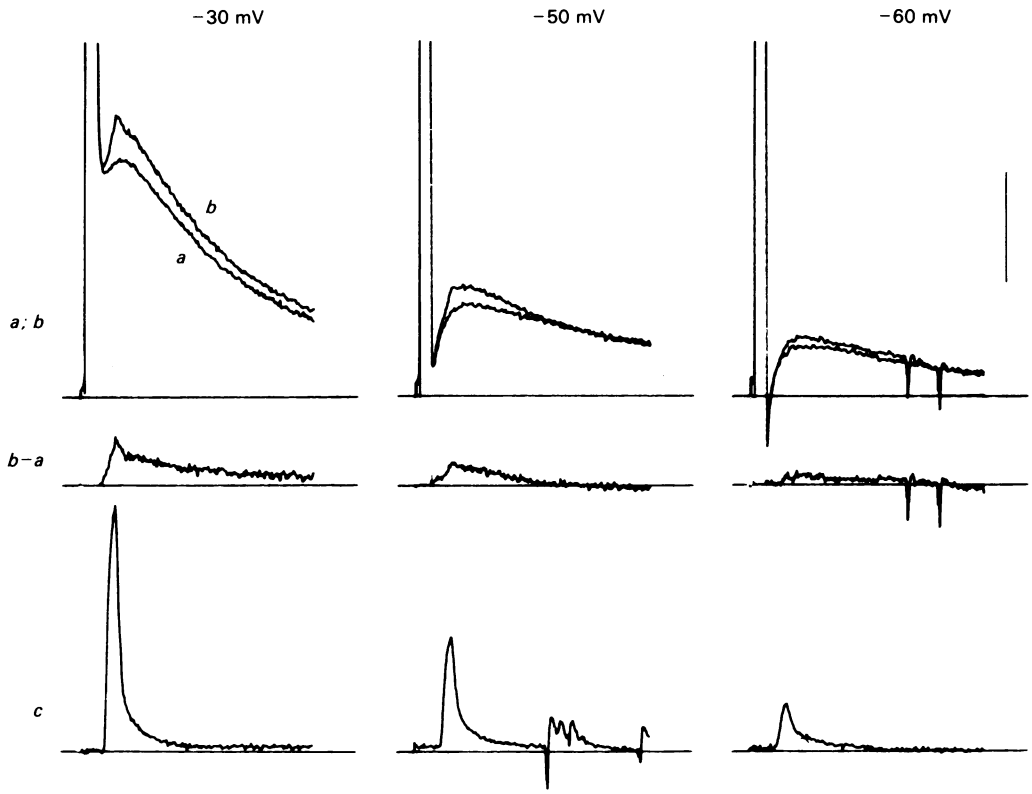


Fig. 7. Effect of a brief depolarization of the medial cell on the subsequently activated, cholinergic, K-dependent synaptic response. In the top row of traces are shown the depolarization-induced outward currents elicited with (*b*) or without (*a*) subsequent activation of the cholinergic presynaptic neurone IV recorded at three different holding potentials (−30, −50 and −60 mV). The records in the middle row indicate the computed difference between the two upper traces (*b*−*a*). The cholinergic K-dependent responses (*c*) elicited by the firing of presynaptic neurone IV when the synaptic stimulation had not been preceded by a depolarization of the post-synaptic cell are presented in the bottom row. These records should be compared with 'blocked' cholinergic responses revealed by the computed differences (*b*−*a*) shown in the middle row. The percentage block of the cholinergic K-dependent synaptic response caused by the preceding depolarizing voltage step in the post-synaptic cell (i.e. the ratio between *b*−*a* and *c*) is the same at all holding potentials tested. In this experiment, the cholinergic K conductance has been pharmacologically isolated by using 100 μM-curare to eliminate the Cl-dependent element of the synaptic response. Calibration: 2 nA, 5 s. Depolarizing voltage step: 2 s to +10 mV. Duration of presynaptic firing: 2 s.

Possible role of cyclic AMP in the synaptic diminution of the depolarization-induced K conductance

Block by IBMX of the depolarization-induced K conductance of the medial cells. Since the data of the preceding paper (Kehoe, 1985) suggested a possible role for cyclic AMP as a mediator of the synaptic diminution of the cholinergic K conductance, it seemed reasonable to assume that a similar role be played in the diminution, by

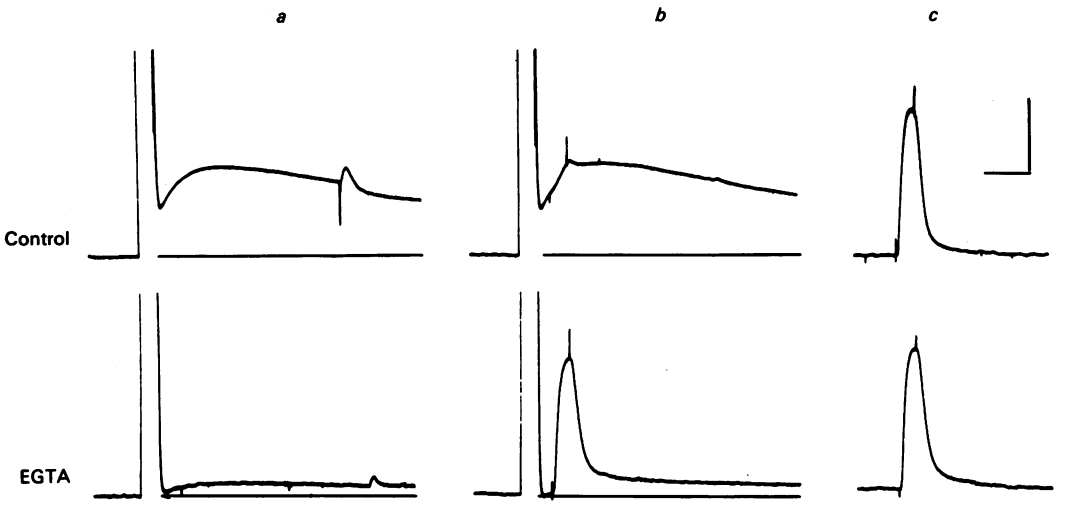
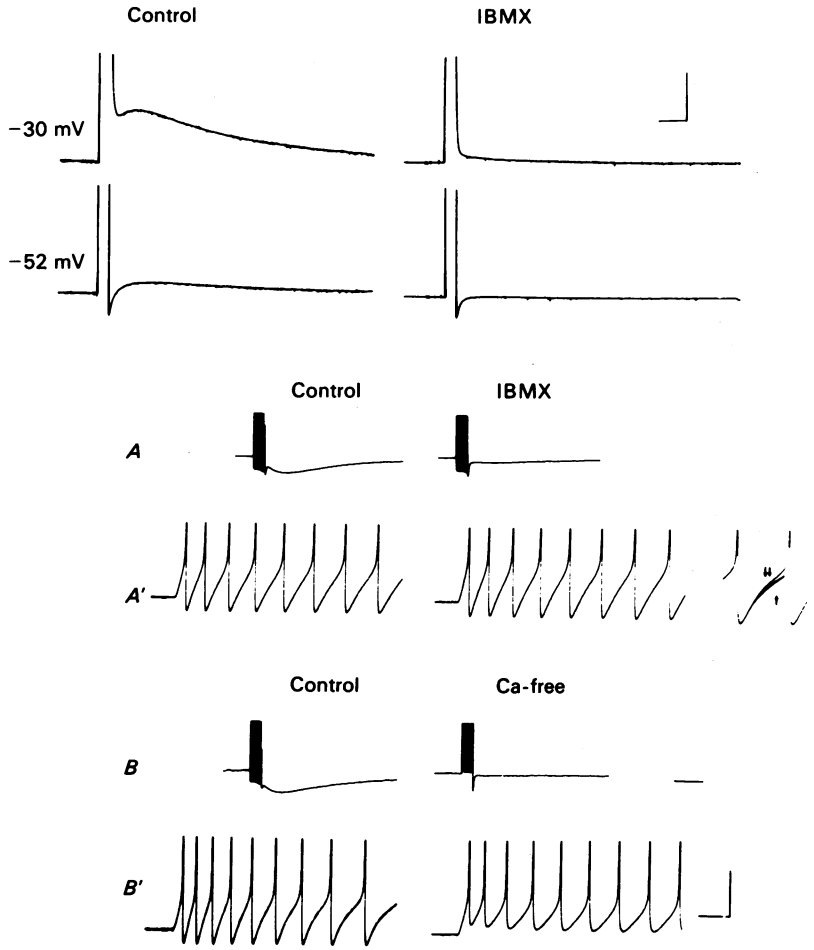


Fig. 8. Effect of EGTA loading of a curarized (200 μM-curare) medial cell on the depolarization-induced K conductance and on the depolarization-induced block of the cholinergic K conductance in that cell. A 60 nA, 45 min intracellular injection of EGTA in the medial cell eliminates the depolarization-induced K conductance (control *vs.* EGTA, column *a*), as well as the depolarization-induced block of the synaptically activated cholinergic K conductance (control *vs.* EGTA, column *b*) in that same cell. Note that the EGTA injection did not alter the amplitude or form of the cholinergic synaptic response, itself (control *vs.* EGTA, column *c*). Calibration: 2 nA, 5 s. Holding potential: −40 mV. Presynaptic stimulation: 2 s.

Fig. 9. Upper records: block of the depolarization-induced K current (at −30 and −52 mV) by a 10 min exposure to 100 μM-IBMX. Calibration: 2 nA, 5 s. Depolarizing voltage step: 2 s to +20 mV. Lower records: recordings transcribed on a Brush recorder from a tape recording of the response of a current-clamped medial cell to a 2 s, 4 nA depolarizing pulse from a −36 mV pre-pulse potential. *A*, control: eighteen action potentials (with the upper portion cut off) that were elicited during the depolarizing voltage step, followed by the depolarization-induced hyperpolarizing wave. *A*, IBMX: eighteen action potentials (with the upper portion cut off) that were elicited during another 4 nA depolarizing pulse in the same medial cell a few minutes after 100 μM-IBMX was added to the bath. A comparison of the records taken in IBMX with those taken in control conditions reveals that the depolarization-induced hyperpolarization was eliminated by IBMX, whereas the after-hyperpolarization associated with individual action potentials seems to remain unaffected. *A'*, control and IBMX: that this drug causes no change in the after-hyperpolarization is best seen in the transcription of the same recordings with an expanded time scale. In the right-hand record of *A'*, in which the inter-spike voltage trace from the control record has been superimposed upon that taken in IBMX, it can be seen that by the end of the 2 s pulse, a slight change in slope appears about 100 ms following the negative peak of the action potential. The single arrow indicates the trace from the control records; the double arrow, that from the IBMX records. No IBMX-induced differences could be measured in the action potential duration or in its peak-to-peak amplitude. In the records labelled *B*, control, a 2 s, 4 nA depolarization from a −35 mV pre-pulse potential elicited, in another medial cell of another ganglion, a similiar series of action potentials (upper portion cut off) followed by a similar post-pulse hyperpolarizing wave. This record can be compared with that obtained in the same cell shortly after the normal sea water was replaced by Ca-free sea



water (see *B*, Ca-free). Removing the Ca clearly blocked the post-pulse hyperpolarizing wave. Furthermore, unlike the case with IBMX (*A*, *A'*), removing Ca also induced a clear alteration of the individual action potentials elicited during the depolarization. Furthermore, Ca-free sea water markedly altered the excitability of the cell such that the number of action potentials elicited by the same pulse increased from fifteen to twenty-three. Even when the pulse was reduced to 2·5 nA so that a similar number of action potentials were elicited by the 2 s depolarization (see *B*, Ca-free), the same conclusions could be drawn concerning the effects of Ca deprivation on the post-depolarization hyperpolarization and on the transformation in the action potential. It can be seen that even with similar firing frequencies under the two conditions, the depolarization-induced hyperpolarization is blocked, and the action potential form (shown for the first 600 ms of the pulse) is markedly altered. This alteration, which is similar to that seen when Cd is added to the sea water (not shown), should be compared with the failure of IBMX to affect the action potential itself. Calibration: *A* and *B*: 20 mV; duration of depolarizing pulse, 2 s: *A'* and *B'*: 100 ms, 20 nA.

the same presynaptic neurones, of the depolarization-induced K conductance. In an effort to establish such a role for cyclic AMP, the nucleotide was first injected into the soma of the cell. However, a somatic injection of cyclic AMP was found to have no effect on the depolarization-induced K conductance. Since a major component of that conductance appears to originate in the axon far from the point of injection, these negative results were not too surprising. To increase the probability of cyclic AMP reaching the site of origin of the Ca-dependent K conductance, IBMX was added to the sea water. It was found that this agent, even when unaccompanied by an injection of cyclic AMP, blocked the depolarization-induced K current (see Fig. 9). Similar results (not shown) were obtained with aminophylline. The IBMX block of the depolarization-induced current occurs at all potential levels, and is not accompanied by a diminution in transmitter liberation, as measured by post-synaptic responses to the firing of any of the presynaptic neurones used in this study. It was thus concluded that the IBMX-induced block cannot be explained by an interference at the level of the Ca influx during the depolarization, but rather represents an interference at some point in the chain after Ca enters the cell. Evaluation of the action potentials of the medial cells before and after IBMX (100 μM) confirmed that this phosphodiesterase inhibitor changed neither the action potential amplitude, nor its duration (not shown). Only at the level of the slowly developing after-potential (which corresponds to the slowly developing K conductance triggered by an influx of Ca), and only near the end of a 2 s depolarization, could an alteration be detected (see Fig. 9, *A* and *A'*, IBMX). In contrast, bathing the ganglion in Ca-free sea water caused a very marked transformation in the action potential that is never seen in IBMX (see *B*, *B'*, Ca-free, and Figure legend for details). Records very similar to those seen in Ca-free solutions are seen when 500 μM-Cd is added to the bath.

Block of the depolarization-induced K conductance by a permeant cyclic AMP analogue. It was shown in the preceding paper that the permeant cyclic AMP analogue *p*-chlorophenylthioadenosine 3',5'-monophosphate (CPT-cyclic AMP) imitates the action of the blocking neurones in diminishing the cholinergic K conductance. To analyse further the hypothesis that the synaptic diminution of both the cholinergic and depolarization-induced K conductances is mediated by cyclic AMP, the effect of the permeant cyclic AMP analogue was tested on the depolarization-induced K conductance. As can be seen in Fig. 10, a block of this conductance occurs after a 30 min exposure to 500 μM CPT-cyclic AMP. Since synaptic transmission persists in the presence of this analogue, as it did in the presence of IBMX, it is assumed that the block of the depolarization-induced K conductance cannot be attributed to a drug-induced interference with Ca influx.

Persistence of the depolarization-induced block of the cholinergic synaptic K conductance in IBMX or CPT-cyclic AMP. It was shown above (Fig. 8) that when the depolarization-induced K conductance was eliminated by an EGTA-induced chelation of the inflowing Ca, the interaction between the depolarization and the cholinergic K conductance disappeared. In contrast, when the depolarization-induced K conductance is blocked by either IBMX or CPT-cyclic AMP, there is no alteration in the depolarization-induced block of the synaptic cholinergic K conductance. The persistence of this interaction in the presence of either IBMX or CPT-cyclic AMP can be seen in the upper and lower records, respectively, of Fig. 11.

These data reinforce the conclusion that the cyclic-AMP-related drugs are not interfering with the influx of Ca, but rather with the K conductance itself.

Effect of IBMX and CPT-cyclic AMP on the depolarization-induced block of the arecoline-activated K conductance. It was shown in Figs. 7 and 8 that the synaptically-

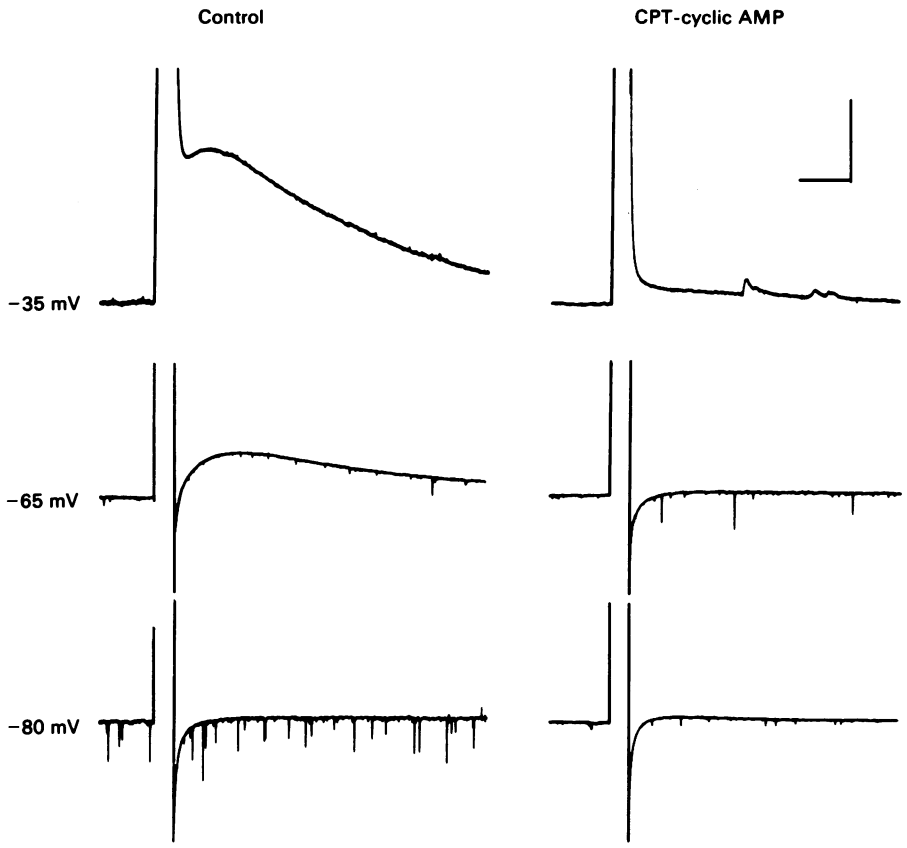


Fig. 10. Block of the depolarization-induced K conductance in a medial cell following a 30 min exposure of the ganglion to 500 μM-CPT-cyclic AMP. Note that the block occurs at all membrane potentials tested. Calibration: 2 nA, 5 s.

activated cholinergic K conductance is blocked by a depolarization of the post-synaptic cell. In Fig. 12 it can be seen that there is a similar block by depolarization of the arecoline-induced K conductance, and that the block clearly occurs on either side of E_K. By repeated voltage steps from -40 to -100 mV ($E_K = -80$ mV) one can see that prior to the addition of arecoline to the bath, depolarization elicits a net current that is outward at -40 mV and inward-going, though very brief, at -100 mV. After application of arecoline to the bath, and the development of a steady-state K current, the depolarizing voltage step is followed (at -40 mV) by a net inward-going current (relative to the steady-state current in arecoline), and, at -100 mV, by a net outward-going current. This diminution in K conductance is followed a few seconds later by a small but regularly occurring increase in K

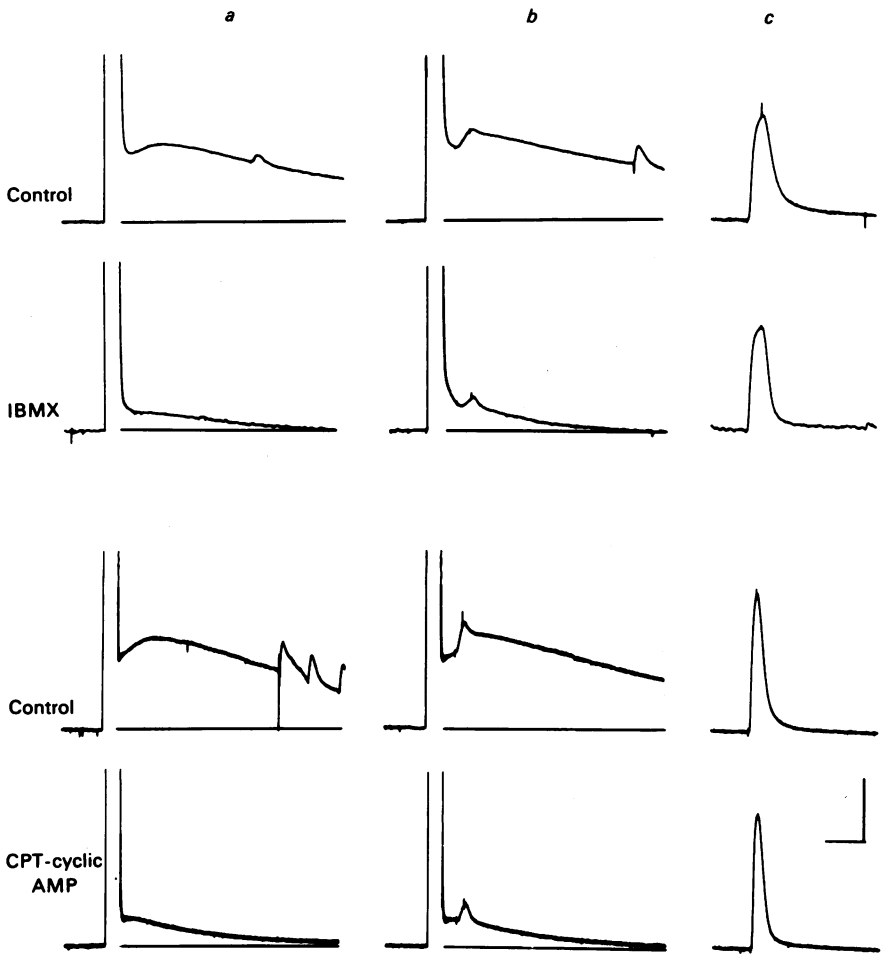


Fig. 11. Failure of either IBMX or CPT-cyclic AMP to affect the depolarization-induced block of the cholinergic K conductance in a curarized (200 μM-curare) medial cell. Upper records: failure of IBMX (100 μM), which eliminates the depolarization-induced K conductance (control *vs.* IBMX, column *a*), to affect either the depolarization-induced block of the cholinergic K conductance (control *vs.* IBMX, column *b*) or the cholinergic K conductance, itself (control *vs.* IBMX, column *c*). Lower records: failure of CPT-cyclic AMP (30 min exposure to 500 μM), which eliminates the depolarization-induced K conductance (control *vs.* CPT-cyclic AMP, column *a*), to affect either the depolarization-induced block of the cholinergic K conductance (control *vs.* CPT-cyclic AMP, column *b*) or the cholinergic K conductance, itself (control *vs.* CPT-cyclic AMP, column *c*). Calibration: 2 nA, 5 s. Holding potential: −40 mV. Post-synaptic voltage step: to +20 for 2 s; presynaptic stimulation: 2 s in upper records; 1 s in lower records.

conductance. The increase corresponds to the Ca-dependent K conductance that, in the later period, dominates the block of the cholinergically activated K conductance.

That this interpretation is the correct one is suggested by comparing the results shown in the last column of rows 1 and 2. In IBMX (cell 2, column 3), the depolarization-induced diminution in K conductance is of greater amplitude than

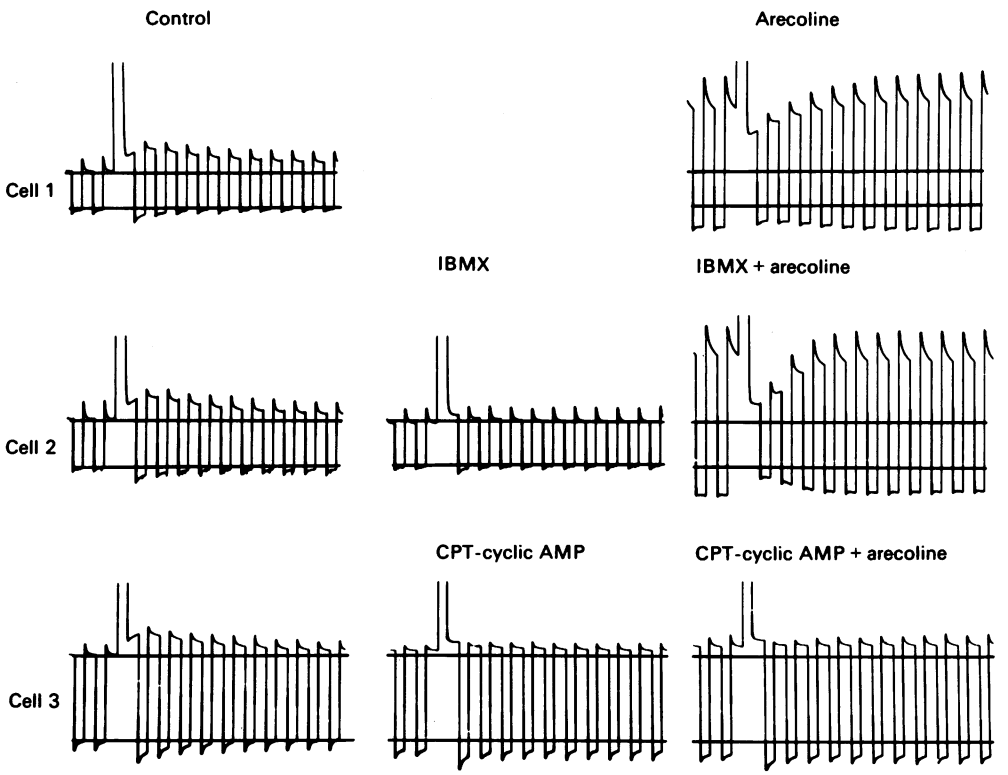


Fig. 12. Depolarization-induced block of the K conductance activated in the medial cells by 20 μM-arecoline. To study the effect of depolarization on the arecoline-induced conductance at more than one potential level, a constant train of 2 s hyperpolarizing voltage steps, separated by a 2 s interpulse interval, was used as a base-line control. When a test was to be made of the effect of membrane depolarization on the membrane conductance, one of the hyperpolarizing voltage steps was replaced by a depolarizing voltage step of the same amplitude, thereby bringing the cell to +20 mV for a 2 s period. Three different cells, each from a different ganglion, were used for the three experiments shown here. Experiment 1: if the control record from experiment 1 is compared with that taken in 20 μM-arecoline, it can be seen (by comparing the current responses of the first two voltage steps in each record) that in the presence of arecoline a steady-state current develops that is outward at −40 and inward at −100 mV. This K-dependent current is momentarily reduced by a 2 s depolarization of the cell. Experiment 2: experiment 2 illustrates that IBMX (100 μM) blocks the depolarization-induced K conductance, but impedes neither the development of the arecoline-induced K conductance nor its diminution by a brief depolarization of the arecoline-activated cell. A comparison of the data obtained from experiments 1 and 2 in arecoline shows, in fact, that the presence of IBMX causes an enhancement of the depolarization-induced block in the arecoline-activated K conductance. This enhancement simply reflects the absence, in IBMX, of the antagonistic increase in K conductance normally activated by depolarization of the cell (see also Fig. 13, cell 3). Experiment 3: in the last row of records, it is shown that a 30 min exposure to CPT-cyclic AMP (500 μM) blocks both the depolarization-induced K conductance and the arecoline-activated K conductance. Since the arecoline-activated K conductance does not develop in the presence of CPT-cyclic AMP, no diminution in membrane current appears following the depolarization of the cell. Holding potential: −40 mV. Calibration: 5 nA, 5 s.

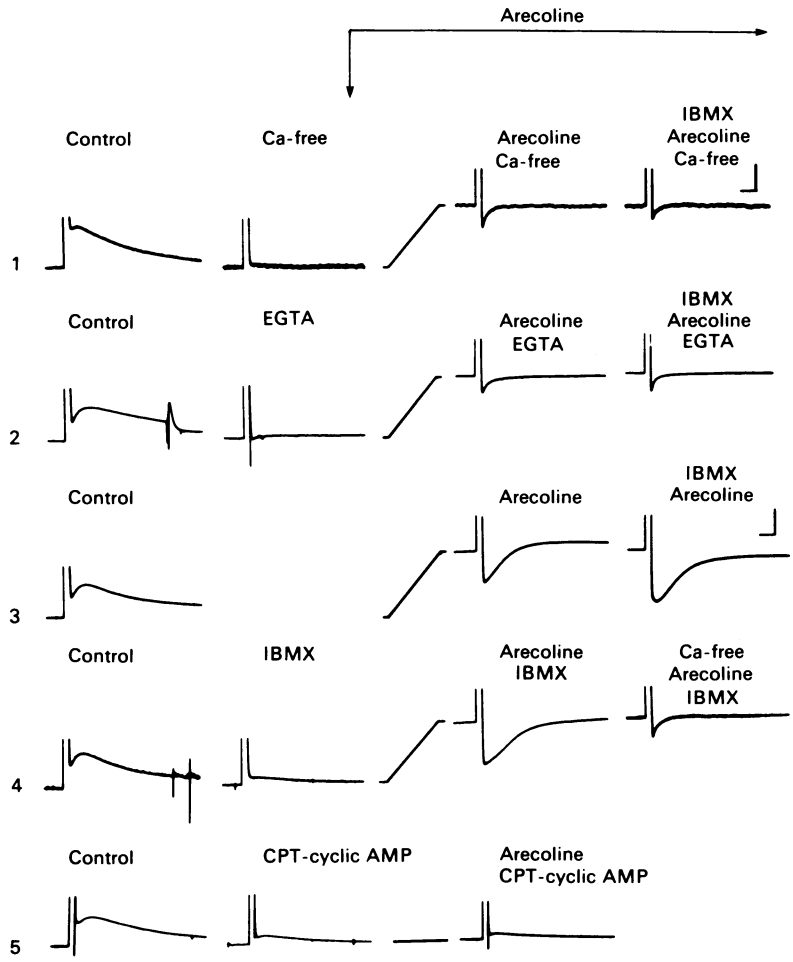


Fig. 13. Comparison of the effects of Ca-free sea water, a 45 min, 60 nA intracellular injection of EGTA, IBMX (100 μM), and CPT-cyclic AMP (500 μM) on the depolarization-induced K conductance and on the depolarization-induced block of the arecoline-activated (20 μM) K conductance. Experiment 1: replacement of the $CaCl_2$ in the sea water by $MgCl_2$ (Ca-free) resulted in the elimination of the depolarization-induced K conductance, but did not impede the development of the arecoline-activated K conductance (see change in current required for holding the cell at -40 mV in the presence of arecoline). In the Ca-free sea water, however, the 2 s depolarizing voltage step failed to cause a diminution in the arecoline-activated K conductance such as is normally observed, and shown in experiment 3 of this Figure. Even when IBMX was added to the Ca-free sea water, no depolarization-induced diminution in arecoline-activated conductance appeared. Experiment 2: an intracellular injection of EGTA likewise caused an elimination of the depolarization-induced K conductance and impeded the development of a depolarization-induced diminution in the arecoline-activated conductance which, itself, was unaffected. Experiment 3: this experiment shows the enhancement, by IBMX, of the depolarization-induced block of the arecoline-activated K conductance, which is easily explained by the elimination, by IBMX, of the depolarization-induced K conductance. Experiment 4: IBMX is shown here to eliminate the depolarization-induced K conductance, but to have no effect on either the development of the arecoline-activated K conductance, or its diminution by the depolarization of the post-synaptic cell. In the final record it is shown

in a cell that has not been exposed to IBMX (cell 1, arecoline). The enhancement of the depolarization-induced block of the arecoline-activated K conductance in the presence of IBMX (also visible in Fig. 13, cell 3) can be readily explained. Since IBMX blocks the depolarization-induced K conductance (see middle column, experiment 2), the diminution in the arecoline-induced K conductance is no longer counteracted by a current of the opposite direction, and the depolarization-induced blocking effect is enhanced (cell 2, last column). A similar accentuation of the depolarization-induced block was seen when IBMX was added to the arecoline-containing sea water in cell 1 (not shown). The records of this Figure also show that the cholinergic conductance is unaffected by IBMX if this drug application is unaccompanied by an intracellular cyclic AMP injection (note the usual arecoline-induced current in the presence of IBMX cell 2, last column; see also Fig. 21, Kehoe, 1985).

The last row of records (cell 3) shows that both the depolarization-induced and arecoline-activated K conductances are blocked by bath application of the cyclic AMP analogue CPT-cyclic AMP. The block of the cholinergic K conductance by CPT-cyclic AMP can be seen by the failure of the steady-state current, at either -40 or -100 mV, to change significantly when arecoline is applied to the bath. Furthermore, since no arecoline-induced current develops in CPT-cyclic AMP, there is no additional effect of depolarization seen in the arecoline-bathed cell. It should be noted that CPT-cyclic AMP often elicits a weak steady-state inward current.

Comparison of the effects of Ca deprivation and cyclic-AMP-related drugs on the depolarization-induced K conductance and the depolarization-induced block of the arecoline-activated K conductance. The records in 1 and 2 of Fig. 13 show the changes in the depolarization-induced conductance that occur under conditions that eliminate depolarization-induced increases in intracellular free Ca. These records can be compared, in the same Figure, with those taken in IBMX or in CPT-cyclic AMP seen in records 4 and 5, respectively.

As can be seen in the records of the second column (experiments 1 and 2), either removal of Ca from the sea water bathing the ganglion (1) or injection of EGTA in the medial cell (2) eliminates the post-depolarization outward current seen in the control records, but does not impede the normal activation of the arecoline-induced K conductance (see 5–6 nA increase in steady-state outward current when 20 μM-arecoline is added to the bath). The records taken in the presence of arecoline have been shifted to the level corresponding to the arecoline-induced current seen at the -40 mV holding potential. In spite of the presence of a normal arecoline-induced conductance in these 'Ca-deprived' cells, a depolarizing voltage step did not produce the long-lasting inward-going current indicative of a diminution in the arecoline current (compare arecoline record in Fig. 13, 3 *versus* 1 and 2).

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that removal of Ca from the arecoline-, IBMX-containing sea water causes an elimination of the depolarization-induced block of the arecoline-activated K conductance. Experiment 5: a 30 min exposure to CPT-cyclic AMP is shown here to block both the depolarization-induced K conductance and that activated by arecoline. Since no arecoline current develops in the presence of CPT-cyclic AMP, there is no diminution in K conductance following the depolarization of the cell in the presence of arecoline. Holding potential: -40 mV. Calibration: 2 nA, 5 s.

In contrast, when the depolarization-induced K conductance was blocked by IBMX rather than by Ca deprivation (see experiment 4, Fig. 13) the depolarization-induced diminution in the arecoline-activated conductance persists (experiment 4, arecoline+IBMX) and is in fact larger than that in arecoline without IBMX (experiment 3, arecoline).

CPT-cyclic AMP likewise eliminates the depolarization-induced, Ca-dependent K conductance. However, since it also blocks the development of the arecoline-induced K conductance (note no increase in outward current between the control and CPT-cyclic AMP records, experiment 5, and Kehoe, 1985), there is no appearance of a slow inward-going current following a depolarizing voltage step in a CPT-cyclic AMP–arecoline-bathed cell (see records in arecoline, experiment 5).

The finding that IBMX blocks the depolarization-induced K conductance at first suggested that this might be the result a depolarization-induced increase in cyclic AMP which, in the presence of IBMX, was sufficient for blocking the subsequent Ca-dependent K current. However, if cyclic AMP is indeed activated by depolarization, and if the prolonged presence of cyclic AMP is sufficient for blocking the cholinergically activated K conductance (see Fig. 14, Kehoe, 1985), one would expect that addition of IBMX to the sea water bathing a 'Ca-deprived' cell would permit the triggering of a depolarization-induced diminution in arecoline-activated conductance. That this is not the case can be seen in records of the final column of experiments 1 and 2, Fig. 13. In experiment 1, IBMX has been added to the Ca-free sea water, and in experiment 2, to the sea water bathing the EGTA-injected cell. In neither case did IBMX make manifest, in a Ca-deprived cell, the development of a depolarization-induced block of the arecoline-induced current. Experiment 4, performed in the opposite order, also shows that the presence of IBMX is not a sufficient condition for a depolarization-induced block of the cholinergic K conductance to appear. In that experiment, Ca-free sea water was added after IBMX had caused the enhancement of the depolarization-induced block in arecoline-activated conductance. Note the disappearance, in the Ca-free sea water, of the long-lasting inward-going current, in spite of the continued presence of IBMX.

The rapid inward current that occurs just following a depolarizing voltage step in the arecoline-bathed, Ca-deprived cell was not analysed, but since it increases with membrane hyperpolarization, it clearly is not the manifestation of a diminution in K conductance.

Ionic dependencies and pharmacological sensitivities of the response of medial cells to an intracellular ionophoretic application of Ca ions

Description of the response of the medial cells to an intracellular ionophoretic injection of Ca ions. The response of the medial cells to an intracellular ionophoretic application of Ca is almost always a multicomponent response, the complexity of which varies with the parameters of the Ca injection, the electrode used for injecting, the position of that electrode in the cell, the ganglion, and the membrane potential.

Fig. 14 illustrates most of the types of conductance change that can be elicited in these cells by an ionophoretic injection of Ca. The multicomponent response includes at least four distinct elements (labelled, *a*, *b*, *c* and *d*). Two early currents can be detected, although at −40 mV they are difficult to separate. However, a notch

on the upward swing of the early outward current corresponds to the onset of a not quite so early inward current that is only momentarily dominant at that membrane potential, and that is apparently isolated by hyperpolarization beyond −50 mV. The early outward current, showing marked rectification, is greatly reduced even at −60 mV, and hence does not interfere with the evolution of the inward current, which

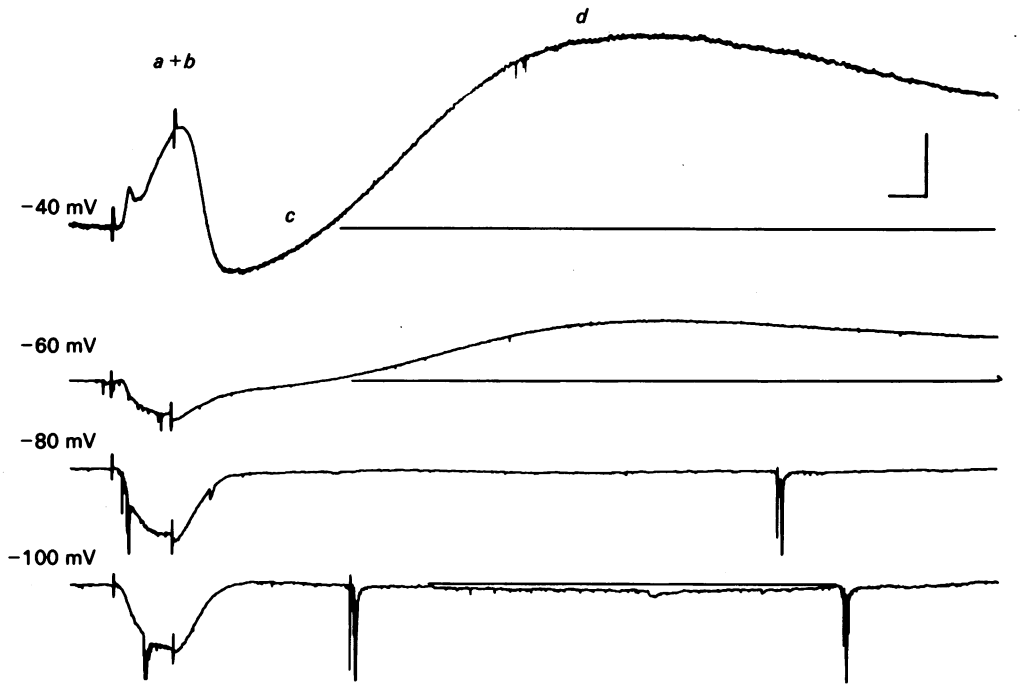

Fig. 14. Currents recorded in a voltage-clamped medial cell in response to an intracellular ionophoretic injection of Ca recorded at four different holding potentials (−40, −60, −80 and −100 mV). Four elements can be distinguished in the upper records. At −40 mV, an early outward current (*a*) hides another early current (*b*) which is the predominant current seen at membrane potentials more negative than −50 mV. The early inward current (*b*) can in fact be detected in the record at −40 mV in which a downward notch is seen on the rising phase of the outward current. The third element (*c*) of the response, which follows the early components, is inward-going at −40 mV and at −60 mV, is in equilibrium at −80 mV, but is inverted (though of very small amplitude) at −100 mV. This element reflects a diminution in membrane conductance that inverts at E_K. The final, slowly developing, long-lasting element (*d*) reflects an increase in conductance; outward at potentials less negative than E_K, and inward at potentials more negative than E_K. Calibration: 2 nA, 5 s. Ca injection: 11 s, 100 nA.

increases weakly with further hyperpolarization. At −40 mV, another, delayed inward-going current is seen and has been labelled *c*. This inward-going current becomes outward-going, though small, when the membrane is hyperpolarized beyond E_K, revealing that the current is due to a diminution in K conductance. Finally, many seconds after the end of the 11 s pulse, there appears (at −40 mV) a slowly developing, long-lasting outward current labelled *d*. This current, like *c*, inverts

at -80 mV (E_K) and is very small at -100 mV. It probably corresponds to the slow outward current observed by Hofmeier & Lux (1981) and to the long-lasting hyperpolarization seen by Meech (1972, 1974*a*, *b*) in response to an intracellular injection of Ca.

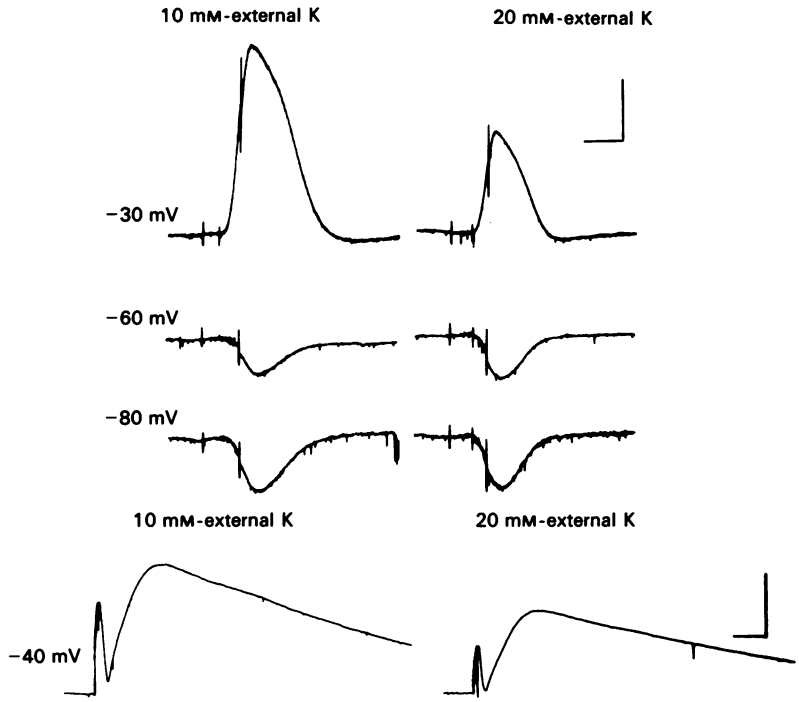

Fig. 15. Effect of doubling external K concentration on the response to an intracellular injection of Ca (4 s, 70 nA) as a function of membrane potential. Upper records: that the response to a Ca injection is not always a pure K conductance increase is shown in this experiment by the inward current measured at potentials less negative than or equal to E_K (-80 mV), i.e. at -60 and -80 mV respectively. The response at -40 mV was markedly reduced when external K was doubled, but there was no significant change in the responses measured at -60 or -80 mV. Calibration: 2 nA, 5 s. Ca injection: 2 s, 200 nA. Lower records: both the early outward and the slowly developing, long-lasting outward current (elements *a* and *d* of Fig. 14) were similarly reduced by a doubling of external K (from 10 mM to 20 mM). Calibration: 5 nA, 30 s. Ca injection: 400 nA, 4 s.

Effect of doubling the extracellular concentration of K on the response to an intracellular injection of Ca ions. In spite of the complexity of the usual response to Ca injection, it is often possible to find injection parameters that produce a very dominant early K-dependent response at -30 to -40 mV, i.e. the membrane potentials at which the depolarization-induced, Ca-dependent K conductance was studied.

For such a response, as shown in the upper records of Fig. 15, it can be seen that, when the external concentration of K is changed, there is a marked reduction in the outward current measured at -40 mV, whereas the inward currents measured at -60 and -80 mV are unchanged. These data confirm the finding of other authors (e.g. Gorman & Hermann, 1979; Hermann & Hartung, 1982) that the increase in

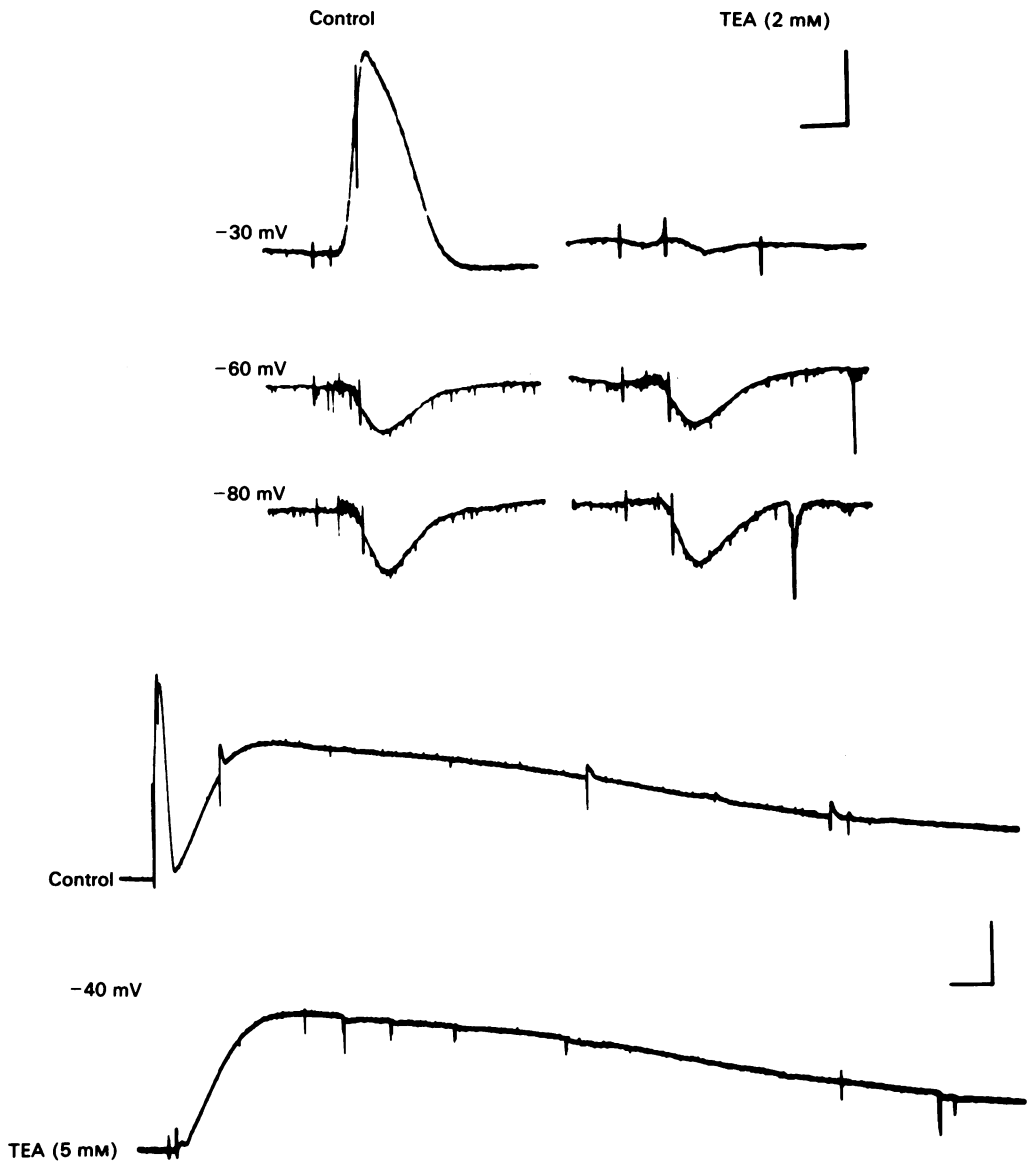

Fig. 16. Effect of TEA on the two K-dependent conductance increases activated by an intracellular injection of Ca. Upper records: 2 mM-TEA completely eliminates the 'early', outward element of the response to a 70 nA, 4 s injection of Ca into a medial cell (see record at −30 mV). Note, in contrast, that the early inward current, evaluated at −60 and −80 mV, is unaffected. Lower records: the late, slowly developing, long-lasting increase in K conductance triggered by an intracellular ionophoretic injection of Ca (400 nA, 5 s) and measured in a medial cell voltage clamped at −40 mV is unaffected by 5 mM-TEA, whereas the rapid, early K-dependent element of the response in the same cell is completely eliminated. Calibration: upper records: 2 nA, 5 s; lower records: 5 nA, 20 s.

K conductance induced by an intracellular injection of Ca ions is highly voltage dependent, disappearing at hyperpolarized membrane potentials.

The slowly developing, long-lasting *d* wave, almost always present when the intensity or duration of the Ca pulse is high, also shows a similar marked reduction in amplitude at −40 mV when the K in the sea water is doubled (from 10 to 20 mM). No attempt was made to try to evaluate this response over a number of membrane potentials in different K concentrations because its very slow time course and marked sensitivity to minor changes in the position of the Ca electrode made such a study unfeasible.

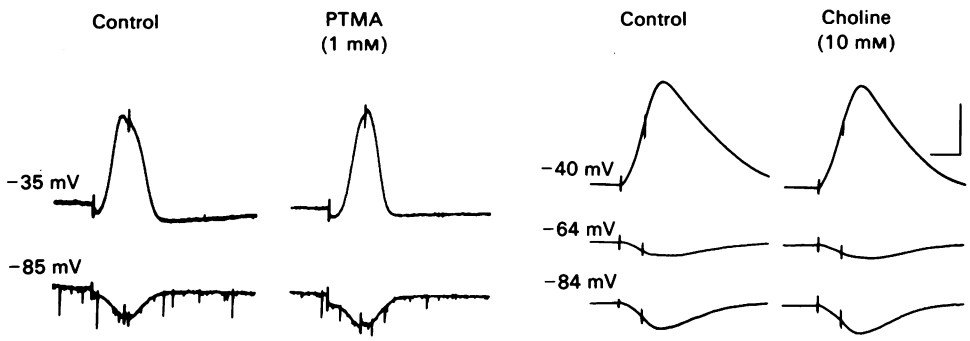

Fig. 17. Failure of the cholinergic antagonists PTMA (1 mM) and choline (10 mM) to affect either the early K-dependent element or the early inward element of the response to ionophoretically applied Ca in medial cells. Calibration: PTMA experiment: 2 nA, 5 s; choline experiment: 5 nA, 5 s.

Selective block by TEA of the early K-dependent response to an intracellular injection of Ca ions. Reinforcing the conclusion that the early, K-dependent element of the response of the medial cells to an intracellular injection of Ca corresponds to the response studied by a number of authors (e.g. Gorman & Hermann, 1979; Hermann & Hartung, 1982) is the finding shown in Fig. 16 showing the total elimination of this element of the complex response by 2 mM-TEA. The selective elimination of the outward element of the two early components of the response (upper records, Fig. 16) confirms the almost pure nature of the rising phase of the early outward current. The more surprising finding shown in the same Figure (lower records) is the failure of TEA (5 mM) to have any effect on the slow increase in K conductance activated in another medial cell by a longer duration and larger amplitude injection of Ca. The persistence of the slow K-dependent response in the presence of TEA can be compared with the total block by the quaternary ammonium ion of the early outward component in response to the same Ca injection. Similar results were obtained with 10 mM-TEA.

Insensitivity of both the early and late K-dependent elements of the 'Ca response' to PTMA and choline. In the preceding paper (Kehoe, 1985), it was shown that the three 'blocking' neurones studied in this paper cause a diminution in the cholinergically activated K conductance that itself can be eliminated by TEA, PTMA, choline and cyclic AMP. TEA was shown above to block the early Ca-activated K response in the medial cells, confirming a finding made in other cells by other investigators (see

Gorman & Hermann, 1979 and Hermann & Hartung, 1982). However, that this action of TEA is independent of its anticholinergic action can be seen in Fig. 17, in which it is shown that other cholinergic antagonists (PTMA, choline) have no effect on this same response to an intracellular injection of Ca. Not shown is a similar negative finding concerning the effect of these cholinergic antagonists on the slow K-dependent component of the 'Ca response'.

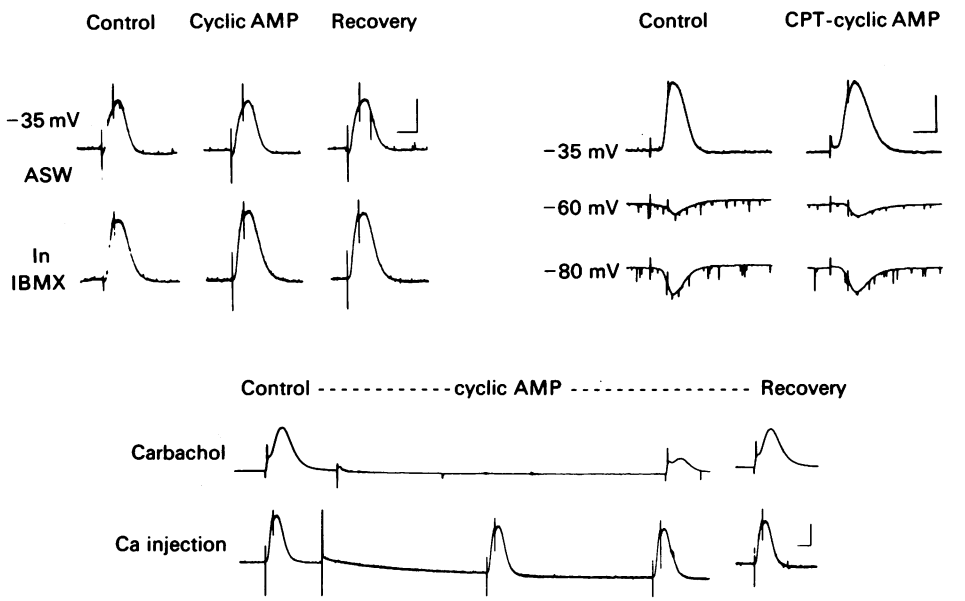

Fig. 18. Upper left: failure of an intracellular injection of cyclic AMP (200 s, 40 nA) (see cyclic AMP) to affect the early K-dependent response to a 3 s, 40 nA intracellular application of Ca, even in the presence of the phosphodiesterase inhibitor IBMX (100 μM) (see second row of records). 'Recovery' was measured 200 s following the end of the cyclic AMP injection. Holding potential: −35 mV. Upper right: failure of bath-applied cyclic AMP analogue CPT-cyclic AMP (1 mM, 40 min) to affect the response elicited by a 70 nA, 4 s intracellular injection of Ca. Lower records: comparison of the effects of a 2 min cyclic AMP intracellular injection (40 nA) on the cholinergic K conductance activated by an ionophoretic application of carbachol (100 nA, 1 s) and the early K conductance activated by an intracellular injection of Ca (40 nA, 2·5 s). Whereas the K-dependent element of the carbachol response is practically eliminated after a 2 min cyclic AMP injection, the early K response to a Ca injection is completely unaffected. Recovery of the cholinergic K conductance is still not complete 4 min after the end of the cyclic AMP injection. Holding potential: −35 mV. Calibration: 2 nA, 5 s.

Insensitivity of the early K-dependent element of the 'Ca response' of the medial cell to cyclic AMP, IBMX, and CPT-cyclic AMP. The purpose of studying the K-dependent response of the medial cells to an intracellular injection of Ca was to see to what extent this K conductance resembles that activated by Ca flowing across the membrane during a depolarizing voltage step. It was shown above that the depolarization-induced increase in K conductance is blocked by bath application of either IBMX or CPT-cyclic AMP. It can be seen in Fig. 18 that neither of these agents

blocks the rapid K conductance activated by an ionophoretic intracellular injection of Ca (upper left- and upper right-hand records, respectively). Likewise, it was found that whether or not IBMX was present, an intracellular injection of cyclic AMP had no effect on that response (upper left-hand records).

The failure of cyclic AMP to block this response cannot easily be attributed to a failure of the cyclic AMP and the Ca to reach the same zone, since both the Ca and the cyclic AMP injections were made in the soma of the cell. In the last set of two lines in Fig. 18 it is possible to compare, in the same cell over the same time period, the block by cyclic AMP of the cholinergic K conductance with the lack of effect of the same cyclic AMP injection on the early K current activated by an intracellular injection of Ca.

Although not shown in the Figure, experiments were also performed in which the slowly developing, long-lasting K-dependent response to an intracellular injection of Ca (*d* in Fig. 14), was studied in the presence of IBMX or CPT-cyclic AMP. These compounds almost always caused a diminution in this response, but in none of the five experiments performed for studying each of the agents was there as clear-cut an elimination of the response activated by a Ca injection as there was of the current activated by membrane depolarization. The effects of a cyclic AMP injection on the slow response activated by a Ca injection were not evaluated.

Evaluation of FMRFamide as a possible transmitter of the synaptic diminution in depolarization-induced K conductance

Data gathered by Cottrell *et al.* (1984) suggested that FMRFamide, applied to the soma of an identifiable snail neurone, caused a diminution in a K current activated by an intracellular injection of Ca. In view of these findings, the possibility was considered that each of the blocking neurones liberates, in addition to a cell-specific transmitter, the common co-transmitter FMRFamide which in turn mediates the common post-synaptic effect, i.e. the diminution in K conductance. To make a first test of this hypothesis, FMRFamide was injected on the medial cells during 'rest', and during the course of the depolarization-induced K conductance. It was found, however, that the response to an injection of FMRFamide (a multicomponent one, consisting of an early inward current and a delayed increase in K conductance) was the same, whether or not it occurred during the depolarization-induced K conductance. Likewise, no transformation in the response occurred when the peptide was applied on an arecoline-activated cell. Similar conclusions were drawn from experiments (not shown) in which FMRFamide (50 μM concentration) was applied to the bath rather than being locally injected. Hence, nothing supported the hypothesis that FMRFamide is the transmitter mediating the diminutions in K conductance caused by firing of the 'blocking neurones'.

DISCUSSION

The three presynaptic neurones that block the cholinergic K conductance also block a depolarization-induced, Ca-activated, K conductance

In the preceding paper (Kehoe, 1985), three neurones presynaptic to the medial cells were shown to be capable of diminishing the cholinergically controlled K conductance of those cells. In this paper, it has been shown that the same three

'blocking' neurones can also reduce, in the same post-synaptic cells, another K conductance: that activated by the influx of Ca occurring during a 1–2 s depolarizing pulse. Since this synaptic diminution of the Ca-dependent K current occurs even when the presynaptic cell is fired many seconds after the depolarizing voltage step, it is clear that the synaptic block is not a secondary effect of an interference with the influx of Ca.

The presynaptic neurone (V), shown in the preceding paper (Kehoe, 1985) to have no effect on the cholinergic K conductance, was likewise found to have no effect on the depolarization-induced K conductance studied here.

The two K conductances that are diminished by the blocking neurones are independently elicited. The similarity of the slow excitatory post-synaptic potential (e.p.s.p.) representing a diminution in the cholinergic K conductance (Kehoe, 1985) and that representing a diminution in the depolarization-induced conductance studied here suggests that the two synaptic events share a common underlying mechanism. In spite of this presumed common link, the evidence strongly suggests that the two K conductances, themselves, are independently elicited. The depolarization-induced conductance disappears when Ca is eliminated from the extracellular medium or when EGTA is injected into the cell. The cholinergically activated K conductance, on the other hand, is maintained in such Ca-deprived cells. The independence of the two K responses is also demonstrated by their differential sensitivity to TEA. Whereas the cholinergically activated K conductance is completely blocked by 1 mM-TEA, the depolarization-induced, Ca-dependent K conductance persists in sea water containing 10 mM-TEA. Similarly, other agents previously shown to block the receptor mediating the cholinergic K conductance in these cells (1 mM-PTMA or choline) have also been shown to have no effect on the depolarization-induced conductance.

That conductances of different origins can be diminished in the same cell by the same transmitter (or by the same synaptic input) has not been observed very frequently. It has however been established, for example, that ACh in hippocampal cells (Halliwell & Adams, 1982) can block both the M-current (Adams, Brown & Constanti, 1982) and the Ca-dependent K conductance (Cole & Nicoll, 1983; see also Bernardo & Prince, 1982). Likewise, many investigators working on bull-frog sympathetic ganglion cells find that there remains a K conductance that is sensitive to muscarinic agonists in a voltage range over which the M-current, also transmitter sensitive, is no longer present (e.g. Katayama & Nishi, 1982; Jan & Jan, 1982). In the same neurones, it has recently been shown that muscarine also reduces, though weakly, a Ca-activated K conductance (Adams, Jones, Pennefather, Lancaster, Galvan & Satin, 1984). Likewise, Brown & Selyanko (1985) have shown in rat sympathetic neurones that, in addition to the K-dependent 'M'-current, muscarine also diminishes a Cl-dependent current. Finally, it has recently been shown in snail neurones that both FMRFamide in certain cells (Colombaioni, Paupardin-Tritsch, Vidal & Gerschenfeld, 1985) and dopamine (DA) in others (Paupardin-Tritsch, Colombaioni, Deterre & Gerschenfeld, 1985) can each diminish more than one ionic conductance.

The depolarization-induced block of the cholinergic K conductance and the synaptically activated diminution of that same conductance appear to be independent. In the EGTA-injected cell, the depolarization-induced block of the cholinergic K conductance

disappears (see also Ascher & Chesnoy-Marchais, 1982; Chesnoy-Marchais & Ascher, 1983). In contrast, when the depolarization-induced K conductance is blocked by either IBMX or CPT-cyclic AMP, which presumably do not block the voltage-gated entrance of Ca ions into these cells, the depolarization-induced block of the cholinergic K conductance persists. Consequently, the block in the cholinergic conductance by depolarization appears to be dependent upon the increase in intracellular free Ca, and hence of a different origin than the synaptic diminution in that same conductance (caused by the blocking neurones), which persists in the EGTA-injected cell.

Comparison of the depolarization-induced K conductance with that elicited by an intracellular, ionophoretic injection of Ca

The effects of a Ca injection in the medial cells were studied in order to obtain a better understanding of the Ca-activated, depolarization-induced outward current that is diminished by the blocking neurones.

It was found that the response to an intracellular injection of Ca can be quite complex, often consisting of at least four elements (see Fig. 14). An early K-dependent current is superimposed upon an inward current that can be detected when the K-dependent element is eliminated either by hyperpolarization of the cell, or by addition of 1 mM-TEA to the bath. These two early conductance changes are those previously reported and studied by a number of investigators, e.g. Gorman & Hermann (1979), Hermann & Hartung (1982), and Hofmeier & Lux (1981). The third element in the response of the medial cell to a Ca injection is a delayed inward current that reflects a diminution in K conductance. This response element is followed by a much more slowly developing increase in K conductance. Although this final element is probably the same as that observed originally by Meech (1972, 1974*b*), only the early K conductance increase studied later by other investigators has been pharmacologically characterized.

When, in the medial cells, the K currents elicited by a Ca injection are compared with the depolarization-induced, Ca-dependent K current, it can be seen that although TEA at 1 mM completely blocks the early, rapid element of the response to ionophoretically applied Ca, it has no effect, even at 10 mM, on the slow K-dependent element of that response or on the depolarization-induced K conductance. On the other hand, IBMX and CPT-cyclic AMP, which completely eliminate the depolarization-induced K conductance, have no effect on the early K-dependent response to a Ca injection, but reduce (though sometimes only slightly) the delayed K increase to the same injection. These data suggest that a parallel, albeit limited, can be drawn between the delayed increase in K conductance activated by a Ca injection and the depolarization-induced K conductance.

TEA-resistant, Ca-activated K conductances. TEA-resistant, Ca-dependent K conductances have been previously observed in response to a depolarizing pulse in voltage-clamped neurones in a variety of preparations, e.g. frog motoneurones (Barrett & Barrett, 1976), neuroblastoma cells (Moolenaar & Spector, 1979), bull-frog sympathetic ganglion neurones (Pennefather, Lancaster, Adams & Nicoll, 1985) and molluscan neurones (e.g. Thompson, 1977; Aldrich, Getting & Thompson, 1979; Smith & Zucker, 1980; Barish & Thompson, 1983; Deitmer & Eckert, 1985).

Furthermore, the method of internal perfusion in molluscan neurones has revealed similar TEA-resistant Ca-activated K currents, whether in response to an influx of Ca by voltage-dependent channels, or by increases in Ca in the internal perfusion medium (Kostyuk, Doroshenko & Tsyndrenko, 1980). Using patch-clamp recording methods a channel that might be the one carrying such TEA-resistant currents has recently been observed (Lux, Neher & Marty, 1981; Ewald, Williams & Levitan, 1985).

In contrast, in many of the studies of the effects of an intracellular injection of Ca in which long-duration, K-dependent responses were seen (e.g. Meech, 1972, 1974*b*; Hofmeier & Lux, 1981), the sensitivity of the conductance change to TEA was not evaluated.

The data presented here on the pharmacology of the response to intracellularly injected Ca therefore reinforce the conclusions recently drawn from studies of the Ca-dependent K currents elicited by depolarizing pulses in bull-frog sympathetic ganglion neurones (Pennefather *et al.* 1985): that two different types of Ca-activated K conductances can exist in the same cell. Furthermore, in both cases the current showing the faster kinetics and the higher voltage dependence was found to be more sensitive to TEA.

Significance of the block of the depolarization-induced K conductance by IBMX and CPT-cyclic AMP, and the possible role of cyclic AMP as mediator of the synaptic diminution

As was shown in Figs. 9, 10 and 11, both IBMX and CPT-cyclic AMP block almost completely the depolarization-induced K conductance. Similar results were obtained with aminophylline. These effects do not appear to be a secondary effect of an interference with the depolarization-induced influx of Ca since (1) synaptic transmission is in no way diminished in the presence of these drugs, as estimated from the persistence of normal amplitude post-synaptic potentials in response to all of the six presynaptic neurones tested, and (2) the action potential of the medial cells does not appear to be affected by IBMX or aminophylline (the effect of CPT-cyclic AMP on the action potential not having been evaluated). These data suggest that IBMX and CPT-cyclic AMP either interfere with the gating of the K channels or block these channels themselves. That the drugs capable of blocking this current are either phosphodiesterase inhibitors (IBMX and aminophylline) or an analogue of cyclic AMP (CPT-cyclic AMP) suggests that cyclic AMP is the mediator of the synaptically activated diminution in this depolarization-induced K conductance.

However, it must be remembered that cyclic AMP injected into the soma of a medial cell has no effect on the depolarization-induced K conductance. This finding might be explained by the fact that the cyclic AMP cannot reach the site of action before being degraded. That the site at which the conductance change occurs is indeed not primarily somatic is shown by the finding that little of the slowly developing, long-lasting depolarization-induced K conductance remains when the cell body is isolated.

It seemed possible, therefore, that the IBMX block of the depolarization-induced conductance reflects the combined action of the phosphodiesterase inhibitor and accumulating, endogenous cyclic AMP. Could not the depolarization itself trigger

an increase in intracellular cyclic AMP? If this were the case, one would expect that in an EGTA-injected cell, addition of IBMX would cause a reappearance of a depolarization-induced block of the cholinergic response; this time mediated by cyclic AMP, activated by the depolarization and now protected from destruction. As can be seen in Fig. 13, this IBMX-induced transformation did not occur. However, it might be possible either that Ca and cyclic AMP must both be present for such a diminution in the K conductance to take place, or that an increase in intracellular Ca induces an increase in cyclic AMP.

Cyclic AMP has been shown to be the likely mediator of many transmitter-induced diminutions in K conductance (see Castellucci, Kandel, Schwartz, Wilson, Nairn & Greengard, 1980; Deterre, Paupardin-Tritsch, Bockaert & Gerschenfeld, 1981; Deterre, Paupardin-Tritsch, Bockaert & Gerschenfeld, 1982; Siegelbaum, Camardo & Kandel, 1982; Madison & Nicoll, 1982). In the experiments of Madison & Nicoll (1982), the K conductance being diminished by cyclic AMP is activated by intracellular Ca. Furthermore, in bag cells of *Apylsia*, cyclic AMP appears to diminish a Ca-activated K conductance (Kaczmarek & Strumwasser, 1984) as well as the rapidly inactivating, voltage-gated 'A'-current (Strong, 1984).

On the other hand, it should be recalled that addition of the catalytic subunit of cyclic-AMP-dependent protein kinase to internally perfused molluscan neurones has been shown to increase a Ca-dependent net outward current (de Peyer, Cachelin, Levitan & Reuter, 1982). Furthermore, Ewald & Eckert (1983) have recently demonstrated that cyclic AMP analogues and cholera toxin potentiate a rapid K current activated by an intracellular injection of Ca. The conclusions drawn by these investigators have been strengthened by recent studies (Ewald *et al.* 1985) in which the activity of Ca-sensitive single K channels recorded from isolated membrane patches has been increased by exposure of the patch to the catalytic subunit of cyclic-AMP-dependent protein kinase (accompanied by ATP). These channels of low conductance (20 pS) appear to be the same as those observed by Lux *et al.* (1981) in a cell-attached patch (associated with a spontaneous action potential or a depolarizing voltage step). These data concerning the sensitivity of Ca-activated K conductances to cyclic AMP, which lead to seemingly opposite conclusions from those drawn from the experiments cited above (e.g. Madison & Nicoll, 1982) or from the data presented here, might reflect that the action of cyclic AMP is cell dependent (able to potentiate a given conductance in one cell and to inhibit that same conductance in another), or that the Ca-dependent K conductances showing differential sensitivity to cyclic AMP are, themselves, different (see below).

Are the effects of TEA and cyclic AMP on Ca-activated K conductances correlated?

It is difficult to establish at this time whether the separation of Ca-activated K conductances into TEA-sensitive and TEA-insensitive currents parallels the distinction between cyclic-AMP-enhanced and cyclic-AMP-inhibited Ca-dependent K conductances, since in the investigations in which the effects of the catalytic subunit of cyclic-AMP-dependent protein kinase (de Peyer *et al.* 1982; Ewald & Levitan, 1984) or cyclic AMP injections (Madison & Nicoll, 1982) were studied on a Ca-activated K conductance, no evaluation was published concerning the TEA sensitivity of that conductance.

In the study of Ewald & Eckert (1983) it appears clear that the K conductance

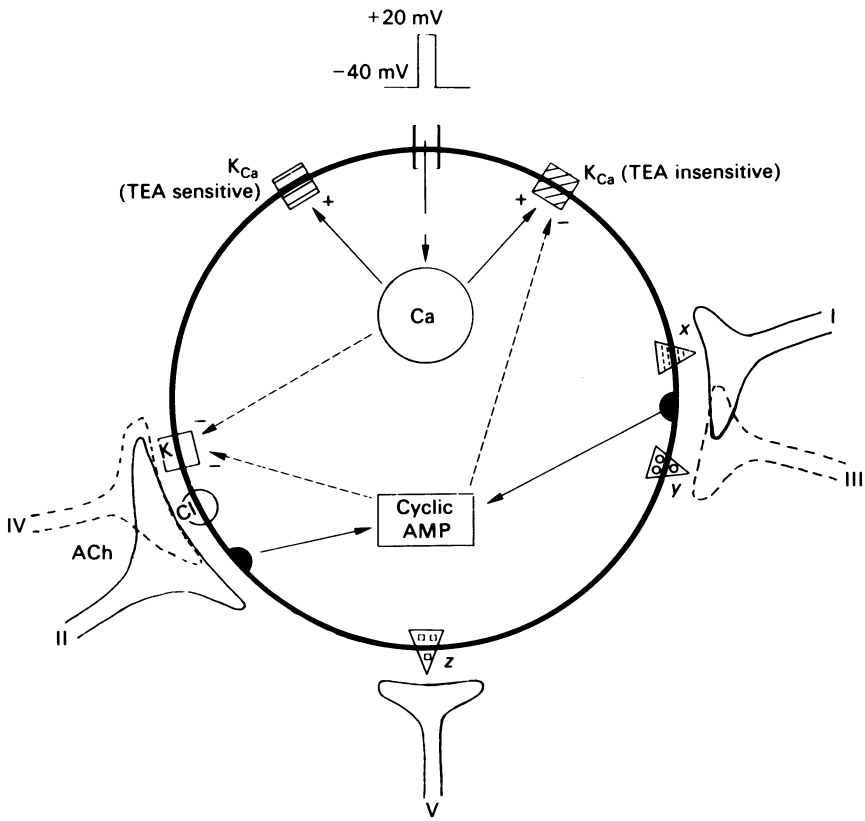

Fig. 19. Schematic description of the phenomena studied in this and the preceding paper and the mechanisms presumed to underly their interactions. The cholinergic presynaptic neurones (II and IV) open independent Cl and K channels. Cholinergic presynaptic neurone II is assumed, in addition, to stimulate (via a third, independent pathway) an increase in intracellular cyclic AMP. Cholinergic presynaptic neurone IV, on the other hand, has practically no access to the cyclic-AMP-associated receptor. The blocking neurones I and III both activate a cyclic-AMP-associated receptor, but presumably have no other common actions. These two neurones have purely excitatory actions of unknown ionic mechanisms (x, y), and therefore activate neither Cl-specific nor K-specific channels. Depolarization activates a Ca channel through which Ca enters the cell and activates two distinct K channels (K_{Ca}): one blocked by 1 mM-TEA (TEA sensitive); the other unaffected by 10 mM-TEA (TEA insensitive). In addition, the Ca that enters the cell blocks the ACh-activated K channel. Intracellular cyclic AMP, increased by the the three blocking neurones (I, II, III), inhibits both the ACh-activated K channel and the Ca-activated, TEA-insensitive K channel. The Ca-induced and the cyclic-AMP-induced block of the cholinergic K channel are assumed to be independent. Presynaptic neurone V, which excites the post-synaptic cell by an unknown ionic mechanism (z), blocks neither of the two K conductances studied.

triggered by a Ca injection, and enhanced by cyclic AMP analogues, corresponds to the TEA-sensitive response studied by Gorman & Hermann (1979) and Hermann & Hartung (1982), as well as to the TEA-sensitive element *a* of the response of Fig. 14 in this paper. In the medial cells, this early, K-dependent response was unaffected by both cyclic AMP and phosphodiesterase inhibitors.

Consequently, from the limited data that have been published on the subject, it

appears that the sensitivity to TEA and cyclic AMP are not necessarily correlated, and that cyclic AMP does not necessarily have the same effect on the same conductance in different cells, even in the same nervous system.

In summary, the delayed, long-lasting K current elicited in the medial cells by an ionophoretic injection of Ca resembles in many ways the depolarization-induced K current that is synaptically diminished. However, only after better understanding the means by which IBMX and CPT-cyclic AMP interfere with this current will it be possible to determine whether a synaptically activated increase in intracellular cyclic AMP is or is not responsible for the synaptic diminution in that current. The marked similarity in the slow e.p.s.p.s representing the diminution in the two K conductances (depolarization induced and cholinergically activated) makes it difficult to imagine that different mechanisms could underlie these two synaptic events.

Fig. 19 summarizes schematically the different K currents studied in this and the preceding paper (ACh and Ca activated), the effects of TEA on these various currents, their sensitivity to intracellular Ca, and the presumed role of synaptically activated cyclic AMP in their modulation.

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