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INTERACTIONS AMONG THE EFFECTS OF NORMORPHINE, CALCIUM AND MAGNESIUM ON TRANSMITTER RELEASE IN THE MOUSE VAS DEFERENS

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1 Excitatory junction potentials (e.j.ps) were recorded with intracellular electrodes from smooth muscle cells of the mouse vas deferens.

2 The dependence of the e.j.p. amplitude on the extracellular calcium ion concentration was determined in the absence or presence of normorphine (50 nM–1 μM) or magnesium (1.2–4.8 mM).

3 The interaction between normorphine and calcium was non-competitive beyond a dose-ratio of 1.5, whereas the interaction between magnesium and calcium was competitive up to the highest dose-ratio investigated (1.9).

4 It is suggested that inhibition by normorphine occurs at least partly by a mechanism different from that of magnesium.

Introduction

Normorphine depresses noradrenaline release in the mouse vas deferens (Henderson, Hughes & Kosterlitz, 1972) and this action can be readily measured by recording excitatory juction potentials (e.j.ps) from the smooth muscle cells (Henderson & North, 1976). The concentration of calcium ions in the perfusing solution also critically affects transmitter release and the e.j.p. amplitude (Bennett & Florin, 1975). The possible interaction between normorphine and calcium ions has been the subject of two previous studies. Illes, Zieglgänsberger & Herz (1980) found that reducing the calcium concentration increased the sensitivity of the e.j.p. to depression by normorphine, while higher calcium concentrations reduced the inhibitory effect of normorphine. However, in those experiments the stimulus voltage was varied over a wide range and there was little or no overlap between the population of nerves excited in the presence and absence of normorphine (e.g. Figure 1b, Illes *et al.*, 1980). Bennett & Lavidis (1980) kept the stimulus voltage constant in their studies of the calcium dependence of the e.j.p. in the absence and presence of morphine. They interpreted their results on the assumption that the e.j.p. amplitude was proportional to the fraction of release sites occupied by calcium ions (the 'occupancy' assumption) and concluded that morphine competitively inhibits calcium action. In the present experiments we have studied the interaction between normorphine and calcium ions under conditions in which (it is assumed) the same population of nerve fibres is excited throughout each experiment, but we have avoided the occupancy assumption by the comparison of equal responses. The interaction between calcium and magnesium was studied using similar methods.

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Methods

Mice (CF-1 strain) were killed by a blow to the head and the vasa deferentia dissected out of the abdominal cavity. A single vas deferens was pinned into a Perspex bath and superfused at 1.5 ml/min with Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, KH_2PO_4 0.93, $CaCl_2$ 2.54, $MgSO_4.7H_2O$ 1.19, glucose 11 and $NaHCO_3$ 25. This solution was gassed with 95% O_2 and 5% CO_2 and heated so that the temperature at the recording site was 35 to 37°C. Drugs and solutions of different ionic composition were applied by changing the perfusing solution. E.j.ps were recorded from single smooth muscle cells in the central one-third of the vas, by techniques previously described (Henderson & North, 1976; North & Vitek, 1980). E.j.ps were evoked by single rectangular pulses (500 μs in duration) which were repeated every 30 s. These stimuli were applied through two platinum ring electrodes placed around the muscle on either side of the site of recording. In preliminary experiments, the stimulus voltage was adjusted so that e.j.p. amplitude was between 10 and 25 mV before normorphine was

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applied (voltages of 15–100 V). In later experiments, the stimulus voltage was always set at the beginning of the recording so as to give a 15 mV e.j.p. in the control Krebs solution described above (voltages of 20–80 V). The calcium concentration was then progressively changed in increments of 0.5 mM, while recording from the same smooth muscle cell. Changes were made in increasing concentrations or in decreasing concentrations in approximately equal numbers of experiments. This procedure was repeated in the presence of either normorphine or an altered magnesium concentration, selecting that range of calcium concentrations which gave an e.j.p. amplitude between 10 and 20 mV. For each normorphine concentration and magnesium concentration, the calcium concentration vs. e.j.p. amplitude curve was determined on at least six different tissues. Solutions containing each different calcium ion concentration superfused the tissue for 15 min; the mean e.j.p. amplitude was calculated from measurements made in the last 3 of these 15 min. Drugs used were normorphine sulphamate (Dr A.E. Jacobsen) and naloxone hydrochloride (Endo). Drug concentrations refer to the salt. When $CaCl_2$ and $MgCl_2$ were altered the NaCl content was changed to maintain the same osmolarity.

Statistical procedures for calculation of regression lines (multiple Y at each X) and comparison of slopes are fully described by Sokal & Rohlf (1969).

Results

Preliminary experiments

The action of normorphine on the e.j.p. was similar in time course, reversibility and naloxone sensitivity to that described previously (Henderson & North, 1976). In the range of normorphine concentrations from 100 nM to 3 μM, the relation between percentage inhibition (I) and normorphine concentration (M) [NM] was of the form $I = 53.6 \log [NM] + 394$ ($r = 0.937$, $n = 24$, $P \ll 0.001$), giving an EC_{50} of 382 nM (Figure 1). Elevating the calcium concentration from 2.54 to 5.08 mM increased the e.j.p. amplitude to 244% ± 20% (mean ± s.e.mean; $n = 5$) of its control value. The stimulus strength was then reduced so as to return the e.j.p. to its original amplitude and a concentration-response curve to normorphine was constructed (Figure 1). The normorphine EC_{50} value calculated from the regression line was 421 nM. Analogous experiments in which the calcium concentration was reduced to 1.27 mM (which reduced the e.j.p. to 34 ± 5% ($n = 6$) of its control value) gave an EC_{50} of 274 nM. These results imply that normorphine is more effective in low calcium solutions. However, it is necessary to assume

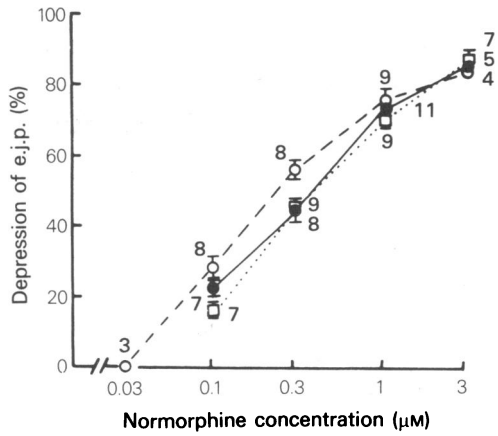

Figure 1 Depression of e.j.p. amplitude as a function of normorphine concentration. Experiments were performed in three different calcium concentrations (1.27 mM, ○; 2.54 mM, ●; 5.08 mM, □). Bars are s.e.mean for number of cells indicated.

that the fibres excited at different stimulus strengths have equal morphine sensitivity and some evidence has been presented that this may not be so (Illes & Schulz, 1980). For this reason, all subsequent experiments were performed by adjusting the stimulus voltage to produce an e.j.p. of 15 mV in a solution containing 2.5 mM calcium, 1.2 mM magnesium and no normorphine, and left at this value throughout the experiment (see Methods).

Interaction between normorphine and calcium

In the absence of normorphine, a step-wise increase in the calcium concentration from 1 to 4 mM caused an increase in the amplitude of the e.j.p. (Figure 2, control). The relation between the e.j.p. amplitude (y) and the calcium concentration [Ca] could be described by a straight line using either semilogarithmic or double logarithmic transformations. The regressions were ($n = 55$) $y = 36.5 \log [Ca] + 0.703$ ($r = 0.954$, $P \ll 0.001$) and $\log y = 1.22 \log [Ca] + 0.673$ ($r = 0.937$, $P \ll 0.001$). In the presence of normorphine, the e.j.ps of the original control amplitude could be restored by increasing the calcium concentration. The concentration-effect curve for calcium was shifted to the right and became flattened at higher normorphine concentrations: the curves in the presence of 300 nM and 1 μM normorphine had significantly different slopes from the curve in the absence of normorphine (Figure 1). This indicates that the interaction between normorphine and calcium is non-competitive when higher normorphine concentrations are used.

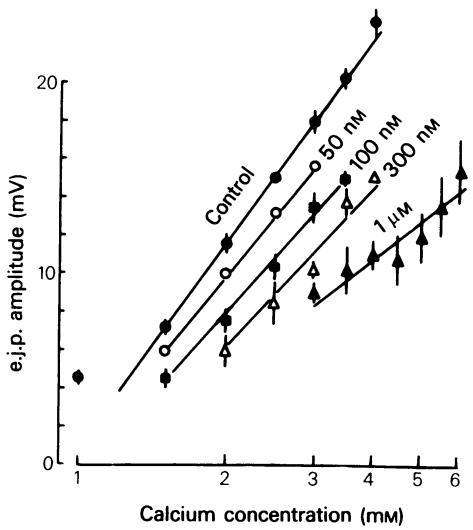

Figure 2 E.j.p. amplitude as a function of calcium concentration; effect of normorphine (concentrations indicated). The points indicate the mean e.j.p. amplitude (with s.e.mean) recorded at a given calcium concentration (number of determinations for each point is between 3 and 16; mean 6). Lines are fitted by least squares to raw data. Where error bars are not shown, they are smaller than the size of the point. For each line, the slope and its 1% and 5% confidence limits were determined. The slopes of the lines for 50 and 100 nM did not differ from the slope of the control line. The slope of lines for 300 nM and 1 μM did differ from the control ($P < 0.05$ and $P < 0.001$ respectively).

Interaction between magnesium and calcium

In the control solution (magnesium 1.2 mM), the relationship between calcium and e.j.p. amplitude was similar to that described above ($n = 43$, $y = 36.7 \log [Ca] + 0.716$ ($r = 0.929$, $P \ll 0.001$) or $\log y = 1.25 \log [Ca] + 0.682$ ($r = 0.931$, $P \ll 0.001$). This relation between calcium and the e.j.p. amplitude was shifted to the left by removal of magnesium and to the right by increasing the magnesium concentration (Figure 3). The slopes of the lines were parallel up to a dose-ratio of 1.9 (Table 1) and the Schild plot had a slope which did not differ from unity (0.95 ± 0.06) (Figure 4). The estimate of the dissociation constant for magnesium (K_{Mg}) was 5.5 mM, with 5% confidence limits of 2.5 to 12.5 mM. The requirement to keep the calcium concentration below 6 or 7 mM precluded the use of higher magnesium concentrations and thereby limited the accuracy of the estimation of K_{Mg}.

Discussion

It is evident from these results that, in the same range of calcium concentrations and at similar dose-ratios, the relationship between extracellular calcium and magnesium appears competitive but that between calcium and normorphine does not.

It is likely that magnesium inhibits transmitter release by competition with calcium at external sites of ion entry, as has been rather fully demonstrated at the neuromuscular juction (Jenkinson, 1957; Kharasch, Mellow & Silinsky, 1981; Silinsky, 1981). Bennett & Florin (1975) concluded that the interaction between calcium and magnesium at the mouse vas deferens was competitive on the basis of a

Table 1 The concentration of calcium (mM) required to give an e.j.p. amplitude of 15 mV.

	Normorphine (nM)					*Magnesium* (mM)			
	0	50	100	300	1000	0	1.2	2.4	4.8
Independent function[a]	2.50	2.52	2.82	3.27	3.75	1.80	2.50	2.84	3.80
Observed[b]	2.50	2.82	3.01	4.04	6.40	2.07	2.50	2.87	3.79
Competition[c]						2.07	2.52	2.97	3.88

[a] is the value calculated on the basis that e.j.p. amplitude (y) is the sum (in mV) of the two independent effects of calcium (Ca) and normorphine (NM), or calcium and magnesium (Mg) concentrations. These effects are given by the three functions (in the range of concentrations used): $\log y = 1.25 \log [Ca] + 0.716$ ([Mg] = 1.2, [NM] = 0, same data as Figure 3), $y = -7.5 \log [NM] - 40$ ([Mg] = 1.2 [Ca] = 2.5, same data as Figure 1) and $y = 3.2 [Mg] + 20$ ([Ca] = 2.5, [NM] = 0, unpublished observations). These functions all give significant fits to the data (least squares regression $P < 0.05$) within the range tested). [b] is the observed value calculated from the regression lines. [c] is the value calculated from $[Ca]/[Ca]_o - 1 = [Mg]/5.5$ where $[Ca]_o$ is measured from Figure 3 as 2.07. Note that the magnesium results observed are compatible with competition (see Schild plot of Figure 4), but they can also be predicted within these concentration ranges by two empirical independent functions. By contrast, more calcium is needed to overcome the normorphine depression that would be expected from the independent functions.

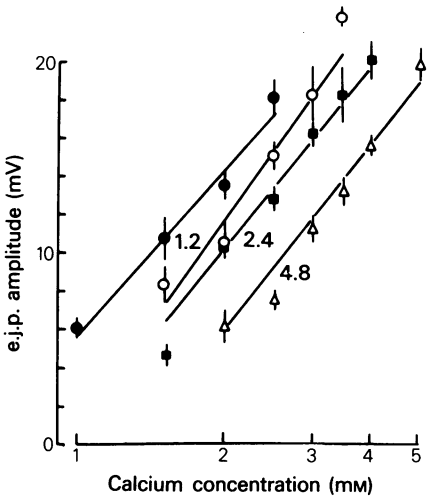

Figure 3 E.j.p. amplitude as a function of calcium concentration; effect of magnesium (concentrations indicated, mM). The points indicate the mean e.j.p. amplitude (with s.e.mean) recorded at a given calcium concentration (number of determinations for each point is between 3 and 31; mean 6). Lines are fitted to raw data by least squares. For each line, the slope and its 1% and 5% confidence limits were determined. None of the slopes of the lines differed from that in the absence of magnesium.

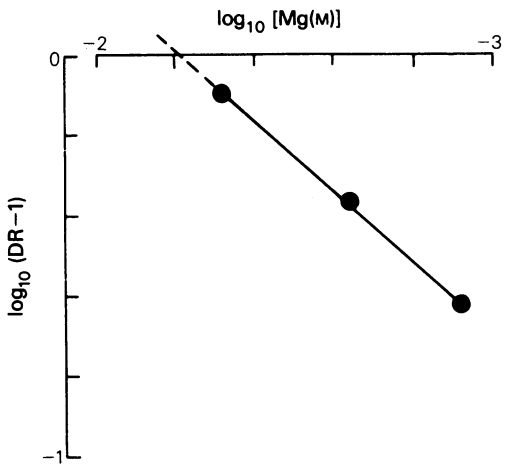

Figure 4 Schild plot of interaction between calcium and magnesium. Data from regression lines of Figure 3. The line is fitted by least squares and does not deviate significantly from linearity ($r = 0.9983$, $P < 0.05$). The calculated intercept on the abscissa gives a K_{Mg} of 5.5 mM.

Lineweaver-Burk plot. Their analysis retained the occupany assumption and the further assumption that the initial slope of the log [e.j.p. amplitude] versus log [Ca] plot gives an indication of order of the reaction between calcium ion and the hypothetical receptor to which it binds in order to initiate transmitter release. Our finding that the Schild plot is a straight line with a slope close to unity is based on neither of those assumptions. It provides an estimate of the magnesium dissociation constant (K_{Mg}) of about 5 mM.

In their study of the normorphine-calcium interaction, Bennett & Lavidis (1980) continued to assume that the regression coefficient of log [e.j.p. amplitude] on log [Ca] indicated the reaction order. This is theoretically unlikely for the e.j.ps of 3–18 mV which they used. Their results, while compatible with competition, therefore provide no evidence for it. In contrast, our results provide evidence against a competitive interaction. The parallelism of the slope of the e.j.p. amplitude versus log [Ca] plot (Figure 2) for low normorphine concentrations implies that at a low calcium concentration the percentage depression by normorphine will be greater. This agrees with the results of Illes *et al.* (1980) and with our own preliminary observations in which the stimulus strength was changed. At higher normorphine concentrations, the nonparallel lines indicate that the percentage depression of the e.j.p. by normorphine will be less sensitive to changes in calcium concentration, as was observed (Figure 1).

Many difficulties surround the interpretation of the present and similar experiments. First, the amplitude of the e.j.p. cannot be taken as a direct measure of transmitter release because of the very substantial corrections which would be necessary for non-linear summation of voltage increments. We attempted to avoid this by comparing equal e.j.p. amplitudes. Second, the assumption must be retained that changes in calcium, magnesium and normorphine do not affect the population of nerves excited and thereby contribute to changes in the e.j.p. amplitude. As elevations in divalent cations (Frankenhauser & Hodgkin, 1957) and normorphine (North & Tonini, 1977) both reduce neuronal excitability, confidence in this assumption is limited. Third, a Schild plot with unit slope offers strong evidence for competition only when the dose-ratio is large (Stephenson & Barlow, 1970). In the present study, the range of divalent cation concentrations was severely limited by considerations of their other actions, such as surface charge effects, fibre excitability changes, postsynaptic membrane potential changes and solubility of bicarbonate salts. Over the concentration range studied, the results for the calcium/magnesium interaction could be closely simulated by two empirical and independent functions which do not assume a common site of

action of the ions (Table 1). These considerations limit the application of drug-receptor theory to divalent cation interactions unless a clearly saturable response is measured (see Silinsky, 1981).

Four factors of interest control the e.j.p. amplitude: the stimulus voltage, the calcium concentration, the magnesium concentration and the normorphine concentration. With constant stimulus voltage and no normorphine present, the relation between calcium and magnesium is probably competitive. With constant stimulus voltage and magnesium concentration, the relation between calcium and normorphine was not competitive. Moreover, this relation could not be readily predicted as the sum of the two independent effects of calcium and normorphine (Table 1), implying that calcium does interfere with the ability of normorphine to depress the e.j.p. Sites of interaction might be the excitation of the sympathetic nerves by the electrical stimulus, the propagation of the action potential into varicosities, the entry of calcium into the varicosity, or within the varicosity at the site where calcium initiates transmitter release. It is difficult to see how experiments of the present type can distinguish among these several possibilities.

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