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NEUROTRANSMITTERS DECREASE THE CALCIUM CONDUCTANCE ACTIVATED BY DEPOLARIZATION OF EMBRYONIC CHICK SENSORY NEURONES

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SUMMARY

Several neurotransmitters including noradrenaline (NA), γ-aminobutyric acid (GABA) and serotonin (5-HT), and also certain peptides, decrease the duration of the Na^+–Ca^{2+} action potential recorded in cell bodies of embryonic chick dorsal root ganglion neurones maintained in cell culture. To determine if these agents decreased action potential duration by affecting Ca^{2+} channels (inward current) or K^+ channels (outward current) membrane currents were recorded in voltage-clamped sensory neurone somata.

1. Depolarization produced a prominent inward Na^+ current and a smaller and slower inward Ca^{2+} current (I_{Ca}). The inactivation of I_{Ca} was not simply dependent on membrane potential but apparently required prior entry of Ca^{2+}. Two components of outward current, voltage-activated and Ca^{2+}-activated, were evident in most cells.

2. The effect of NA, and also of GABA and 5-HT, was shown to result from a direct effect on I_{Ca} because: NA decreased the TTX-resistant tail current recorded at E_K and also the inward current recorded in the presence of 125 mM-TEA and TTX (in which Na^+ and K^+ currents were blocked).

3. The decrease in I_{Ca} is most likely due to an effect on the number of available Ca^{2+} channels and/or the single Ca^{2+} channel conductance rather than to a shift in either the kinetics of channel activation or the Ca^{2+} equilibrium potential.

4. No effect of the several transmitters on the voltage-dependent Na^+ and K^+ currents was observed.

5. Implications of I_{Ca} modulation for the phenomenon of presynaptic inhibition are discussed.

INTRODUCTION

Certain neurotransmitters and neuropeptides can alter the shape of neuronal action potentials even though they do not produce a change in resting membrane potential or conductance. Noradrenaline (NA), γ-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT), enkephalin and somatostatin all reversibly decrease the duration of action potentials recorded in embryonic chick dorsal root ganglion (d.r.g.) neurones

maintained *in vitro* (Dunlap & Fischbach, 1978; Mudge, Leeman & Fischbach, 1979). NA decreases the duration of rat superior cervical gangion spikes (Horn & McAfee, 1979, 1980). 5-HT prolongs action potentials recorded in *Aplysia* sensory neurones (Klein & Kandel, 1978), and an unidentified transmitter, released on stimulating one of the inputs to the test ganglion, decreases spike duration in other ganglion cells (Shapiro, Castellucci & Kandel, 1980). Each type of action potential examined to date is a mixed Na^+–Ca^{2+} spike. The inward Ca^{2+} current rises slowly compared to the Na^+ current, and it inactivates slowly. Therefore, a change in spike duration implies a change in Ca^{2+} influx. In principle a neurotransmitter-induced change in spike duration might be due to an effect on inward Ca^{2+} current or outward K^+ current. Our first experiments with chick sensory neurones suggested that NA, GABA, and 5-HT decreased the inward Ca^{2+} current, but an increase in outward K^+ current could not be ruled out. In this paper, a further analysis of membrane currents measured directly in voltage-clamped d.r.g. cell bodies demonstrates that the transmitters do, in fact, exert a direct effect on voltage-sensitive Ca^{2+} channels. NA was studied in most experiments, but GABA and 5-HT act in the same way. A preliminary account of some of these experiments has been published (Dunlap & Fischbach, 1979).

The notion that neurotransmitters can modulate voltage-sensitive channels is not new. In the heart, NA increases an inward Ca^{2+} current and also a delayed outward K^+ current (Tsien, Giles & Greengard, 1972; Reuter, 1974) during the ventricular action potential; ACh decreases inward Ca^{2+} current during the atrial spike (Giles & Noble, 1976). Thus, actions on voltage-sensitive channels in the myocardium may account, in part, for the positive inotropic effect of NA and the negative inotropic effect of ACh. In neurones, this type of transmitter action at sites of transmitter release may effectively modulate synaptic efficacy.

METHODS

Cell culture. Dissociated cell cultures of embryonic chick sensory neurones were prepared with slight modification of techniques described by Dichter & Fischbach (1977). Dorsal root ganglia dissected from 10-to 12-day-old embryos were incubated at 37 °C for 30 min in a Ca^{2+} and Mg^{2+}-free saline (Pucks D_1G), and then mechanically dissociated into single cells by repeated passage through a fire-polished Pasteur pipette. Proteolytic enzymes were not used. The cells were plated in Eagle's Minimum Essential Medium (MEM) supplemented with heat-inactivated horse serum (10 %, v/v), chick embryo extract (5 %, v/v), 2·5 S Nerve Growth Factor (1 μg/ml.), penicillin (50 U/ml.), and streptomycin (50 μg/ml.). NGF was purified from mouse salivary glands and kindly supplied by Dr Anne Mudge. Multiplication of fibroblasts and glia was halted by adding cytosine arabinoside to the medium at 5×10^{-6} M for 72 hr beginning 12–24 hr after plating. Cultures between 2 and 6 weeks of age were used in this study. During electrophysiological experiments the cells were viewed with phase-contrast optics, and they were maintained at room temperature (about 25 °C).

Voltage clamp. After 2 weeks in culture, the largest neurones could be penetrated with two 10–20 MΩ electrodes, one for recording and the other for injecting current. The current electrode was painted with silver to within 50 μm of the tip and insulated with carbowax. The silver shield was connected directly to ground. The membrane potential was controlled with a feed-back amplifier loaned (donated) by Dr Charles Stevens. The capacitative surge and other artifacts were negligible within 0·5 msec of the onset of 50–100 mV command pulses. Several criteria indicate adequate voltage control of the impaled soma: the decay of the inward current following peak activation was free of notches or distortions; the maximum inward current was a continuous, gradual function of membrane potential over a 30–40 mV range; current tails following cessation

of a command pulse decayed according to a smooth exponential (Cole, 1968; Johnson & Lieberman, 1971).

An estimate of series resistivity (R_s) was made by dividing the apparent time constant of decay of the capacitative charging curve (τ_c) by the specific membrane capacitance (C_m) (Hodgkin, Huxley & Katz, 1952). The capacitative transient was examined following small (5–20 mV) voltage steps from the holding potential of −50 mV. At an optimal gain setting of the feed-back amplifier there was some oscillation of the current trace immediately after the voltage step, but a smooth curve could be drawn through the current decay phase. The time constant of such curves was invariably less than 10 μsec. The total membrane capacity was estimated by dividing the integrated capacitative current by the voltage displacement. All cells used to estimate R_s had a smooth, circular outline under phase-contrast illumination. Estimates of cell area used in the calculation of C_m were based on the assumption that the cells were perfect spheres. In four cells, the mean C_m was 1·7 μF/cm^2, so R_s was taken as approximately 5·9 Ω cm^2. The largest total TTX-resistant, inward currents observed in this study were about 25 nA. Assuming a mean cell diameter of 40 μm, this current density of 520 μA/cm^2 flowing across R_s would result in an error in the membrane potential measurement of only 3 mV. This must be considered an upper limit.

Solutions. The standard recording solution used during electrophysiological experiments contained 10 mM-$CaCl_2$, 125 mM-NaCl, 5·9 mM-KCl, 0·8 mM-$MgCl_2$, 25 mM-HEPES and 5 mM-glucose. Variations in the standard solution are noted in the text. The pH of each solution was adjusted to 7·3. Tetrodotoxin (TTX) and 4-aminopyridine (4-AP) were added in some experiments at 10^{-7} g/ml. and 10^{-3} M, respectively, as indicated.

Drug application. Drugs were applied in known concentration by pressure ejection from a 3–5 μm tip 'puffer' pipette (Choi, Farb & Fischbach, 1978; Choi & Fischbach, 1981). Control experiments have shown that when the puffer is positioned *ca.* 50–100 μm from the impaled soma, ejection at 1–2 lb/in^2 rapidly (within 1 sec) 'bathes' the cell in the same concentration of drug as is present in the pipette to within 10% (Choi & Fischbach, 1980). The pressure pulse is often accompanied by a small (2–5 mV) hyperpolarization (see Fig. 1*B*) that quickly returns to base line when the pulse is terminated. This is an artifact of the pressure ejection technique: ejection of recording medium without added drug is associated with the same shift in potential whether the recording electrode is inside the cell or immediately outside it. Drug effects described in this paper were measured several seconds after the puffer artifact.

RESULTS

Embryonic chick d.r.g. neurones maintained in cell culture generate action potentials that are relatively prolonged. Under our conditions of culture, distinct plateaus during the repolarization phase were evident at all times up to 6 weeks after plating; they were especially prominent in high Ca^{2+} (5·4–10·0 mM) media. As previously reported, NA applied by pressure ejection at concentrations between 5×10^{-7} and 10^{-4} M rapidly and reversibly decreased the duration of the plateau. A typical result is shown in Fig. 1*A* where in each panel the briefer of the two superimposed spikes was recorded a few seconds after application of 10^{-5} M-NA. When the initial Na^+ component of the spike was blocked with TTX, NA decreased the rate of rise and the amplitude of the spike as well as the duration (Fig. 1*A*, right). The NA-induced decrease in spike size and duration was never associated with a change in resting membrane potential or input resistance (Fig. 1*B*). The small hyperpolarization evident in Fig. 1*B* is an artifact coincident with the pressure ejection pulse (see Methods).

The effect of NA on spike duration did not desensitize rapidly. As shown in Fig. 2 the decrease in duration was maintained throughout a 4-min-long application of 10^{-5} M-NA. Spike duration usually returned to control values within 30–60 sec following ejection of 10^{-5} M or lower concentrations of NA, but recovery was slower (2–3 min) following ejection of higher (10^{-4} M) concentrations.

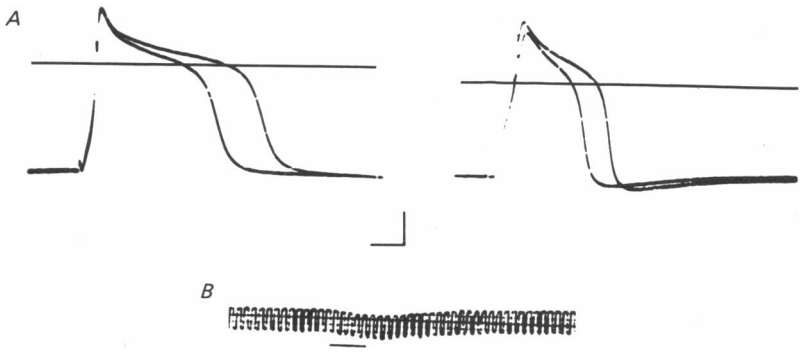

Fig. 1. NA decreases d.r.g. spike duration. *A*, action potentials recorded from two different d.r.g. cells were evoked by 1 msec square current pulses passed through the recording electrode using an active bridge circuit. Left: control solution containing 5·4 mM-Ca^{2+}; right: another cell bathed in 5·4 mM-Ca^{2+} plus 10^{-7} TTX g/ml. *B*, resting membrane resistance was tested by injecting 100 msec pulses of inward current before, during and after application of 10^{-5} M-NA. Horizontal bar indicates period of NA application. Calibrations *A*: 20 mV, 2 ms; *B*: 10 mV, 1 sec.

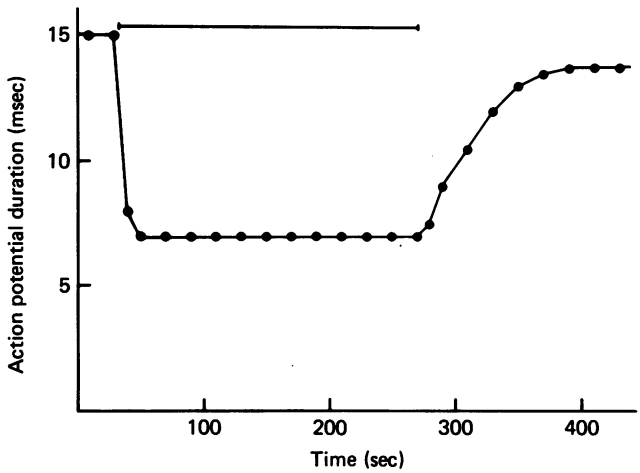

Fig. 2. Lack of NA desensitization. One action potential was evoked every 10 sec before, during and after a 4 min application of 10^{-5} M-NA (horizontal bar). Action potential duration was measured as time from peak to half fall.

Membrane currents recorded in voltage-clamped d.r.g. cell bodies

The following brief description of membrane currents recorded in voltage-clamped d.r.g. neurones is a necessary preface to our further analysis of NA action. The resting membrane potential of d.r.g. neurones adequately penetrated with a single microelectrode ranges between −40 and −60 mV. Many cells larger than 35 μm in diameter could be penetrated with two 20 MΩ electrodes and voltage-clamped at −50 mV for more than 30 min without significant change in the holding current or in the phase contrast appearance of the soma.

Inward Na^+ and Ca^{2+} currents. In control medium, a step depolarization from −50

to +10 mV activated a transient inward current that reached a peak in 1–2 msec, followed by a large and maintained outward current (Fig. 3, upper traces). Most of the inward current is probably carried by Na^+ ions (I_{Na}) since only a small inward current remained in the presence of 10^{-7} TTX g/ml. (Fig. 3, middle traces). In some cells bathed in TTX, there was an early minimum in the net membrane current recorded at +10 mV, but the current was outward at all times. However, the presence of a slowly rising, TTX-resistant inward current was always revealed when 4-AP, a drug that partially blocks voltage-dependent K^+ currents in other cells, was ejected along with TTX (Fig. 3, lower traces). Action potentials recorded in TTX and 4-AP were prolonged compared to control.

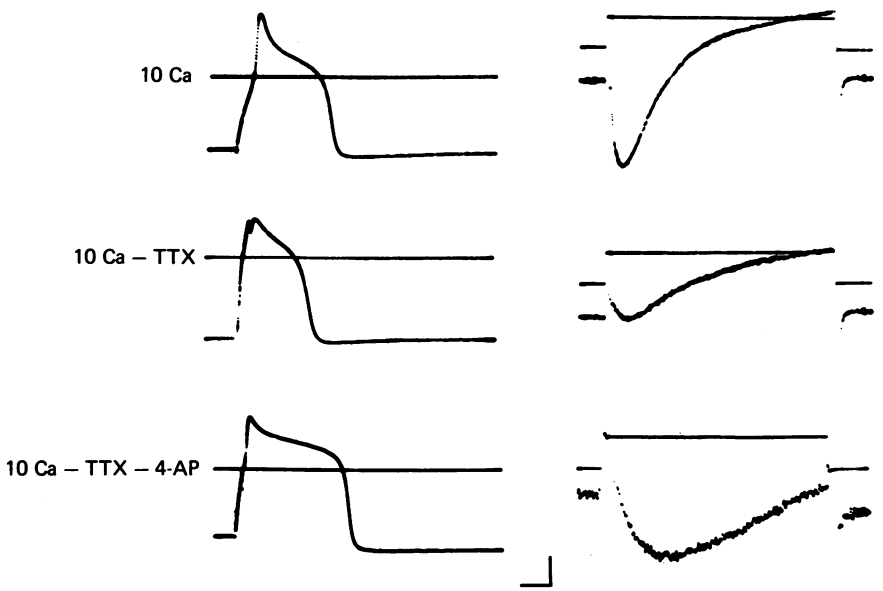

Fig. 3. Action potentials (left) and membrane currents (right) recorded from the same cells. Immediately after the spikes were recorded (in the indicated bathing medium), the cells were voltage-clamped and the membrane currents recorded during a command voltage step of 60 mV (from −50 to +10 mV). Top panels: 10 mM-Ca^{2+}; middle panels: 10 mM-Ca^{2+} plus 10^{-7} TTX g/ml.; and lower panels: 10 mM-Ca^{2+}, 10^{-7} TTX g/ml. and 1 mM-4-AP. Horizontal lines in the action potential panels indicate 0 mV. In the voltage-clamp panels, the upper traces represent membrane potential and the lower membrane current. Calibrations: 20 mV, 5 msec (left, top and middle); 20 mV, 10 msec (left, lower); 10 nA, 50 mV, 2 msec (right, top, and middle); 5 nA, 50 mV, 2 msec (right, lower).

The suggestion that Ca^{2+} ions carry a large part of the TTX-resistant inward current is supported by the observation that this current was markedly reduced when extracellular Ca^{2+} was decreased from 10 to 1 mM. Moreover, the slow inward current was completely blocked when Ca^{2+} was replaced with 10 mM-Co^{2+} (Fig. 4). In 1 mM-Ca, the 'threshold' for activation of the inward current, as well as the maximum inward current and the extrapolated reversal potential (E_R) of the inward current all occurred at more negative membrane potentials than in 10 mM-Ca^{2+}. This shift in E_R is expected if the current is carried predominantly by Ca^{2+} ions, but it should be emphasized that E_R is probably not an accurate estimate of E_{Ca}. The TTX-resistant

current rises slowly, and a significant outward K^+ current may be activated by the time it reaches its peak, even in the presence of 4-AP. Moreover, some TTX-resistant channels are not completely impermeable to Na^+ ions (Reuter & Scholz, 1977*a*). The null potential estimated in TTX and 4-AP may therefore represent a multi-ionic equilibrium. Despite these reservations, we shall for convenience, call the slow, TTX-resistant inward current 'I_{Ca}'.

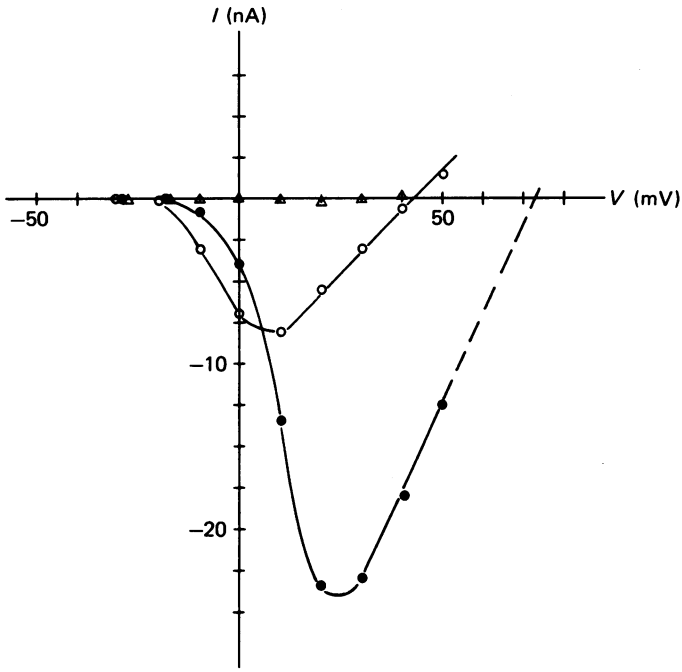

Fig. 4. Voltage–current relationships showing Ca^{2+} dependence of TTX-resistant inward current. Peak inward current was measured at several voltages in a cell bathed in 10 mM-Ca^{2+}-TTX–4-AP (filled circles), 1 mM-Ca^{2+}–TTX–4-AP (open circles) and 10 mM-Co^{2+}–TTX (triangles). In these experiments leakage resistance, measured ± 20 mV around the holding potential of -50 mV, was assumed to be linear and subtracted from the net inward current at each potential.

Ca^{2+}-activated outward current. Several invertebrate and vertebrate neurones exhibit a Ca^{2+}-activated K^+ conductance, $g_K(Ca)$, in addition to the usual voltage-dependent K^+ conductance, $g_K(V)$, (Meech, 1976). In voltage-clamp experiments, an effect of Co^{2+} on the late outward current is taken as evidence for an underlying $g_K(Ca)$. In twenty-two of twenty-nine embryonic chick sensory neurones tested in medium containing 10 mM-Co^{2+} (replacing 10 mM-Ca^{2+}), the outward current measured near 0 mV was clearly reduced (Fig. 5*A*). Thus in most of the sensory neurones a component of the K^+ conductance was apparently activated by prior influx of Ca^{2+} ions. In the remaining seven cells, Co^{2+} did not affect the outward current even though I_{Ca} was eliminated (Fig. 5*B*).

I_{Ca} inactivation. The rapid decay in inward current in the presence of 4-AP and TTX (Fig. 3) probably reflects activation of $I_K(V)$ or $I_K(Ca)$ rather than inactivation

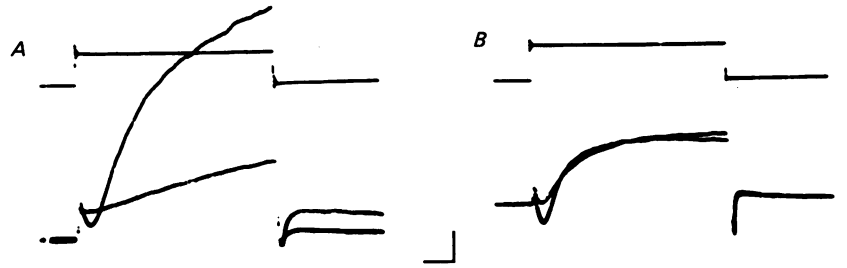

Fig. 5. Ca-activated K^+ current–$I_K(Ca)$. Membrane currents produced by a 60 mV depolarization in two cells bathed in 10 mM-Ca^{2+}–TTX. Holding potential was −50 mV. 10 mM Co^{2+} (substituted for Ca^{2+}) decreased the outward current in most cells (left) but not all (right). Note that Co^{2+} eliminated the TTX-resistant inward current in both cases. Calibrations: 20 nA, 50 mV, 5 msec.

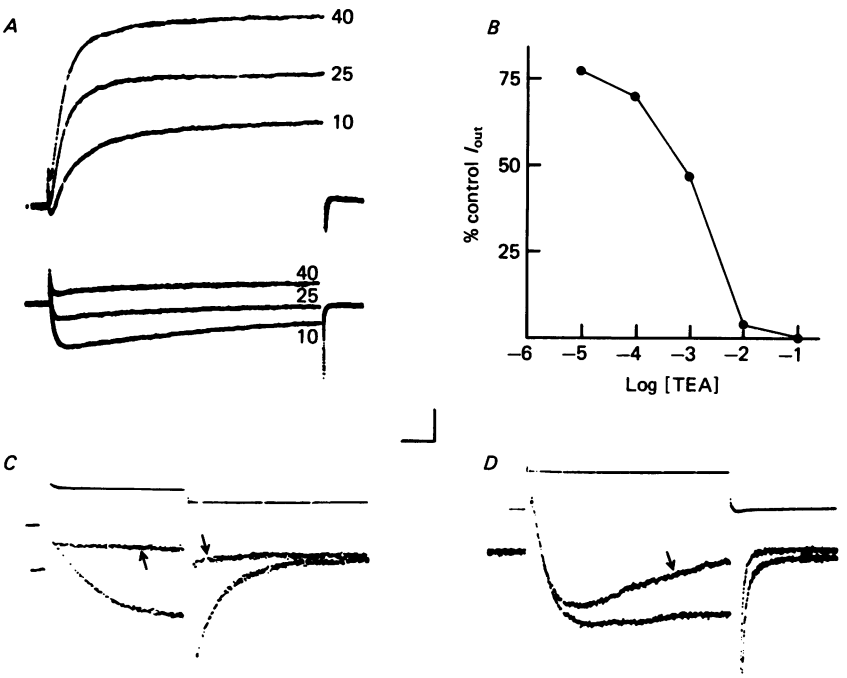

Fig. 6. TEA blocks time-dependent outward current. *A*, membrane currents produced by depolarizing voltage steps (to the values noted at the right of the traces) in a solution containing 10 mM-Ca^{2+}–TTX (upper) and 10 mM-Ca^{2+}–TTX–125 mM-TEA (lower). Holding potential was −40 mV. *B*, steady-state outward currents following a 30 msec depolarization to 0 mV were measured in the presence and absence of TEA. The % control current is plotted against TEA concentration. *C*, membrane currents recorded in 10 mM Ca^{2+}–TTX–125 mM-TEA following depolarization to +10 mV and repolarization to −10 mV are completely blocked by substituting Co^{2+} for Ca^{2+} (arrows). Holding potential was −50 mV. *D*, membrane currents during a 60 mV depolarization in the presence of 10 mM-Ca–TTX–TEA and 30 sec following cessation of the TEA puff (arrow). Calibrations: *A*, 20 nA, 10 msec; *C*, 5 nA, 50 mV, 1 msec; *D*, 10 nA, 50 mV, 2 msec.

of I_{Ca}. At 1 mM, 4-AP may not completely block $I_K(V)$ at 0 mV or more positive membrane potentials (cf. Yeh, Oxford, Wu & Narahashi, 1976). Little is known about the effect of 4-AP on I_K(Ca). Therefore we tested tetraethylammonium (TEA), another K^+-channel blocker. In *Helix* (Meech & Standen, 1975) and *Aplysia* (Hermann & Gorman, 1979) neurones TEA at 50 mM blocks nearly all voltage-dependent and Ca^{2+}-activated K^+ channels.

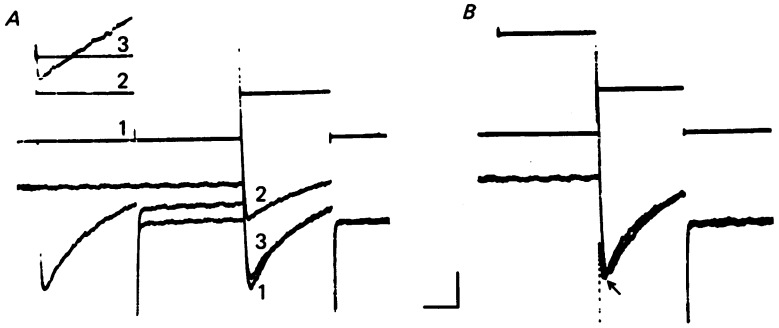

Fig. 7. I_{Ca} inactivation. Membrane currents recorded in 10 mM = Ca–TTX–125 mM-TEA. *A*, a conditioning depolarization of variable amplitude (0 mV (1), 70 mV (2), 120 mV (3)) was followed by a fixed amplitude test pulse (70 mV). The inward current during the test pulse varied depending upon the amplitude of the conditioning pulse. Each current trace associated with a test pulse is labelled with the number corresponding to the conditioning pulse which preceded it. *B*, another cell in which a conditioning pulse to +100 mV was followed by repolarization to +10 mV (the value of the test pulse in the absence of the conditioning pulse). The conditioning pulse tail current (arrow) was virtually identical to the test pulse current (measured in the absence of a conditioning pulse), indicating little or no inactivation of I_{Ca} produced by the large amplitude conditioning pulse. Holding potential was −60 mV for both *A* and *B*. Calibrations: 10 nA, 50 mV, 20 msec. The large persistent inward currents following repolarization to the holding potential evident in both *A* and *B* were seen in some but not all of the d.r.g. cells. They probably correspond to the long lasting (100–200 msec) after-spike depolarization reported by Dichter & Fischbach (1977).

High concentrations of TEA rapidly depolarized the sensory neurones to about −20 mV. Therefore rather than add TEA to the bath, the drug was applied by pressure ejection to individual voltage-clamped cells. In the presence of 125 mM-TEA, the time constant of inward current decay following a step depolarization to +10 mV was about 40 msec (Fig. 6*A*). This is probably a more accurate estimate of I_{Ca} inactivation since there was no evidence of a time-dependent outward current even at membrane potentials as positive as +50 mV, and there was no sag at all in the current trace during the first 10 msec following a step depolarization. Thus I_{Ca} inactivation in cultured d.r.g. neurones is a relatively slow process.

The complete TEA dose–response curve is shown in Fig. 6*B*. Outward currents were reduced by 50% in 1 mM-TEA which is slightly lower than the ED_{50} of 10 mM observed by Meech & Standen (1975). As expected, the inward current recorded during a depolarizing pulse to +10 mV in 125 mM-TEA (and TTX) was completely blocked by Co^{2+}, as was the inward tail current observed after shifting the membrane potential back to −10 mV (Fig. 6*C*).

The effect of high concentrations of TEA was reversible. One of the two current

traces in Fig. 6*D* was recorded during application of 125 mM-TEA. The other, which exhibited a prominent outward current 'sag' was recorded 30 sec after termination of the pressure pulse.

Our preliminary studies using paired pulses to investigate I_{Ca} inactivation are consistent with the suggestion that inactivation of Ca^{2+} channels is in some way related to the prior entry of Ca^{2+} ions (Brehm & Eckert, 1978; Tillotson, 1979). Inactivation during a constant-amplitude, 50 msec test pulse was measured 50 msec after conditioning pulses of different amplitudes. Inactivation during the test pulse was maximal following conditioning pulses that produced maximal inward Ca^{2+} currents, whereas larger conditioning depolarizations, near E_{Ca}, that were associated

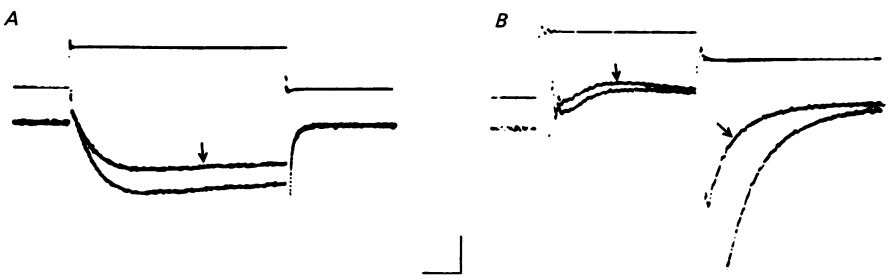

Fig. 8. NA decreases I_{Ca}. Membrane currents in 10 mM-Ca–TTX–TEA with and without 10^{-4} M-NA. *A*, step depolarization of +60 mV from a holding potential of −50 mV. Current in the presence of NA noted by arrow. *B*, depolarization from a holding potential of −50 mV to +40 mV, followed by repolarization to +10 mV, produced large inward current tails. NA decreased the tail current (arrow). Current flowing during the pulse in the presence of NA is also marked by an arrow. Calibrations: *A*, 10 nA, 50 mV, 5 msec; *B*, 5 nA, 50 mV, 1 msec.

with little or no slow inward current, produced no additional inactivation during the test pulse (Fig. 7*A*). Thus I_{Ca} inactivation in d.r.g. cells is not a simple monotonic function of voltage but seems to depend on Ca^{2+} influx. The possibility of a voltage-dependent inactivation of I_{Ca} that decays rapidly following larger depolarizations is ruled out in Fig. 7*B*. Here, there is no interval between the two pulses, but the conditioning depolarization was followed by repolarization to the same membrane potential achieved during the test pulse. The size and the rate of decay of I_{Ca} *during* the test depolarizing pulse (evoked without a preceding conditioning pulse) were identical to the size and rate of decay of the conditioning pulse I_{Ca} *tail* current.

A direct effect of NA on I_{Ca}

Inward current in the presence of TEA. Since high concentrations of TEA apparently block all K^+ current, whether voltage-dependent or Ca^{2+}-activated, we assayed effects of NA on I_{Ca} by ejecting the drug along with 125 mM-TEA. In each case a several-second long 'conditioning' pulse of 125 mM-TEA (delivered from a second puffer pipette) preceded the test NA–TEA combination. As shown in Fig. 8*A*, 10^{-4} M-NA decreased I_{Ca} throughout the duration of a 30 msec depolarization. In one group of ten neurones, the peak TEA- or TTX-resistant, inward current (measured at +10 mV) was 16·9±2·3 nA (mean±S.E.), and the decrease in inward current

induced by 10^{-4} M-NA ranged between 19 % and 57 % with a mean of 31 %. NA also decreased the inward tail current recorded in the presence of TEA (Fig. 8*B*).

Inward tail currents measured at E_K. A direct effect of NA on I_{Ca} was also shown in the *absence* of TEA by studying TTX-resistant tail currents recorded at E_K, the membrane potential at which all K^+ currents are eliminated (whether voltage-dependent or Ca^{2+}-activated). E_K was taken as that potential at which the tail current reversed polarity in a medium containing 10 mM-Co^{2+} and 10^{-7} TTX g/ml. (Fig. 9*A*). E_K defined in this manner ranged between −70 and −80 mV. When Co^{2+} was replaced with Ca^{2+} a small inward tail current that could be distinguished from the capacitative discharge was evident in most cells.

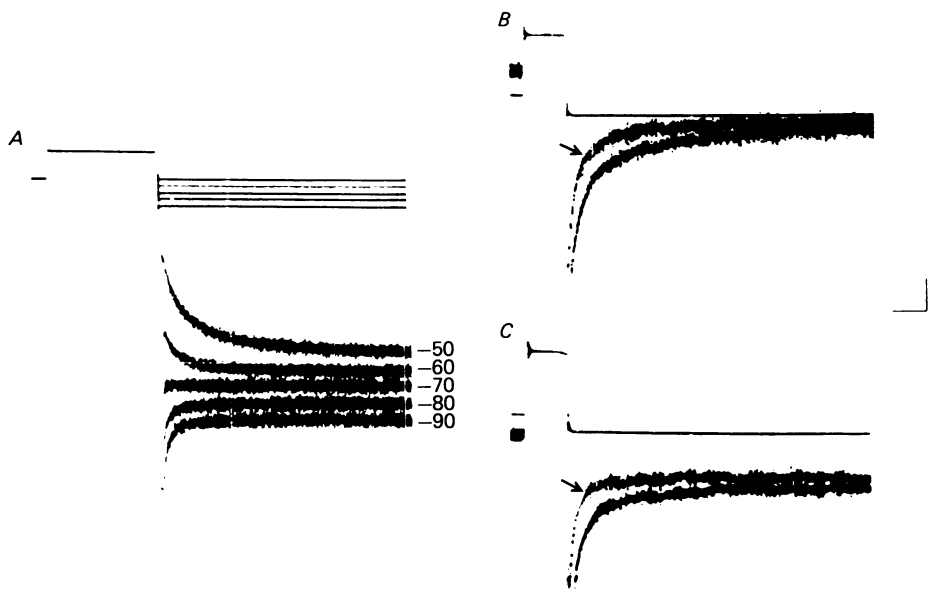

Fig. 9. Tail currents recorded at E_K. *A*, depolarizations to −10 mV for 60 msec were followed by successive repolarization to −50, −60, −70, −80 and −90 mV. The tail currents following repolarization are correspondingly labelled. The cell was bathed in 10 mM-Co^{2+}–TTX. The reversal potential was taken as E_K. Holding potential was −50 mV. *B*, another cell (bathed in 10 mM-Ca^{2+}–TTX) which was depolarized to +50 mV for 2 msec and repolarized to E_K (−70 mV), produced an inward tail current at E_K that was decreased with 10^{-4} M-NA (arrow). *C*, this tail current (same cell as *B*) was eliminated with 10 mM-Co^{2+} (arrow). Holding potential in this cell was −40 mV. Calibrations: *A*, 5 nA, 50 mV, 20 msec; *B* and *C*, 2 nA, 50 mV, 2 msec.

According to the simplest model of channel activation (see below) the rate constants governing channel opening (α) and closing (β) are voltage-dependent. The small size and relatively rapid decay of the Ca^{2+} tail current at E_K can be accounted for by assuming that α is negligible and β is large at this level of hyperpolarization (Reuter, 1974; see Noble, 1975 for review). Ca^{2+} tail currents in chick d.r.g. neurones are more prominent at more positive potentials (cf. Figs. 8*B* and 9*B*).

In each of the six cells tested, NA at 10^{-4} M rapidly and reversibly decreased the E_K tail current (Fig. 9*B*). Fig. 9*C* shows that ejection of Co^{2+} abolished this tail current in the same cell.

NA does not change the kinetics of I_{Ca} *activation*. Both the rise and fall of I_{Ca} followed

a simple exponential curve so, as a first approximation, it can be assumed that the opening and closing of Ca^{2+} channels is a two-state, first-order process. Thus, in the steady state, the fraction of channels in the open state is $y_{ss} = \alpha/(\alpha+\beta)$, where α and β are voltage-dependent rate constants.

Ignoring Ca^{2+} channel inactivation, which is a slow process, a general description of the inward Ca^{2+} current is:

$$I_{Ca} = \bar{g}_{Ca} y (E_m - E_{Ca}),$$

where $\bar{g}_{Ca}$ is the maximum Ca^{2+} conductance, y is the voltage-and time-dependent variable defined above, E_m is the membrane potential and E_{Ca} is the Ca^{2+} equilibrium potential. Thus a NA-induced decrease in I_{Ca} might be due to an effect on $\bar{g}_{Ca}$, on the rate constants α and/or β, or on E_{Ca}.

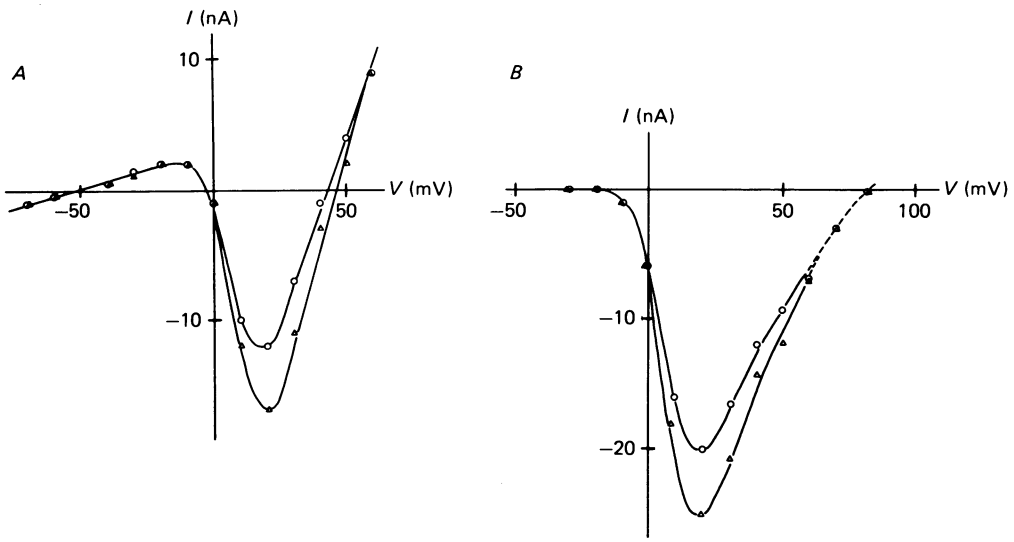

Fig. 10. NA effect on V–I_{Ca} relationship. Maximum inward currents plotted against voltage for a cell bathed in 10 mM-Ca–TTX–TEA in the presence (circles) and absence (triangles) of 10^{-4} M-NA. The curve in *A* was not corrected for leakage conductance. The result of subtracting the leakage conductance is shown in *B*. Time-invariant outward leakage currents that remained in the presence of 10 mM-Co^{2+}–TTX–TEA were averaged in three cells. This 'average leakage curve' which was concave upward was subtracted from the data in *A*. The null potential was approximated by extrapolation.

The voltage–I_{Ca} relation was determined in the presence of 125 mM-TEA and 10^{-7} TTX g/ml. NA at 10^{-4} M decreased I_{Ca} throughout the voltage range examined: there was no change in the I_{Ca} 'threshold' or in the potential at which I_{Ca} was maximal (Fig. 10*A*). When the control and NA curves were normalized to the same maximum I_{Ca}, the negative resistance regions of the plots (an index of y_{ss}) were superimposed. As shown in Fig. 11, NA did not change the time constant of I_{Ca} activation. Since y_{ss} and τ are unchanged, it can be assumed that the rate constants α and β are not altered by NA.

The effect of NA on I_{Ca} is apparently not due to a decrease in E_{Ca}. The null potential shown in Fig. 10*A* determined by interpolation was the same in the presence of 10^{-4} mM-NA as in control medium.

The issue of other ions contributing to the null potential (see above) is not of concern here: Na^+ was replaced and I_K was eliminated by application of 125 mM-TEA. Nevertheless, these estimates of E_R cannot be simply equated with E_{Ca} because the leakage resistance (the inverse slope of the voltage–current curve measured in TTX, TEA and Co^{2+}) was not linear at potentials above +20 mV. When the leakage resistance, including the non-linear region, was taken into account, the inward current did not reverse polarity even at +80 mV (Fig. 10*B*). A similar decrease in leakage resistance at positive membrane potentials was reported by Kostyuk & Krishtal (1977). Despite this reservation about the true value of E_{Ca}, it is the difference in E_{Ca}, plus and minus NA, that is important here. If the intracellular free Ca^{2+} is 10^{-7} M (cf. Baker, Hodgkin & Ridgway, 1971; Meech & Standen, 1975) then E_{Ca} must be near +150 mV. To account for our observed 30% decrease in I_{Ca}, E_{Ca} would have to decrease by over 40 mV. A shift of this magnitude would have been detected even in the face of a non-linear leakage conductance.

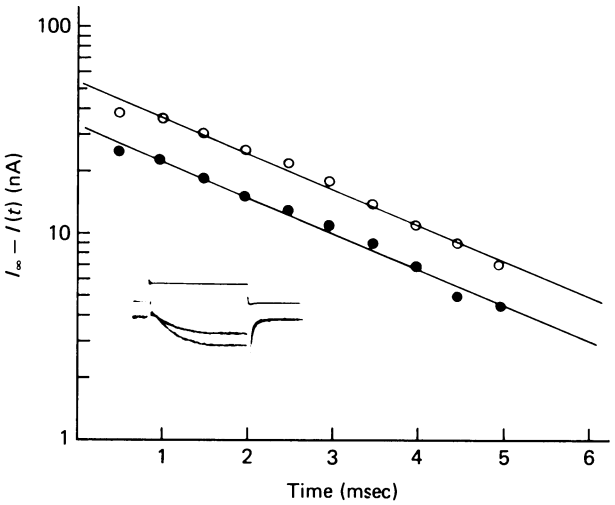

Fig. 11. NA effect on I_{Ca} time course. Semilogarithmic plot of current amplitude against time. Measurements were made from the data shown in the inset. Currents were recorded from a cell bathed in 10 mM-Ca–TTX–TEA with (●) and without (○) 10^{-4} M-NA. Holding potential was −50 mV; the 60 mV depolarization lasted 12 msec. I_∞ is the maximum current achieved during the pulse.

Thus, since NA did not alter the voltage dependence or the kinetics of Ca^{2+} channel activation or the Ca^{2+} reversal potential, we conclude that it must decrease $\bar{g}_{Ca}$.

NA does not affect I_{Na} or $I_K(V)$. When I_{Ca} is eliminated with Co^{2+}, the soma spike is relatively brief, and there is only a slight break in the falling phase (Fig. 12*A*) or none at all. NA at 10^{-4} M did not further decrease the duration of this 'Na^+ spike'. As expected, the large, rapid inward Na^+ current recorded in the presence of Co^{2+} was not decreased by 10^{-4} M-NA (Fig. 12*B*). It is also evident in Fig. 12 that NA did not increase the magnitude or alter the kinetics of the outward K^+ current. In the presence of 10 mM-Co^{2+}, the outward current reflects $I_K(V)$ only. We have not obtained a direct measure of I_K(Ca).

GABA and 5-HT also decrease I_{Ca}

We have done fewer experiments with GABA and 5-HT than with NA, but it is clear that these drugs also can decrease the soma spike duration by a direct effect on I_{Ca}. Pressure ejection of 10^{-4} M-GABA in 125 mM-TEA produced an average 32%

decrease in I_{Ca} ($n = 4$ cells) measured at +10 mV. The inward tail current measured at −20 mV was markedly reduced by 10^{-4} M-GABA. The effect of GABA on complete voltage–current curves in the presence of 125 mM-TEA was determined in three other cells. The maximum I_{Ca} was reduced by 39% and, as with NA, there was no shift in the voltage dependence of I_{Ca}.

High concentration of NA, GABA or 5-HT ejected alone did not completely eliminate I_{Ca} or the action potential plateau. The effects of these drugs were additive

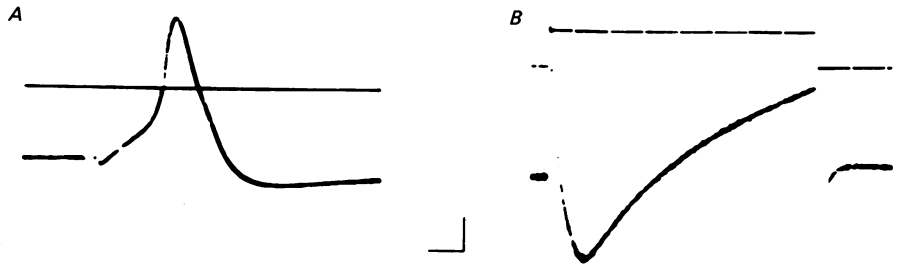

Fig. 12. NA does not alter Na^+ spike or voltage-dependent Na^+ and K^+ currents. Action potentials (*A*) and membrane currents (*B*) recorded in a cell bathed in 10 mM-Co^{2+}. Oscilloscope records shown are actually two superimposed traces in the presence and absence of 10^{-4} M-NA. Horizontal line in *A* represents 0 mV. Holding potential in *B* was −50 mV. Calibrations: *A*, 20 mV, 2 msec; *B*, 10 nA, 50 mV, 2 msec.

however. In one group of four cells 10^{-4} M-NA decreased spike duration from 6·8 to 2·9 msec. Application of 10^{-4} M-NA plus 10^{-4} M-GABA from a second pipette immediately after terminating the NA pulse further reduced spike duration to 2·1 msec. In another group of four cells 10^{-4} M-GABA, applied first, reduced spike duration from 6·8 to 4·0 msec, and a second GABA plus NA pulse reduced it further to 2·6 msec.

The effect of GABA was not as consistent as that of NA. In some cultures only a small effect on spike duration or no effect at all was observed. We cannot yet explain this variability. It may be a matter of time (or development) *in vitro* because, in several experiments, the percentage of responsive cells in different culture dishes increased with time after plating.

DISCUSSION

We have shown that NA shortens the duration of the d.r.g. cell body action potential by decreasing a voltage-sensitive, slow inward Ca^{2+} current. It is important to emphasize that this effect was not associated with a drug-induced change in membrane potential or resting membrane conductance.

The strongest evidence for a direct effect on I_{Ca} is that NA decreases the TTX-resistant, inward tail current recorded at E_K. It might be argued that this inward current measured in the cell body is not due to a local change in Ca^{2+} conductance but reflects an outward current generated in some distant and perhaps poorly clamped region of the neurone. But in that case, a NA-induced decrease in inward soma current would reflect a decrease in this outward current which is extremely unlikely: a decrease in outward current would lengthen the action

potential duration rather than shorten it as observed. Additional evidence for a direct effect of NA on I_{Ca} is that NA decreased the inward current in the presence of high concentrations of TEA (125 mM) that were sufficient to block all time-dependent K^+ channels.

We did not detect an effect of NA, GABA or 5-HT on the fast inward (TTX-sensitive) current or on the outward current recorded in the presence of 10 mM-Co^{2+}. Therefore these drugs probably do not alter I_{Na} or $I_K(V)$. We cannot, however, claim that the action of NA is restricted to I_{Ca}: an effect on I_K(Ca) has not been ruled out.

In *Helix* neurones, I_K(Ca) is an important determinant of action potential duration (Meech & Standen, 1975), and it may serve the same function in embryonic chick neurones as well. To maintain the hypothesis that NA increases I_K(Ca) one must recognize that this must occur despite a 30 % decrease in Ca^{2+} influx. Also, since NA did not alter the time course of the slow inward current, one must maintain that the kinetics of I_K(Ca) are indistinguishable from those of I_{Ca}. It is noteworthy that in adult superior cervical ganglia the NA-induced decrease in spike duration is accompanied by a decrease rather than an increase in the Ca^{2+}-dependent after-hyperpolarization (Horn & McAfee, 1979, 1980).

NA did not abolish I_{Ca} even when applied at 10^{-4} M. In fact, the 25–30 % decrease in I_{Ca} seems small considering that the same concentration of NA reduced the duration of the action potential by about 65 %. However, the membrane conductance during the plateau phase of the spike is low, so a small change in inward current would produce a relatively large change in membrane potential. For example, in five cells the mean membrane conductance, estimated from currents recorded with the membrane clamped at 0 mV, was $4{\cdot}2 \times 10^{-7}$ mho. A 5 nA (30 %) decrease in inward current would, therefore, repolarize the membrane by 12 mV. This, in turn, would decrease g_{Ca} further and speed repolarization.

Our data indicate that NA decreases I_{Ca}, not by altering the driving force on Ca^{2+} ions or the voltage dependence of Ca^{2+} channels but by decreasing the maximum available Ca^{2+} conductance, $\bar{g}_{Ca}$. Since $\bar{g}_{Ca} = ng_0$, where n is the number of available Ca^{2+} channels and g_0 is the single Ca^{2+} channel conductance, NA must decrease either n or g_0. Perhaps the simplest explanation is that NA binds to and blocks Ca^{2+} channels in the same way that TTX blocks Na^+ channels. However, the observations that (1) several different agents (GABA, 5-HT, enkephalin and somatostatin) produce the same effect, (2) at least two of them (NA and enkephalin) are blocked by specific antagonists (Dunlap & Fischbach, 1978; Mudge *et al.* 1979) and (3) the effects of the different drugs are additive, are more consistent with the notion that each agent binds to a unique receptor that in some way alters Ca^{2+} channel function. The mechanism of this control remains to be determined.

A role for cyclic AMP in the modulation of voltage-dependent channels has been suggested in studies of heart muscle (Tsien *et al.* 1972; Reuter, 1974) and *Aplysia* neurones (Klein & Kandel, 1978). In heart ventricular muscle, NA increases rather than decreases $\bar{g}_{Ca}$. Reuter & Scholz (1977*b*) suggested that this cyclic AMP-dependent action of NA was due to an increase in the number of Ca^{2+} channels rather than to an effect on channel conductance. This conclusion was based on indirect evidence: the ion selectivity of the voltage-dependent Ca^{2+} channel was not altered. Preliminary experiments indicate that neither dibutyryl cyclic AMP nor dibutyryl cyclic GMP

added to the bath at 1 mM decrease sensory neurone spike duration (K. Dunlap, unpublished observations).

Another mechanism of NA action might involve an accumulation of intracellular Ca^{2+} that is too small to alter E_{Ca} but that is sufficient to inactivate Ca^{2+} channels. Ca^{2+}-dependent inactivation of Ca^{2+} channels was originally described in *Paramecium* (Brehm & Eckert, 1978) and in *Aplysia* neurones (Tillotson, 1979), and we have documented a similar phenomenon in chick d.r.g. neurones. However, neither the time course of NA action nor the maximum NA effect depend on repetitive activation of d.r.g. spikes (K. Dunlap, unpublished observations). This lack of 'use dependence' does not support a Ca^{2+}-dependent Ca^{2+} inactivation hypothesis.

If the neurotransmitters studied here affect I_{Ca} in sensory nerve terminals in the same way that they do in the cell bodies, then they may decrease transmitter release. This suggests a rather novel mechanism for the phenomenon of presynaptic inhibition. A strong case for a relation between direct transmitter action on I_{Ca} and presynaptic inhibition has been made in *Aplysia*. Shapiro *et al.* (1980) studied presynaptic inhibition of L_{10} neurones by recording p.s.p.s in L_{10} follower cells while simultaneously recording membrane currents in voltage-clamped L_{10} somata. The decrease in p.s.p. size was paralleled by a decrease in I_{Ca}.

Each of the agents so far shown to decrease I_{Ca} in chick sensory neurones have been implicated in presynaptic inhibition of vertebrate afferent axons. NA and 5-HT are contained in neurites that originate in the locus ceruleus and Nucleus Raphé Magnus respectively and descend to terminate in the dorsal horn (Carlsson, Falck, Fuxe & Hillarp, 1964; Basbaum, Clanton & Fields, 1976). GABA and enkephalin are present in local dorsal horn interneurones (Barber, Vaughn, Saito, McLaughlin & Roberts, 1978; Elde, Hökfelt, Johannson & Terenius, 1976).

The most accepted explanation for presynaptic inhibition at vertebrate sensory nerve terminals (reviewed in Burke & Rudomin, 1977) is that the inhibitory neurotransmitter produces an increase in presynaptic membrane conductance that decreases the size of (shunts) the invading action potential. In this model, the decrease in Ca^{2+} influx and transmitter release are secondary consequences of inhibitory transmitter action. In the spinal cord, presynaptic inhibition is associated with depolarization of afferent terminals (primary afferent depolarization; p.a.d.). Indeed the similar time course of presynaptic inhibition and p.a.d. is cited in support of a presynaptic shunt. However, this evidence and also related pharmacological data are not conclusive (see review by Krnjević, 1979). It is significant in this regard that NA, 5-HT, GABA and enkephalin, which do not alter the resting membrane potential or membrane conductance of embryonic d.r.g. neurones in 2- to 3-week-old cultures, are all capable of reducing the evoked release of substance P from the same cells (Mudge *et al.* 1979; Mudge, 1979).

GABA does depolarize and increase the membrane conductance of adult sensory neurones (DeGroat, 1972; Nishi, Minota & Karczmar, 1974) and an increase in conductance can be measured in dissociated embryonic chick sensory neurones grown alone (without central or peripheral targets) in cell culture (Choi & Fischbach, 1981). But the GABA-induced increase in conductance is evident only during the first week after plating in this culture system, and we never observed it in the 2- to 6-week-old cultures studied here.

The ability to visualize synaptic contacts in unfixed d.r.g. spinal cord co-cultures might allow direct assay of the relative contributions of the shunt mechanism and of direct Ca channel modulation to the phenomenon of presynaptic inhibition.

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