Bull. Environ. Contam. Toxicol. (1999) 62:570-577

Bulletin of Environmental Contamination and Toxicology

Toxic Action of the Herbicide 2,4-D on the Neuroepithelial Synapse and on the Nonstimulated Skin of the Frog *Caudiverbera caudiverbera*

M. Suwalsky,[1] L. Quevedo,[2] B. Norris,[2] M. Benites[1]

[1] Faculty of Chemical Sciences, University of Concepción, Casilla 160-C Concepción, Chile
[2] Faculty of Biological Sciences, University of Concepción, Chile

Received: 20 November 1998/Accepted: 3 March 1999

Herbicides are compounds manufactured for the sole purpose of destroying weeds and to control woody plants, in an attempt to increase crop production, and their use has increased markedly in the past two decades (Klaassen 1996). Some of these compounds are highly toxic and have caused human fatalities. There is an increase in concern about the health risks of herbicides because they enter into the drinking water supply. 2,4-D (2,4-dichlorophenoxyacetic acid) is one of the most widely used herbicides (Clausen et al. 1990). In Vietnam it was employed to defoliate jungle areas, as a component of the "agent orange" (Macilwain 1993). Extensive research has not resolved its putative carcinogenicity, mutagenicity and genotoxicity (Macilwain 1993) and very little work has been done on its effects on the peripheral nervous system. Its 2.81 partition coefficient (expressed as log K (o/w), Noble 1993) and solubility in non polar solvents suggest that this compound accumulates in membrane lipids and may cause toxic effects primarily by inducing changes in membrane organization (Blasiak 1995, Antunes-Madeira et al. 1993, 1994). Neurotoxic actions of some chemicals are due to interference with nerve transmission (Quevedo et al. 1988; Montoya and Quevedo 1990; Tilson 1993; Blusztajn and Davis 1995; Kilburn and Thornton 1995).

In the course of in vitro systems search for the toxicity screening of chemicals, different cellular models have been applied to examine the adverse effects of pesticides in isolated organs (McCarthy and Shugart 1990). A model for ion movements across epithelia is the frog nerve-skin preparation. Electric stimulation of the synapse between sympathetic nerve endings and skin mucous glands has long been known to induce a transient rise in the bioelectric parameters. These are the transmembrane potential difference (PD) and the short-circuit current (SCC) across the skin (Norris et al., 1993; Norris and Quevedo, 1995a; Norris et al., 1995b). The rise is due to an increase in active Cl^- transport by the mucous glands (Thompson and Mills 1981). The PD and SCC are kept at a steady basal level by means of active Na^+ transport (see review by Ussing 1994). This work examines the effect of 2,4-D on the response of the frog neuroepithelial synapse to electrical stimulation and on the bioelectric parameters of the non-stimulated skin.

Correspondence to: M. Suwalsky

MATERIALS AND METHODS

Experiments were performed on frogs of the species *Caudiverbera caudiverbera* (180-350 g) collected from fresh water ponds during the spring and summer months (October to March). The amphibians were kept in tap water at room temperature (18-22°C) at least 24 hr before use, and fed on sow bugs (*Oniscus asellus*). The frogs were pithed; the cutaneous branch of the tibial nerve supplying part of the skin of the hindleg was isolated together with the attached piece of skin and mounted between perspex Ussing chambers. An area of 1.33 cm^2 was exposed to 3.5 mL phosphate-buffered (pH 7.5) Ringer's solution on both surfaces and oxygenated with a stream of air. The composition of the solution was (mM): Na^+ 114, K^+ 2.5, Cl^- 117.5, Ca^{2+} 2.0, HCO_3^- 2.3 and glucose 11. The SCC was monitored with non-polarizable Ag/AgCI electrodes placed at 15 mm distance from the epithelium and connected to a voltage-clamp circuit (G. Métraux Electronique) set to keep the PD across the skin at zero mV. The PD was measured with calomel-agar electrodes at intervals of 2 min for 4 sec. Both parameters were displayed on a two channel Cole-Parmer recorder. Experiments were started 30 min after the bioelectric parameters of the preparation had reached a steady level. For electric stimulation the nerve was placed on a pair of Ag electrodes connected to the isolation unit of a Grass S44 stimulator. Square wave pulses of 4 msec duration at a rate of 10 Hz and 10 V for 30 sec were used. Preparations were stimulated at regular intervals (30 min). The herbicide from Aldrich (99% purity) was used without further purification and was added in an aqueous suspension to the solution bathing the inner (serosal) surface of the skin in the final equivalent concentrations indicated in the text. Values throughout the work refer to means ±S.E.M. for each neuroepithelial synapse and for each non-stimulated skin. Statistical analysis was performed using Student's *t* test for paired samples.

RESULTS AND DISCUSSION

Although a few synapses showed responses of slightly different form and amplitude, for each preparation, the control responses, consisting of a transient increase in the bioelectric parameters, were stable (Fig.1 and Table 1). Therefore the electrical changes were expressed as percentages of the control values (Table 2). Each experiment lasted eight to ten hr and usually only one set of readings was made for each neuroepithelial synapse. At least thirty percent of the neuroepithelial synapses stored overnight at 0°C showed similar responses the next day; these second readings have not been included in the statistics, as the SCC often decreased to half the original value. Fig. 1 shows that the addition of aliquots of Ringer's solution to the inner surface of the preparation did not alter the effect of stimulation.

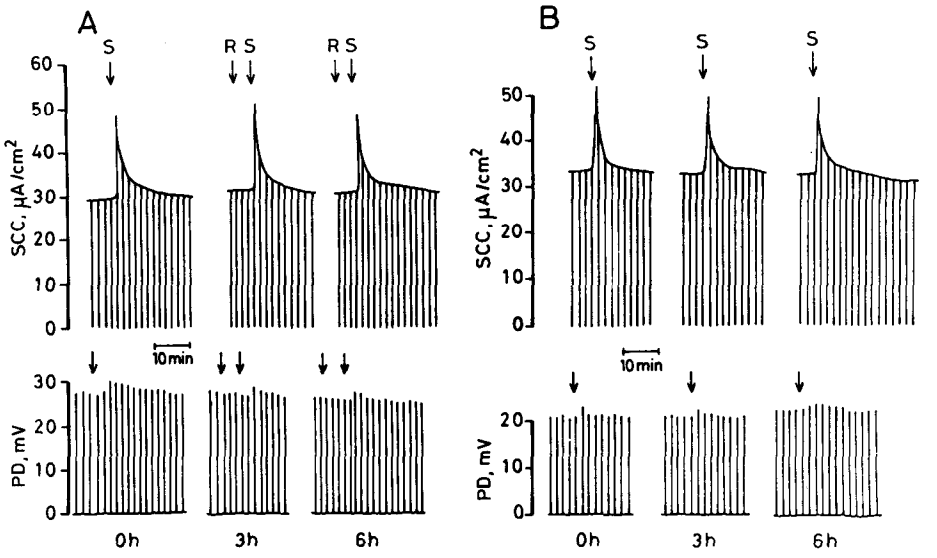

Figure 1. Control experiment showing records of the responses of the frog neuroepithelial synapse to electrical stimulation. A) Tracing taken during the first day. B) Tracing taken during the second day after overnight storage at 0°C. SCC=short-circuit current; PD=potential difference; S=stimulation; R=aliquot of Ringer's solution applied in the inner bathing solution.

Table 1. Stimulatory responses of frog *C. caudiverbera* neuroepithelial synapse to electric stimulation during a time period of 8 hours.

	Stimulatory responses		
	0 h	4 h	8 h
SCC, µA/cm²	10.5 ± 1.3 (45.7 ± 2.5)	10.8 ± 1.4	10.6 ± 1.4
PD, mV	3.8 ± 0.4 (34.0 ± 2.7)	3.8 ± 0.5	3.8 ± 0.6

Values are means ± SEM, n=12. Figures in parentheses are the values of the bioelectric parameters of the skin in the non-stimulated preparation. SCC=short-circuit current, PD=potential difference.

The increase in SCC usually consisted of two main components. The first one was a rapid rise in current from 36.4 ± 4.4 µA/cm² to 45.8 ± 4.34 µA/cm² (Fig. 2). The peak was reached in 0.39 ± 0.03 min and the duration of the rapid rise was 1.02 ± 0.07 min (n = 22). The second component consisted of a slow rise when the first component was

declining: the peak was very variable, usually smaller than that of the first rise. The profile of the rise in PD was similar although always smaller in magnitude than that of the rise in SCC: a rapid initial component rose from 33.6 ± 3.1 mV to 36.2 ± 3.2 mV. Since the slow component was nearly continuous with the rapid component and very difficult to measure, it was not further analysed. The values throughout the work refer to the initial rapid rise in SCC and in PD. In 12 synapses, stimulation of the nerve every 30 min for a period of 8-10 hr induced repetitive responses which did not decline significantly in magnitude (Table 1).

Increasing concentrations (equivalent to 0.01 m*M* up to 1.0 m*M*) of 2,4-D produced a concentration-dependent reduction of the response to stimulation. Smaller concentrations had no effect. Fig. 2 shows a single experiment illustrating this reduction. Table 2 reveals a 69% decrease in the bioelectric parameters; this decrease was reached after 132.0 ± 16.5 min (n = 14). These effects were usually reversible (in 12 out of 14 experiments) after removal of the chemical by a threefold washout. Observation of the records showed that the second component disappeared in most of the experiments when blockade was nearly complete. On the other hand, the bioelectric parameters of the non-stimulated skin were not significantly altered, although they decreased slightly (Table 2).

The acute effects reported for the concentrations used may be connected to toxic effects in human subjects after 2-3 hr exposure to 2,4-D; lethal doses ranging from a serum concentration of 0.26 mM in one patient, to 3.7 mM in another, have been described (Durakovic et al. 1992); necrospermia was found in a patient with a urinary concentration of 0.03 mM; and a fatal outcome was described after ingestion of about 0.3 mM.

The effects of most pesticides appear to be membrane-connected due to their lipophilicity and the dynamic function of biomembranes (Jain 1988; Suwalsky et al. 1990; Antunes-Madeira and Madeira 1993; Antunes-Madeira et al. 1994). In fact, partition studies indicate that membrane fluidity and pesticide structure are main parameters affecting pesticide incorporation and potential toxicity (Videira et al. 1996). The lipid-protein interfaces are apparently accessible to pesticides which then affect protein structure and function (Antunes-Madeira et al. 1994). Lundbaek et al. (1996) showed that structural perturbation of lipids affects the functioning of ion channels. The effect of a pesticide on the membrane properties could be due to one or more of four mechanisms: a) entirely lipid with no protein involved, that is, a loss of bilayer integrity and a decreased resistance across the bilayer; b) a lipid perturbing effect, changing the bulk properties of the lipid bilayers in a way that alters the lipid-protein interaction such as to favour certain protein conformational states; c) specific interaction with a part of a protein receptor facing the

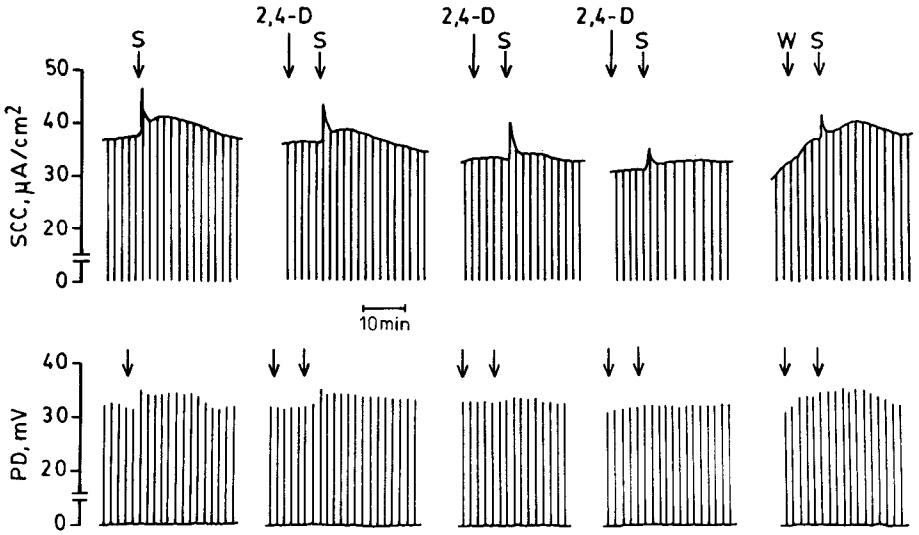

Figure 2. Single experiment illustrating effects of increasing (0.01m*M*, 0.1 m*M* and 1.0 m*M*) concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) applied in the inner bathing solution, on the response of the frog neuroepithelial synapse to electrical stimulation. PD=potential difference; SCC=short-circuit current; S=stimulation; W=washout.

Table 2. Effect of increasing concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D, inner solution) on the frog neuroepithelial synapse stimulatory response after electrical stimulation, and on the bioelectrical parameters of the non-stimulated skin.

	Neuroepithelial synapse		Non-stimulated skin	
Pesticide	% decrease in the PD stimulatory response	% decrease in the SCC stimulatory response	% decrease in PD	% decrease in SCC
2,4-D				
0.01 mM	26.6±2.9^{NS}	12.4±1.6^{NS}	3.8±0.9	3.6±0.9
0.1 mM	47.2±3.1*	39.9±3.6*	6.7±1.1	6.9±1.2
1.0 mM	68.9±3.4**	69.6±3.3**	9.2±1.8^{NS}	9.7±1.5^{NS}

Results are expressed in percent decrease ± SEM, n=14, for the stimulatory response of the PD (potential difference) and of the SCC (short-circuit current) over the basal values of the non-stimulated synapse: and for the values of the electrical parameters of the non-stimulated skin. Significantly different from the response to nerve stimulation in the absence of pesticide: *p<0.05; ** <0.01; response of non-stimulated skin: NS = not significant (Student's paired t test).

surrounding bilayer, and d) specific interaction with a part of a protein receptor facing the cytoplasm. The first mechanism may be discarded since the electrophysiologic studies in the current work did not show significant changes in resistance. Evidence which favours the second, non-specific mechanism, comes from studies on phospholipid multilayers and liposomes by X-ray diffraction and fluorescence spectroscopy, respectively (Suwalsky et al. 1996). In fact, it was shown that 2,4-D deeply perturbed the structure of dimyristoylphosphatidylcholine, a class of phospholipid present in the outer monolayer of cell membranes (Devaux and Zachowsky 1994). On the other hand, the same authors found that 0.01, 0.1 and 1.0 m*M* 2,4-D induced human erythrocyte crenation, an effect explained by the pesticide insertion into the outer monolayer of the lipid moiety of the membrane. Therefore 2,4-D, which has a high partition coefficient, can dissolve readily in the lipid moiety of the nerve fiber membrane and interfere with ion transport (Chefurka et al. 1987).

The experiments on the neuroepithelial synapse are in agreement with the interference of 2,4-D with active Cl^- secretion in the mucous glands. Unpublished data from our laboratory showed that isethionate Ringer's solution blocked the response to nerve stimulation. Gonzalez et al. (1989) found that Cl^- transport could be largely accounted for by cAMP dependent processes and that it is contingent on a Na^+-K^+-2Cl^- co-transport system in the basolateral membrane; this process is driven by Na^+/K^+ ATPase (Butt et al. 1994). At the other membrane, Cl^- exits passively via Cl^- channels; however, variations on this basic theme may be observed in several epithelial tissues (see review by Fong et al. 1995). Epithelial cells harbour a wide variety of Cl^- channels which display a spectrum of biophysical and regulation characteristics (Begenisich and Melvin 1998). Among important pathways are the channels regulated by G proteins and by cAMP. If 2,4-D affects integral membrane protein conformation, then channel function will be altered, in accord with the third mechanism. On the other hand, inhibition of ATPase enzymes by 2,4-D has not been excluded (Antunes-Madeira et al. 1993). Norris et al. (1993) found that amiloride did not affect the rapid component of the response; however, the slow component often disappeared, a finding which was also observed in the presence of 2,4-D and which points to decreased Na^+ transport. Because there are several putative mechanisms of action, it appears that the effects of the herbicide are non-specific and that it does not destroy the phospholipid. The slight effect on the non-stimulated skin may be considered in the light of the importance of the glandular mechanism in the overall values of the bioelectric parameters.

To conclude, 2,4-D caused a dose-dependent reduction in the response of a sympathetic junction of the frog Caudiverbera caudiverbera to nerve stimulation, possibly by a non-specific lipid-perturbation mechanism and

interference with membrane properties such as protein conformation and /or interaction with protein receptors leading to glandular Cl^- channel inhibition.

Acknowledgments. This study was supported by grants from FONDECYT (1960680) and DIUC (94.33.75-1 and 98.24.19-1).

REFERENCES

Antunes-Madeira MC, Madeira VMC (1993) Effect of DDT on the fluidity of model and native membranes: implications for the mechanisms of toxicity. Biochim Biophys Acta 1149: 86-92.

Antunes-Madeira MC, Videira RA, Madeira VMC (1994) Effects of parathion on membrane organization and its implications for the mechanisms of toxicity. Biochim Biophys Acta 1190: 149-154.

Begenisich T, Melvin JE (1998) Regulation of chloride channels in secretory epithelia. J Membrane Biol 163: 77-85.

Blasiak J (1995) Changes in membrane fluidity evoked by organophosphorus insecticide bromfenvinfos and its methylated analogue. Comp Biochem Physiol 110c: 15-21.

Blusztajn JK, Davis RD (1995) Use of differentiated cholinergic and second messenger endpoints to evaluate cholinesterase inhibitors. Clin Exp Pharmacol Physiol 22: 368-369.

Butt AG, McLaughlin CW, Bowler JM, Purves RD, Macknight ADC (1994) Cell Cl and transepithelial Na transport in toad urinary bladder. J Membrane Biol 142: 9-20.

Chefurka W, Chatelier RC, Sawyer WH (1987) Perturbation of phospholipid layers by DDT. Biochim Biophys Acta 896: 181-186.

Clausen M, Leier G, White I (1990) Comparison of the cytotoxicity and DNA-damaging properties of 2,4-D and U 46 D fluid (dimethylammonium salt of 2,4-D). Arch Toxicol 64: 497-501.

Devaux PF, Zachowsky A (1990) Maintenance and consequences of membrane phospholipid asymmetry, Chem Phys Lipids 73: 107-120.

Durakovic Z, Durakovic A, Durakovic S, Ivanovic D (1992) Poisoning with 2,4-dichlorophenoxyacetic acid treated by hemodialysis. Arch Toxicol 66: 518-521.

Fong P, Jentsch TJ (1995) Molecular basis of epithelial Cl channels. J Membrane Biol 144: 189-197.

Gonzalez CS, Norris BC, Concha JB, Contreras GM (1989) Comparative effects of catecholamines, angiotensin II and antidiuretic hormone on chloride transport in toad skin. Cell Mol Biol 35: 55-61.

Jain M (1988) In: Introduction to Biological Membranes, pp. 10-51. Wiley, New York.

Kilburn KH, Thornton JC (1995) Protracted neurotoxicity from chlordane sprayed to kill thermites. Environ Health Perspect 103: 690-694.

Klaassen CD (1996) Nonmetallic environmental toxicants. In: The Pharmacological Basis of Therapeutics, pp. 1673-1696. 9th ed. Eds.: Hardman, J.G. and Limbird, L.E. McGraw Hill, New York.

Lundbaek JA, Birn P, Girschman J, Hansen J, Andersen OS (1996) Membrane stiffness and channel function. Biochemistry 35: 3825-3830.

Macilwain C (1993) US Congress urged to back further Agent Orange studies. Nature 364: 373.

McCarthy J, Shugart LR, editors (1990) Biological markers of environmental contamination, pp. 3-14. In: Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, Florida, OH.

Montoya G, Quevedo L (1990) The effects of pentachlorophenol (PCP) on the toad neuromuscular function. Comp Biochem Physiol 995: 193-197.

Noble A (1993) Partition coefficients (n-octanol-water) for pesticides. J Chromatogr 642: 3-14

Norris B, Concha J, Contreras G, Contreras E (1993) Calcium channel blockers apparently decrease noradrenaline release from nerve-skin terminals in *Caudiverbera caudiverbera.* Gen Pharmacol 24: 971-976,

Norris B, Quevedo L (1995a) Adverse effects of polluted continental water bodies in Chile on frog adrenergic synapse. Bull Environ Contam Toxicol 57: 640-647.

Norris B, Núñez G, Contreras G, Contreras E (1995b) Diazepam decreases the response to the electrical stimulation of the nerve-skin preparation of the toad *Caudiverbera Caudiverbera.* Gen Pharmacol 26: 1607-1611.

Quevedo L, Neumann V, Schmidt E, Cárdenas H (1988) Action of lycorine on noradrenergic response of a nerve-skin preparation. Cell Mol Biol 34: 295-302.

Suwalsky M, Espinoza MA, Bagnara M, Sotomayor CP (1990) X-ray and fluorescence studies on phospholipid bilayers. IX. Interactions with pentachlorophenol. Z. Naturforsch 45c: 265-272.

Suwalsky M, Benites M, Villena F, Aguilar F, Sotomayor CP (1996) Interaction of 2,4-dichlorophenoxyacetic acid (2,4-D) with cell and model membranes. Biochim Biophys Acta 1190: 149-154.

Thompson IG, Mills JW (1981) Isoproterenol-induced current changes in glands of frog skin. Am J Physiol 241 (Cell Physiol 10): C250-C257.

Tilson HA (1993) Neurobehavioural methods used in neurotoxicological research. Toxicol Lett 68: 231-240.

Ussing HH (1994) Does active transport exist? J Membrane Biol 137: 91-98.

Videira AR, Antunes-Madeira MC, Madeira VMC (1996) Interaction of ethylazinphos with the physical organization of model and native membranes. Biochim Biophys Acta 1281: 65-72.