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THE EFFECTS OF pH CHANGES ON THE FREQUENCY OF MINIATURE END-PLATE POTENTIALS AT THE FROG NEUROMUSCULAR JUNCTION*

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SUMMARY

1. As reported by Landau & Nachshen (1975), a decrease in extracellular pH at the frog neuromuscular junction leads to an increase in min.e.p.p. frequency.

2. Decreasing the extracellular pH still increases the min.e.p.p. frequency when the bathing Ringer contains 10 mM-Ca^{2+}, in place of the usual 2·5 mM. At the mammalian neuromuscular junction, the elevated Ca^{2+} blocks the effect of the pH change on the min.e.p.p. frequency (Hubbard, Jones & Landau, 1968).

3. In Cl^--free solution (isethionate or methylsulphate substitution) min.e.p.p. frequency is no longer a monotonic function of decreasing pH. Instead there is an optimum pH for spontaneous release between pH 6·6 and 8·6.

4. This suggests that in Cl^- containing Ringer min.e.p.p. frequency increases with increasing extracellular acidity because there is a change in the P_{Cl} of the nerve terminal leading to a depolarization. In agreement with this idea, in low Ca^{2+} Ringer, acid pH has little effect on the min.e.p.p. frequency.

5. Decreasing the intracellular pH by raising P_{CO_2} produces substantial increases in the min.e.p.p. frequency. The effects are much greater than the effects of equal changes of H^+ in the extracellular solution.

6. Possible explanations for the effects of increased P_{CO_2} are discussed. Although release of Ca^{2+} from mitochondria or other unknown effects of intracellular pH change or molecular CO_2 are possible, the results do give

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some support to the hypothesis that an important step in transmitter release involves an electrostatic repulsion between fixed membrane surface charges on the transmitter containing vesicles and the inner face of the nerve terminal. The surface charge density would be decreased by a lower pH in the axoplasm, and this would increase the rate of spontaneous transmitter release, in agreement with the observations.

INTRODUCTION

The past 20 years have seen a vast increase in the understanding of synaptic function. None the less, the steps involved in the liberation of transmitter from a vesicle in the axoplasm of the nerve terminal into the synaptic cleft are still only poorly understood. One hypothesis suggests that a major factor controlling the release of acetylcholine (ACh) at the neuromuscular junction is an energy barrier created by the presence of an excess of negative charges on the surface of the vesicles and on the inner surface of the nerve membrane. The negative charges on the membranes produce an electrostatic repulsion, which might act to prevent contact between the ACh-containing vesicles and the release sites on the inner face of the axolemma (Blioch, Glagoleva, Lieberman & Nenashev, 1968; Kita & Van der Kloot, 1971; Van der Kloot & Kita, 1973).

An obvious experimental test of this hypothesis is to alter the surface potentials. If the model is correct, an increase in the surface potential would increase the energy barrier opposing vesicle–membrane contact, and thereby decrease the miniature end-plate potential (min.e.p.p.) frequency. A lowering of the surface potential would increase the rate of spontaneous release. In practice, this goal is not readily accomplished in an unambiguous fashion, since any substance that alters the fixed surface charges on the vesicles on the inner face of the axolemma would also produce similar effects on the external surface of the nerve terminal. A change in the surface potential on the external surface will change the concentrations of ions at the membrane–solution interface and the voltage gradient within the membrane. Such changes would markedly complicate the interpretation of the experimental results.

This problem can be circumvented by changing H^+ concentrations, because cell membranes are relatively impermeable to H^+. Therefore alterations in extracellular pH have little effect on intracellular pH (Thomas, 1974). On the other hand, CO_2 passes readily through cell membranes and alters intracellular pH rapidly.

This paper reports the effects of both extracellular and intracellular pH changes on spontaneous quantal release at the frog neuromuscular junction. The effects of pH changes on post-synaptic sensitivity to ACh were

studied by del Castillo, Nelson & Sanchez (1962). Their results showed that the alterations in post-synaptic ACh sensitivity produced by changing pH are not of sufficient magnitude to complicate the detection and counting of min.e.p.p.s.

A brief account of some of the results presented here was communicated previously (Cohen & Van der Kloot, 1973).

METHODS

The experiments were performed on the sartorius muscle of the frog, *Rana pipiens*. The muscle was pinned in a bath with a capacity of about 12·0 ml. Fresh solution flowed into the chamber at a rate sufficient to replace the contents in about 1 min.

Min.e.p.p.s were recorded with an intracellular micro-electrode, amplified and recorded on magnetic tape. Min.e.p.p. frequencies were measured by: (1) counting events from recording made with an inkwriter; (2) detecting min.e.p.p.s with an amplitude discriminator and adding the counts over a set time period, and (3) using a detecting and timing program on a PDP–11 computer.

A number of variations of Ringer solution were used in the experiments. For clarity they will be described along with the results. Ca^{2+} and buffers were varied for obvious experimental reasons; NaCl concentration was varied in order to change the osmotic pressure of the bathing solution to establish min.e.p.p. frequencies that were readily and accurately counted. The solutions all contained neostigmine methyl sulphate (10^{-6} g/ml.) to increase the min.e.p.p. amplitudes. The freezing point of each of the solutions was measured with a Fiske osmometer, to insure against changes in the osmolarity that might change frequencies between control and experimental solutions.

RESULTS

Extracellular pH changes

The effects of changing the extracellular pH with Tris maleate buffers in the range 5·2–8·6 are shown in Fig. 1. A decrease in pH resulted in an increase in min.e.p.p. frequency. A change from pH 8·6 to 5·2 brought about a roughly threefold increase. Obviously the buffering capacity of the Tris-maleate changed appreciably over this range of pHs, but since the volume of buffered Ringer was very large relative to the volume of the tissue, this factor is unlikely to have influenced the results.

Extracellular pH and Ca^{2+}

Hubbard *et al.* (1968) studied the effects of changes in pH on min.e.p.p. frequency at the mammalian neuromuscular junction. The pH was shifted by changing the HCO_3^- concentration in the physiological saline, so the resultant pH changes could be intracellular as well as extracellular. Shifting the external pH from 7·3 to 6·5 produced a 1·8-fold increase in min. e.p.p. frequency. When the extracellular Ca^{2+} concentration was increased to about 10 mM, a level that causes the maximum stimulation that Ca^{2+} can produce on the min.e.p.p. frequency, lowering the pH had little or no effect on spontaneous transmitter release. They therefore concluded that

H^+ acts by mimicking Ca^{2+}, binding to a Ca^{2+} receptor on the external surface of the nerve membrane and causing transmitter release. As more of the receptor sites were occupied, the min.e.p.p. frequency rose. When all of the sites were bound with Ca^{2+}, so that the receptor was saturated, an increase in the concentration of H^+ could have no additional effect on the min.e.p.p. frequency.

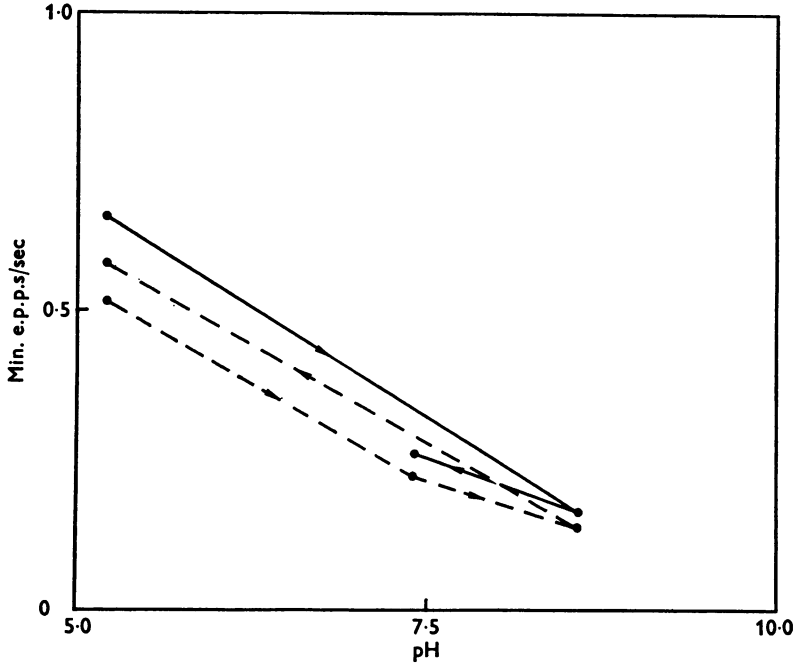

Fig. 1. The effects of three different pHs on the min.e.p.p. frequency. Two experiments performed at different end-plates of the frog sartorius muscle. For clarity in the presentation estimates of the S.D. of the points have not been included but the changes in mean min.e.p.p. frequency were significant ($P < 0{\cdot}001$, Student's t test).

We tested this hypothesis at the frog neuromuscular junction by varying the pH in Ringer containing 10 mM-Ca^{2+} and comparing the results with the same changes in the usual 2·5 mM-Ca^{2+} Ringer. The experimental results are shown in Fig. 2. The slope of the curve relating min.e.p.p. frequency to pH was at least as high in 10 mM-Ca^{2+} as in the 2·5 mM-Ca^{2+} Ringer. Similar results were obtained in two additional experiments. Apparently at the frog neuromuscular junction a marked elevation in the Ca^{2+} concentration does not antagonize the effects of lowered pH. There are other significant differences between our results in the frog and the results on the mammalian end-plate. These will be described shortly.

The results just presented show that in the frog H^+ does not compete with Ca^{2+} for a binding site on a 'saturatable receptor'. Such a receptor was proposed to account for the observation that in the rat hemidiaphragm neuromuscular junction min.e.p.p. frequency increases as the external Ca^{2+} concentration, $[Ca^{2+}]_o$, is increased until the concentration reaches 10 mM; additional Ca^{2+} does not increase min.e.p.p. frequency (Hubbard *et al.* 1968). If this observation means that a Ca^{2+} receptor is saturated and producing a maximal effect, then yet another receptor must be involved in stimulation, which can still increase release rates many thousand-fold. Instead of postulating two receptors, it seems much more likely that increasing the Ca^{2+} concentration has two opposing effects on Ca^{2+} influx. The two obvious candidates are an increase in the driving force for Ca^{2+} entry into the terminal and

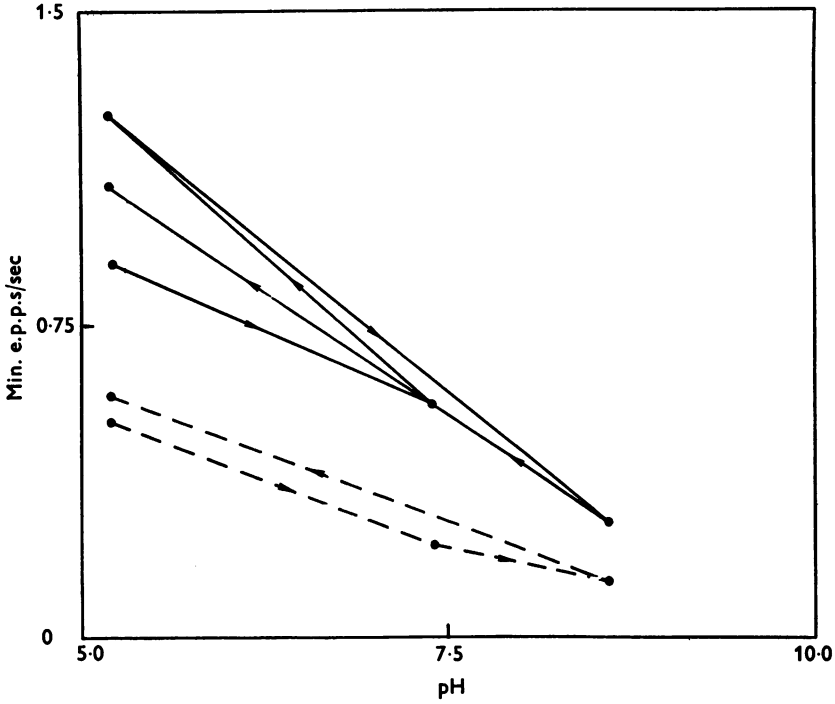

Fig. 2. An experiment on the effects of pH on min.e.p.p. frequency at a single end-plate. Dashed line, in normal (2·5 mM-Ca^{2+}) Ringer. The arrow indicates the sequence of solution changes. Continuous line, in Ringer containing 10 mM-Ca^{2+}.

simultaneously a decrease in the total number of Ca^{2+} channels available at the resting potential, owing to the effects of Ca^{2+} on the surface potential. The net result could be a greater movement of Ca^{2+} through the few channels available for entry, and this might cause little or no change in the internal Ca^{2+} concentration. Differences between the properties of the Ca^{2+} channel might well be responsible for the differences in the response of the rat and frog to increased extracellular Ca^{2+}.

Although the uncertainties in the interpretation of this experiment make it an incomplete counter-example to the model proposed by Hubbard *et al.* (1968), it does

serve to illustrate the difficulties in the original hypothesis. The model can only be tested at large outputs during near saturation of the Ca^{2+} receptor (if such saturation ever exists). However, Landau & Nachshen (1975) have shown that lowering the pH reduces stimulated output, which is inexplicable on the basis of the above model, but may well be due to blockage by H^+ of a means for Ca^{2+} entry into the nerve terminal. These experiments are also difficult to interpret as pH changes may change the shape of the action potential at the nerve terminal and it was for this reason that the present study did not include the effects of pH on stimulated release.

Effect of chloride concentration on min.e.p.p. frequency

Changes in pH markedly affect the Cl^- permeability in the membranes of many excitable cells. Hutter & Warner (1967) found that lowering the external pH decreased the P_{Cl} in frog skeletal muscle, and thereby produced a depolarization. Walker & Brown (1970) showed that lowering the pH raised the P_{Cl} of *Aplysia* neurones. Decreasing the external pH is also known to slightly depolarize rat nerve (Dettbarn & Stämpfli, 1957). It is possible, therefore, that lowered pH alters the P_{Cl} of the motor nerve terminals, causing a depolarization and a consequent increase in the min.e.p.p. frequency. The magnitude of the depolarization need only be a few millivolts because of the steep relation between min.e.p.p. frequency and nerve terminal polarization (Liley, 1956).

One way to test this idea is to replace the Cl^- in the Ringer with an impermeant anion. Then the increase in P_{Cl} should not produce a depolarization. We used methylsulphate and isethionate as Cl^- substitutes, since they are believed not to permeate many excitable membranes.

In Cl^--free Ringer the min.e.p.p. frequency was maximal at pH 7·4 and was less at both pH 8·6 and 5·2 (Fig. 3). The limited number of points in each experiment was a result of the well-known tendency of muscle fibres in Cl^--free Ringer to contract spontaneously, thereby ejecting the electrode (Hodgkin & Horowicz, 1959). If the electrode is then re-inserted into the fibre the resting potential is often no more than -40 mV (Erlij & Van der Kloot, 1967).

Therefore a second series of experiments, using solutions at pH 5·2, 6·0, 6·6 and 7·4, was performed to test the sharpness of the apparent pH optimum at 7·4. In Cl^--free Ringer, shifting the pH from 7·4 to 6·6 caused a marked fall in the min.e.p.p. frequency (Fig. 4), while decreases in pH below 6·6 had little or no effect. Together with the results in Fig. 3, those in Fig. 4 show that in Cl^--free Ringer there is a clear optimum for spontaneous quantal release between pH 6·6 and 8·6.

Extracellular calcium and the response to lowered pH

If lowered extracellular pH causes nerve terminal depolarization, then in the absence of extracellular Ca^{2+} there should not be an increase in the min.e.p.p. frequency, since depolarization acts by opening channels

that allow Ca^{2+} to enter the nerve terminal. Hubbard *et al.* (1968) reported that min.e.p.p. frequency increases at the rat neuromuscular junction when the pH is lowered, even in Ringer containing no added Ca^{2+} and with added EDTA.

Our experimental design was as follows: the preparation was soaked for at least 1 hr in Mg^{2+} Ringer at pH 7·6 (in mM:NaCl, 120; KCl, 2·0; $MgCl_2$, 2·5; and Tris maleate, 2·0), then the solution was changed to Mg^{2+}

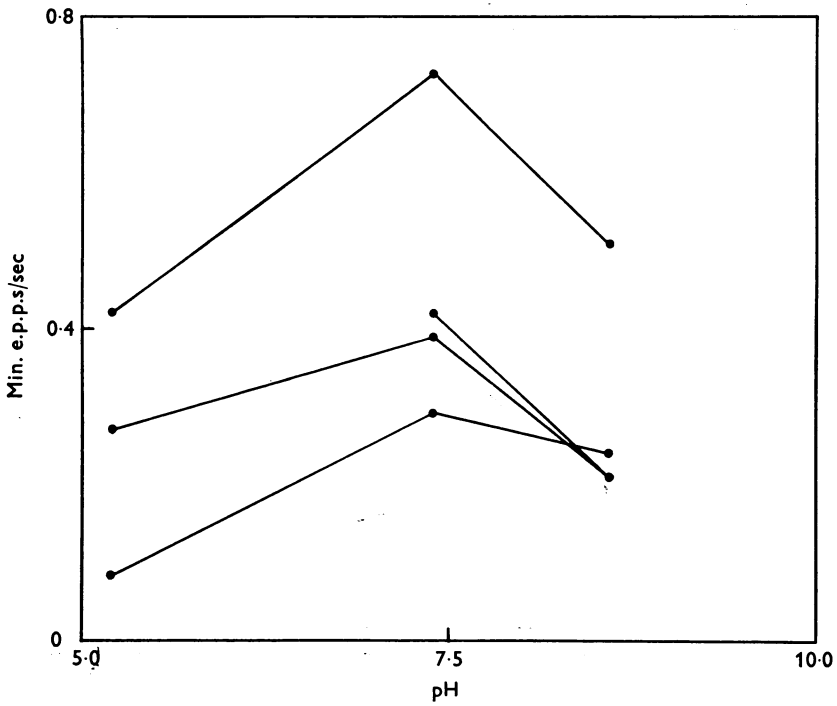

Fig. 3. The effects of changes in pH on min.e.p.p. frequency in Cl^--free solution (isethionate and methylsulphate substitution).

Ringer at pH 6·4 and, finally, to 2·5 mM-$CaCl_2$ Ringer, first at pH 6·4 and then at pH 7·6. The number of min.e.p.p.s in each of a series of four second bins was counted and the counts were used to estimate the frequency. The results of one experiment are shown in Fig. 5. An analysis of variance showed that the difference in the frequency of min.e.p.p.s in Ca^{2+} Ringer and Mg^{2+} Ringer at pH 6·4 was significant ($P < 0·01$). Similar results were obtained in two additional experiments.

Therefore we conclude that the increase in min.e.p.p. frequencies caused by acidic extracellular pH changes is negligible unless Ca^{2+} is present in the Ringer. Landau & Nachshen (1975) reported that the min.e.p.p.

frequency at pH 6·0 is insensitive to changes in the bathing Ca^{2+} between 0·5 and 1·0 mM. The data in their Fig. 4, however, suggest that there is a rise in min.e.p.p. frequency of roughly 2·5-fold with a tenfold rise in the Ca^{2+} concentration. However, insufficient evidence is given to allow one to judge the statistical reliability of this conclusion.

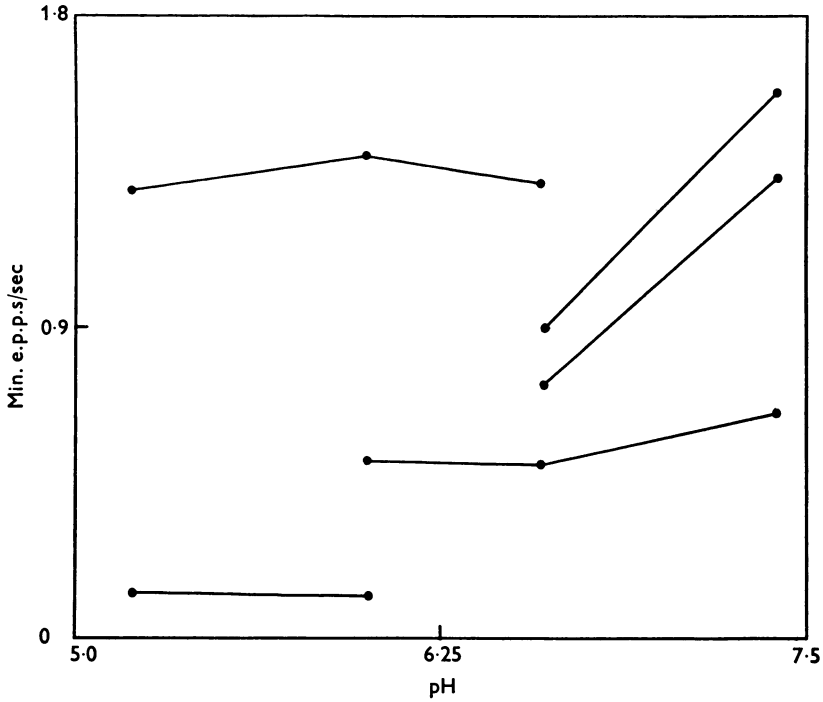

Fig. 4. The effects of pH on min.e.p.p. frequency at pH 5·2, 6·0, 6·6 and 7·4 in Cl^--free solution.

Effects of CO_2

To study the effects of simultaneous extracellular and intracellular pH changes, the preparations were bathed in a buffered solution and the min.e.p.p. frequency determined and they were then exposed to a solution at the same pH but containing an elevated partial pressure of CO_2. In most of these experiments the ionized Ca^{2+} concentration was maintained at a constant level by adding some bicarbonate to all solutions, since it determines to some extent the amount of ionized Ca^{2+} present (Hubbard *et al.* 1968). Slight errors in the amount of ionized Ca^{2+} could in no way account for an effect of the magnitude presented below, as the effect of Ca^{2+} on the frequency of min.e.p.p.s at the frog neuromuscular junction is small and inconsistent (Fatt & Katz, 1952).

Fig. 6*A* shows the results of an experiment in which the muscle was first bathed in an acetate buffered solution at pH 5·7 and then exposed to a solution of the same pH equilibrated with 81·4% CO_2 and 18·6% O_2. The CO_2 containing solution elicited a substantial increase in min.e.p.p.

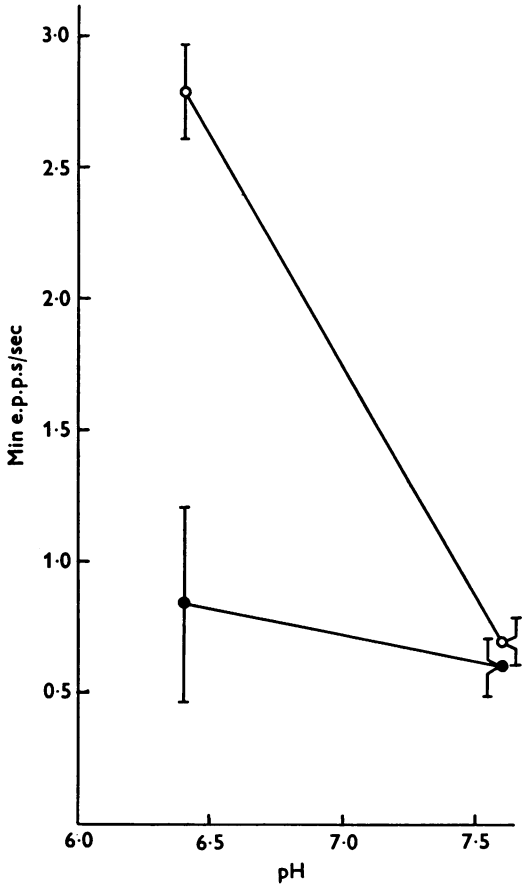

Fig. 5. The effects of pH on the min.e.p.p. frequency in the presence of bathing Ca^{2+} (open circles) and when 2·5 mM-Mg^{2+} is substituted for Ca^{2+} (filled circles). The bars indicate the S.E. of mean at each point. For more details see text.

frequency. The rise in frequency occurs so promptly that we cannot distinguish between the time required for the replacement of the solution outside the nerve terminal and the time required for the onset of the increased min.e.p.p. frequency. When the CO_2 is replaced by acetate buffer, again at the same pH, the min.e.p.p. frequency returned to the control level with a half-time of about 85 sec. Similar results were obtained

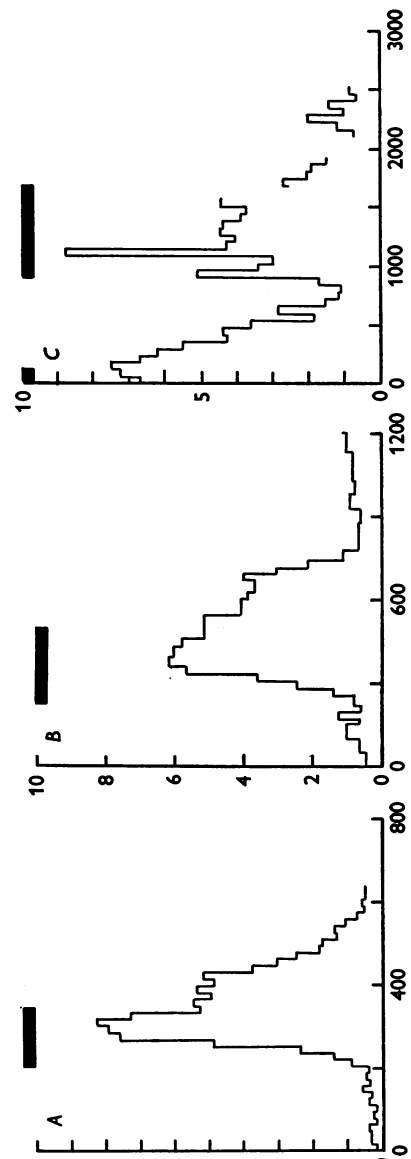

Fig. 6. *A*, The response to elevated P_{CO_2}. Initially the muscle was in a solution containing (in mM): NaCl, 95·0; $CaCl_2$, 2·5; KCl, 2·0; $NaHCO_3$, 5; Na acetate, 5, at pH 5·7. During the interval indicated by the filled horizontal bar, the muscle was bathed with a solution containing (in mM): NaCl, 100; $CaCl_2$ 2·5; KCl, 2·0; $NaHCO_3$ 5·0, which was equilibrated with 81·4 % CO_2 and 18·6 % O_2. The pH of this solution was also 5·7. *B*, the response of a muscle to a solution containing 20·1 % CO_2 and 79·9 % O_2. Initially the muscle was in a solution containing (in mM): NaCl, 110; $CaCl_2$, 2·5; and Tris maleate buffer, 5·0, pH 6·3. During the interval indicated by the bar the solution contained 5·0 mM-$NaHCO_3$ in place of the Tris maleate and was equilibrated with the CO_2–O_2 mixture. *C*, the response of a muscle to 5 % CO_2 and 95 % O_2. When indicated by the bar the muscle was in (in mM): NaCl, 134; $CaCl_2$, 2·5; KCl, 2·0; and $NaHCO_3$, 5; equilibrated with the gas mixture. In the intervening periods (without bar) the muscle was in (in mM): NaCl, 130; $CaCl_2$, 2·5; KCl, 2·0; $NaHCO_3$, 5 and Tris maleate buffer 4·0 at pH 6·7. The breaks in the line show intervals during which the min.e.p.p.s were not counted because the muscle was undergoing contractures.

in two additional experiments except that the half-times for recovery were longer: 320 and 500 sec. This may be related to the longer period of exposure to CO_2 containing solution.

The interpretation of these results seems straightforward. Changes in extracellular pH produce modest effects on min.e.p.p. frequencies by altering P_{Cl} and thereby depolarizing the terminal. In CO_2 containing solutions at the same pH, the CO_2 rapidly diffuses through the nerve terminal membrane. The partial pressure of CO_2 within the terminal increases. We cannot say with certainty whether the resulting increase in spontaneous quantal release is due to a direct effect of CO_2 or to a fall in intracellular pH, nor can we estimate accurately the intracellular pH, since so little is known about the buffering capacity of the axoplasm or the ability of the cell to transport H^+ actively.

Increases in min.e.p.p. frequency were also observed following exposure to lower partial pressures of CO_2. Fig. 6*B* shows an example with 20% CO_2–80% O_2. There was a prompt twelvefold increase in the min.e.p.p. frequency, which declined after return to the control solution with a half-time of about 250 sec. In six experiments with 20% CO_2 the increase in min.e.p.p. frequency was $4{\cdot}1 \pm 1{\cdot}7$-fold (mean ± S.E. of mean). Control experiments showed that even 100% O_2 had no detectable effects on min.e.p.p. frequency, so the observed changes are caused by the change in CO_2, not the O_2.

Fig. 6*C* shows an example exposed to 5% CO_2, which demonstrates about a fivefold increase in min.e.p.p. frequency following exposure to CO_2. In eight experiments the increase was $3{\cdot}7 \pm 2{\cdot}0$ times the control level (in one of these trials 5% CO_2 had no detectable effect).

DISCUSSION

It appears that there is an optimal extracellular pH, somewhere between 6·6 and 8·6, for spontaneous quantal transmitter release. The factors involved in producing this optimum are unknown. One obvious possibility is that H^+ affects the properties of a mechanism regulating Ca^{2+} entry into the nerve terminal (Landau & Nachshen, 1975).

However, this optimum is largely obscured in the usual Cl^- containing Ringer, in which the min.e.p.p. frequency appears to be a monotonic function of decreasing pH, at least over the range studied (5·2–8·6). The results strongly support the idea that H^+ alters the P_{Cl} of the terminal membrane and when present in sufficiently high concentration produces a slight depolarization that leads to an increase in min.e.p.p. frequency by increasing the Ca^{2+} influx. Landau & Nachshen (1975) rejected this interpretation because they found little difference in min.e.p.p. frequency

at pH 6·0 when the extracellular Ca^{2+} was varied between 0·5 and 1·0 mM. As was mentioned previously, their data does suggest that a change in Ca^{2+} could produce a change in min.e.p.p. frequency and, in any event, slight changes in Ca^{2+} concentration are not as convincing a test as shifts between normal Ringer and a solution containing no added Ca^{2+}.

Hubbard *et al.* (1968) found that min.e.p.p. frequencies rose as the pH was decreased, even in Ringer made without Ca^{2+} and containing EDTA. The difference with our results may be owing to differences between amphibian and mammalian neuromuscular junctions, or they may reflect the possibility that Hubbard *et al.* (1968) were experimenting with solutions that might change the intracellular as well as the extracellular pHs.

There is a second difference between the frog and the mammal. Hubbard *et al.* (1968) found that in a solution containing 10 mM-Ca^{2+} a decrease in the pH did not increase the min.e.p.p. frequency. This suggested that H^+ acts by mimicking Ca^{2+} in binding to a Ca^{2+} receptor on the nerve terminal. In the frog, Landau & Nachshen (1975) found that the response to low pH was quite insensitive to the divalent cation concentration of the medium. Our results show that increasing Ca^{2+} in the Ringer had little effect on the action of pH. Once again, a species difference may be involved or the discrepancy might come from the solutions used by Hubbard *et al.* (1968) which might shift both extracellular and intracellular pHs. At present there seems scant evidence supporting the idea that a Ca^{2+} receptor on the external surface of the nerve terminal is important in regulating the rate of spontaneous quantal release.

Our results with solutions containing an elevated partial pressure of CO_2 strongly suggest that intracellular pH changes have a much greater effect on spontaneous quantal release rates than extracellular changes. They also suggest that the assumption made by Landau & Nachshen (1975), that their buffers were producing intracellular pH changes, is probably incorrect. Their assumption disagrees both with direct measurements of intracellular pH in nerve cells (Thomas, 1974) and with our results with elevated P_{CO_2}.

The results do not rule out the idea that molecular CO_2 has a direct action within the cell on quantal release but this requires a hitherto undescribed effect of CO_2 and for this reason we regard an indirect action of CO_2 working by altering intracellular pH as a more likely explanation. A change in intracellular pH is unlikely to alter Cl^- permeability, since the factors controlling P_{Cl} have been shown to reside near the external membrane surface of both nerve and muscle membranes (Hutter & Warner, 1967; Walker & Brown, 1970).

There are a number of ways in which a decrease in intracellular pH might cause an increase in the rate of spontaneous quantal release.

Increased acidity might decrease the rate of Ca^{2+} transport into mitochondria (Van Breeman, Farinas, Casteels, Gerba, Wuytack & Deth, 1973) or out of the terminal. This interpretation could be evaluated by raising the concentration of abnormal divalent cations within the terminal by tetanic stimulation (Hurlbut, Longenecker & Mauro, 1971; Kita & Van der Kloot, 1973) and seeing whether CO_2 has any effect on the decline of min.e.p.p. frequency, which presumably reflects the sequestering or the outward transport of the divalent ions.

Another possibility is that an increase in intracellular H^+ decreases the surface charges on the vesicles and near the release sites on the inner face of the nerve terminal. This would lower the energy barrier and increase the likelihood of a vesicle-terminal membrane collision. Further experiments with other agents that should alter the fixed surface charges on intracellular membranes in known directions will be required before a conclusive statement can be made on the validity of the surface charge model.

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