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ALTERATION OF Na^+, K^+-DEPENDENT ATPase ACTIVITY AND INOTROPISM BY POLYETHYLENE GLYCOL 300 IN GUINEA-PIG ATRIA

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Abstract—Polyethylene glycol 300 (PEG 300) elicited a dose-related negative inotropic response in guinea-pig left atria at 37°. However, when the tissue was washed and a second dose–response relationship determined, PEG 300 then elicited a marked positive inotropic response. The positive response was not the result of catecholamine release since it was still observed in reserpine-pretreated as well as in propranolol treated tissues. Atropine, phentolamine and triprolidine had no effect upon this result.

PEG 300 had little effect upon dose–response curves to Ca^{2+} but in the presence of low (1/3) Na^+ solutions, both dose–response curves were negative. The involvement of Na^+ in the bizarre inotropic responses to PEG 300 was substantiated by the findings that intracellular cardiac action potential amplitudes and the maximal rate of depolarization were markedly depressed while the resting membrane potential amplitude was unchanged.

In histochemical studies, PEG 300 (7% v/v) was found to cause a 14 per cent increase in the activity of Na^+, K^+-dependent ATPase but did not affect the Mg^{2+}-activated or mitochondrial Ca^{2+}-activated ATPases in guinea-pig left atrial strips. Washing the PEG 300 from the tissues caused the enzyme to return to control levels. A second addition of PEG 300 to these tissues did not elicit any changes in the activity of Na^+, K^+-dependent ATPase.

Biochemical studies on isolated enzyme preparations demonstrated that PEG 300 had no direct stimulatory effect upon the activity of Na^+, K^+-dependent ATPase. It was suggested that PEG 300 increases the number of sites of enzyme activity, similar to deoxycholate treatment.

THERE are many reports in the literature of chemical and pharmaceutical agents which inhibit Na^+, K^+-dependent ATPase, of which the most well known and studied group are cardiac glycosides.[1–4] Other agents which inhibit this enzyme include oligomycin,[3,4] sodium fluoride,[2] dibenamine,[5] dibenzyline,[5] *n*-ethyl maleimide,[2,4] tributyltin-chloride,[4] octylguanidine,[4] chlorhexidine,[6] salmine sulphate,[2] dimethylsulphoxide,[7] dimethylformamide[7] and dimethylacetamide.[7]

Cardiac glycosides in low concentrations (10^{-12}–10^{-9} M) are the only agents reported to stimulate Na^+, K^+-dependent ATPase,[8–10] but the amount of stimulation was small and has not been generally confirmed.[1,3,4] Digitonin, bee venom and sphingosinphosphorylcholine have been reported[11] to stimulate Mg^{2+}-activated ATPase prepared from guinea-pig heart muscle. All of these agents, however, in-inhibited the Na^+, K^+-dependent ATPase. One brief report[12] states that a pteridine derivative increases the activity of both Na^+, K^+-dependent and independent ATPases in erythrocytes, while others only increase the activity of the Na^+, K^+-dependent ATPase. Those effects were not thought to be due to a direct stimulation of the enzyme.

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During studies on organic solvents routinely used in our laboratory we observed some unusual inotropic responses to polyethylene glycol 300 (PEG 300). Polyethylene glycols are polymers of ethylene oxide with the general formula: $HOCH_2 (CH_2OCH_2)_n CH_2OH$. These compounds are referred to by a number which represents the average molecular weight, e.g. polyethylene glycol 200, 300, etc. Lower members of the series are colourless liquids and higher members (1000 and above) are white solids. The liquid members have been used as solvents of medicinal compounds for several years[13–15] and are relatively non-toxic.[14–17]

In order to explain the inotropic results it seemed reasonable to suggest that PEG 300 either stimulated Na^+, K^+-dependent ATPase directly or increased the number of sites of enzyme activity, similar to deoxycholate. Upon investigation, evidence was found which supported the latter hypothesis.

METHODS

Male guinea-pigs (300–500 g) were killed by a blow on the head and their hearts rapidly removed and placed in a beaker containing Krebs-bicarbonate solution, which was well oxygenated with a 95% O_2:5% CO_2 mixture. Left atria were dissected free and mounted vertically in a 20 ml muscle chamber maintained at 37°. Tissues were stimulated with rectangular pulses of 1 msec duration at a frequency of 60 pulses/min at a voltage which was 10 per cent above threshold. Resting tension equalled one gram and was not affected by PEG 300. Atrial contractility was measured isometrically on a polygraph (Devices). Krebs-bicarbonate solution was used to wash the atria by the "overflow" method, allowing 200 ml per wash.

PEG 300 (Union Carbide) concentrations are expressed in % v/v. Concentrations of other substances in terms of grams of the salt per ml of medium in the muscle chamber unless otherwise specified. In experiments in low (1/3) Na^+ containing solutions, ionic balance was maintained with choline chloride. PEG 300 is a fairly hygroscopic solvent and caution was taken to only use fresh undiluted solvent in these experiments.

Transmembrane potentials were obtained at 32° using glass capillary electrodes filled with 3M KCl. Electrode resistances varied between 10 and 25 megohms. The signal picked up by the microelectrode passed through an agar filled cell with tungsten wire and was amplified by neutralized input capacity preamplifier (BAK standard wide band electrometer–Electronics for Life Sciences), and then passed simultaneously through a d.c. input audioindicator and to the upper beam of the oscilloscope. Film records were made routinely. The maximal rate of depolarization was obtained by passing the signal through a differential circuit with time constants that ranged from 0·1 to 1·0 msec. The differentiated spike was then compared with the known differential of the calibrated pulse in order to determine the rate of depolarization in volts per second. Contractile activity was recorded with an RCA 5734 transducer tube. A voltage and time calibration signal was triggered by a Digitimer at the start of every sweep and was applied to the agar filled indifferent electrode in the bath.

For histochemical determinations of Na^+, K^+-dependent ATPase, left atria of guinea-pigs were dissected free and cut lengthways into two equal segments. Three atrial strips served as controls and were immediately quenched in liquid nitrogen at −196°. The number of strips studied represents the number of different animals used in each experiment and strips were selected so that there was an equal number of right

and left halves. Eleven strips were placed in well oxygenated Krebs solution at 37°. Twenty strips were placed in another chamber containing oxygenated Krebs solution at 37° into which PEG 300 (7% v/v) was added. Twelve min later five of the strips in the Krebs solution and seven strips in the solution containing PEG 300 were removed and quenched in liquid nitrogen. Both chambers were then washed for 20 min, at which time three strips from the Krebs and six from the chamber which had been treated with PEG 300 were removed and quenched. PEG 300 was then reintroduced (7%, v/v) into the chamber from which it was washed and two strips removed and quenched 2 min later. The remaining three strips in Krebs-bicarbonate solution and five strips in the PEG 300 treated chamber were removed and quenched 12 min after the second addition of PEG 300. These experiments were performed on two separate occasions, although the numbers of strips represent the total number involved.

After 24 hr storage in liquid nitrogen, 5 μm sections were prepared in a cryomicrotome at −20°. Two unfixed sections from each atria studied were attached to clean glass slides and transferred into a 100 ml incubation solution consisting of 5 mM ATP, (Na^+ free), 24 mM tris–HCl buffer (pH 7·3), 5 mM $MgSO_4$ and 1·5 mM $Pb(NO_3)_2$. A range of ATP concentrations was studied and 5 mM was not found to cause an inhibitory effect or to produce nonspecific hydrolysis products with the concentration of Pb used. The incubation solution was divided into two aliquots and 80 mM NaCl and 15 mM KCl were added to one aliquot and 15 mM KCl to the other. Concentrations of Na^+ below 60 mM did not stimulate the Na^+, K^+-dependent ATPase. It was thus felt that the Na^+ levels in the thin sections could not be responsible for the stimulation of ATPase observed in the presence of K^+ alone.

Half of the serial sections from each tissue were incubated for 10 min at 30° in the NaCl containing solution and the other half were incubated under the same conditions in the other solution. Sections were then rinsed in distilled water, reduced for 60 sec in 5% $(NH_4)_2S$, rinsed in distilled water and mounted under coverslips in Farrants media. Sites of Na^+, K^+-dependent ATPase were observed as brown to black deposits of PbS. Studies were also performed in the absence of Na^+ and K^+ to determine the activity of Mg^{2+}-dependent ATPase alone.

Control studies were also performed with ouabain. In these experiments, sections were placed directly in ouabain (10^{-4} M) at 20° for 15–30 min, followed by the method described above to confirm the inhibition of the enzyme by this agent.

Mitochondrial Ca^{2+}-dependent ATPase was determined by previously described methods[18] modified to contain Mg^{2+}. Final concentration of the Ca^{2+} was 30 μM. This enzyme was also stimulated by Mg^{2+} (5 mM).

All reagents were dissolved in deionized glass distilled water and were freshly prepared each day.

Following microscopic examination, the sections were analyzed with a Reichert microspectrophotometer using the two wavelength method of microspectrophotometry.[19–21] The maximal extinction (Eb) was determined at 462 nm and the half maximal extinction (Ea) at 510 nm. The transmittance of 50 random areas of plasma membranes (eight measurements per area) was determined on one section of each tissue studied, using both wavelengths. The amount of PbS colouring was expressed in arbitrary units (γ) which was determined using "two-wavelength" tables.[19] The distributional error of the 50 areas was less than 1·5 per cent and thus not significant.

Our method for the determination of Na^+, K^+-dependent ATPase has considered

major criticisms of histochemical techniques by previous workers.[23–36] The adoption of a low Pb^{2+} and high substrate concentration, and short incubation times coupled with incubation at 30° on unfixed material are technical modifications said to lead to a more reliable histochemical demonstration of the enzyme.[36–39] Na^+, K^+-dependent ATPase was also found to be inhibited in the presence of Ca ions (15 mM). The activation of Na^+, K^+-dependent ATPase and K^+-dependent ATPase by Mg ions was assumed.

Na^+, K^+-dependent ATPase activity was also determined biochemically. The enzyme was prepared from guinea-pig heart muscle by previously described methods.[2] Total ATPase activity was measured in a medium containing 25 mM imidazole ·HCl (pH 7·0); 4 mM $MgCl_2$; 4 mM tris-ATP; 1 mM-tris-EGTA; 100 mM NaCl and 5 mM KCl in a total volume of 1 ml. The incubation was terminated by the addition of 0·5 ml ice cold 15% w/v trichloroacetic acid. Inorganic phosphate in the protein-free filtrate was determined by the method of Fiske and Subbarow.[40] The Na^+, K^+-dependent ATPase activity was taken as the difference between the total ATPase activity and that in the absence of added Na^+ and K^+.

RESULTS

Dose–response curves to PEG 300 in guinea-pig left atria at 37° demonstrated a dose-related negative inotropic response (lower curve in Fig. 1). However, when the

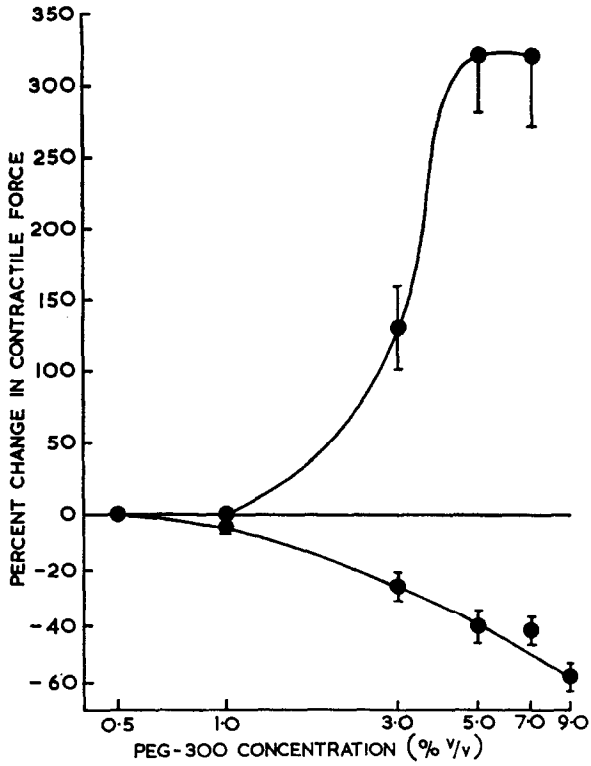

FIG. 1. Dose–response curves for PEG 300 on the contractility of guinea-pig left atria. The lower curve represents the first dose–response determination to PEG 300 (n = 15) and the upper curve represents the second dose–response determination to PEG 300 (n = 11).

tissue was washed and control contractile levels reestablished, a second dose–response curve to PEG 300 invariably showed marked positive inotropic responses (upper curve in Fig. 1). A third dose–response curve was also strongly positive and was indistinguishable from the second. The time interval from addition of PEG 300 to equilibration, when measurements were made, was between 5 and 10 min for all concentrations. In order to determine whether release of catecholamines would account for the positive responses, beta-adrenoceptive receptor blockade was produced by propranolol (5×10^{-7} for 40 min) after the first dose–response curve was obtained. The second dose–response curve under these conditions revealed positive inotropic activity ($n = 7$), with an average maximal increase of 96 per cent at 7% PEG 300. The magnitude of the response was thus not as marked as in Fig. 1. Guinea-pigs were pretreated with reserpine (1 mg/kg i.p. for each of 3 days) to deplete catecholamine stores and dose–response curves to PEG 300 obtained. Similar results were obtained as those shown in Fig. 1.

Atropine (1×10^{-7} for 2 min) did not affect the dose–response curves to PEG 300 whether it was added to the muscle bath before the first or before the second dose–

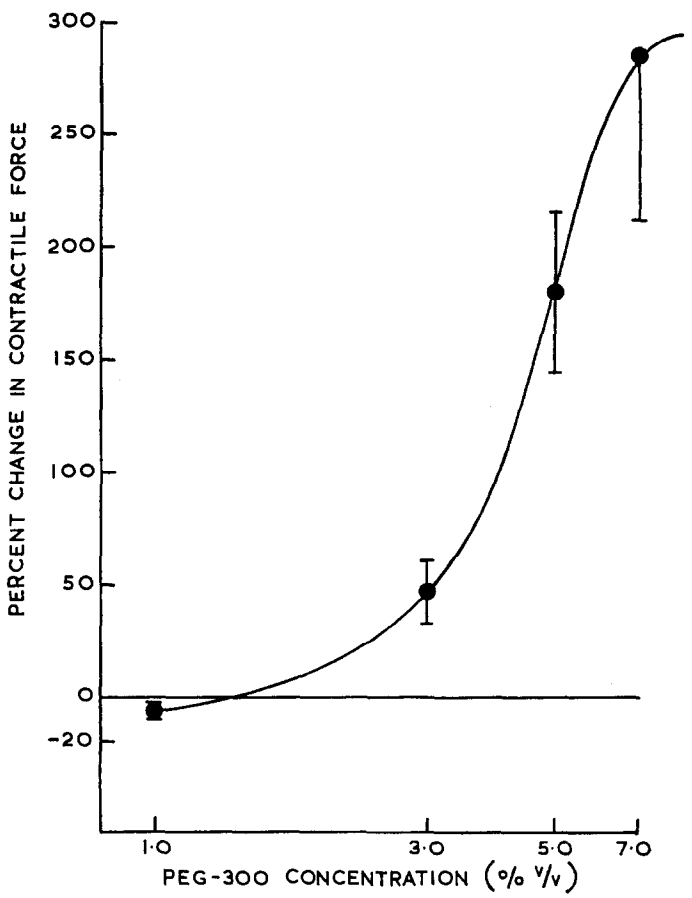

FIG. 2. Dose–response curve to PEG 300 after initial incubation of the tissue with 7% PEG 300 for 30 min ($n = 5$). The value for PEG 300 (1%) is not significantly different from control.

response determination ($n = 3$ in both cases). The antihistamine triprolidine ($3{\cdot}4 \times 10^{-6}$) and the alpha adrenoceptive-receptor blocking agent phentolamine (1×10^{-6}) also had no effect upon the dose–response curves to PEG 300 ($n = 3$ in each case).

Initial incubation of the atria with 7% PEG 300 for 30 min (either with or without stimulation) followed by washing of the muscle chamber, affected the tissue so that subsequently the first dose–response curve to PEG 300 was strongly positive (Fig. 2). However, when guinea-pigs were pretreated with PEG 300 (5 ml/kg i.p. for 24 hr) the first dose–response to PEG 300 was the same as the negative one shown in Fig. 2. Eight atria were treated with 7% PEG 300 and all stopped beating within 90 min and could not be revived by washing the tissue or by increasing the stimulation voltage. The contractile tension declined steadily in six of eight atria studied but in the other two a brief positive response was observed after 10 min.

In order to determine whether PEG 300 was affecting calcium ions, dose–response curves were obtained to $CaCl_2$ before and after the atria were incubated with 7% PEG 300 ($n = 7$). The dose–response curve for Ca^{2+} (0·1 to 6·0 mM), was slightly depressed after PEG 300 was washed from the tissues, but attained the same maximal response and was thus not markedly different from the first curve.

To examine possible effects upon sodium ions, dose–response curves were performed in both high and low Na^+ concentrations. Dose–response curves using Krebs solution with twice the normal concentration of Na^+ demonstrated the same effects as those in normal Na^+. However both the first and second dose–response curves in low (1/3) Na^+ Krebs solution were negative (Fig. 3). There were no differences between these two curves. Contractile responses were abolished by 4–5% PEG 300 in the low Na^+ Krebs, whereas 10–15% PEG 300 was required to suppress contraction in normal Na^+ Krebs.

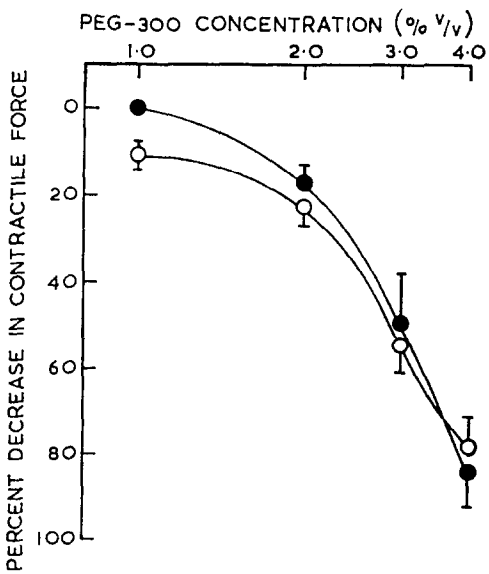

FIG. 3. Dose–response curves to PEG 300 in low (1/3) Na^+ Krebs solution. The open circles represent the first dose–response curve ($n = 6$) and the closed circles represent the second dose–response curve ($n = 6$) to PEG 300. The values at 1% PEG are significantly different ($0{\cdot}01 > P$).

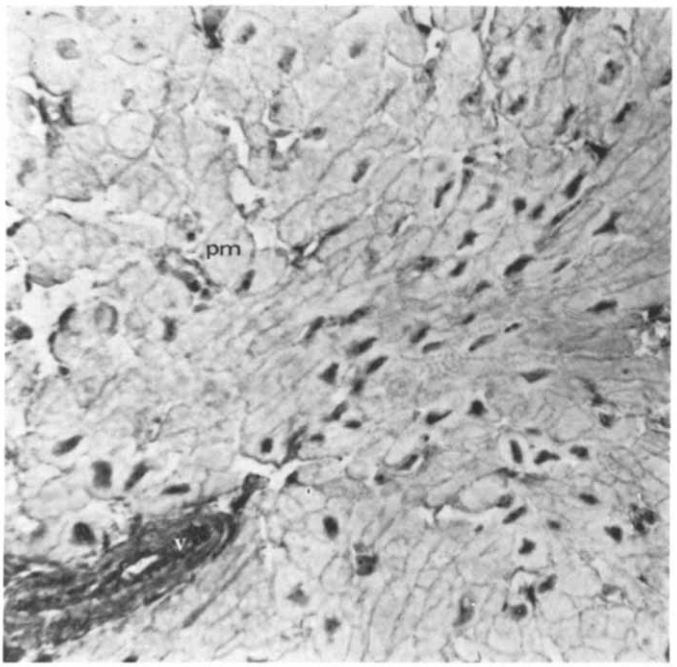

FIG. 4. Na^+, K^+-dependent ATPase in untreated guinea-pig left atria. The enzyme is localized in plasma membranes (PM) and vasculature (V). Darker areas represent greatest enzyme levels. Magnification ×160. Figures 1–4 were all developed to the same degree of contrast. Exposure, developing, printing times and conditions were identical.

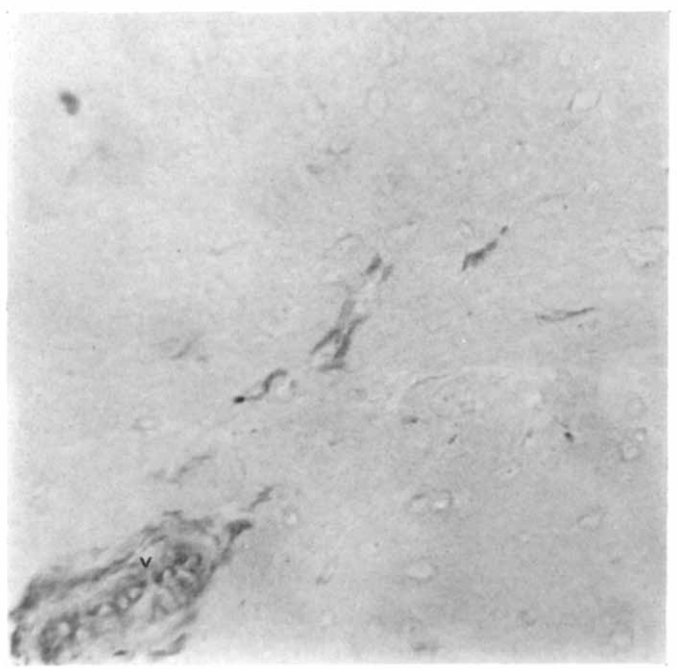

FIG. 5. Na^+, K^+-dependent ATPase levels after treatment with ouabain (10^{-4} M) for 15 min. Activity localized in vasculature (V) is primarily Mg^{2+}-dependent ATPase. Magnification ×160. This is a serial section to that used in Fig. 4.

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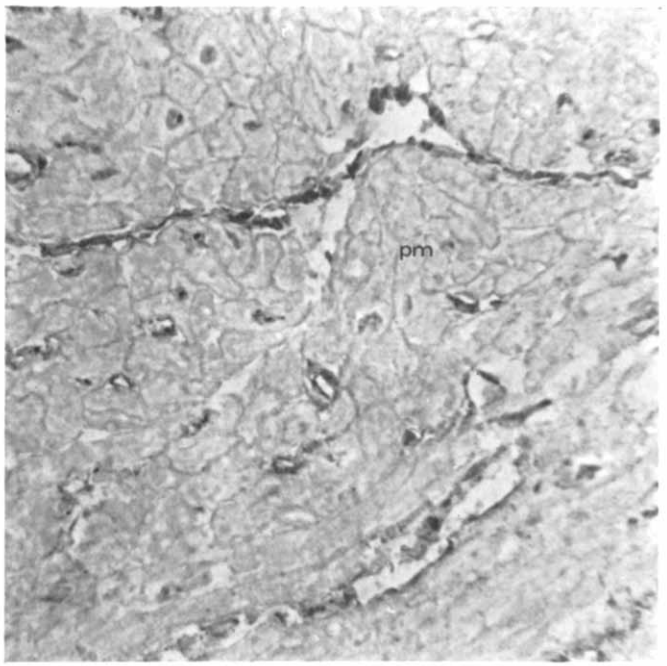

FIG. 6. Na^+, K^+-dependence ATPase levels after incubation with PEG 300 (7% v/v) for 12 min. The enzyme is localized in plasma membranes (PM) and vasculature. Darker areas represent greatest enzyme levels. Magnification ×160.

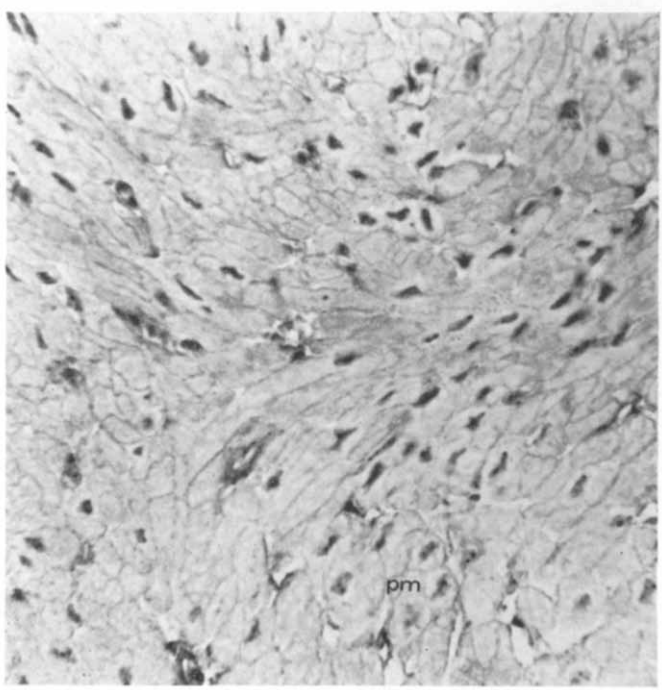

FIG. 7. Na^+, K^+-dependent ATPase levels after the tissues were incubated for the second time in PEG 300 (7% v/v). The enzyme is localized in plasma membranes (PM) and vasculature. Darker areas represent greatest enzyme levels. Magnification ×160.

TABLE 1. EFFECTS OF POLYETHYLENE GLYCOL 300 (PEG 300) ON GUINEA-PIG ATRIAL INTRACELLULAR POTENTIALS AND CONTRACTILITY*

Experiment	Resting membrane potential (mV)	Action potential (mV)	Maximal rate of depolarization (V/sec)	Time for repolarization to be		Contractile amplitude (g)	Time to peak tension (msec)
				10% complete (msec)	90% complete (msec)		
Control	65·9 ± 3·9	85·1 ± 4·3	102·2 ± 7·3	19·9 ± 2·0	102·4 ± 4·1	0·73 ± 0·09	60·6 ± 5·3
7% (v/v) PEG 300							
5 min	64·3 ± 2·5	70·0 ± 5·7	76·4 ± 2·6†	17·3 ± 3·9	80·4 ± 7·9†	0·60 ± 0·07	61·6 ± 3·4
10 min	66·0 ± 5·4	54·0 ± 5·1‡	59·2 ± 7·7‡	12·9 ± 4·2	60·4 ± 15·2‡	0·57 ± 0·08	56·7 ± 1·8
Control (after 1 hr wash)	65·7 ± 2·6	82·4 ± 3·9	97·5 ± 8·5	11·8 ± 2·2†	72·7 ± 8·5	0·30 ± 0·05‡	47·5 ± 3·2†
7% (v/v) PEG 300 (second addition)							
5 min	68·2 ± 3·3	74·5 ± 5·6	100·9 ± 7·5	13·5 ± 2·6	67·5 ± 12·6	0·35 ± 0·06	51·5 ± 4·3
10 min	63·2 ± 3·0	58·5 ± 5·3‡	48·4 ± 2·4‡	3·5 ± 0·01‡	40·2 ± 5·3‡	0·47 ± 0·07†	50·4 ± 3·9
Recovery (after 1 hr wash)	72·6 ± 3·0	76·6 ± 4·4	74·0 ± 7·5†	10·4 ± 1·8‡	60·8 ± 11·4	0·20 ± 0·06	45·1 ± 7·2

* Each value is the average of between 10 and 15 measurements in a total of three atria ± standard error.
† $0{\cdot}1 < P < 0{\cdot}05$.
‡ $= P < 0{\cdot}01$. The values for the second addition of PEG 300 and for the recovery were compared with the second control values.

Intracellular membrane potentials were recorded in guinea-pig atria at 32° after PEG 300 (7%) was perfused through the muscle bath. PEG 300 decreased contractile amplitude and caused a marked depression of action potential amplitude, maximal rate of depolarization and shortened the duration of the action potential but did not significantly alter the resting membrane potential amplitude, or time to peak tension (Table 1). Control values were only partly restored after the tissue was washed for 1 hr. The second perfusion of 7% PEG 300 through the muscle bath had the same effects as the first one on all parameters measured, except that contractile tension was significantly increased (Table 1). These values partially returned to control after a further wash of 1 hr. Identical results were obtained in each of three atria studied.

Levels of Na^+, K^+-dependent ATPase and K^+-dependent ATPase were determined histochemically in each left atrial strip studied. The two ATPases maintained identical levels in tissues incubated in Krebs solution for 12, 32 and 45 min as in the non-incubated controls (Fig. 4). Exposure, developing and printing times of Figs. 4–7 were identical. Figure 4 illustrates the presence of ATPase activity in both vasculature and plasma membranes. The darker areas represent the greatest concentration of the enzyme. There is more Mg^{2+}-dependent ATPase than Mg^{2+}, Na^+, K^+-dependent ATPase in heart muscle, due in part to the high proportion of Mg^{2+}-dependent ATPase located in the vasculature.[24,37] Ouabain (10^{-4} M) caused a complete inhibition of Na^+, K^+-dependent ATPase in plasma membranes, but did not inhibit the Mg^{2+}-dependent ATPase in the vasculature (Fig. 5).

Microscopic examination of seven atria incubated with PEG 300 (7% v/v) for 12 min showed a small but definite increase in Na^+, K^+-dependent ATPase activity in plasma membranes (Fig. 6). No differences in the activity of Mg^{2+}-dependent ATPase or an ATPase dependent on K^+ were observed in tissues incubated with PEG 300. The increased activity of Na^+, K^+-dependent ATPase by PEG 300 was evidenced by an increased deposition of PbS, which appear as dark areas. The microspectrophotometer was used to quantify the Na^+, K^+-dependent ATPase reaction product (PbS). The control value was $43{\cdot}7 \pm 0{\cdot}24\ \gamma$ and for PEG 300 treated tissue the mean value was $49{\cdot}9 \pm 0{\cdot}18\ \gamma$. This is significant at the $P = 0{\cdot}002$ level and represents an increase in activity of 14 per cent.

After washing the tissues for 20 min, the levels of Na^+, K^+-dependent ATPase returned to pre-incubation control values. A second treatment with PEG 300 (7% v/v) of tissues which had been previously exposed to PEG 300, did not alter the activities of either ATPase after two ($n = 2$) or twelve ($n = 5$) min (Fig. 7).

To determine whether PEG 300 would increase the activity of other ATPases in heart, mitochondrial Ca^{2+}-dependent ATPase activity was also determined in these tissues. No differences from control levels were observed.

In order to determine whether the increased activity of Na^+, K^+-dependent ATPase by PEG 300 was the result of a specific stimulation of the enzyme, biochemical studies on isolated enzyme preparations were performed. No increases in enzymatic activity with PEG 300 (7% v/v) were observed.

DISCUSSION

The present experiments demonstrated that the first inotropic dose–response curve to PEG 300 was negative and that the second dose–response curve was positive. A third dose–response curve was indistinguishable from the second. The positive res-

ponse was not the result of catecholamine release since it was still observed in reserpine-pretreated as well as propranolol-treated tissues. Atropine, phentolamine and the antihistamine triprolidine had no effect upon this result.

PEG 300 had little effect upon dose–response curves to calcium, but in the presence of low (1/3) Na^+ solutions, both dose–response curves to PEG 300 were negative. In high (2×) Na^+ solutions, however, the second dose–response to PEG 300 was positive. The involvement of Na^+ in the bizarre inotropic responses to PEG 300 was substantiated by the finding that intracellular cardiac action potential amplitudes and the maximal rate of depolarization were markedly depressed while the resting potential amplitude was unchanged. The former parameters are well known to be associated with Na^+, while the resting potential is largely dependent upon the K^+ equilibrium potential.

It seemed reasonable to suspect that PEG 300 was either blocking the inward movement of Na^+ through the membrane during depolarization which might account for the effects observed on action potentials, or else that PEG 300 was affecting Na^+, K^+-dependent ATPase in some way. When these possibilities were tested by histochemical techniques, it was found that PEG 300 increased the activity of Na^+, K^+-dependent ATPase by 14 per cent, but did not affect the Mg^{2+}-dependent ATPase alone. Mitochondrial Ca^{2+}-ATPase was also unaffected by PEG 300. In order to determine whether the increased enzyme activity was due to a direct stimulation, a biochemical assay of Na^+, K^+-dependent ATPase was performed and was found to be unaffected by PEG 300.

The increased activity of Na^+, K^+-dependent ATPase after PEG 300 in atrial sections might be due to an effect on the membrane whereby PEG 300 increases the number of sites of enzyme activity, similar to deoxycholate treatment. In support of this view was the observation that PEG 300 could not increase the activity after it was washed from the tissue and then reintroduced. The initial negative inotropic response to PEG 300 may have been partly the result of these membrane effects. Upon washing PEG 300 from the tissue, certain factor(s), to which PEG 300 had been bound, may have also been washed from the tissue or altered, so that the second dose–response curve to PEG 300 was positive. Schwartz and Laseter[41] reported the presence of an inhibitor of Na^+, K^+-dependent ATPase in heart muscle, so that it is also possible that PEG 300 inhibited this inhibitor protein to increase the enzyme activity.

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