Table 2. Effects of paraquat (PQ) on histamine and isoproterenol induced changes of cyclic nucleotides in rat lung slices at different times following the incubation

	Cyclic nucleotide levels in lung slices plus medium (pmoles/mg protein)*					
	2 min		4 min		10 min	
	cAMP	cGMP	cAMP	cGMP	cAMP	cGMP
Histamine						
KRBB (control)	114.28	6.82	131.96	6.65	128.57	5.76
Histamine (10^{-5} m)	152.77	8.21	161.78	7.14	118.74	6.22
PQ (10^{-4} M)+ Histamine (10^{-5} M)	115.65	4.11	127.27	4.39	131.22	5.05
Isoproterenol						
KRBB (control)	179.81	9.46	158.09	8.82	160.03	11.22
Isoproterenol (10^{-5} M)	317.80	9.60	232.57	7.14	244.49	6.76
PQ (10^{-4} M)+ Isoproterenol (10^{-5} M)	232.75	5.89	223.33	5.02	243.13	5.61

* Each value is the mean of 2 slices.

terenol (10^{-5} M). The incubation was terminated by 1 ml of 25% TCA (cold) followed by homogenization of tissue plus medium with a Brinkman Polytron. After centrifugation, the precipitate was dissolved in 0.2 N NaOH and the protein content determined using bovine serum albumin as a standard[9]. The supernatant fraction was used to determine cAMP and cGMP by the radioimmunoassay method of Frandsen and Krishna[10].

Results and discussion. The effects of paraquat ion (10^{-4} M) on cAMP and cGMP, levels of lung slices at varying times following incubation are shown in table 1. Paraquat in general had no effect on cAMP and cGMP levels of lung slices at anytime except that a slight reduction in cGMP level was noted at 6 and 12 min.

The effects of paraquat (10^{-4} M) on histamine and isoproterenol induced changes in cyclic nucleotides in the lung slices are summarized in table 2. Incubation of lung slices with histamine (10^{-5} M) was found to increase the cAMP level by 34 and 23% at 2 and 4 min respectively and the cGMP level by 20% at 2 min. The finding in the present study that incubation of lung slices with histamine increased the cAMP level is consistent with the finding of other investigators who have previously reported an increased cAMP level in rat lung slices following treatment with histamine[6]. The interesting finding in the present study was that the presence of paraquat (10^{-4} M) in the incubation mixture abolished the histamine mediated elevation of cAMP level. In case of cGMP, paraquat, not only abolished the histamine mediated slight elevation, it caused a further reduction in cGMP level. Consequently, the cGMP level in lung slices treated with paraquat (10^{-4} M) and histamine (10^{-5} M) at 2 and 4 min following the incubation were 50 and 63% of the levels obtained with histamine treatment alone, respectively.

The adrenergic beta agonist, isoproterenol is shown to raise the cAMP level of lung slices but has no effect on cGMP level[7]. In the present study, isoproterenol produced a similar effect on cyclic nucleotides levels in lung slices. For instance, isoproterenol at 10^{-5} M concentration raised the cAMP level of lung slices by 77, 47 and 52% at 2, 4 and 10 min respectively over that of corresponding controls but has little or no effect on cGMP level at 2 and 4 min. The preincubation of lung slices with paraquat (10^{-4} M) inhibited the cAMP elevating effect of isoproterenol only at 2 min and also caused a reduction in the cGMP level by 50% (table 2). How this inhibitory effect of paraquat against the histamine and isoproterenol induced changes of the cyclic nucleotide levels in the lung relates to pathophysiology of this lung toxicant is not understood.

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Ouabain potentiation of Ca release from fragmented cardiac sarcoplasmic reticulum from isolated cat heart

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Summary. The present study was performed to determine the effect of ouabain on Ca release from fragmented sarcoplasmic reticulum (FSR) isolated from cat cardiac muscles. The results clearly demonstrate that ouabain potentiates the Ca release from FSR by changing the ionic environment.

We have previously reported that microinjection of ouabain into crab muscle fibres produced a marked positive inotropic effect[1]. Furthermore, ouabain potentiated the contractile response in cardiac muscles under the condition of a Ca-free medium prepared by pretreatment with Dowex A-1, Ca-chelete resin[2,3]. These findings suggest that ouabain may produce a increase of Ca release from fragmented sarcoplasmic reticulum (FSR) isolated from cardiac muscles. Recently, it was reported that changing the ionic environment results in a potent release of Ca^{2+} from FSR and the effect is probably due to depolarization of skeletal FSR-membrane[4]. In the present study we tested whether or

not 1. Ca-release from cardiac FSR occurs by changing the ionic environment, and 2. ouabain potentiates the release.

Methods. FSR was isolated from cat cardiac muscles as microsomal fractions obtained biochemically by means of ultracentrifugation at 8000–35,000 × g by Weber's method[5] with modifications. After sufficient time (10 min) ^{45}Ca-uptake by FSR in a potassium methan-sulfonate (KMS) medium (KMS; 0.3 M, $MgCl_2$; 2 mM, Mg-ATP; 3 mM, total $CaCl_2$($CaCl_2$ + $^{45}CaCl_2$); 30 µM, Tris-maleate; 20 mM, pH 6.8, FSR; 0.2 mg protein/ml), 1 ml of the reaction mixture was filtered through a Millipore filter (0.45 µm). By this treatment almost all FSR adhered to the filter. 3 ml of potassium chloride washing medium (KCl; 0.3 M, MgCl; 2 mM, Tris-maleate; 20 mM, pH 6.8) was added in order to change the ionic environment and to remove remaining free ^{45}Ca, and 1 min later the washing medium was passed through the filter. The remaining ^{45}Ca in FSR was mesured. A KMS washing medium containing 30 mM caffeine was used in order to induce Ca release by caffeine. In the case of ouabain, a washing medium containing ouabain (2×10^{-6} M) was used. The time of ouabain treatment was 1 min.

Results. It was ascertained by electron microscopy that the SR preparation of these experiments contained almost completely 'intact' vesicles. Each vesicle exhibited tails and many granules (figure 1). The amount of total Ca bound by FSR was 42.1 ± 5.0 n moles/mg protein. As shown in figure 2, ouabain potentiated Ca release from FSR by changing the ionic environment. In control preparations, the amount of Ca release was about 10%, whereas in the presence of ouabain the amount of released Ca increased to one-half of the totally bound Ca. The amount of calcium released by caffeine was half the totally bound Ca, irrespective of whether ouabain was present or not, i.e. the caffeine-induced Ca release was not potentiated by ouabain.

Discussion. The results demonstrated that 1. Ca release from cardiac FSR occurs by changing the ionic environment, as those of skeletal FSR, and 2. ouabain potentiates the release. It was postulated using skeletal FSR, that depolarization-induced Ca release by changing the ionic environment is produced as follows: 1. membrane potential of SR is considered to be inside positive so that active transport of Ca^{2+} takes place through SR membrane from outside to inside. 2. MS^- must be less permeable than Cl^-. 3. Depolarization of FSR membrane caused by the replacement of MS^- with Cl^- brought about the release of Ca^{2+} (Kasai and Miyamoto[4]). It could be said that in cardiac FSR the depolarization-induced Ca release from FSR also occurs. The maximal possible Ca release from FSR isolated from rabbit leg muscles amounted to about one-half of the totally bound Ca[4]. The releasing capacity of ^{45}Ca from FSR may be one-half of the totally bound Ca. Ouabain potentiates Ca release from FSR by changing the ionic environment to the maximal level (figure 2). According to recent views, among the various means of inducing Ca release from SR, depolarization of SR membrane might play the most important role in physiological E-C coupling in skeletal muscles[6]. In the case of mammalian cardiac muscle, this view may also be applicable[7]. The depolarization-induced Ca release might indicate that the SR membrane has characteristics similar to those of excitable membranes[6]. It has often been pointed out that the positive inotropic action of cardiac glycosides appears to be due to an action on the process in membrane excitation[8–11]. Ouabain might act on 2 different excitable membranes in the same manner. However, the exact mechanism remains unknown. Several investigators have reported that cardiac glycosides decrease the rate of Ca-binding by FSR or increase the endogenous Ca fraction in FSR[12–14]. These phenomena might be related to our present findings. We showed already that, in cat papillary muscles driven electrically (6 times/min), ouabain of 2×10^{-6} M produces the maximum positive inotropic effect within 30 min under the condition of 20 °C[3]. Lee also used this concentration in his study on the effect of ouabain on FSR isolated mammalian heart[15]. It follows, then, that the concentration of ouabain used in this experiment is the optimum one to produce the positive inotropic action. Previous results from our laboratory demonstrated that ouabain occupies a considerable part of the cellular space of hearts when a positive inotropic

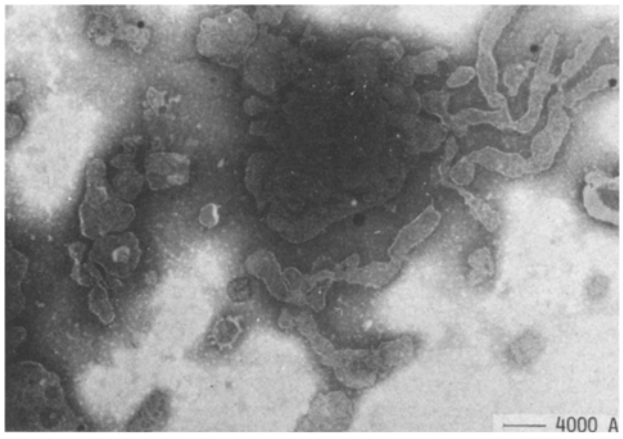

Fig. 1. Electron micrographs of negatively stained SR vesicles prepared from cat cardiac muscles (potassium phosphotungstate-stained). Each vesicle had tails and many granules.

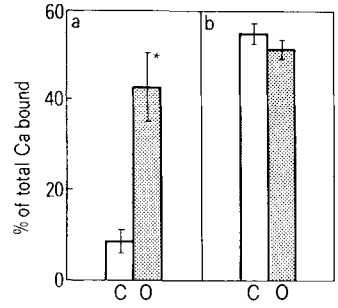

Fig. 2. Effect of ouabain (2×10^{-6} M, 1 min) on the Ca release from fragmented cat cardiac SR by changing the ionic environment (a) and by caffeine of 30 mM (b). Each column represents the mean values based on 6 observations and vertical bars indicate SEM. *$p < 0.01$ compared to control. C, control; O, ouabain. Further explanations see the text.

action is monitored[1,16,17]. In conclusion, it may be stated that ouabain potentiates Ca release from SR by changing the ionic environment and that this phenomenon is responsible for the positive inotropic effect of ouabain.

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Purification and properties of a heat-resistant exotoxin produced by *Macrophomina phaseolina* (Tassi) Goid in culture

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Summary. A partially purified preparation of a water-soluble, heat-resistant, nonspecific exotoxin produced by a strain of *Macrophomina phaseolina,* isolated from *Phaseolus mungo* L. could reduce Cu^{++} ions and produced a red colour with 2,4-dinitrophenyl hydrazine reagent. It caused inhibition of seed germination, wilting of cut seedlings, stunted growth of young seedlings and loss of permeability of the cell membrane. Seedlings of *P. mungo,* grown in presence of the toxin showed a slight increase in the contents of protein and total RNA over control, but a significant increase in the specific activities of F-1, 6-BP aldolase and G-6-P isomerase.

An internally seed-borne fungus, found to cause serious damping-off of seedlings of *Phaseolus mungo,* was isolated and identified as *Macrophomina phaseolina* (Tassi) Goid. The fungus produced an exotoxin and was also strongly cellulolytic. Production of a nonspecific extracellular toxin by *M. phaseolina* and its role in pathogenesis and inhibition of seed germination have already been reported by Mathur[1] and Chan and Sackston[2]. The present communication deals with partial purification of the toxin, some of its physical and chemical properties, and some biochemical changes induced by it.

Material and methods. The fungus was isolated from surface sterilized (0.1% $HgCl_2$ solution) seeds of *P. mungo* L. (var. L22) and was brought into pure culture by dilution plating on Czapek's agar. It was identified as *M. phaseolina.* The toxin was produced in Czapek's liquid medium (pH 6.8) by the fungus in stationary culture. The culture filtrate was found to be also strongly cellulolytic. For isolating the toxin, the fungus was grown in 250-ml Erlenmeyer flasks containing 50 ml of the liquid medium at 30 °C for 15 days, and the mycelial mat was removed by filtration through Whatman No. 1 filter paper. The culture filtrate was treated with activated charcoal (Norit, E. Merck, 20 g/l) for 1 h at room temperature. The charcoal with the adsorbed toxin was collected by filtration and dried at 40 °C in an air-oven and then extracted with chloroform. The eluate was filtered and the filtrate was evaporated to dryness under reduced pressure at 30 °C. The residue was dissolved in a minimal quantity of ethanol and diluted with distilled water, filtered to remove insoluble matters and shaken with equal volume of diethylether in a separating funnel to eliminate fatty substances. The aqueous phase was collected and evaporated to dryness under reduced pressure at 80 °C to obtain a viscous liquid. The preparation so obtained was used for determining the properties of the toxin. It was free from cellulolytic activity.

The hypocotyl regions of treated and untreated seedlings were cut out and 1 g (fresh weight) of each was homogenized in a prechilled pestle and mortar with analytical sand. The homogenate was extracted with 5 ml of tris-HCl buffer (pH 7.4) and the extract after centrifugation at 12,000 × g for 10 min was dialyzed against the same buffer for 2 h at 4 °C. The dialyzed extract was used for enzyme assays and for protein determinations. F-1, 6-BP aldolase and G-6-P isomerase were assayed following the methods of Sibley and Lehninger[4] and Nosoh[5], respectively. Protein and total RNA were determined by Folin reagent[6] and the method of Markham[7], respectively. The isozyme patterns of the following enzymes were studied by polyacrylamide gel-electrophoresis, according to the methods of Arnisson and Boll[8]: peroxidase, polyphenol oxidase, catalase, glutamate dehydrogenase and malate dehydrogenase.

Results and discussion. The purified preparation of the toxin was found to be chromatographically homogenous, because it produced a single spot on thin-layer chromatograms using n-butanol:acetic acid:water (8:2:2), or ethanol:ammonia:water (80:5:15) as developing solvents and 5% chromic acid solution as spraying reagent. Paper chromatograms developed with the same solvent systems and sprayed with 2,4-dinitrophenyl hydrazine (DNPH) solution (0.1% in 2N HCl) followed by 10% NaOH solution also showed single spots, reddish in colour and having a R_f value of 0.85. That the spots were due to the toxin was confirmed by eluting parallel spots in distilled water and using the solution for seed germination test. The reaction with the DNPH solution indicated the presence of a carbonyl group in the toxin compound.

An aqueous solution of the toxin had a pH value of 4.8, and it did not lose its toxic property when autoclaved at 121 °C for 15 min. It was found to have reducing property, as indicated by its ability to reduce Cu^{++} to Cu^{+} ions using Somogyi's reagent[3].