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EFFECTS OF DIVALENT CATIONS ON RESPONSES OF A SYMPATHETIC GANGLION TO 5-HYDROXYTRYPTAMINE AND 1,1-DIMETHYL-4-PHENYL PIPERAZINIUM

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1 The effects of raising or lowering $[Ca^{2+}]_o$ or $[Mg^{2+}]_o$ on potential changes evoked by 5-hydroxytryptamine (5-HT) and by the nicotinic agonist, 1,1-dimethyl-4-phenyl piperazinium (DMPP) have been investigated.

2 Changes in membrane potential were measured at the ganglion or in postganglionic axons by the sucrose-gap technique. The ganglionic response to both 5-HT and DMPP was a depolarization followed by an after-hyperpolarization (AH). AH decayed exponentially over most of its time course; the time constant of decay for 5-HT responses was 4.4 ± 0.3 min (mean ± s.e.mean, rate constant 0.23 min^{-1}) and that for DMPP responses was not significantly different, being 3.9 ± 0.3 min (rate constant 0.26 min^{-1}).

3 Increasing $[Ca^{2+}]_o$ to 5.1 or 7.6 mM caused some hyperpolarization of the ganglion, reduced the amplitude of depolarizations evoked by 5-HT by 29% and usually potentiated responses to DMPP (average 12%). Ca-free solutions caused a depolarization of the ganglion, increased the amplitude of depolarizations evoked by 5-HT by 23% and reduced that of depolarizations to DMPP by 32%. $[Mg^{2+}]_o$ 12.7 and 25.4 mM caused depolarizations of the ganglion and reduced the amplitude of depolarizations evoked by 5-HT by 34 and 84%, respectively, and those to DMPP by 10 and 75%, respectively. Mg-free solutions or low $[Mg^{2+}]_o$ caused a slow depolarization of the ganglion and reduced the amplitude of depolarizations to both 5-HT and DMPP by approx. 20%. Ca/Mg-free solutions produced a slow depolarization of the ganglion, increased the amplitude of depolarizations evoked by 5-HT by 78% and reduced those to DMPP by 58%.

4 Increasing $[Ca^{2+}]_o$ reduced the amplitude of AH evoked by 5-HT by 50% and increased that to DMPP by 73%, while prolonging AH duration and increasing the time constant of decay. Ca-free solutions had complex effects on AH evoked by 5-HT, which were increased on average by 116%, and depressed AH evoked by DMPP; in both cases there was a decrease in the time constant of decay. $[Mg^{2+}]_o$ 12.7 mM reduced the amplitude of AH evoked by 5-HT more than that evoked by DMPP, and increased the rate of decline of the exponential phase. Low Mg solutions reduced in amplitude the AH evoked by 5-HT by 56% and the AH evoked by DMPP by 38%. The time constant of decay was increased. Ca/Mg-free solutions reduced AH amplitude in both 5-HT and DMPP responses. The effects on time constant are consistent with the generation of AH by an electrogenic sodium pump, the ATP-ase of which is Mg^{2+}-dependent and inhibited by Ca^{2+}.

5 Responses to 5-HT could be recorded from postganglionic axons and consisted of a rapid depolarization, sometimes followed by an AH whose time constant of decay was smaller than that of ganglionic responses. Full dose-response curves in control and test media could be obtained. In Ca/Mg-free solutions, 5-HT depolarizations were potentiated but no significant shift in the curve was observed.

6 It is suggested that divalent cations modulate the coupling between 5-HT receptor and ion channel, an increase in $[Ca^{2+}]_o$ reducing the coupling or stabilizing the ion channel in the closed conformation. Ca^{2+} and Mg^{2+} may compete for the same binding site. This mechanism does not appear to be involved at nicotinic receptors and their related ion channels.

Introduction

The responses of ganglion cells to the nicotinic agonist 1,1-dimethyl-4-phenyl piperazinium (DMPP) and 5-hydroxytryptamine (5-HT) are similar in several respects (Wallis & Nash, 1980). For instance, both are rapid depolarizations generated by an increase in membrane conductance to sodium and other cations

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(Wallis & North, 1978) and both responses may show an after-hyperpolarization. The nicotinic depolarization is dependent on an increase in G_{Na} and G_K (see Nishi, 1977), whereas that induced by 5-HT is dependent on an increase in G_{Na} and probably also in G_K and G_{Ca} (Wallis & Woodward, 1975; Wallis & North, 1978). There is evidence suggesting that nicotinic and 5-HT receptors may be in relatively close association in the membrane (Wallis & Nash, 1980; Nash & Wallis, 1980). Nevertheless, antagonists can effect a selective blockade of one species of receptor without diminishing responses elicited by ligands acting at the other species of receptor (Lansdown, Nash, Preston, Wallis & Williams, 1980). In view of the finding (Smith, 1966) that raised extracellular Ca^{2+} has a selective action in reducing responses of the cat superior cervical ganglion (s.c.g.) *in situ* to 5-HT while leaving unaffected those evoked by DMPP, and reports that Ca^{2+} or Ca^{2+} and Mg^{2+} are involved in agonist-receptor interactions (Tuttle & Moran, 1969; Burgen & Spero, 1970; Takagi, Takayanagi & Liao, 1972), the effects of divalent cations on ganglionic responses to the nicotinic ligand, DMPP, and to 5-HT have been reinvestigated. The sucrose-gap method of recording changes in membrane polarization was used, since the extracellular ion concentrations can easily be changed and responses can be evoked over periods of many hours.

The exact manner in which calcium or other divalent cations modify ganglionic responses to depolarizing agents is far from clear. Takeshige & Volle (1964) injected a solution of $CaCl_2$ close-arterially towards the s.c.g. of the cat and observed blockade of responses to a muscarinic agonist, while Smith (1966) showed that this depressant action of $CaCl_2$ extended to responses elicited by 5-HT or histamine. He also demonstrated that a slow infusion of calcium-free Ringer solution to the ganglion brought about a potentiation of responses to 5-HT, histamine or a muscarinic agonist. In contrast, either Ca^{2+} or Mg^{2+} in physiological concentrations are required for the binding of agonists with their receptors in taenia of guinea-pig and in rat vas deferens (Takagi *et al.*, 1972) but are not involved with the binding of competitive antagonists at these receptors; α-adrenoceptors, muscarinic and histamine receptors were examined. Burgen & Spero (1970) concluded that both Ca^{2+} and Mg^{2+} are involved in the combination of agonists with muscarinic receptors in the guinea-pig ileum. Increases or decreases in Ca ion concentration, $[Ca^{2+}]_o$, from an initial concentration of 2.5 mM resulted in a shift to the right of the dose-response curve for contraction of the ileum. The apparent affinity constant for carbachol fell in altered $[Ca^{2+}]_o$, but was also affected by changes in $[Mg^{2+}]_o$. The authors postulated that divalent cations were necessary for a conformation of the receptor which displayed a high efficiency of coupling to the contractile response. There are also indications that Ca^{2+} are intimately involved with the action of nicotinic ligands in skeletal muscle. Lièvremont & Pascaud (1972) suggested that one component of the receptor-ion channel complex is a Ca^{2+}-binding lipoprotein; the critical step in the mediation of the response is displacement by the agonist, perhaps by allosteric action, of Ca^{2+} bound to the lipoprotein, which serve to maintain the ion channels in the closed state. Increase in $[Ca^{2+}]_o$ would be likely to stabilize ion channels in the closed conformation. Taylor (1973) proposed that agonist-divalent cation exchange is of central importance at the muscle endplate.

In the experiments described in this paper, $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ were raised and lowered by altering their concentration in the superfusion medium. Since in their actions on the transmitter release mechanism, calcium and magnesium act in an antagonistic fashion (Blackman, Ginsborg & Ray, 1963; Bennett, Florin & Pettigrew, 1976), in some experiments the ratio of Ca:Mg was deliberately varied. The results describe changes in both the evoked depolarizations and after-hyperpolarization (AH). An AH consistently follows a depolarization evoked by DMPP and though of smaller magnitude, usually follows a 5-HT depolarization. The AH in both cases may be the consequence of the electrogenic extrusion of Na^+ from the cell (Wallis & Woodward, 1975).

The axons of s.c.g. somata which run in the nerve accompanying the internal carotid artery also display a brisk depolarization to 5-HT in many preparations (Wallis, 1979). Responses are of shorter duration than in the ganglion and full dose-response curves are more readily obtained. In some experiments axonal responses were examined.

Methods

Preparation

Superior cervical ganglia were removed from adult rabbits anaesthetized with urethane (1.25–1.5 g/kg i.v. as a 25% w/v solution) and prepared as described by Wallis, Lees & Kosterlitz (1975) for insertion into a sucrose-gap apparatus. In this version of the apparatus, the sucrose compartment is separated from adjacent chambers by rubber membranes (Kosterlitz & Wallis, 1966; Wallis *et al.*, 1975). Potential changes induced by 5-HT or DMPP were amplified and displayed on a potentiometric chart recorder (Servoscribe R.E. 511.20.). Ganglia were superfused with Krebs solution at 20–22°C at a rate of 2–3 ml/min. To avoid the tachyphylaxis that follows

superfusion of the tissue with a solution of 5-HT in an effective concentration, injections of 5-HT dissolved in a small volume of Krebs solutions were made into the superfusion stream to the ganglion (Wallis & Woodward, 1975) or, in a few experiments, into the superfusion stream to the internal carotid nerve. At the ganglion, 0.2 μmol (81 μg) of 5-HT produced a depolarization approximately two-thirds maximum and this quantity was used to evoke a standard response. A response of similar amplitude was evoked by 0.1 μmol (32 μg) DMPP in many experiments, while in some 0.2 μmol DMPP was required. Reproducible responses could be obtained if the flow of Krebs solution to the ganglion was controlled with the aid of a perfusion pump and drop chamber and if the rate of injection was kept relatively constant at 1.5 ml/min. Injections of 5-HT or DMPP were made from 1 ml syringes mounted in a Perspex block through which the superfusion medium flowed. The syringe needles penetrated this Perspex block and were positioned so that their tips ejected material into the superfusion stream just upstream of the ganglion. All agonists were dissolved in Krebs solution.

Measurement of potentiation and depression

The amplitudes of the depolarizing and hyperpolarizing phases of the responses to 5-HT or DMPP were measured from a projection of the trace preceding the response. In earlier experiments (Wallis & Woodward, 1975), it was established that responses remained relatively constant for several hours provided that a sufficient interval was left between each injection. The amplitudes of responses evoked during superfusion with a modified Krebs solution were expressed as a percentage of the final control.

The after-hyperpolarization evoked by a nicotinic ligand decays exponentially after an initial slower component (Lees & Wallis, 1974). The amplitude of this after-hyperpolarization was measured each minute from its peak and plotted on semi-logarithmic paper. The linear portion of the curve was extrapolated back to $t = 0$, and time and rate constants calculated according to Rang & Ritchie (1968).

Solutions and drugs

All solutions were made from distilled water passed through a deionizer. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2 and glucose 11; it was gassed with 5% CO_2 and 95% O_2. The concentration of the sucrose solution superfusing part of the internal carotid nerve was 315 mM and taken to be isotonic.

The following modified Krebs solutions were used: (1) calcium-rich solution (Ca rich) in which the amount of $CaCl_2$ was raised 2 fold (5.1 mM) or 3 fold (7.6 mM). In some experiments the osmolarity change was minimized by an appropriate reduction in the amount of NaCl, while in others the small increase in osmolarity was ignored. Modified Krebs made by either procedure seemed to produce very similar effects. (2) Calcium-free solution (Ca-free) in which the $CaCl_2$ was omitted from normal Krebs and the small change in osmolarity ignored or compensated by an appropriate increase in the concentration of NaCl. Both modified Krebs solutions seemed to produce similar effects. (3) Magnesium-rich solutions in which the amount of $MgSO_4$ was increased 10 fold (12.7 mM) or 20 fold (25.4 mM). At the lower concentration, the osmolarity change was minimized by reducing the concentration of NaCl, but at the higher concentration the reduction in NaCl concentration required would be expected to depress any sodium-dependent depolarization induced by a drug. In the latter experiments, therefore, the increased osmolarity attributed to $MgSO_4$ was only partly compensated by reducing the concentration of NaCl. (4) Low magnesium solutions (low Mg) in which $MgSO_4$ was omitted from normal Krebs or reduced to change the ratio of calcium to magnesium from approximately 2 to 20 (0.13 mM $MgSO_4$); the small change in osmolarity was ignored. Krebs modified by either procedure appeared to produce very similar effects. (5) Calcium and magnesium-free solution (Ca/Mg free) in which $CaCl_2$ and $MgSO_4$ were omitted from normal Krebs and the small change in osmolarity ignored.

The calcium concentration in Krebs solution could not be increased beyond 7.6 mM because of precipitation of insoluble calcium salts. In order to test the effect of higher concentrations of calcium in the superfusion medium, a modified Locke solution was used of the following composition (mM): NaCl 118, Na_2SO_4 12.5, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.2, $MgSO_4$ 1.2, Tris HCl 10 and glucose 11; it was gassed with O_2. The pH of the Tris buffer solution was adjusted to 7.4 by the addition of HCl. A calcium-rich variant of this solution contained 5 times the normal $CaCl_2$ concentration (12.7 mM).

The drugs used were 5-hydroxytryptamine creatinine sulphate (Sigma) and 1,1-dimethyl-4-phenyl piperazinium iodide (Koch-Light). Drugs were made up in normal or modified Krebs solution or in modified Locke solution as appropriate.

Results

Responses evoked by 5-hydroxytryptamine and 1,1-dimethyl-4-phenyl piperazinium

Chart records of membrane potential changes

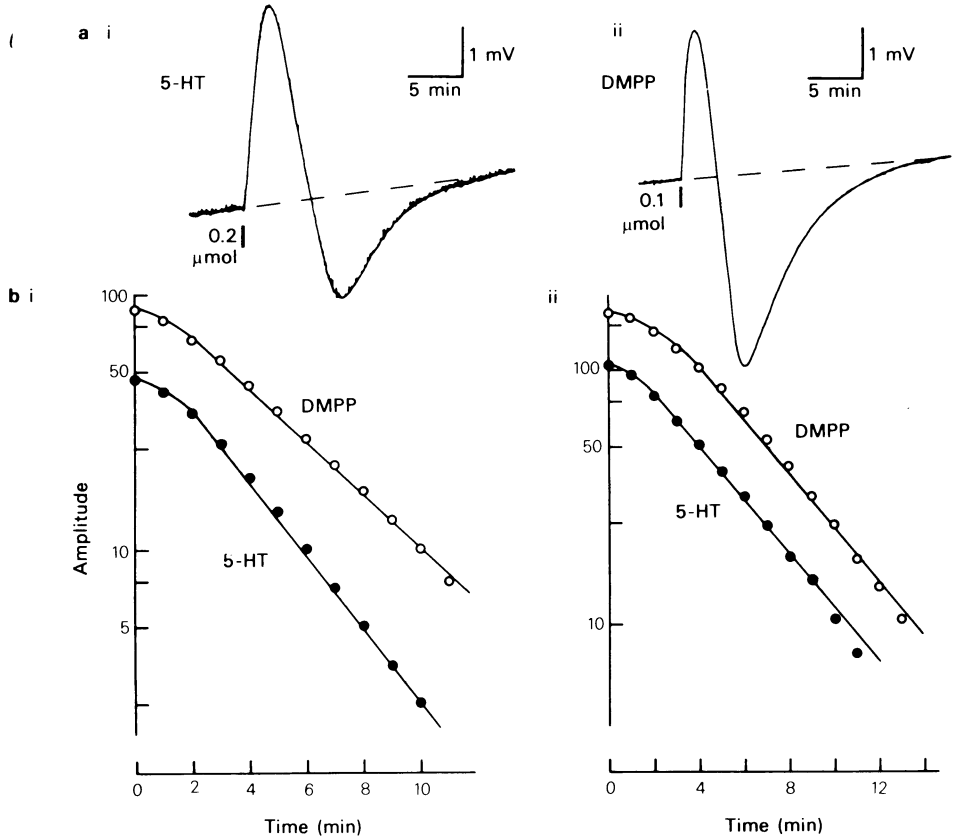

Figure 1 Responses evoked by 5-hydroxytryptamine (5-HT) and 1,1-dimethyl-4-phenyl piperazinium (DMPP) and the time courses of return to resting membrane potential during the hyperpolarizing phase of the response. (a) Chart records of membrane potential change induced by: (i) 5-HT (injection of 0.2 μmol in 0.2 ml Krebs solution); (ii) DMPP (injection of 0.1 μmol in 0.1 ml Krebs solution), depolarization of ganglion upwards. The black bars indicate the duration of the injections; dashed lines are projections of the baseline preceding the responses indicating the extent of baseline drift. (b) Decay of after-hyperpolarization plotted on a semi-logarithmic scale against time. Ordinates: hyperpolarization in arbitrary units, logarithmic scale. (i) (●) 5-HT and (o) DMPP responses shown above; (ii) (●) 5-HT and (o) DMPP responses from another experiment.

evoked from the ganglion by injection of 0.2 μmol 5-HT or 0.1 μmol DMPP in a typical experiment are shown in Figure 1. Characteristically 5-HT induced a brisk depolarization and repolarization, which was followed by a phase of hyperpolarization (Figure 1 ai). In this particular experiment, the after-hyperpolarization (AH) was of greater magnitude than usual, but typically it was smaller than the AH induced by DMPP. DMPP induced a rapid depolarization and repolarization followed by a large AH (see also Wallis & Nash, 1980) (Figure 1 aii). In most experiments it was found that injections of 0.2 μmol 5-HT and 0.1 μmol DMPP produced depolarizations of approximately equal magnitude.

Lees & Wallis (1974) found that after superfusing the rabbit s.c.g. with a solution of acetylcholine the tissue repolarized rapidly and a large AH developed. The decay of this AH was found to be exponential after an initial slower rate of decay. The decay of the hyperpolarizing phase of the responses of Figure 1(a) is shown graphically in Figure 1 (bi). Although in these experiments responses were generated by injection of agonists into the superfusion stream, it can be seen that the decay of AH was exponential after an initial slower rate of decay. The time constants of the exponential portions of the two curves were 3.0 and 4.3 min for the 5-HT and DMPP responses, respectively; corresponding rate constants were 0.33 and 0.23 min^{-1}. The rate of decay of AH evoked by 5-HT, when this response was large enough for accurate measurement to be feasible, was not consistently greater than that of DMPP responses. In some exper-

iments, DMPP responses appeared to display somewhat faster rates of decay than 5-HT responses, while in others the rates of decay were identical (Figure 1 bii). The time constant of the exponential portions of both curves of Figure 1 (bii), which shows decay of AH evoked by 5-HT and by DMPP in the same experiment, was 4.0 min (rate constant 0.25 min^{-1}). Comparing responses from a series of experiments, the mean time constant of decay for 5-HT responses was 4.4 ± 0.3 min (mean $\pm$ s.e.mean, $n = 13$), while that for DMPP responses was not significantly different, being 3.9 ± 0.3 min ($n = 17$). The corresponding rate constants were 0.23 and 0.26 min^{-1}.

Effect of divalent cations on depolarizations evoked by 5-hydroxytryptamine and 1,1-dimethyl-4-phenyl piperazinium

Ca-rich solutions On superfusing the ganglion with a modified Krebs solution in which the $CaCl_2$ concentration was increased to 5.1 or 7.6 mM, changes in potential were recorded which were sometimes biphasic; the principal change was a small, slow hyperpolarization which persisted for many minutes. With this method of recording, initial changes of potential may be due, at least in part, to changes in junction potential arising at the interface between solutions (Wallis *et al.*, 1975). Slower changes are likely to reflect changes in ganglion cell membrane potential. Doubling the external Ca^{2+} concentration, $[Ca^{2+}]_o$, caused a slow hyperpolarization of 0.9 ± 0.3 mV ($n = 4$); somewhat larger changes were seen on trebling $[Ca^{2+}]_o$.

Responses evoked by both 5-HT and DMPP were altered in Ca-rich solutions. The kind of effects observed can be seen in Figure 2. $[Ca^{2+}]_o$ 5.1 mM reduced in amplitude responses evoked by 5-HT (Figure 2 aii) without changing the character of the response to any significant degree; partial reversal of the depression was normally seen on returning to

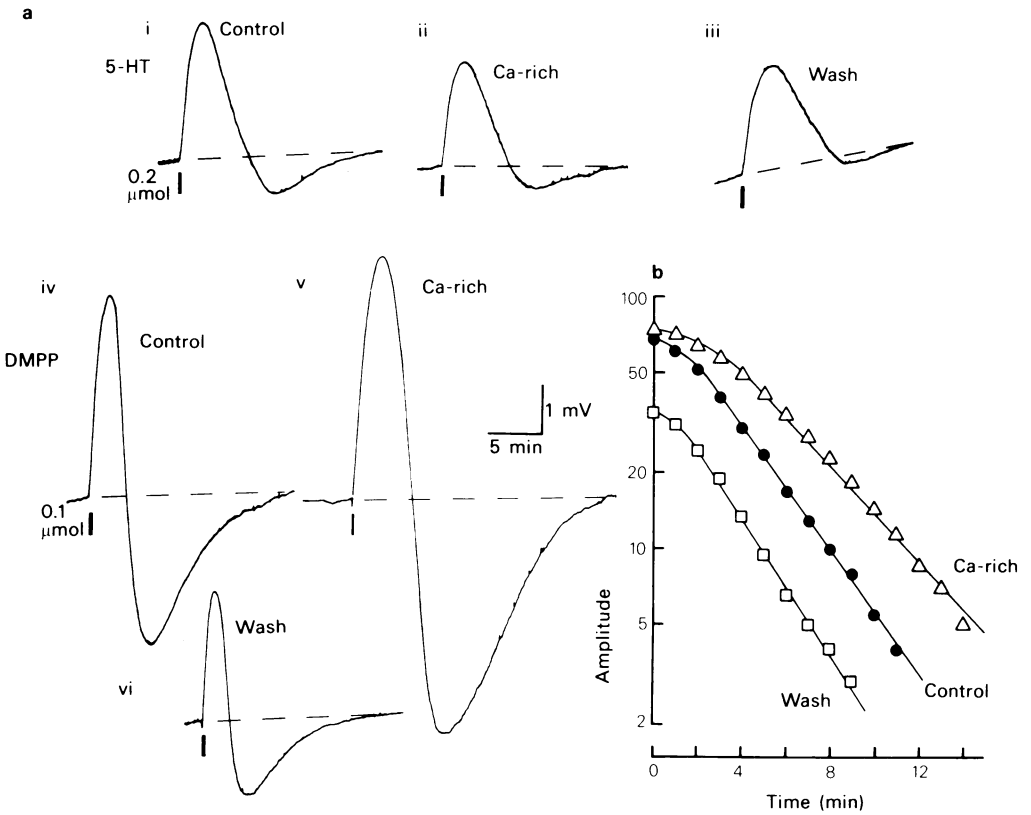

Figure 2 Effect of Ca-rich solution on responses to 5-hydroxytryptamine (5-HT) and 1,1-dimethyl-4-phenyl piperazinium (DMPP). (a) Chart records of responses to 5-HT or DMPP as in Figure 1: (i) 5-HT control; (iv) DMPP control; (ii) response to 5-HT after superfusing with Ca-rich solution (5.1 mM) for 67 min; (v) response to DMPP after superfusing with Ca-rich solution (5.1 mM) for 36 min; (iii) and (vi) after washing with normal Krebs solution for 12 and 36 min, respectively. (b) Decay of after-hyperpolarization plotted as in Figure 1 for responses to DMPP (iv, v, vi): (●) control; (△) Ca-rich medium; (□) wash.

normal Krebs solution. $[Ca^{2+}]_o$ 7.6 mM produced comparable effects which were not consistently greater in magnitude. For analysis, results from experiments in which 5.1 and 7.6 mM $[Ca^{2+}]_o$ were used have been pooled and are summarized in a histogram (Figure 5). Mean reduction in depolarization amplitude was 29%. In contrast, responses to DMPP were usually potentiated by Ca-rich solutions (Figure 2 av), although there was considerable variability between experiments. The potentiation was reversed on returning to normal Krebs solution. $[Ca^{2+}]_o$ 7.6 mM produced comparable effects to 5.1 mM $[Ca^{2+}]_o$, which were not consistently greater in magnitude. Pooled results from all experiments are summarized in Figure 5. Depolarization amplitude was increased on average by 12%.

A modified Locke solution in which $[Ca^{2+}]_o$ was raised to 12.7 mM had inconsistent effects on responses evoked by 5-HT and DMPP. However, the Tris buffer solution employed in the Locke or some other constituent of this solution itself depressed the amplitude of depolarizations evoked by 5-HT. Thus, when the ganglion was superfused with modified Locke solution containing $CaCl_2$ (2.54 mM), following a control period in Krebs solution, a substantial reduction in amplitude was observed.

Ca-free solutions On superfusing the ganglion with a

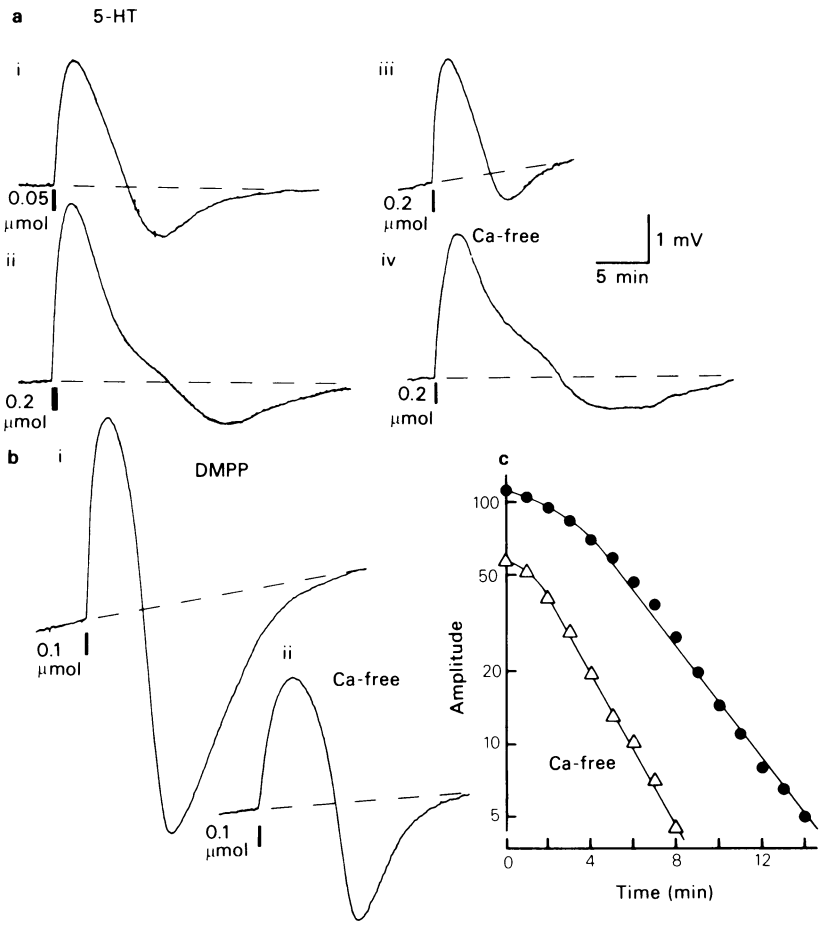

Figure 3 Effect of calcium-free solution on responses to 5-hydroxytryptamine (5-HT) and 1,1-dimethyl-4-phenyl piperazinium (DMPP). (a) Chart records of responses as in Figure 1 showing similarities in the responses of a ganglion which showed a high sensitivity to 5-HT to those obtained in another ganglion on superfusion with Ca-free medium: (i) and (ii) responses to 0.05 and 0.2 µmol 5-HT, respectively, in sensitive ganglion, (iii) and (iv) responses from another ganglion to 0.2 µmol 5-HT in normal Krebs (iii) and after superfusion with Ca-free solution for 38 min (iv). (b) Chart record of responses to DMPP: (i) control and (ii) after superfusion with Ca-free solution for 125 min. (c) Decay of after-hyperpolarization plotted as in Figure 1 for responses to DMPP (bi, bii): (●) control, (△) Ca-free medium.

modified Krebs solution without $CaCl_2$, a slow depolarization was recorded of 2.3 ± 0.3 mV ($n = 7$). Responses to both 5-HT and DMPP were altered by this medium, but whereas 5-HT responses were enhanced (Figure 3 aiv), DMPP responses were reduced in amplitude (Figure 3 bii). A small minority of ganglia superfused with normal Krebs solution displayed an abnormally high sensitivity to 5-HT, so that a smaller amount of 5-HT, e.g. 0.05 μmol (Figure 3 ai), evoked a typical response, while 0.2 μmol evoked a maximal or near maximal response. It was typical of maximal responses that a late, slower phase of repolarization was seen as a shoulder on the falling phase of the depolarization (Figure 3 aii). In several experiments, Ca-free solution had the apparent effect of increasing the sensitivity of the ganglion. Thus, 0.2 μmol 5-HT evoked a characteristic response in normal Krebs solution (Figure 3 aiii), but in Ca-free medium evoked a larger depolarization which displayed a slower phase of repolarization on its falling phase (Figure 3 aiv). On average, depolarizations evoked by 5-HT were increased by 23% and those evoked by DMPP reduced in amplitude by 32% (Figure 5).

Mg-rich solutions Increasing the concentration of $MgSO_4$ 10 or 20 fold produced triphasic changes in recorded potential. An initial rapid hyperpolarization was assumed to arise from a change in diffusion potential. A subsequent, slow depolarization was taken to be a membrane potential change, confirming the depolarizing action of Mg-rich solutions on ganglion cells reported by Guerrero & Riker (1973). The depolarization was about 2.5 mV in amplitude on superfusing with 12.7 mM $[Mg^{2+}]_o$ and 15–30 mV in amplitude on superfusing with 25.4 mM $[Mg^{2+}]_o$. The depolarization slowly decayed producing a very slow change of baseline potential in the hyperpolarizing direction. The converse of these potential changes was observed on changing from a Mg-rich medium to normal Krebs solution. Although depolarizations to both 5-HT and DMPP were reduced in amplitude by Mg-rich solutions, 5-HT responses appeared to be affected to a greater extent. $[Mg^{2+}]_o$ 12.7 mM reduced 5-HT and DMPP responses by 34 and 10%, respectively (Figure 5); $[Mg^{2+}]_o$ 25.4 mM caused a large reduction in 5-HT and DMPP responses, the former being reduced by 84 and the latter by 75% (Figure 5).

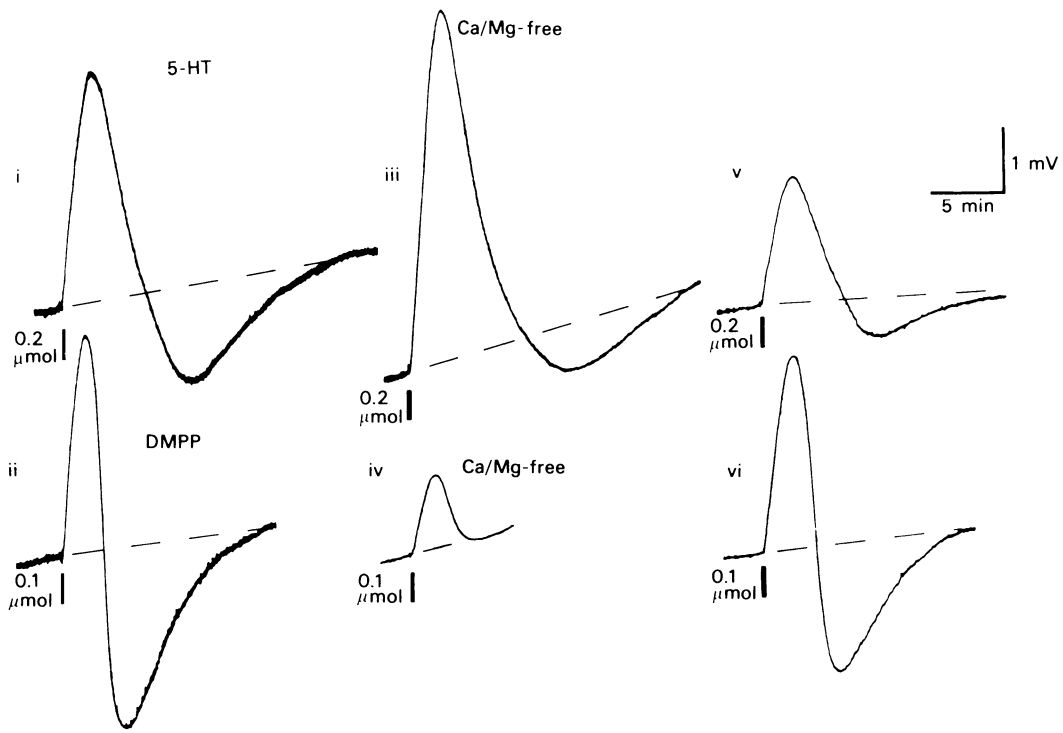

Figure 4 Effect of calcium-, magnesium-free solution on responses to 5-hydroxytryptamine (5-HT) and 1,1-dimethyl-4-phenyl piperazinium (DMPP). Chart records as in Figure 1: (i) 5-HT control; (ii) DMPP control; (iii) response to 5-HT and (iv) response to DMPP after superfusing with Ca/Mg-free medium for 17 and 86 min, respectively; (v) response to 5-HT and (vi) response to DMPP after washing with normal Krebs solution for 60 and 77 min, respectively.

Low Mg solutions Reducing the concentration of $MgSO_4$ to 0.13 mM (Ca:Mg ratio, 20) or omitting $MgSO_4$ from the superfusion medium had similar effects. Results from both experiments were pooled. On superfusing the ganglion with low Mg solution a slow depolarization was recorded, which varied in magnitude considerably between different preparations. A slow hyperpolarization was recorded on returning to normal Krebs solution. Depolarizations evoked by both 5-HT and DMPP were reduced in magnitude to a similar extent by low Mg solution, the former by 20% and the latter by 22% (Figure 5).

Ca/Mg-free solutions The largest changes in the depolarizations evoked by 5-HT or DMPP were produced by Ca/Mg-free medium. On superfusing the ganglion with this medium, a slow depolarization was observed; the effects on 5-HT and DMPP responses can be seen in the records shown in Figure 4. The depolarization evoked by 5-HT was substantially increased in amplitude, while that evoked by DMPP was greatly reduced, changes which were reversed on returning to normal Krebs solution. On average, responses to 5-HT were potentiated by 78%, while those to DMPP were reduced by 58% (Figure 5).

Effect of divalent cations on after-hyperpolarizations (AH) evoked by 5-hydroxytryptamine or 1,1-dimethyl-4-phenyl piperazinium

Changes in the concentration of divalent cations also affected the hyperpolarizing phases of the responses.

Ca-rich solutions AHs evoked by 5-HT were often small and showed a tendency to decline throughout the experiment. Ca-rich solutions accelerated this effect by reducing AH amplitude considerably (Figure 2a). Results were pooled from experiments where 5.1 and 7.6 mM $[Ca^{2+}]_o$ were used. The mean reduction in AH amplitude was 50% (Figure 6). In contrast, AHs evoked by DMPP were increased in amplitude (Figure 2a) during superfusion with Ca-rich medium, an effect that was reversed on returning to normal Krebs solution. On average, AH amplitude was increased by 73% (Figure 6). AHs showed some prolongation of their time course during superfusion with Ca-rich solution (cf. Figure 2 aiv and v). The decay of hyperpolarizing phases of the responses evoked by DMPP is shown graphically, plotted on a semi-logarithmic scale against time in Figure 2b. The time constants of the exponential portions of the curves are 3.3, 4.4 and 3.0 min for control, response in Ca-rich medium and response on washing, respectively. This increase in time constant and decrease in rate constant were consistently observed for DMPP responses in Ca-rich medium (Table 1). Responses to 5-HT in this medium displayed small AHs of insufficient magnitude for accurate analysis.

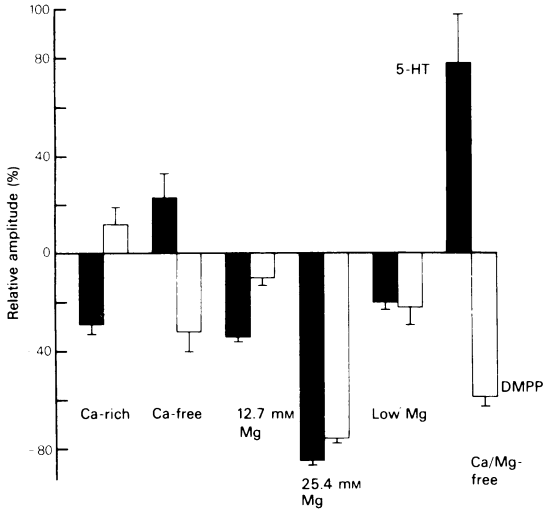

Figure 5 Histogram summarizing the effects of change in concentration of calcium or magnesium ions on amplitude of depolarizations evoked by 5-hydroxytryptamine (5-HT) and 1,1-dimethyl-4-phenyl piperazinium (DMPP). Ordinate scale: relative amplitude compared to last control; negative values indicate a reduction in amplitude. Columns show mean and bars show s.e.mean for all responses generated in modified superfusion medium. Solid columns, responses to 5-HT; open columns, responses to DMPP.

Ca-free solutions The effects of this medium on AHs evoked by 5-HT were variable, partly because a slower phase of repolarization appeared in some preparations which partially occluded AH (Figure 3a), and also because there was a time-dependent effect on amplitude. The time-dependent effect can be seen in Figure 6 in which response amplitude is plotted against time. The amplitude of AH induced by 5-HT was initially increased in Ca-free solution but subsequently declined. There was also an increase in amplitude on returning to normal Krebs solution. Nevertheless, there was on average a 116% increase in AH amplitude in Ca-free solutions (Figure 6). AHs evoked by DMPP were depressed in Ca-free solutions (Figure 3b), on average by 52% (Figure 6), and it was noticeable that the rate at which AH decayed was increased. Decay of AH of Figure 3b is plotted on a semi-logarithmic scale against time in Figure 3c. The time constants of the exponential portions of the curves were 3.7 and 2.8 min for responses in Krebs solution and Ca-free medium, respectively; the corresponding rate constants were 0.27 and 0.36 min^{-1}. AHs generated by both DMPP and 5-HT were similarly affected, the rate of decay increasing; changes were reversible on washing (Table 1).

Mg-rich solutions The hyperpolarizing as well as the depolarizing phase of responses evoked by 5-HT or

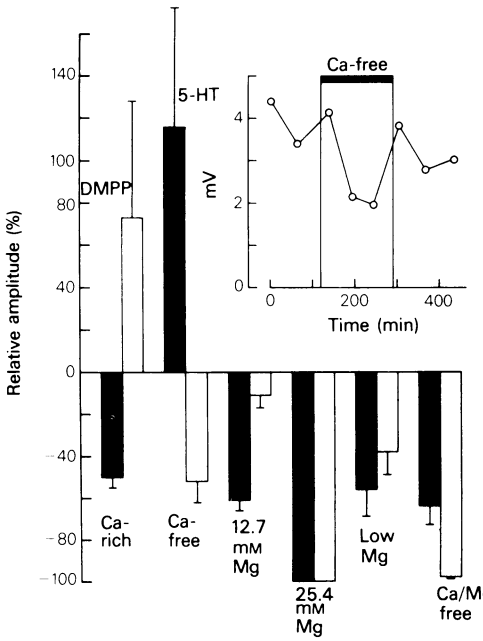

Figure 6 Histogram summarizing the effects of change in concentration of calcium or magnesium ions on amplitude of after-hyperpolarizations evoked by 5-hydroxytryptamine (5-HT) and 1,1-dimethyl-4-phenyl piperazinium (DMPP). Ordinate scale as in Figure 5, columns show mean and bars show s.e.mean for all responses generated in a modified superfusion medium. Solid columns, responses to 5-HT; open columns, responses to DMPP. The graph shows amplitude (mV) of the AH evoked by 5-HT plotted against time (min) for one particular experiment in which the ganglion was superfused with Ca-free medium for the period indicated by the black bar.

DMPP were depressed by Mg-rich solutions. $[Mg^{2+}]_o$ 12.7 mM had a greater effect on 5-HT AHs which were reduced on average by 61% than on DMPP AHs which were reduced by 11% (Figure 6). On superfusion of the ganglion with 25.4 mM $[Mg^{2+}]_o$, AHs evoked by either 5-HT or DMPP were completely suppressed (Figure 6). $[Mg^{2+}]_o$ 12.7 mM changed the rate of decay of AH but the effect was not a simple one. After an initial 1 min or 2 min of decline, an exponential decay began, the time constants of which are given in Table 1 for three different preparations in which responses to DMPP were analysed. It can be seen that the rate of decay increased in Mg-rich medium. However, there were indications in all three experiments that a late, slower phase of decay ensued after 6–8 min which did not appear to be exponential.

Low Mg solutions The hyperpolarizing as well as the depolarizing phase of responses evoked by 5-HT or

Table 1 Effect of change in the concentration of calcium or magnesium ions on amplitude and on time and rate constants of decay of after hyperpolarizations induced by 5-hydroxytryptamine and 1,1-dimethyl-4-phenyl piperazinium

			Control			*Experimental solution*			*Wash*	
Experiment		*Agonist*	*Amplitude (mV)*	*Time constant (min)*	*Rate constant (min^{-1})*	*Amplitude (mV)*	*Time constant (min)*	*Rate constant (min^{-1})*	*Amplitude (mV)*	*Time constant (min)*	*Rate constant (min^{-1})*
Ca-rich	A	DMPP	3.4	4.8	0.21	2.2	9.8	0.1	1.5	4.7	0.21
	B	DMPP	2.1	2.2	0.45	1.5	3.5	0.29	2.2	2.3	0.43
	C	DMPP	3.0	3.3	0.3	3.3	4.4	0.23	1.5	3.0	0.33
Ca-free	A	5-HT	3.4	4.8	0.21	3.2	2.2	0.45	3.0	5.3	0.19
	B	DMPP	4.7	5.0	0.2	2.2	3.5	0.29	1.6	7.3	0.14
	C	DMPP	4.8	3.7	0.27	2.6	2.8	0.36	2.5	4.5	0.22
Mg 12.7 mM	A	DMPP	3.8	5.8	0.17	4.0	4.5	0.22	2.4	5.5	0.18
	B	DMPP	2.7	4.4	0.23	2.9	3.5	0.29	1.9	4.3	0.23
	C	DMPP	3.1	4.3	0.23	2.5	3.4	0.29	2.6	4.4	0.23
Low Mg	A	DMPP	3.3	3.4	0.29	1.0	4.3	0.23	3.5	3.7	0.27
	B	DMPP	3.7	4.3	0.23	1.4	4.7	0.21	2.3	3.7	0.27
	C	DMPP	5.7	3.2	0.31	3.8	4.2	0.24	3.3	3.7	0.27

Results from 3 different preparations are shown for each modified superfusion medium.

DMPP were depressed in low Mg solutions. On average, AHs evoked by 5-HT and DMPP were reduced in amplitude by 56 and 38%, respectively (Figure 6). The rate of decay of AH was also somewhat changed, although this could only be measured accurately in DMPP responses because 5-HT responses were too small. As can be seen from Table 1, there was a consistent tendency for the rate of decay to be slowed in low Mg solutions.

Ca/Mg-free solutions Removal of both species of divalent cation caused large changes in the amplitude of AHs evoked by 5-HT or DMPP. Despite the large increase in the amplitude of the depolarization evoked by 5-HT, AH was reduced in size (Figure 4), the mean reduction being 64% (Figure 6). Depolarizations evoked by DMPP were greatly reduced in Ca/Mg-free medium and the hyperpolarizing phase was often completely absent (Figure 4). On average AHs evoked by DMPP were reduced by 98%.

Responses of post-ganglionic axons to 5-hydroxytryptamine

In most preparations, the axons of the internal carotid nerve, if the sheathing material had been carefully removed, also responded to 5-HT and displayed a rapid depolarization; the response to DMPP was more variable. 5-HT responses were normally of much shorter duration than those evoked from the ganglion (Figure 7a). Often no appreciable AH was detectable, at least in normal Krebs solution. Because of these features of the response, different quantities of 5-HT could be tested at shorter intervals

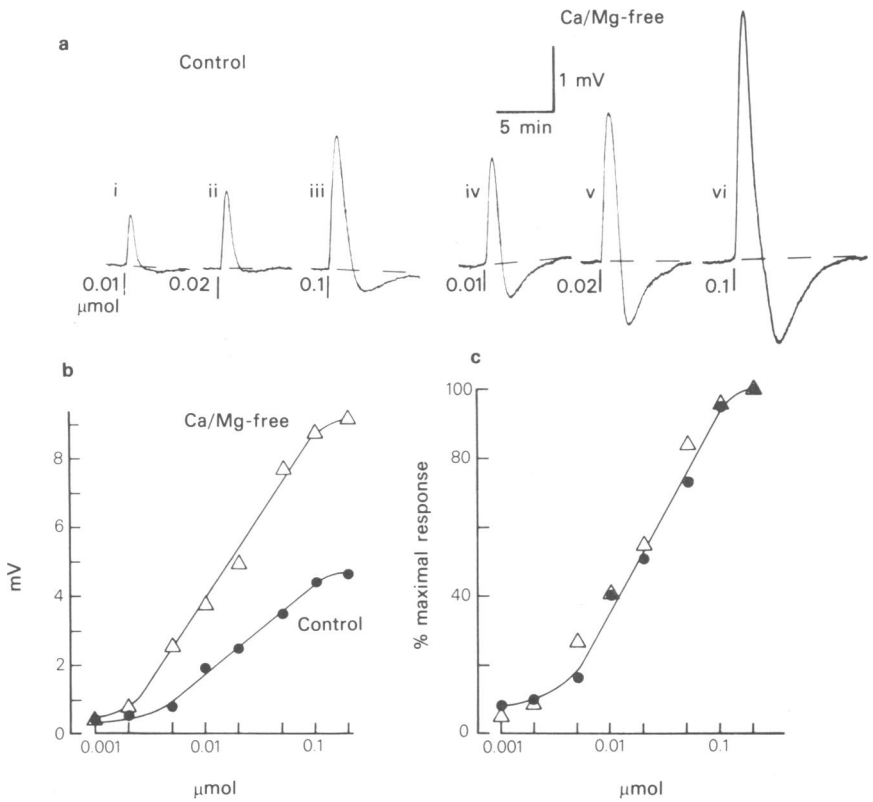

Figure 7 Responses of fibres in the internal carotid nerve to 5-hydroxytryptamine (5-HT) and the effect of superfusion with a calcium-, magnesium-free medium. (a) Chart records of membrane potential change induced by 5-HT, depolarization upwards: (i), (ii) and (iii) control responses to 0.01, 0.02 and 0.1 μmol 5-HT, respectively; (iv), (v) and (vi) responses to 0.01, 0.02 and 0.1 μmol 5-HT during superfusion with Ca/Mg-free medium. (b) and (c) results from another preparation shown graphically. In (b), depolarization amplitude (mV) is plotted against the quantity of 5-HT injected into the superfusion stream and in (c) control responses are plotted relative to the largest control response and responses in Ca/Mg-free medium plotted relative to the largest response in this medium against the quantity of 5-HT injected. (●) Control responses; (△) responses in Ca/Mg-free medium.

than on the ganglion. Approximately 10–15 min were required between injections of the larger amounts of 5-HT if tachyphylaxis was to be avoided. It was possible in a few experiments to obtain full dose-response curves in control and test media on the axonal preparation.

As can be seen from Figure 7a, an AH was discernible as part of the response in this preparation. In two experiments this was sufficiently large for the decay to be measured accurately. The time course of the decay was considerably shorter than was observed for ganglionic potentials. The time constants of the exponential phases of decay were 1.15 and 1.25 min in these two preparations, corresponding rate constants being 0.8 and 0.87 min^{-1}.

Chart records of responses evoked by 0.01, 0.02 and 0.1 μmol 5-HT from axons can be seen in Figure 7. The response evoked by 0.1 μmol was just submaximal. The amplitudes of responses from another experiment are plotted against the quantity of 5-HT injected on a semi-logarithmic scale in Figure 7b. The ED_{50} for this set of observations was 0.02 μmol. The observations are replotted in Figure 7c in the conventional way as a percentage of the maximum.

On the assumption that axonal 5-HT receptors display the same characteristics as somatic receptors, some preliminary experiments on the effect of Ca/Mg-free solution were done. In each of 3 experiments, the depolarizations evoked by 5-HT were greatly potentiated. Chart records of responses to 0.01, 0.02 and 0.1 μmol 5-HT during superfusion with Ca/Mg-free solution are shown in Figure 7a (iv–vi). It can be seen that not only was depolarization amplitude greatly increased, but that AH became much larger and more pronounced. Depolarization amplitude is plotted in Figure 7b and relative amplitude is plotted in Figure 7c. The latter graph suggests that no significant shift in the position of the curve has occurred.

Discussion

Changes in depolarization amplitude can be considered separately from changes in AH, because the depolarization arises directly from the ligand-receptor interaction and subsequent permeability change, whereas AH is believed to be a response to a change in $(Na^+)_i$ (Kerkut & York, 1971; Lees & Wallis, 1974).

Depolarizations

The observations on depolarization amplitude confirm by a direct method and extend findings on the cat s.c.g. *in situ* (Takeshige & Volle, 1964; Smith, 1966). Increases in $(Ca^{2+})_o$ produced a clear differential effect, reducing the amplitude of 5-HT responses and usually potentiating responses to DMPP. However, because of the variability of these effects and the apparent inability of modified Locke solution to support normal responses, it was not possible to demonstrate a concentration-dependence of the depression or potentiation. Superfusion with Ca-free solutions had the effect of potentiating responses to 5-HT and reducing responses to DMPP. Ca^{2+} might affect a drug-induced depolarization in at least four ways: (a) indirectly, by altering resting membrane potential, (b) by altering the relative magnitude of the ionic conductances underlying the drug-induced depolarization, (c) by altering the affinity of receptor for agonist and (d) by some action on the coupling of agonist-receptor complex to effector mechanism.

Thus, it might be postulated that changes in $(Ca^{2+})_o$ would alter the amplitude of responses evoked by 5-HT and DMPP since these changes appeared to alter resting membrane potential. Increase in $(Ca^{2+})_o$ appeared to cause a small hyperpolarization and decrease in $(Ca^{2+})_o$ a depolarization of the membrane. The well-attested influence of Ca^{2+} on the resting sodium conductance (G_{Na}) (Frankenhauser & Hodgkin, 1957) probably underlies these changes in potential. Mg^{2+} might have a similar effect (Brown, Brownstein & Scholfield, 1972), and this could account for the depolarization observed in Mg-free solutions. Decreased membrane resistance might also attenuate drug-induced responses. The AH evoked by acetylcholine is reduced in Ca-free medium and Lees & Wallis (1974) suggested that this was a consequence of decreased membrane resistance (see also Brown *et al.*, 1972). Thus, membrane depolarization and decreased membrane resistance might be expected to occlude a drug-induced depolarization. In spite of this, depolarizations evoked by 5-HT were potentiated in Ca-free solutions. An indirect action of Ca^{2+} on membrane potential or resistance cannot explain this effect. It might explain the reduction in responses to DMPP, even though the ionic mechanism of this response appears similar to that underlying 5-HT responses.

An effect of Ca^{2+} on the conductance increase responsible for the fast (nicotinic) e.p.s.p. of amphibian ganglion cells has been reported (Kuba & Koketsu, 1978). Ca^{2+} reduced preferentially the increase in G_{Na} with respect to G_K and the reversal potential for the e.p.s.p. became more negative when $(Ca^{2+})_o$ was increased 10 fold. Again, our results are not consistent with this kind of effect, since a reduction in sodium current during a nicotinic response ought to result in a decrease in the depolarization evoked by DMPP. In fact, DMPP responses were usually potentiated in Ca-rich medium. Although this kind of mechanism could explain the changes in responses evoked by 5-HT, it is difficult to see why

5-HT responses should be affected preferentially in this way.

The apparent increase in sensitivity to 5-HT, seen in some experiments wih Ca-free solutions, might suggest that Ca^{2+} altered the binding of 5-HT to its receptors. It has been postulated that a Ca^{2+} binding site might be intimately linked to the 5-HT receptor (Wallis, 1979). Increased affinity in Ca-free medium ought to manifest itself as a shift to the left of the dose-response curve. Unfortunately, it is technically very difficult to obtain repeated, full dose-response curves of ganglionic responses but by using responses from axons which appear to possess identical receptors it was possible to make these observations. The substantial potentiation seen in the absence of both divalent cations was used in order to maximize the effect to be measured. Potentiation of responses to all quantities of 5-HT, including the maximal response, was evident in each experiment but there was no significant shift of the curves to the left. Thus, experiments on axonal responses failed to confirm that the affinity of the 5-HT receptor had altered.

The fourth mode of action might best explain the changes observed in response to 5-HT, for these seem consistent with an association of Ca^{2+} and a membrane binding site which modulates the coupling between receptor and ion channel (see Burgen & Spero, 1970). From our experiments it must be admitted, however, that this mode of action cannot readily be distinguished from an effect on the relative magnitude of the transmitter-induced ion conductances. In terms of the mechanism proposed by Lièvremont & Pascaud (1972), the Ca^{2+} may be bound at membrane sites which serve to maintain ion channels in the closed conformation. An increase in $(Ca^{2+})_o$ would be likely to stabilize ion channels in this conformation. Such an idea is consistent with the increase in the maximum response to 5-HT observed with axonal preparations in Ca/Mg-free solutions. Paradoxically, this role for calcium binding sites was proposed for nicotinic receptors of skeletal muscle (Lièvremont & Pascaud, 1972; Taylor, 1973). However, it has recently been shown that, for the nicotinic receptors of *Torpedo* electroplax, increases in $(Ca^{2+})_o$ induce an increase in receptor affinity for cholinergic ligands; maximal enhancement was achieved with 5 mM $(Ca^{2+})_o$ (Cohen, Weber & Changeux, 1974).

We suggest that the membrane site stabilizing ion channels in the closed conformation can also bind Mg^{2+}, Ca^{2+} and Mg^{2+} competing for this site. An increase in $(Ca^{2+})_o$ would then depress responses to 5-HT as might an increase in $(Mg^{2+})_o$, since in both cases the total concentration of divalent cations having a stabilizing action would have increased. An additional action contributing to the strong depressant effect of magnesium might be the depolarization evoked by Mg-rich solution, especially 25.4 mM $(Mg^{2+})_o$. The decrease in responses to 5-HT in Mg-free solutions seems inconsistent with this hypothesis unless it is postulated that Mg^{2+} are of lower efficacy at the stabilizing site. In that case, absence of Mg^{2+} would allow Ca^{2+} freer access because of the absence of competing ion species. A similar action of Ca^{2+} and Mg^{2+} explains the greater potentiation of 5-HT responses observed on omitting both divalent cations from the superfusion medium compared to the effect of Ca-free solutions.

The changes observed in DMPP responses are puzzling but might be related to the effect of Ca^{2+} on the binding of ligands at nicotinic receptors (Cohen *et al.*, 1974). In the absence of Ca^{2+}, responses to DMPP were depressed. Since, when both Ca^{2+} and Mg^{2+} were omitted from the superfusion medium, a somewhat greater depression was observed, it is possible that Mg^{2+} can substitute to some extent for Ca^{2+} in this action, or that both ions are necessary for efficient coupling of ligand-receptor complex to ion channel, as suggested by Burgen & Spero (1970). Either mechanism might explain why responses to DMPP were reduced in Mg-free solutions. In Mg-rich solutions, DMPP responses were substantially depressed only by 25.4 mM $(Mg^{2+})_o$, an effect that might be attributed entirely to the membrane depolarization induced by this concentration of magnesium (cf. Guerrero & Riker, 1973).

After-hyperpolarizations

Pronounced alterations in the AH were produced on changing $(Ca^{2+})_o$ and, to a lesser extent, on changing $(Mg^{2+})_o$. AH amplitude is believed to depend on a number of factors, such as amount of sodium to be transported (sodium load), membrane resistance across which the potential is generated and activity of membrane Na-K-ATPase. Extrusion of sodium which has entered the cell during the depolarizing phase of the response to acetylcholine appears to be achieved by an electrogenic sodium pump (Lees & Wallis, 1974). Depolarizations evoked either by 5-HT or by a nicotinic receptor agonist are partially dependent on an influx of Na^+ (Wallis & Woodward, 1975; Wallis & Nash, 1980). That the mechanism underlying the AH induced by 5-HT or DMPP may be the same is suggested by the finding that the rate constants of decay of AH were the same, even though the magnitude of AH induced by DMPP was usually considerably greater than that induced by 5-HT.

In some preparations, responses recorded from the unmyelinated axons of the postganglionic trunk also displayed an AH in response to 5-HT. The presence of an electrogenic sodium pump in the axonal membrane is well documented (see Kerkut & York, 1971). The rate constant of the decay of these responses appeared to be considerably greater than

that observed for ganglion cells, being 0.8–0.87 min^{-1} compared to 0.26 min^{-1}, and also somewhat greater than the maximal rates reported for garfish olfactory nerve fibres (0.47 min^{-1}, Straub & Ritchie, 1974; 0.55 min^{-1}, McDougal & Osborn, 1976). The faster rates observed in axons compared to ganglion cells may reflect the larger surface-to-volume ratio pertaining to the former. Rate is also known to increase with increased sodium load.

Changes in the concentration of divalent cations might alter AH amplitude because the depolarizations, and presumably sodium load, were altered. However, divalent cations have direct actions on sodium pumping, because the Na-K-ATPase involved is Mg^{2+}-dependent (Skou, 1960). Further, activation of ATPase by Mg^{2+} can be inhibited by Ca^{2+} both competitively and non-competitively (Skou, 1960). A direct action on ATPase activity should manifest itself as a change in the rate of decay of AH. Ca-rich solutions decreased in amplitude AH evoked by 5-HT, but increased AH evoked by DMPP, changes that probably reflect differences in the amount of sodium entering the cells. The rate of decay of AH evoked by DMPP was slower in Ca-rich solutions, despite the fact that sodium load probably increased. A reduction in rate constant is consistent with Ca^{2+} inhibiting Na-K-ATPase. In Ca-free solutions, AH evoked by 5-HT was increased while AH evoked by DMPP was reduced in amplitude, probably reflecting changes in sodium load. Previous work had indicated that AH is greatly attenuated by short-circuiting in Ca-free medium as a result of reduced membrane resistance (Brown *et al.*, 1972; Lees & Wallis, 1974). Changes in membrane resistance might have contributed to the complex changes in amplitude of AH evoked by 5-HT, but AH whether evoked by 5-HT or DMPP persisted in Ca-free medium. The rate of decay of AHs evoked by both 5-HT and DMPP was increased in this medium, consistent with a reduction in the inhibition by Ca^{2+} of Na-K-ATPase. Alterations in $(Mg^{2+})_o$ confirmed that both species of divalent cation could affect the rate of decay of AH. Although 12.7 mM $(Mg^{2+})_o$ depressed the amplitude of AH evoked by DMPP, it somewhat increased the rate of decay of AH, consistent with increased activation of Na-K-ATPase. The slower component of decay seen in Mg-rich solutions has not been analysed further. In low Mg solutions, AH amplitude also declined but the rate of decay was reduced, consistent with reduced activation of Na-K-ATPase. It is unclear from these experiments, however, whether these effects of divalent cations on the rate of decay of AH are the result of an interaction at extracellular or intracellular sites.

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