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HARMALINE DISTRIBUTION IN SINGLE MUSCLE FIBRES AND THE INHIBITION OF SODIUM EFFLUX

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Harmaline, a known inhibitor of the ($Na^+ + K^+$)-ATPase in cell membranes, inhibited 50% of the ^{22}Na efflux from barnacle muscle fibres at an extracellular concentration of 2.4 mM. Injected harmaline inhibited 50% of the efflux at an estimated intracellular concentration of about 8 mM · kg^{-1}, assuming complete equilibration with no binding. Total fibre harmaline was measured in separate fibres by ultraviolet spectrophotometry. Fibres in 3 mM harmaline saline accumulated harmaline with a half-time of 17 min and a final total fibre concentration of 6–12 mM · kg^{-1}. In harmaline-free saline this accumulated harmaline was lost exponentially with a half-time of 35 min; injected harmaline was lost exponentially from fibres with a half-time of 50 min. It is proposed that harmaline crosses the fibre membrane as the uncharged base and that its apparent accumulation against a concentration gradient is mainly due to intracellular binding with an additional contribution from a transmembrane pH gradient. It is concluded that, in fibres exposed to harmaline saline, the intracellular concentration can reach a sufficiently high value, as judged from the results of the injection experiments, to inhibit Na^+ efflux at an interior-facing site on the fibre membrane. In contrast, harmaline appears to inhibit the Na^+-dependent uptake of L-glutamate at an extracellular site.

Introduction

The alkaloid harmaline is known to inhibit the active extrusion of Na^+ from the squid axon [1] and red blood cell [2], as a result of an inhibition of the membrane ($Na^+ + K^+$)-ATPase activity [1,3,4]. In contrast to the action of the cardiac glycoside, ouabain, which is thought to inhibit the Na^+ pump on the exterior membrane surface of cells at a K^+-dependent site [5,6], the results from squid axons and red cells suggested that harmaline inhibits the Na^+ pump at a Na^+-sensitive site on the interior membrane surface [1,2]. However, there is no documented evidence that extracellular harmaline can actually penetrate the cell membrane to reach this site. In addition, the complexing of harmaline with ATP [3], and inhibitory effects of harmaline on both the K^+-activation of the Na^+ pump [7] and the activity of other ATPases [4] have been found which cast doubts that harmaline exerts its effects on Na^+ transport exclusively through competition with Na^+ ions.

In this paper we present confirmation that in barnacle muscle fibres both extracellularly applied and intracellularly injected harmaline can inhibit Na^+ efflux. More importantly, measured uptake rates of harmaline by the fibres are found to be consistent with the hypothesis that extracellular harmaline can penetrate the cell membrane and act on the Na^+ pump at an interior-facing site. The inhibition by harmaline of the Na^+-dependent uptake of L-glutamate by the fibres, on the other hand, appears to take place at the exterior Na^+-sensitive sites, as has been reported in a variety of epithelia for other Na^+-dependent transport systems [8–11].

Abbreviation: Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid.

Materials and Methods

^{22}Na efflux. Single muscle fibres of the barnacle *Balanus nubilus* were cannulated and injected axially with $^{22}NaCl$ (1 μCi/μl in 100 mM Tes, pH 7.3) and the effluent isotope was collected for given periods of time in vials containing 2.5 ml physiological saline [12–14]. ^{22}Na was counted in the vials by conventional gamma spectrometry. Na^+ efflux was expressed as a fractional loss of isotope defined as follows, for a collection period, t (min):

$$\frac{^{22}\text{Na lost in period } t \text{ (cpm)}}{t \times \text{mean } ^{22}\text{Na in fibre during the period (cpm)}} \ (\text{min}^{-1})$$

[^{14}C]Glutamate influxes. L-[^{14}C]Glutamate influxes were measured using bundles of fibres still attached to the shell of the barnacle and immersed in 20 ml saline, containing 2 μCi L-[^{14}C]glutamate, which was continuously stirred. After 15 min, fibres were removed, blotted, measured and then immersed in 0.1 N HNO_3 to elute the isotope which was counted by conventional liquid scintillation methods. Corrections were made for extracellular space.

Measurement of harmaline uptake. Bundles of fibres, still attached to the shell of the barnacle, were immersed in 20 ml saline containing harmaline for various periods. At the end of each period, fibres were cut from the shell, blotted and measured and then soaked in saline (0.5 ml) for 12 h to remove harmaline and washed again. In some cases, elution was in butan-1-ol.

Some uptake experiments were performed on cannulated fibres, with glass weights attached to the tendons, in 2 ml saline. Control fibres were subjected to the same treatment, but in the absence of harmaline.

Harmaline efflux. Effluxes are measured as with ^{22}Na: 0.5 μl harmaline solution (22 mM + 20 mM Tes, pH $\approx$ 7) was injected into cannulated fibres and the effluent collected in vials containing 2 ml saline.

In some experiments fibres were first 'loaded' with harmaline by immersion in 3 mM harmaline saline for 2 h.

Harmaline estimation. Harmaline concentrations were measured on a Unicam SP.800 Ultraviolet spectrophotometer at an activation wavelength of 374 nm. A standard curve was prepared for concentrations up to 10^{-4} M. The absorbance due to the control fibres, which were not immersed in harmaline salines, was subtracted from the absorbance due to harmaline-containing fibres.

Saline. Physiological saline contained (mM): NaCl, 510; KCl, 12.9; $MgCl_2$, 23.6; $CaCl_2$, 11.8; $NaHCO_3$, 2.6; Tes, 2.0; pH 7.2–7.4. Experiments were performed at room temperature, 19–23°C. The condition of each fibre was assessed before and after an experiment by measuring the membrane potential, using 3 M KCl-filled axial electrodes. Values for undamaged muscle fibres ranged from –40 to –60 mV. Harmaline hydrochloride was supplied by Sigma London.

Results

Effects of harmaline on Na^+ efflux

Harmaline, at concentrations greater than 0.1 mM in the external saline, reversibly inhibited ^{22}Na efflux (Figs. 1 and 2). The maximum inhibition observed (56% of the total Na^+ efflux) was obtained at 5 mM; concentrations greater than this caused damage to the fibres and a measurable depolarisation. Na^+ efflux was also inhibited when a concentrated solution of harmaline (30–200 mM, buffered at pH 7.2 by 10 mM Tes) was injected axially into fibres along their length using a glass capillary injector (Fig. 1B). The threshold concentration of harmaline which was required to inhibit Na^+ efflux was greater than 0.4 mM $\cdot$ kg^{-1}, assuming equilibration of the injected harmaline throughout the cross-section of the fibres and only a small loss across the surface membrane (see later).

Inhibition of Na^+ efflux is plotted in Fig. 2 against harmaline concentration for fibres exposed to extracellular harmaline and for fibres injected with harmaline. It is noticeable that a given intracellular, equilibrated concentration of harmaline was less effective at inhibiting Na^+ efflux than the same extracellular harmaline concentration. 50% of the efflux was inhibited by an extracellular harmaline concentration of 3.0 mM, compared with an extrapolated value of about 7.5 mM $\cdot$ kg^{-1} for injected harmaline.

One explanation for this difference might be that injected harmaline inhibits one component of the efflux and extracellular harmaline inhibits another. However, the following experiment does not support this idea. The inhibition of Na^+ efflux due to 2 mM

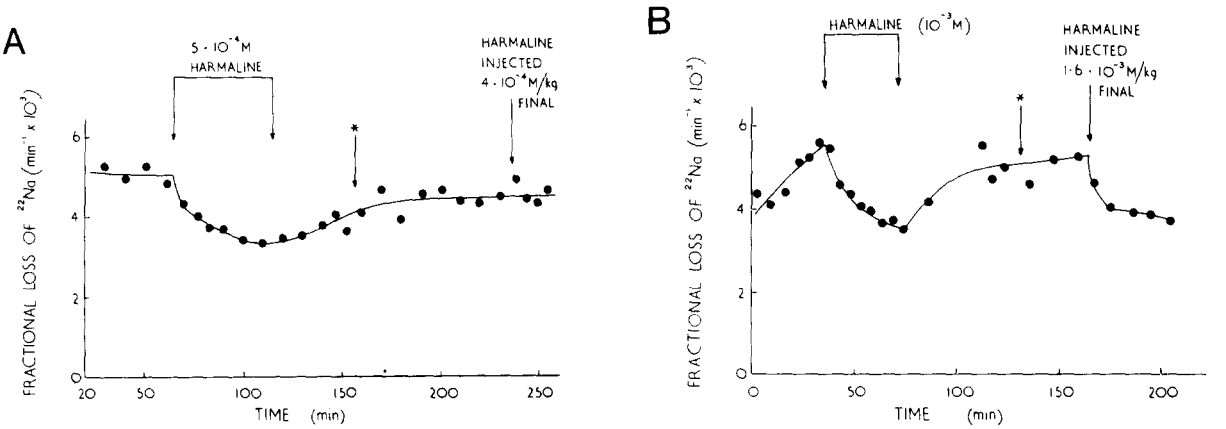


Fig. 1.A. Inhibition of Na^+ efflux by extracellular harmaline ($5 \cdot 10^{-4}$ M) but not by an intracellular concentration of $4 \cdot 10^{-4}$ M · kg^{-1}. The asterisk shows the point at which the injector was inserted into the fibre before injection of 0.03 M harmaline solution. 'Final' intracellular concentration was estimated by assuming equilibration throughout the fibre and no binding or loss across the sarcolemma. Time axis is time after injection of ^{22}Na. Fibre diameter = 1.3 mm. B. Inhibition of Na^+ efflux by extracellular (10^{-3} M) and injected harmaline. Asterisk shows the point at which injector was inserted into the fibre before injection of 0.1 M harmaline solution, to give a final concentration of 1.6 mM · kg^{-1}. Fibre diameter = 1.2 mm.

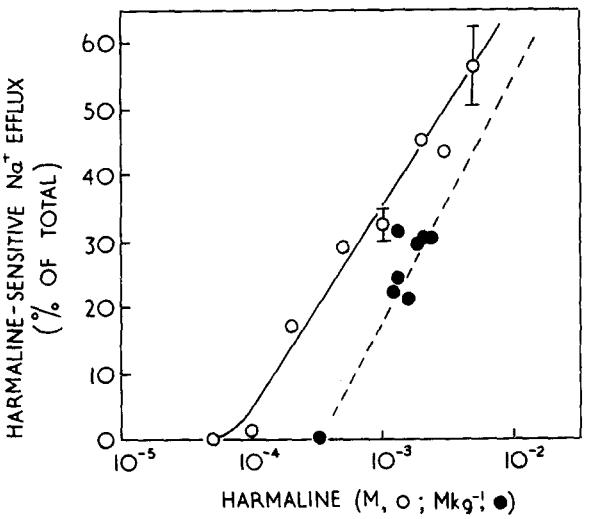


Fig. 2. Dependence of the harmaline-sensitive Na^+ efflux on extracellular harmaline concentration in fibres exposed to harmaline saline (o) and on total intracellular harmaline concentration in fibres injected with 0.03–0.2 M harmaline solution (•). For extracellular harmaline each point is mean of results from two or more fibres (±S.E.) after 40 min in harmaline saline. For injected fibres each point is the result from a single fibre 20 min after injection. The intracellular concentration was estimated assuming equilibration occurs without any binding, but with 18% loss of harmaline across the sarcolemma in 20 min. Dashed line is calculated relation between inhibition of Na^+ efflux by external harmaline and the resulting, total intracellular harmaline concentration (estimated from the data in Table I). Mean rate constant for Na^+ efflux in absence of harmaline = $6.4 \cdot 10^{-3}$ min^{-1} ($\pm 0.3 \cdot 10^{-3}$ S.E.). Fibre diameters = 0.9–1.4 mm.

extracellular harmaline was compared with that in fibres which were injected with harmaline to give an intracellular concentration of approx. 2 mM and then exposed to extracellular 2 mM harmaline. In four fibres, in which the estimated intracellular harmaline concentration following injection varied from 1.9 to 2.8 mM · kg^{-1} and external 2 mM harmaline was applied, Na^+ efflux was reduced by a mean value of 46.8% (±3.5% S.E.), compared with 45.0% in control fibres which were only exposed to extracellular harmaline. If the sodium efflux was made up of one component which was sensitive to only extracellular harmaline and another sensitive to only intracellular harmaline, then injected harmaline should have produced an additional inhibition of the efflux in fibres already exposed to extracellular harmaline.

Use of the cardiac glycosides, strophanthidin and ouabain, has produced evidence that there is more than one component to the Na^+ efflux from barnacle muscle fibres, since part of the efflux is insensitive to maximal concentrations of external strophanthidin [15,16]. Both extracellular (Fig. 3) and injected harmaline reduced the size of the ouabain-insensitive component. Thus in seven fibres, 3–5 mM harmaline in the saline reversibly reduced the efflux in 10^{-4} M ouabain from a mean value 25.3% (±2.8 S.E.) to 14.0% (±1.4 S.E.), both values being expressed as a percentage of the total Na^+ efflux in the absence of ouabain.

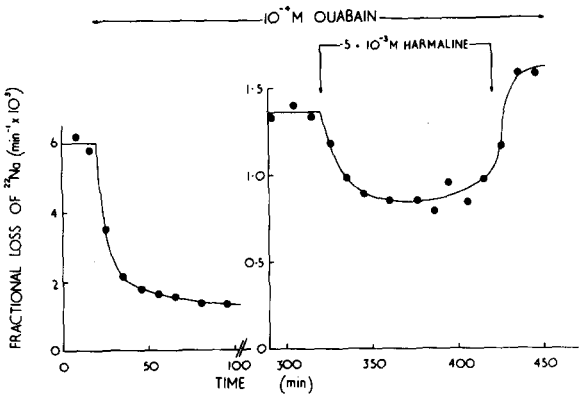


Fig. 3. Inhibition of the ouabain-insensitive Na^+ efflux by 5 mM extracellular harmaline.

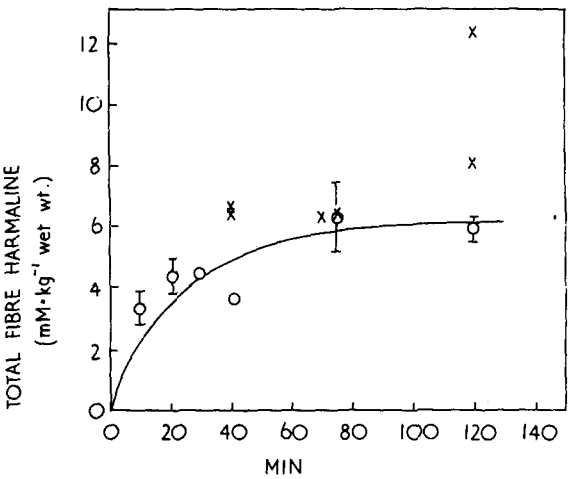


Fig. 4. Uptake of harmaline. Total fibre harmaline plotted against time of immersion of fibres in 3 mM harmaline saline. o: intact fibres retained on barnacle shell, mean results from 3–6 fibres at each time, with bars showing ± S.E. X: single cannulated fibres. Curve is for exponential filling of a single compartment with a time constant of 0.04 min^{-1}. Fibre diameters: 0.88–1.23 mm.

Measurement of harmaline distribution in muscle fibres

The time course of harmaline uptake by both intact and cannulated fibres in 3 mM harmaline saline is shown in Fig. 4. The total fibre harmaline concentration reached a plateau value of 6–12 mM · kg^{-1} after 80 min, with an approximate half-time of 17 min. The extracellular or 'cleft' space in these fibres represents 10% of the fibre volume, with a half-time for filling of about 15 min, as measured with inulin [14]. The appropriate correction will increase the fibre harmaline concentration by 7%. The apparent concentration of harmaline by the fibres against a concentration gradient was also seen at lower saline concentrations (Table I). This could not be attributed to binding of harmaline to the exterior surface of the fibres as the results of measuring the rate of har-

TABLE I

HARMALINE DISTRIBUTION IN MUSCLE FIBRES

Harmaline uptake was measured in cannulated fibres for 40 min. Two fibres were used at each concentration. Individual results did not differ by more than 5% of the mean. Fibre radii: 500–590 μm. The value for pH_i of 7.3 is taken from Boron's measurements [19]. Concentrations are defined as follows: $[harm_T]$, total harmaline; [harm], uncharged; $[harm^+]$, cation; $[harm]_{Bd}$, bound (charged and uncharged). Their values were calculated assuming:

(i) $[harm]_i = 0.8 \times [harm]_o$ after 40 min (from the curve in Fig. 4).

(ii) $pK_a = pH + \log([harm^+]/[harm])$, where $pK_a = 9.8$.

(iii) $[harm_T]_i = [harm^+]_i + [harm]_i + [harm]_{Bd}$.

Extracellular concentration (mM) ($pH_o = 7.4$)		Intracellular concentration (mM · kg^{-1} wet wt.)($pH_i = 7.3$)				Required pH_i for total uptake to be due to pH difference. ($pH_o = 7.4$)
$[harm_T]_o$	$[harm]_o$	$[harm_T]_i$	$[harm]_i$	$[harm^+]_i$	$[harm]_{Bd}$	
0.20	0.00079	0.90	0.00063	0.20	0.70	6.65
1.0	0.004	3.4	0.0032	1.01	2.39	6.77
3.0	0.012	7.1	0.0095	3.00	4.10	6.93

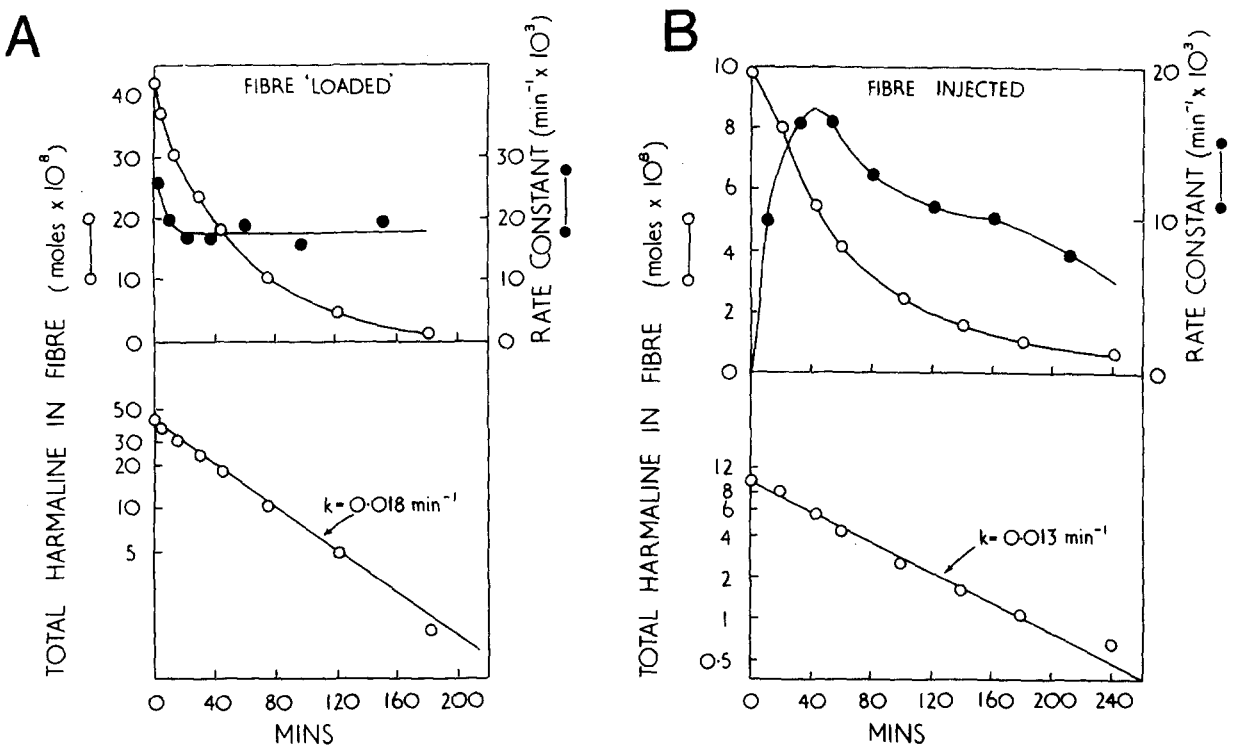
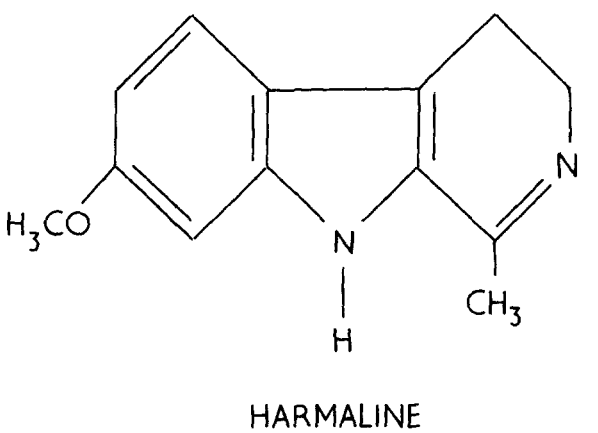


Fig. 5. Efflux of harmaline. Total fibre harmaline (o) is plotted linearly (upper graphs) against time in harmaline-free saline. In A, the fibre was first immersed in 3 mM harmaline saline for 2 h and in B the fibre was first injected with 0.2 M harmaline solution. Both fibres were cannulated. A rate constant for harmaline efflux is also plotted (•) which demonstrates in B a time lag before the peak value is reached, due to radial diffusion of the injected harmaline. Lower graph in A and B is logarithmic plot of same data in upper graph to show the exponential decline in total fibre harmaline. Overall rate constant (k) was estimated from the slope. Fibre diameters, 1.1–1.2 mm.

maline efflux from cannulated fibres showed. Fig. 5 demonstrates that the harmaline concentrations in both fibres, which had been 'loaded' by immersion in saline containing 3 mM harmaline, and fibres which had been injected with 0.2 M harmaline solution, decreased with time in harmaline-free saline according to a single exponential. Its mean rate constant for 'loaded' fibres was 0.020 min^{-1} (two fibres) and for injected fibres 0.014 ± 0.002 min^{-1} (three fibres) (half-time 35 and 50 min, respectively). The efflux component from the extracellular space in 'loaded' fibres was difficult to distinguish, which is not surprising, since a 10% space, containing 3 mM harmaline, would contribute only about 2% of the total harmaline in the fibres at the start of the efflux. The absence of a second, fast component of any significance to the efflux from 'loaded' fibres effectively excludes the possibility of any significant binding to the exterior muscle surface. More likely explanations for the concentration of harmaline against a concen-



HARMALINE

Fig. 6. Structure of the uncharged form of harmaline.

tration gradient are either that it is bound intracellularly or that there is a sufficient pH gradient across the sarcolemma (inside more acid) so as to accumulate harmaline as a weak base (see Discussion). Evidence of intracellular binding comes from the time taken for the fractional loss or rate constant for harmaline efflux to reach a maximum following intracellular injection (Fig. 6B). Using the type of analysis which was employed for ^{22}Na efflux from squid axons [18], an approximate value of the apparent diffusion coefficient for harmaline within the muscle fibres is calculated to be $8 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}. This does assume that fibres can be treated as simple cylinders and that the harmaline is injected as a column of negligible diameter. A precise value for free diffusion of harmaline in solution was unobtainable, but molecules of similar size all have coefficients of $(3–5) \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}. The lower value for the apparent coefficient for harmaline within the muscle fibre is consistent with the occurrence of binding to some non-diffusible components such as the organelles or the contractile filaments.

Effects of harmaline on amino acid transport

The influx of the amino acid L-glutamate into barnacle muscle fibres is dependent on extracellular Na^+ (Na_o^+) [21]: at an extracellular glutamate concentration of 20 μM, 96% of the influx in normal saline ($Na_o^+ = 510$ mM) can be abolished by replacing extracellular Na^+ with Li^+.

Table II shows that 5 mM harmaline had no significant effect on L-glutamate uptake at normal extracellular Na^+ levels but significantly inhibited the uptake by 36% at an extracellular Na^+ level of 102 mM.

Discussion

The distributon of harmaline in muscle fibres

The results of the harmaline influx and efflux experiments show that harmaline can easily cross the fibre membrane and that fibres can apparently accumulate it from the saline against a concentration gradient. This is not due to extracellular binding, but can be attributed to a combination of intracellular binding and a possible pH gradient across the membrane. Harmaline presumably crosses the membrane as the uncharged bases (Fig. 6) since it is known to have a high partition coefficient in isobutanol/H_2O [1], consistent with a high lipid solubility for a non-electrolyte. Although the cationic form of the harmaline molecule will predominate at pH 7.3 (the $pK_a = 9.8$ [17]), it is unlikely that its movement across the fibre membrane will contribute much to the overall transport of harmaline. The initial uptake rate by the muscle fibres at 3 mM harmaline was 1400 pmol $\cdot$ cm$^{-2} \cdot$ s^{-1}, a value which is considerably larger than

TABLE II

EFFECT OF HARMALINE ON Na^+-DEPENDENT GLUTAMATE UPTAKE

Uptake of L-[^{14}C]glutamate was measured in fibres which were still attached to the shell, over a period of 1 h, during which time the fibre [^{14}C]glutamate concentration increases linearly with time. Extracellular glutamate: 24 μM. Values for uptake are mean ± standard error. Extracellular space (10% of fibre volume) corrections have been made – these reduced the influx values by about 4%.

Saline concentration (mM)		L-Glutamate influx (fmol $\cdot$ cm$^{-2} \cdot$ s^{-1})			Number of fibres
Harmaline	Na^+	Control	Harmaline	Harmaline/Control	
1.0	510	394 ± 23	448 ± 33	1.14	7
5.0	510	873 ± 55	806 ± 36	0.92	5
5.0	102 *	180 ** ± 16	116 ** ± 4	0.64	6

* This saline was Ca^{2+}-free to prevent possible Ca^{2+} influx in reduced Na^+ saline. Fibres were first equilibrated in this saline for 30 min before addition of [^{14}C]glutamate. The size of the glutamate uptake is not significantly affected by the removal of extracellular calcium.

** Significantly different, $P < 0.002$ by Student's t-test.

the documented values for smaller cations, e.g., Na^+: 49 pmol · cm^{-2} · s^{-1} and K^+: 29 pmol · cm^{-2} · s^{-1} [15]. The uptake rate of the harmaline cation is presumably smaller than the values for Na^+ and K^+, so that one can assume that its permeability is very small compared to that of the uncharged base and that the distribution of the harmaline molecule as a whole is unaffected by the fibre membrane potential.

Table I shows that for the apparent accumulation of harmaline against a concentration gradient to be due solely to its distribution as a weak base according to the transmembrane pH gradient, the intracellular pH (pH_i) would have to be 6.65–6.93. Such values are well below the range found in barnacle muscle fibres by Boron [19], who gives $pH_i = 7.31 \pm 0.01$ S.E. at $pH_o = 7.5$. Table I also shows that the harmaline accumulated by a pH gradient of 0.1 unit when $pH_i = 7.3$ and $pH_o = 7.4$ is only 22–42% of the intracellular concentration actually found. In practice, the pH gradient was probably less favourable, since pH_o was 7.2–7.4 and harmaline itself probably increased pH_i above 7.3 as a result of the removal of protons from the sarcoplasm by the unprotonated harmaline (cf. NH_4Cl [19]). One can therefore conclude that at least half of the accumulated harmaline is probably bound intracellularly. The harmaline cation may well have an affinity for the fixed charge sites on the contractile filaments which have been proposed to account for Na^+ and K^+ binding in barnacle muscle fibres [22]. This could explain why harmaline is concentrated whereas caffeine, a permeant molecule which is almost totally uncharged at pH 7.2, is not [14]. The concentration against a chemical gradient of another weak base, procaine, by frog muscle fibres has also been interpreted in terms of intracellular binding [20]; like harmaline, it is predominantly cationic at pH 7.3 ($pK_a = 9.0$).

The site of harmaline's inhibitory action

The values for the inhibition of Na^+ efflux obtained at different extracellular harmaline concentrations in Fig. 2 have been replotted against the values for total intracellular harmaline which were obtained in fibres after 40 min in three different saline concentrations (Table I) to give the dashed line in Fig. 2. It can be seen that the points for the harmaline injection experiments lie on this line, a result which is consistent with harmaline's acting at a single site on the interior surface of the fibre membrane and with extracellular harmaline's inhibiting Na^+ efflux by first entering the fibre to reach this site. The possibility that harmaline has an exclusive or additional effect on the exterior surface cannot be rigidly excluded, since it could be argued that injected harmaline crosses the membrane and reaches a significant concentration in the extracellular space so as to inhibit the Na^+ efflux at such a site. However, an interior site is in agreement with the proposed actions of harmaline in competing with Na^+ in the $(Na^+ + K^+)$-ATPase system in squid axons [1] and in inhibiting Na^+ efflux when introduced into red blood cell ghosts [2]. The barnacle data do not support the claim that intracellular concentrations of 10^{-6} M harmaline can inhibit Na^+ efflux from red cell ghosts by up to 60% [2], since 50% inhibition of Na^+ efflux from the fibres required an estimated intracellular harmaline concentration of 7.5 mM · kg^{-1} (half of this could be bound). The 50% inhibition values for harmaline on squid axon and rat brain $(Na^+ + K^+)$-ATPase [1] and on Na^+-dependent uptake of sugars and amino acids by renal brush border vesicles [11] also lie in the millimolar range for Na^+ values of 1–200 mM. The intracellular free concentration of Na^+ in the muscle fibre sarcoplasm is reported to be 14 mM · kg^{-1} [22].

Ouabain-insensitive efflux of sodium

In contrast to the case in barnacle, the ouabain-insensitive Na^+ efflux in red blood cells is not inhibited by harmaline [2], which may mean that this efflux component has different mechanisms in the two types of cell. Certainly it differs in its sensitivity to extracellular Na^+, for in red blood cells Na^+-free saline does not affect it [23], whereas in barnacle muscle Na^+-free saline decreases it by about 70% (Lea, T.J., unpublished results). It is still unknown whether the $(Na^+ + K^+)$-ATPase mediates the ouabain-insensitive efflux, although evidence suggests that in red cells K^+ acts as a counterion [23]. Injected cyclic AMP has been reported to stimulate the ouabain-insensitive efflux of barnacle fibres, and this could indicate the involvement of a membrane adenyl cyclase [24].

Effect of Na^+-dependent transport

The harmaline sensitivity of the Na^+-dependent glutamate uptake of barnacle muscle fibres in saline

containing only 20% of the normal Na^+ concentration is explicable in terms of harmaline inhibition at the Na^+-sensitive site on the exterior surface of the membrane. This appears to be the site of harmaline inhibition of sugar and amino acid uptake in intestinal epithelia and other tissues [8,9] and in brush border membrane vesicles prepared from the jejunum and kidney (containing no $(Na^+ + K^+)$-ATPase) [10,11]. It should be pointed out here that a fraction of the glutamate uptake, measured in the barnacle muscle fibres, is probably into presynaptic nerve terminals attached to the fibres. Sodium-dependent Ca^{2+} efflux from barnacle muscle fibres is also sensitive to extracellular harmaline, although part of the Na^+-dependent efflux is also harmaline-sensitive [25].

Acknowledgements

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