Effect of Cations and Some Compounds on Paraquat Accumulation into Cultured Pneumocytes

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SAITO, K. *Effect of Cations and Some Compounds on Paraquat Accumulation into Cultured Pneumocytes.* Tohoku J. exp. Med., 1986, **148** (1), 41-47 — Paraquat and diquat, bipyridilium herbicides, have been shown to be accumulated into pneumocytes of three species in culture. Paraquat at 10^{-3} M was accumulated to a maximum of 3-7 nmol/mg protein for 20 min. Diquat accumulated more slowly but reached a concentration of 1-6 nmol/mg protein for 60 min. Differences in the ability of various pneumocytes to accumulate paraquat and diquat appear to exist. It was found that the cations, Ca^{++} , Na^{+} and K^{+} , reduced the accumulation of paraquat. Valinomycin stimulated paraquat accumulation significantly. On the other hand, paraquat accumulation was inhibited by 2, 4-dinitrophenol. These results indicate that paraquat may be accumulated, in part, as cation species into pneumocytes and also by a energy-dependent process regulated by cellular ATP. — paraquat; valinomycin; diquat; 2, 4-dinitrophenol; pneumocytes

The herbicide paraquat (1,1'-dimethyl 4,4'-bipyridilium, PQ) has been known to produce wide spread edema and fibrosis in the lung of human and experimental animals (Dasta 1978; Rebello and Mason 1978; Haley 1979; Parkinson 1980; Schoenberger et al. 1984). After the first discovery of PQ accumulation into lung slices by energy-dependent uptake process (Rose et al. 1974), additional experiments have been performed to assess the ability of various compounds to inhibit the accumulation of PQ into lung slices (Drew et al. 1979; Siddik et al. 1979; Rose and Krieger 1981). The mechanism of PQ accumulation into lungs is however not fully understood. Previously, PQ^{++} ion has been demonstrated to be a good electron acceptor because of the stability of the reduced free radical ion, PQ^{+} , and to form charge transfer complex with anions resulting in its precipitation (Nakahara and Wang 1963). It has been suggested that cations are able to displace PQ from melanin and cartilage in mice (Larson et al. 1978). The processes regulating PQ accumulation may play an important role in the etiology of PQ-induced lung damage per se. In the present study, the

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accumulation of PQ and diquat (N, N'-ethylene 2, 2'-bipyridilium, DQ), a compound related to PQ, into cultured pneumocytes and the effects of cations and exogenous compounds on PQ accumulation into the cells were examined.

MATERIALS AND METHODS

Cell culture. Three established cell lines of pneumocytes, L-2 cells derived from adult rat lung (Douglas and Kaigh 1974), AK-D cells derived from embryonic feline lung (Kniazeff et al. 1976) and A-549 cells derived from adult human lung (Lieber et al. 1976) were obtained from American Type Culture Collection (Rockville, MD, USA). L-2 cells and AK-D cells were cultured in F-12K culture medium supplemented with 20% and 15% of fetal calf serum (FCS), respectively, A-549 cells were cultured in Dulbecco's modified minimum essential medium with Earle's salts (MEM) supplemented with 10% FCS in plastic tissue culture flasks at 37°C in an incubator. Tissue culture media were supplemented with 100 units/ml of penicillin G and 100 μ g/ml of streptomycin. The tissue culture media were changed at 24 hr before the experiments of PQ accumulation. The cells grown to confluent were used for the experiments.

PQ and DQ accumulation into the cells. PQ and DQ were dissolved in Tyrode's solution, pH 7.4 at a final concentration of 10^{-3} M. Cells were washed three times with Tyrode's solution (37°C) and were incubated with 10^{-3} M of PQ and 0.05 μ Ci/ml of 14 C-PQ (specific activity; 120 mCi/m mol) or 10^{-3} M of DQ and 0.05 μ Ci/ml of 14 C-DQ (specific activity; 120 mCi/m mol) in Tyrode's solution for indicated times in the text at 37°C in an incubator. At the end of each incubations, cells were washed with ice cold Tyrode's solution containing 10^{-3} M of cold PQ or DQ to prevent the efflux of radioactive PQ and DQ accumulated into the cells. Cells were solubilized in 1N NaOH and aliquots were taken to measure protein concentration by the method of Lowry (Lowry et al. 1951). Protein concentration was standardized with calf serum albumin. Other aliquots were neutralized with 1 N HCl and used to count the cellular radioactivity of 14 C-PQ and 14 C-DQ in POPPOP liquid scintillation cocktail by liquid scintillation techniques. The concentrations of PQ and DQ accumulated into the cells were expressed as nmol per mg protein.

Effect of cations on PQ accumulation. The following solutions were prepared to observe the effect of cations on PQ accumulation into the cells; isotonic sucrose-Tris buffer solution, isotonic sucrose- K_2HPO_4 solution, Tyrode's solution, isotonic NaCl-Tris buffer solution, isotonic KCl-Tris buffer solution, $CaCl_2$ (10^{-2} M and 10^{-3} M) in isotonic sucrose-Tris buffer solution. The pH of these solutions was adjusted to 7.4. The cells were washed three times with each solutions (37°C) and were incubated for 2 hr in the solution containing 10^{-3} M of PQ and 0.05 μ Ci/ml of 14 C-PQ and 0.05 μ Ci/ml of 14 C-DQ at 37°C. At the end of incubation, cells were washed with the same incubation solution containing 10^{-3} M of PQ at 4°C. PQ concentrations per mg protein were determined as described above and expressed as percentages of control concentration in isotonic sucrose-Tris buffer solution.

Effect of exogenous compounds on PQ accumulation. Valinomycin (10^{-3} and 10^{-4} M), putrescine (10^{-3} M), norepinephrine (10^{-3} M), 5-hydroxytryptamine (10^{-3} M) and 2, 4'-dinitrophenol (10^{-3} and 10^{-4} M) were dissolved in isotonic sucrose-Tris buffer solution, pH 7.4. After washing the cells with warm isotonic sucrose-Tris buffer solution (37°C), cells were incubated with 10^{-3} M of PQ and 0.05 μ Ci/ml of 14 C-PQ and with each exogenous compounds at 37°C for 2 hr. PQ concentrations per mg protein were determined as described above, and expressed as percentage of control value. The statistic analysis of the percent uptake of PQ was performed by Student's *t* test. The radioactive 14 C-PQ and 14 C-DQ were obtained from Amersham (Arlington Height IL, USA). Tissue culture materials were obtained from GIBCO (Gland Island, NY, USA). Other chemical reagents were obtained from Sigma Chem. Co., (St. Louis, MO, USA).

RESULTS

PQ and DQ accumulation. The accumulation of PQ and DQ in Tyrode's solution by three types of cultured pneumocytes was examined. L-2 cells accumulated PQ with time to a maximum of 7 nmol/mg protein by 60 min and DQ to that of 6 nmol/mg protein by 180 min (Table 1). AK-D cells accumulated a maximum of 3-4 nmol PQ/mg protein by 30 min, but did not accumulate higher amounts of PQ during incubation up to 60 min. DQ was accumulated by AK-D cells to a maximum of 2 nmol/mg protein by 30 min (Table 1). A-549 cells also attained a concentration of PQ of 3 nmol/mg protein by 20 min. A-549 cells accumulated less DQ, 1 nmol/mg protein in 60 min (Table 1). Cell viability was unaffected for up to 2 hr incubation with either PQ and DQ (trypan blue exclusion).

Effect of cations on PQ accumulation. In all cells examined, PQ was accumulated to a greater extent in isotonic sucrose (low ionic strengths) solution than in Tyrode's solution. PQ accumulation was reduced to approx. 25% in Tyrode's solution (Table 2). The decreased accumulation appeared to be due to Ca^{++} , but Na^+ and K^+ ions also inhibited the accumulation of PQ during 2 hr incubation. PQ concentration was decreased to 38% by 10^{-2} M of Ca^{++} and to 50% by 10^{-3} M of Ca^{++} in L-2 cells and AK-D cells (Table 2). Isotonic Na^+ and K^+ inhibited PQ accumulation to 45% and 60% in L-2 cells and A-549 cells, respectively (Table 2).

Effects of some compounds on PQ accumulation. Valinomycin (10^{-4} M) was stimulatory for PQ accumulation into L-2 cells and AK-D cells, producing to approx. 180% increase (Table 2). Of the amines tested, putrescine (10^{-3} M) had no effect on PQ accumulation into L-2 cells and A-549 cells (Table 2). Norepinephrine (10^{-3} M) inhibited PQ accumulation in L-2 cells and AK-D cells to approx. 60% (Table 2). 5-Hydroxytryptamine also inhibited PQ accumulation to 50%-

TABLE 1. Accumulation of paraquat and diquat into the cells

	L-2 cells (n=4)		A-549 cells (n=4)		AK-D cells (n=4)	
Time (min)	Concentration (10 ⁻³ M)					
	PQ	DQ	PQ	DQ	PQ	DQ
10	—	—	1.9±0.1	—	2.5±0.2	1.4±0.1
20	—	—	3.1±0.1	—	2.9±0.5	1.9±0.2
30	2.3±0.3	3.2±0.3	3.2±0.1	0.6±0.4	3.2±0.9	2.2±0.2
60	6.8±0.5	4.1±0.9	2.6±0.4	1.1±0.3	2.4±0.1	2.2±0.1
180	7.2±0.8	6.7±0.7	—	—	—	—

The cells were incubated with 10^{-3} M of paraquat and diquat in Tyrode's solution at 37°C for 180 min in L-2 cells and 60 min in A-549 cells and AK-D cells.

The values given are means $\pm$ s.d. of the concentrations (nmol/mg protein) of paraquat or diquat in the cells.

TABLE 2. *Effect of cations and some compounds on PQ accumulation into the cells*

Substances	Uptake of PQ (%)		
	L-2 cells (n=6)	A-549 cells (n=3)	AK-D cells (n=3)
Isotonic sucrose-Tris buffer solution	100.0± 7.5	100.0± 5.0	100.0± 6.9
Isotonic sucrose-K ₂ HPO ₄ solution	66.9± 6.5*	—	—
Tyrodé's solution	23.2± 1.3*	27.9± 1.6*	21.3± 7.2*
Isotonic NaCl-Tris buffer solution	48.8± 3.6*	43.1± 2.2*	—
Isotonic KCl-Tris buffer solution	65.8± 7.8†	63.4± 5.1*	—
CaCl ₂ (10 ⁻² M) in sucrose-Tris solution	37.8± 6.9*	—	38.7± 9.3*
CaCl ₂ (10 ⁻³ M) in sucrose-Tris solution	41.3± 4.2*	—	57.5± 2.8*
Valinomycin (10 ⁻⁴ M)	187.4± 43.5*	—	167.1± 6.7*
Valinomycin (10 ⁻⁵ M)	96.6± 11.9	—	—
Putrescine (10 ⁻³ M)	107.1± 17.0	93.4± 11.2	—
Norepinephrine (10 ⁻³ M)	51.2± 2.8*	—	71.0± 9.8†
5-Hydroxytryptamine (10 ⁻³ M)	47.2± 3.2*	74.4± 4.3†	—
2, 4-Dinitrophenol (10 ⁻³ M)	37.4± 3.9*	—	—
2, 4-Dinitrophenol (10 ⁻⁴ M)	37.0± 2.8*	—	—

* $p < 0.001$; † $p < 0.01$.

Cells were incubated with PQ at 10⁻³ M in ionic solutions and in isotonic sucrose-Tris buffer solution with or without substances indicated in the table for 2 hr at 37°C.

The values given are means ± S.D.

60% in L-2 cells and A-549 cells (Table 2). PQ accumulation was markedly reduced to 37% by a treatment of 2, 4-dinitrophenol at 10⁻³ M and 10⁻⁴ M in L-2 cells (Table 2).

DISCUSSION

In the present study, PQ and DQ were accumulated with time into cultured pneumocytes derived from rat, feline and human lung. It seemed that there are saturable PQ and DQ-binding sites in these cells and that differences in PQ and DQ accumulation might be exist in cell types. It has been suggested that one electron reduction of bipyridilium salts is achieved electrochemically and can be measured spectrophotometrically at $\lambda_{\max}$ 603 nm for PQ and at $\lambda_{\max}$ 760 nm for DQ. Reduction of bipyridilium salts does not stop at one electron stage but can proceed further to give a putative tetrahydro product corresponding to an overall electron reduction, as follows :

Of the cations examined, Na⁺ and K⁺ ions were the potent inhibitors on PQ accumulation into the cells. Valinomycin, known as a K⁺ specific ionophore (Pressman 1976), stimulated PQ accumulation into the cells. This result supports

the hypothesis that extracellular PQ is translocated as a monovalent cation into the cells. Ca^{++} was found to be the most potent cationic inhibitor of PQ accumulation. It is thought that Ca^{++} is bound to endoplasmic reticulum in cells (Moolenaar et al. 1984; Morris et al. 1984) and regulates cell functions (Chafouleas et al. 1984). Phosphatidylcholine of pulmonary surfactant is known to be synthesized at endoplasmic reticulum of alveolar type II cells (Batenburg 1985). PQ has been shown to impair phospholipid metabolism including phosphatidylcholine synthesis in A-549 cells (Saito et al. 1982.) Thus, PQ might antagonize the functions of Ca^{++} in the cells examined.

It has been shown that DQ is not accumulated by lung slices (Rose and Smith 1977). In the present study, DQ was accumulated into all of the cells in a time-dependent manner. The difference between lung slices and cultured cells is not clear, but there is a possibility that DQ may also be accumulated into the cells as cations.

It has been suggested that 2,4-dinitrophenol, a classic mitochondrial uncoupler, alters ATP levels in many cell types (Korhl et al. 1977; Reeves 1975). PQ accumulation into the cells was markedly inhibited by this compound. This result suggests that PQ accumulation into the cells in culture is an energy-dependent process.

The lungs have been known to accumulate a variety of amines (Gillis and Iwasawa 1972; Junod 1972; Lau and Henderson 1978). Other reports have demonstrated that basic amines inhibit the PQ accumulation into lung slices (Lock et al. 1976; Mailing et al. 1978; Drew et al. 1979). In the present study, the effect of amines on PQ accumulation into the cells was examined. Norepinephrine and 5-hydroxytryptamine were found to have an inhibitory effect on PQ accumulation into the cells. The mechanisms of the inhibition of PQ accumulation into the cells by these amines are not clear, a simple competition between PQ and these amines may account for this phenomenon.

In conclusion, PQ appears to be accumulated, in part, as a cation involving an energy dependent (ATP requiring) uptake process.

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