THE EFFECT OF PROTOVERATRINE A ON POTASSIUM AND CALCIUM ION MOVEMENTS IN MUSCLE AND NERVE

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The effects of protoveratrine A on the efflux of potassium ions (K^+) from frog and rat skeletal muscle and the isolated electrically driven rat heart have been studied. No effect was seen upon the rate of efflux from skeletal muscle but that from the heart was increased. Protoveratrine A increases K^+ uptake from frog skeletal muscle and causes this tissue to release more calcium (Ca^{++}). There was no increase in Ca^{++} release from lobster nerve treated with protoveratrine A. The theoretical implications of these findings are discussed.

The mechanism of action of the veratrum alkaloids is still not fully understood. The ester alkaloids are responsible for the antihypertensive actions and probably act on sensory nerve endings in the lungs, heart and great vessels. Direct actions on the brain and nodose ganglion have also been demonstrated[1]. The veratrum alkaloids sensitise sensory nerve endings and may do so by upsetting the normal ionic balance. There is sensitisation to K^+ in nerve and muscle and when sensitised tissues are stimulated, spontaneous repetitive responses occur[2]. Rosenblueth[3] has suggested that veratrine acts upon nerves by preventing the reabsorption of K^+ released during the spike. Shanes[4] has shown that K^+ is released from nerve exposed to veratrine and Gordon and Welsh[5] have suggested that veratrine may displace Ca^{++} from the cell membrane and so alter its permeability to ions. Harris (personal communication) and Khan and Acheson[6] have not been able to show changes in K^+ flux in muscle or nerve after treatment with veratrum alkaloids. Preliminary investigations[7] measuring absolute concentrations of K^+ have indicated that cardiac muscle, unlike skeletal muscle, shows an increase in K^+ efflux after protoveratrine. This investigation was made to determine whether protoveratrine A altered uptake and efflux of Ca^{++} and K^+ from muscle and nerve and to see whether these effects were related.

Materials and Methods

The composition in mM of the bath fluids used was as follows.

Fenn Ringer's fluid[8]. NaCl, 111·20; KCl, 2·50; $CaCl_2$, 1·80; Na_2HPO_4, 2·50; NaH_2PO_4, 0·50. Locke's solution. NaCl, 153·90; KCl, 5·63; $CaCl_2$, 2·16; $NaHCO_3$, 5·95; dextrose, 5·55. Krebs-Henseleit solution. NaCl, 118·30; KCl, 4·69; $CaCl_2$, 2·61; K_2HPO_4, 1·17; $NaHCO_3$, 14·16; $MgSO_4$, 2·41; dextrose, 11·10. Lobster saline (Robertson, personal communication). NaCl, 490·30; KCl, 8·75; $CaCl_2$, 57·6; $MgCl_2$, 27·4; $NaHCO_3$, 2·48; Na_2SO_4, 31·70.

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Radioactive bath fluids were made by replacing some of the KCl or $CaCl_2$ by a calculated amount of ^{42}KCl or $^{45}CaCl_2$. A 1 mg./ml. stock solution of protoveratrine A was made by dissolving the free base in dilute hydrochloric acid and neutralising the excess acid until the pH was 6·8. The solution was stored at 4° in the dark and diluted as required with the appropriate saline solution.

$^{42}K^+$ Loading of Muscles

A sterile 1·15 per cent w/v solution of ^{42}KCl with a specific activity of 0·15 mc./ml. was obtained from A.E.R.E. Harwell. 1 ml. of this was injected into the dorsal lymph sac of male or female frogs weighing from 25 to 50 g. Male or female albino rats weighing between 150 and 200 g. were given 2 ml. of the solution by intraperitoneal injection. A 2-hour equilibration period was allowed before the animals were killed and the sartorius or soleus muscles removed.

$^{42}K^+$ Release from Frog Sartorius Muscle

The frog sartorius muscles are thin and flat: they can be easily removed intact and with care paired muscles can be obtained which differ in weight by less than 2 mg. $^{42}K^+$ efflux was measured by suspending the muscles in three successive 10 ml. samples of Fenn Ringer's fluid followed by five successive samples containing 2 or 10 μg./ml. of protoveratrine A, allowing 10 minutes for each immersion. The control muscle was passed through a parallel series without protoveratrine A. Each muscle was then blotted dry and dissolved in 10 ml. of concentrated nitric acid and the total radioactivity of the solution determined. The radioactivity of the bathing solution was estimated by means of an Ekco M6 Geiger-Muller tube using a scaler of conventional pattern. The readings were corrected for background and lost counts and expressed as counts per minute.

$^{42}K^+$ Release from Rat Soleus Muscle

The soleus muscle was chosen because it has a thin flattened structure. Muscles were suspended in successive 10 ml. volumes of Krebs-Henseleit solution at 37° aerated with 95 per cent O_2 and 5 per cent CO_2. The method used was similar to that for frog sartorius muscle.

$^{42}K^+$ Release from Rat Cardiac Muscle

Hearts were rapidly removed from ^{42}K-loaded rats, washed free from blood and perfused with oxygenated Locke's solution at 37° by Langendorff's method. To avoid drug-induced variations in rate the heart was driven electrically at a rate of 120 per minute by means of square pulses of 10 volts amplitude and 10 msec. duration. The radioactive effluent from the heart was allowed to flow under slight negative pressure through an Ekco F 10 liquid flow counting tube connected to a recording ratemeter. Variations in radioactivity were shown as changes in the gradient of the recorded curve. When the rate of exchange between the $^{42}K^+$ of the heart and the K^+ of the perfusion fluid was constant, 2 or 5 μg. of protoveratrine A were injected into the aortic cannula.

$^{42}K^+$ Uptake by Frog Sartorius Muscle

Paired sartorius muscles differing in weight by not more than 2 mg. were used. The control was immersed for 10 or 20 minutes in 10 ml. of Fenn Ringer's fluid containing $^{42}K^+$ at 19° agitated and aerated by a stream of O_2. The test muscle was immersed in a similar solution containing 10 μg./ml. of protoveratrine A. The muscles were removed from the solutions, drained and each flat surface washed for 5 seconds in a stream of Fenn Ringer's fluid to remove $^{42}K^+$ adherent to the surface. The radioactivity of the muscle could now be assumed to be due to intra- or intercellular $^{42}K^+$. Radioactivity was measured by exposing each flattened muscle surface for 1 minute, 1·5 cm. away from an Ekco GM4 aluminium

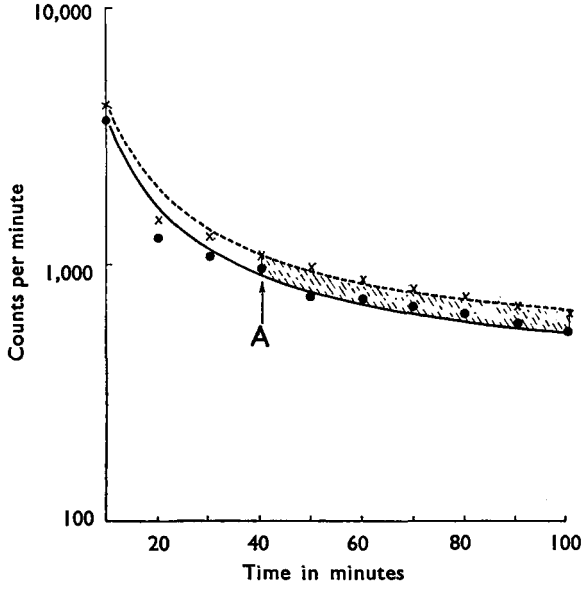

FIG. 1. $^{42}K^+$ release from frog sartorius muscle. At A, 10 μg./ml. protoveratrine A added to the test.
×—× Test. ●—● Control.

end-window counter which was connected to a recording ratemeter. The muscles were then returned to the radioactive solution for a further period of 10 minutes and the procedure repeated. Total time of exposure was 3 hours but in some experiments it was increased to 10 hours. At the end of the experiment the total radioactivity of each muscle was determined by dissolving it in concentrated nitric acid and counting in a liquid counter (see above).

$^{45}Ca^{++}$ Release from Frog Sartorius Muscle

Paired sartorius muscles were soaked for 3 hours at room temperature in oxygenated Fenn Ringer's fluid of which the Ca^{++} had been replaced by $^{45}Ca^{++}$. The muscles were washed to remove adherent $^{45}Ca^{++}$ and

the experiment continued as described for $^{42}K^+$ release but to ensure that $^{45}Ca^{++}$ release was occurring at the same rate from both muscles each was exposed for six successive 10 minute periods to a series of control solutions. The test muscle was then exposed for three successive 10-minute periods to a series of three tubes each containing 10 μg./ml. of protoveratrine A in Fenn Ringer's fluid and finally to two more tubes containing Fenn Ringer. Radioactivity was determined by taking three 0·5 ml. samples from each tube, adding 0·1 ml. of 1 per cent w/v solution of cetrimide (to ensure even spreading) and evaporating to dryness on

TABLE I

$^{42}K^+$ RELEASE FROM FROG SARTORIUS MUSCLE AFTER EXPOSURE TO 10 μg./ml. OF PROTOVERATRINE A

1	2	3	4	5
		Residual $^{42}K^+$ in muscle counts per min.		
Experiment No.	Area between curves cm.^{2}	control	test	Difference (4—3)
1	+ 4·4	9,721	11,127	−1,406
2	− 1·4	33,584	30,024	+3,560
3	− 9·5	6,027	7,405	−1,378
4	+13·0	22,992	23,836	− 844
5	− 4·1	33,665	33,977	− 272
6	+ 2·3	11,945	10,731	+1,214
7	+14·1	5,515	2,031	+3,484
8	−10·0	6,416	6,255	+ 161
9	− 9·2	12,885	15,082	−2,197

a flat aluminium planchette. The mean radioactivity of each group of three samples was determined by counting with an Ekco EW3H mica end-window counter using a conventional scaler. After correction the results were expressed as counts per minute and multiplied by twenty to give the total count for the solution.

$^{45}Ca^{++}$ Release from Lobster Nerve

The nerve from the cheliped of the common lobster (*Homarus vulgaris*) was dissected and halved. One half was used as the control, the other as the test. The nerves were soaked for 1 hour in lobster saline containing $^{45}Ca^{++}$. After loading, the surfaces were washed for 10 minutes with lobster saline to remove adherent $^{45}Ca^{++}$. The control nerve was exposed for ten successive periods of 10 minutes in a series of planchettes each containing 0·5 ml. of lobster saline. The procedure was repeated for the test nerve but each of the last five tubes also contained , 110 or 100 μg. per ml. of protoveratrine A.

RESULTS

$^{42}K^+$ Release from Frog Sartorius Muscle

The amount of $^{42}K^+$ taken up by muscles from different frogs varied widely but the individuals from a pair took up corresponding amounts. Variations in the weights of muscles from different frogs and in the radioactivity of the ^{42}KCl made direct comparisons of results difficult. It has been assumed that $^{42}K^+$ efflux is proportional to the total K^+ flux in the

muscle. Data from each experiment was expressed by plotting $^{42}K^+$ efflux against time. The curves obtained from a typical experiment are shown in Figure 1. Differences in $^{42}K^+$ efflux between test and control were obtained by measuring with a planimeter the area subtended by

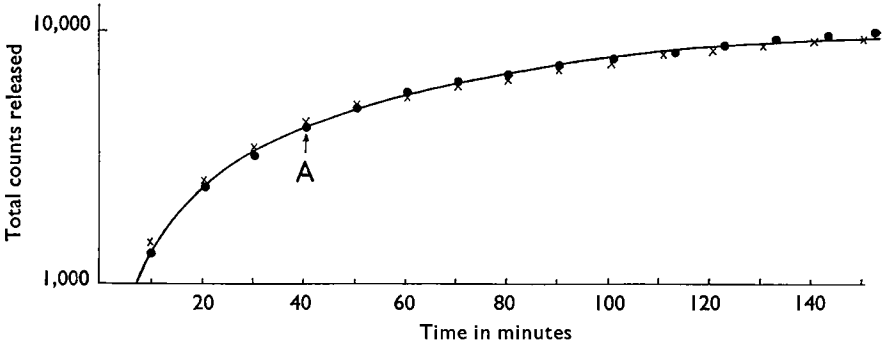

FIG. 2. $^{42}K^+$ release from rat soleus muscle. At A 10 μg./ml. protoveratrine A added to the test. ×—× Test. ●—● Control.

the two curves. When total $^{42}K^+$ efflux from the control was greater than from the test the difference was taken as positive, when less, as negative (see Table I). When analysed using the *t* test the differences in $^{42}K^+$ efflux between control and test were not significant ($P > 0{\cdot}9$). Therefore, 10 μg./ml. protoveratrine A, a concentration reported to be sufficient to influence the physiological and electrochemical behaviour of

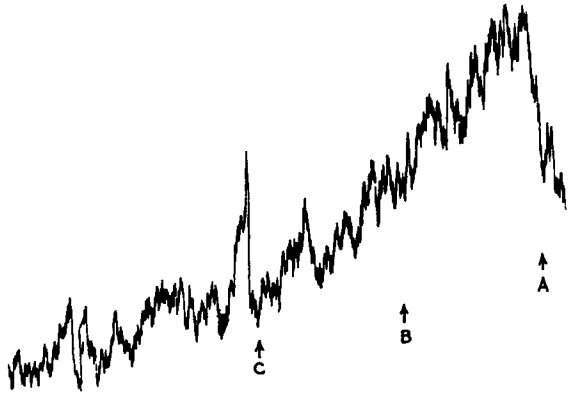

FIG. 3. $^{42}K^+$ release from rat cardiac muscle. Tracing reads from right to left. At A, beginning of experiment. At B, 0·4 ml. control solution. At C, 2 μg. protoveratrine A.

the muscle[1,2] had no effect on $^{42}K^+$ efflux. These results were confirmed using $^{42}K^+$-loaded muscles bathed in Ringer's solution continuously circulated through an FM6 flow counter when bath concentrations of protoveratrine of 1, 10 or 100 μg./ml. failed to increase $^{42}K^+$ efflux.

$^{42}K^+$ Release from Rat Soleus Muscle

The results were treated similarly to those obtained from frog sartorius muscle. 1 or 10 μg./ml. of protoveratrine A had no effect upon $^{42}K^+$ efflux from rat skeletal muscle (Fig. 2). In some experiments muscles were made to contract isometrically by stimulating at a rate of 2 per second with square wave pulses, at 10 volts, 10 msec. duration and a frequency of 100/min. Protoveratrine A had no detectable influence on $^{42}K^+$ efflux in these preparations.

$^{42}K^+$ Release from Rat Cardiac Muscle

Protoveratrine A, 2 or 5 μg., reversibly increased $^{42}K^+$ efflux. As heart rate and cardiac output were constant this effect appeared to be

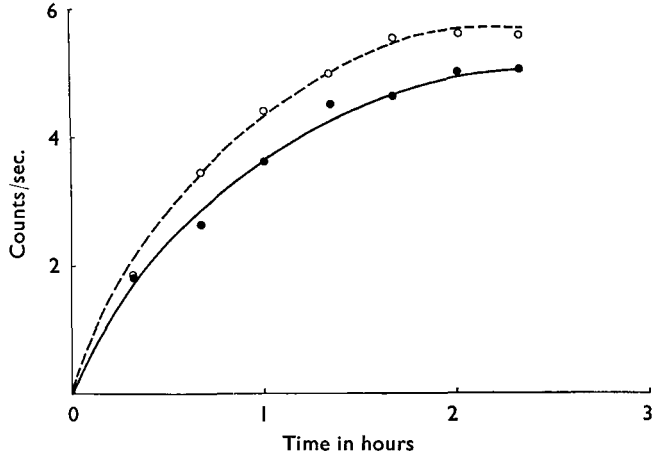

FIG. 4. $^{42}K^+$ uptake by frog sartorius muscle. The test muscle was treated with 10 μg./ml. protoveratrine A.
○—○ Test. ●—● Control.

due to a direct drug action (Fig. 3). These results agree with the findings of Vick and Kahn[10] who used isolated guinea pig hearts treated with veratridine and measured the K^+ concentration of the effluent before and after drug administration.

$^{42}K^+$ Uptake by Frog Sartorius Muscle

The rate and total uptake of $^{42}K^+$ from a labelled medium differed considerably between frogs. The method used here made it possible for the activities of the treated and the control muscles of each pair to be measured and recorded side by side, thus reducing errors of measurement as much as possible. By plotting the total uptake by each muscle against time, curves of an exponential character were obtained (Fig. 4).

In ten experiments the rate and total uptake of $^{42}K^+$ by the protoveratrine treated muscle was always greater than that from the corresponding control muscle. This difference was found to be significant ($P < 0{\cdot}001$).

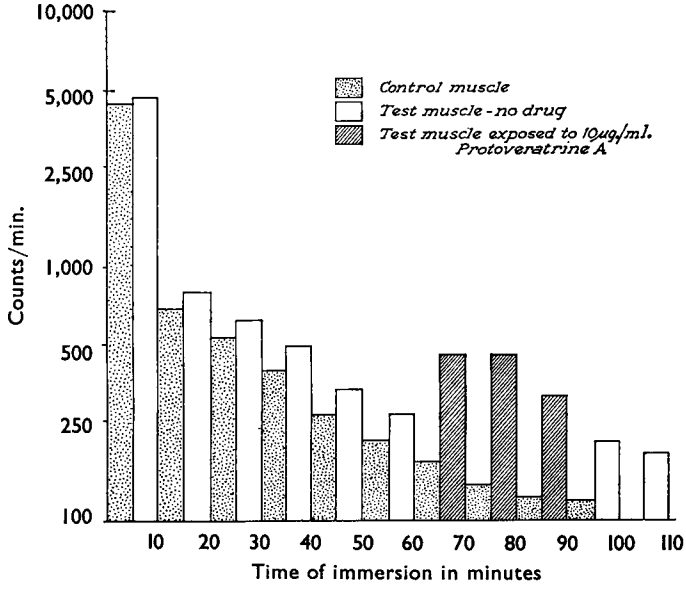

FIG. 5. $^{45}Ca^{++}$ release from frog sartorius muscle.

TABLE II

$^{45}CA^{++}$ RELEASE FROM FROG SARTORIUS MUSCLE BY PROTOVERATRINE A 10 μg./ml.

1	2		3	4	5	6	7	
	Wt. (mg.) of muscle C = Control T = Test		Total number of counts released during			Percentage of total released by		Ratio of results in columns 6 to 7
Expt. No.			run	exposure to drug	exposure to control	exposure to drug	exposure to control	
1	C	80	17,085	2,839	2,361	17·54	13·82	1·27
	T	80	16,180					
2	C	36	7,104	2,569	1,552	30·01	21·84	1·38
	T	37	8,533					
3	C	24	9,918	762	285	6·58	2·87	2·29
	T	24	11,579					
4	C	40	2,044	307	224	1·51	1·09	1·38
	T	38	2,030					
5	C	40	2,436	397	237	1·70	0·97	1·75
	T	42	2,335					
6	C	54	5,289	758	586	1·33	1·10	1·21
	T	52	5,698					
7	C	78	7,099	1,196	433	14·0	6·1	2·29
	T	77	8,543					
8	C	148	2,554	284	153	10·74	5·99	1·79
	T	148	2,644					
9	C	129	8,506	1,114	801	10·07	9·42	1·07
	T	130	11,067					
10	C	27	2,849	293	161	9·28	5·65	1·64
	T	28	3,156					

$^{45}Ca^{++}$ *Release from Frog Sartorius Muscle*

The normal rate of $^{45}Ca^{++}$ release from frog sartorius muscle was exponential and similar to that reported by Harris[9]. In ten experiments exposure of the $^{45}Ca^{++}$ loaded muscle to 10 μg./ml. of protoveratrine A led to more $^{45}Ca^{++}$ being released into the bathing fluid than from the control. In seven of these, maximum $^{45}Ca^{++}$ release occurred during the first 10 minute period of exposure and in the remainder during the second 10-minute period. After returning to the normal bathing solution the rate and total $^{45}Ca^{++}$ release fell approximately to control levels (Fig. 5). Paired muscles were comparable with respect to uptake and release of Ca^{++} but muscles from different frogs of the same weight showed no correlation. The total counts per minute obtained from the muscle during exposure to the drug were expressed as the total counts per cent, released during the entire experiment. In each experiment more $^{45}Ca^{++}$ was released from the protoveratrine-treated muscle than from the control (Table II). The results indicate that 10 μg./ml. protoveratrine A significantly increases ($P < 0{\cdot}001$) the release of $^{45}Ca^{++}$ from frog sartorius muscle.

$^{45}Ca^{++}$ *Release from Lobster Nerve*

Protoveratrine A (1, 10 or 100 μg./ml.) had no significant effect ($P > 0{\cdot}9$) on release of $^{45}Ca^{++}$ from lobster nerve.

Discussion

Early pharmacological studies on the veratrum alkaloids were made with the mixture known as veratrine. This has actions qualitatively similar to those of the purified ester alkaloids which, in the intact animal, cause reflex hypotension, bradycardia and bradypnoea. This reflex is known as the Bezold-Jarisch reflex and is due to sensitisation of receptors in the left heart, great vessels and lungs[11], which respond by firing at an increased frequency. Similar phenomena can be demonstrated in isolated nerve and muscle and have been observed after changes in the ionic composition of the bathing fluid[2]. Initiation of a nerve volley at the sensory endings is accompanied by an alteration in the permeability of the cell membrane with alterations in the relative concentrations of K^+ and Na^+. There is some evidence that these changes are related to alterations in calcium-binding at the cell surface[12]. Reduction of the concentration of Ca^{++} in the external medium causes repetitive firing in nerve and muscle and an increase in the external concentration of Ca^{++} abolishes the sensitising effects of the veratrum alkaloids[2]. From our results it appears that the influence of protoveratrine A on nerve and muscle differs. No increase in K^+ efflux from protoveratrine A-treated skeletal muscle was shown. This is in contrast to the observations of Rosenblueth[3] and Shanes[4] using nerve but agrees with Harris (personal communication), who used frog muscle and Kahn and Acheson[6], who used erythrocytes. The increased K^+ efflux from cardiac muscle after protoveratrine A may reflect differences in the rate of metabolism, in the mechanism of potassium transfer or in the lability of intracellular potassium and its freedom

to exchange, but it must also be remembered that protoveratrine has a direct action on receptors in the heart. Low concentrations of veratrum alkaloids initiate the Bezold-Jarisch reflex by acting on sensory nerve endings in the coronary artery bed[13]. Most (but not all) of the effluent in our experiments has passed through the coronary circuit. K^+ release may occur more readily at this site and this may explain why the Bezold-Jarisch reflex is elicited by doses of protoveratrine which have little or no effect elsewhere. It is difficult to explain why protoveratrine-treated muscles took up more $^{42}K^+$ than the controls.

Protoveratrine in concentrations which displace $^{45}Ca^{++}$ from muscle also promotes uptake of $^{42}K^+$. Displacement of Ca^{++} may free anionic sites on the cell surface which can bind K^+ in excess of that already present. Gordon and Welsh[5] suggested that veratrine acted by displacing Ca^{++} from the cell membrane, but their evidence was indirect and no measurements of Ca^{++} concentrations were made. Our results on muscle support this theory, but we found no release of Ca^{++} from lobster nerve. Monné[14] has suggested that the polypeptide chains of the surface protein may be linked by divalent Ca^{++}. Making and breaking of this link alters the configuration of the protein surface and may account for changes in permeability often associated with alteration in Ca^{++} concentration. Protoveratrine is a highly hydroxylated molecule[15]; it may form hydrogen bonds with the protein of the cell surface preventing Ca^{++} from performing its normal linking function and so induce changes in permeability.

We believe that our results support the view that protoveratrine and the related veratrum alkaloids interfere with the normal metabolism of Ca^{++} on the cell surface, altering membrane permeability and thus ionic balance which is the factor finally responsible for the characteristic actions of the veratrum alkaloids on nerve and muscle.

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