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Short communication

# Diazepam induces tolerance in the isolated skin of *Pleurodema thaul*

Beryl Norris [a,*], Enrique Contreras [b], Gastón Nunez [b], Graciela Contreras [a]

[a] *Departamento de Fisiología, Facultad de Ciencias Biológicas, Universidad de Concepción, Casilla 152-C, Concepción, Chile*
[b] *Departamento de Farmacología, Facultad de Ciencias Biológicas, Universidad de Concepción, Casilla 152-C, Concepción, Chile*

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**Abstract**

The effects of the long-term administration of diazepam on the potential difference and short-circuit current of the isolated skin of the toad *Pleurodema thaul* (*P. thaul*) were investigated. Diazepam applied in a concentration range of $4.6 \times 10^{-6}$ to $5.2 \times 10^{-5}$ M decreased both electrical parameters. This response was unaffected by flumazenil indicating that the action of diazepam is not induced through benzodiazepine receptors. Induction of tolerance to diazepam on its observed effects on potential difference and short-circuit current was obtained by the administration of a single dose of the drug in a slow release preparation. Skins tolerant to diazepam were also tolerant to the acute effects of verapamil on both electric parameters. Tolerance to diazepam effects was partly reversed by increasing $Ca^{2+}$ concentration in the inner bathing solution. The results are consistent with a $Ca^{2+}$ channel blocking effect of diazepam in the *P. thaul* skin.

*Keywords:* Skin, toad; Diazepam; Tolerance; $Ca^{2+}$ channel

## 1. Introduction

The primary effect of benzodiazepines consists in modulation of the chloride channel associated with γ-aminobutyric acid (GABA) in the central nervous system. These drugs also interact with $Ca^{2+}$ channels in peripheral tissues (Hullihan et al., 1983; Macdonald and McLean, 1986). Previous work in our laboratory (Norris et al., 1991) showed that diazepam reduced the stimulatory response to noradrenaline applied to the inner surface of the skin of *Caudiverbera caudiverbera* and partially antagonised the effect of barium which indicates that the drug is acting on epithelial transport.

The aim of the present work was to investigate whether diazepam might induce tolerance to its effects on the electrical parameters of isolated toad skins treated previously with a slow-release preparation of the benzodiazepine. The influence of a high $Ca^{2+}$ concentration on the latter process was also analysed.

## 2. Materials and methods

### 2.1. Animals and preparation

Experiments were performed on *Pleurodema thaul* toads (18–22 g) collected from fresh water ponds in Concepción, Chile, during the spring and summer months. The amphibians were kept in tap water at room temperature (18–22° C) at least 24 h prior to use. They were decapitated and pithed and segments of abdominal skin were 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 gassed with a stream of air. The composition of the solution was (mM): $NaCl_2$ 112; KCl 1.9; $CaCl_2$ 2.0; $NaHCO_3$ 2.3 and glucose 11.

The potential difference across the skin was recorded by means of a Cole-Parmer 2-channel recorder using calomel electrodes connected with the solutions bathing both surfaces of the skin through agar-Ringer bridges and to the first channel of the recorder. The short-circuit current was monitored through Ag-$AgCl^-$ electrodes connected to a voltage clamp circuit (G. Métraux

* Corresponding author. Tel. (56) (41) 234985, fax (56) (41) 245975.


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Electronique) and to the second channel of the recorder.

Experiments were started 30 min after the bioelectric parameters of the skin had reached a steady level.

### 2.2. Drugs and solutions

The following drugs were used: diazepam (Hoffmann-La Roche and Co., Basel, Switzerland) was added to either the inner or the outer solution (serosal or mucosal surface) in the concentrations mentioned in the text. Flumazenil (kindly donated by Hoffmann-La Roche, Basel, Switzerland) and verapamil (Sigma Chemical Co., St. Louis, MO, USA) were used in some experiments.

Chronically treated toads received an intraperitoneal injection of 100 mg/kg diazepam contained in a slow-release preparation, which consisted of 100 mg powdered diazepam dissolved in 4.2 ml liquid paraffin, 0.8 ml sorbitol sesquioleate and mixed with 5 ml physiological saline. A quantity of 0.01 ml/body weight was injected in each toad. The amphibians were kept in bins containing tap water at room temperature and fed on sow bugs (*Oniscus asellus*) during the period of treatment. Control (naive) toads were injected with vehicle and maintained in conditions similar to diazepam-treated toads. No changes in weight were observed either in naive or diazepam-treated toads during the treatment period.

### 2.3. Statistical analysis

Values throughout the work refer to means ± S.E.M. Statistical analysis was performed by a two-way analysis of variance (ANOVA) and the significance was calculated according to the Student-Newman-Keuls test.

## 3. Results

### 3.1. Effect on electrical parameters of isolated toad skin

Diazepam (inner solution) added to the Ussing chamber in cumulative concentrations reduced the electrical parameters. When the concentration reached a value of $9.0 \times 10^{-5}$ M, a 65.4 ± 3.6% reduction in the potential difference and a 60.3 ± 2.2 reduction in the short circuit current across the skin ($n = 9$, $P < 0.01$) were observed. The effect was usually reversible after a 6-fold washout and in three experiments the biolectric parameters did not return to the original values after a 30 min waiting period. Diazepam applied in the outer solution induced a non-significant decrease.

To rule out the possibility that diazepam added to the inner solution might be acting on benzodiazepine receptors, eight skins were exposed to $5.0 \times 10^{-5}$ M flumazenil 15 min previous to the addition of diazepam. Results showed that $5.2 \times 10^{-5}$ M diazepam induced a 52 ± 4.3% reduction in potential difference and a 56 ± 3.0% in short-circuit current after exposure to flumazenil, values in accordance with the above-mentioned reductions found in the absence of the benzodiazepine receptor agonist after a cumulative dose of $9.0 \times 10^{-5}$ M diazepam. No significant difference was found between the effects of diazepam by

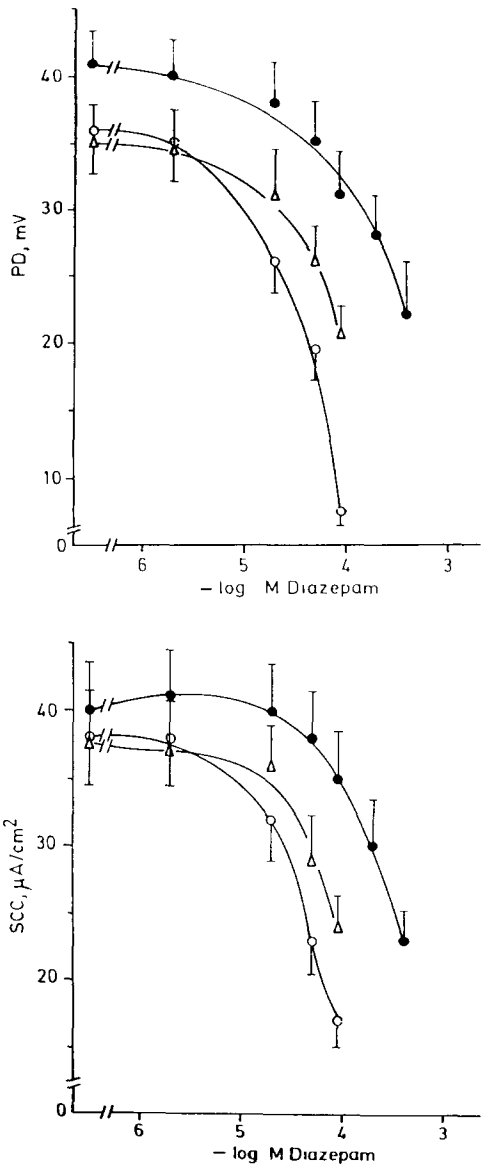


Fig. 1. Decrease of diazepam (D) effect in *P. thaul* skins isolated from toads injected 6 days previously with a slow-release diazepam preparation; partial reversal of the process on increasing inner $Ca^{2+}$ concentration to 6 mM. Values are means ± S.E.M.; $n = 8$. Open circles, naive toads; triangles, diazepam-treated toads, inner $Ca^{2+}$ concentration 6 mM; filled circles diazepam-treated toads. (A) potential difference (PD); (B) short-circuit current (SCC). Values in chronically treated toads (usual $Ca^{2+}$ concentration) are statistically different ($P < 0.05$, Student-Newman-Keuls test).

itself and 15 min after pretreatment with flumazenil in similar concentrations.

### 3.2. Effect of the i.p. administration of 100 mg/kg diazepam, 6 days previously, on the skin response to the drug

Fig. 1 shows induction of tolerance 6 days after treatment with a slow-release diazepam preparation. In eight skins dissected from naive toads, a cumulative concentration of $9.0 \times 10^{-5}$ M diazepam was followed by a $73.8 \pm 4.0\%$ decrease in potential difference and a $70.4 \pm 3.4\%$ decrease in short-circuit current; in contrast, 11 skins dissected from pretreated toads showed a shift to the right of the dose-response curve, a $50.8 \pm 3.7\%$ decrease in potential difference and a $48.6 \pm 1.5\%$ decrease in short-circuit current only after a cumulative concentration of $9.0 \times 10^{-3}$ M diazepam, indicating apparent tolerance to the drug. The figure also shows that when the $Ca^{2+}$ concentration in the inner solution was increased to 6 mM, the tolerance to diazepam decreased significantly ($n = 8$, $P < 0.01$).

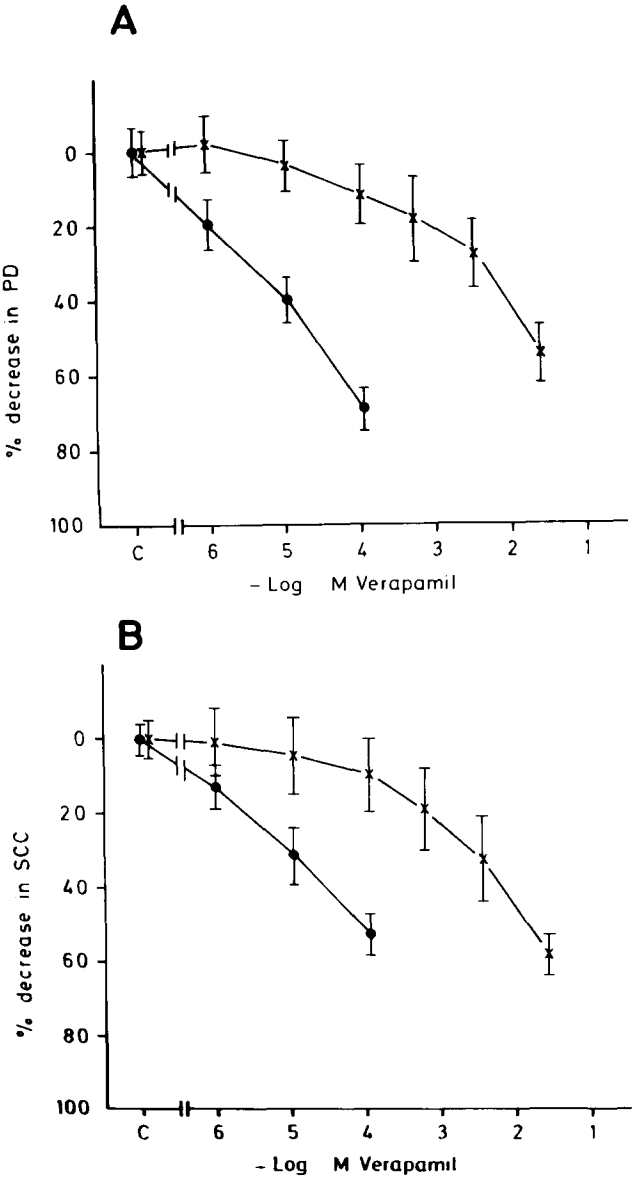


Fig. 2. Apparent cross-tolerance to verapamil in diazepam-treated *P. thaul* skins. C: control; PD: potential difference; SCC: short-circuit current. Results are expressed (means ± S.E.M., $n = 9$) as percent decrease in basal values, which were: (A) potential difference 31.5 ± 4.3 mV; (B) short-circuit current 36.9 ± 3.9 $\mu A/cm^2$. (○) Naive toads; (×) diazepam-treated toads. Values in chronically treated toads are statistically different from those observed in naive toads ($P < 0.05$, Student-Newman-Keuls test).

### 3.3. Effect of pretreatment with the slow-release diazepam preparation on the skin response to verapamil

Fig. 2 shows induction of cross-tolerance to verapamil 6 days after treatment. In skins dissected from naive toads, a cumulative concentration of $1.2 \times 10^{-4}$ M verapamil induced a $65.2 \pm 2.8\%$ reduction in potential difference and a $52.0 \pm 1.4\%$ reduction in short-circuit current, whereas in skins dissected from toads pretreated with diazepam, a cumulative concentration of $2.4 \times 10^{-2}$ M verapamil was necessary to induce a similar decrease, indicating development of cross-tolerance to verapamil ($n = 9$, $P < 0.05$).

## 4. Discussion

Diazepam causes a dose-dependent block of the response of the neuroepithelial synapse of *C. caudiverbera* to electric stimulation (Norris et al., 1991); however, *P. thaul* skin shows greater sensitivity to the effects of this agent on potential difference and short-circuit current. The effect is independent of the benzodiazepine receptor associated with the GABA-chloride channel complex since it is not antagonised by flumazenil. In accordance with Rampe and Triggle (1986) the benzodiazepine effects on $Ca^{2+}$ disposition are also unrelated to peripheral receptors for these drugs. The results obtained by Watabe et al. (1986) and Reuveny et al. (1993) add further evidence as to the existence of benzodiazepine effects on $Ca^{2+}$ channels.

Experiments on skins from toads treated 6 days previously with an i.p. injection of a slow-release diazepam suspension demonstrated induction of tolerance to the drug.

The influence of $Ca^{2+}$ channel antagonists on tolerance and some related contra-adaptive responses to the long-term administration of several central nervous system depressants has been studied by a number of authors (for review, Littleton and Little, 1989). In this respect, work in our laboratory has shown that $Ca^{2+}$

antagonists attenuate opiate tolerance (Contreras et al., 1988, 1993). Therefore, it was tempting to analyse the effect of long-term administration of diazepam on the electric parameters of the toad skin. Benzodiazepines block T and L types of $Ca^{2+}$ channels in mouse neuroblastoma cells (Reuveny et al., 1993), and the first type is resistant to dihydropyridine and related organic $Ca^{+}$ antagonists (Tsien et al., 1991), thus, it seems possible that the induction of tolerance to diazepam and its cross-tolerance to verapamil reside in the L type or in a nonidentified site functionally associated to this type of channel.

The acute effects of diazepam and the process of tolerance in the isolated skin of *P. thaul* clearly indicate a close association with $Ca^{2+}$ concentration. Thus, $Ca^{2+}$ is necessary for the expression of the acute inhibition of the electric parameters induced by diazepam and, in addition, the presence of a high concentration of the cation can revert tolerance induced by sustained exposure to the drug.

To explain the $Ca^{2+}$-induced reversal of tolerance it may be suggested that during chronic treatment with diazepam, $Ca^{2+}$ disposition in skin cells is reduced and a further addition of a high concentration partially restores the inhibition of potential difference and short-circuit current exerted by the acute administration of the drug. The interaction of diazepam with essential processes of the $Ca^{2+}$ channel protein (Krueger, 1989) may explain the cross-tolerance to verapamil.

To summarise, these results, based on electrophysiological and pharmacological evidence, are consistent with a $Ca^{2+}$ channel blocking effect of diazepam on *P. thaul* skin and with the induction of tolerance to this agent after pretreatment with a slow-release suspension of the drug. The results suggest the use of the toad skin as a model to examine the mechanisms which lead to tolerance.

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## References

Contreras, E., L. Tamayo and M. Amigo, 1988, Calcium channel antagonists increase morphine-induced analgesia and antagonize morphine tolerance, Eur. J. Pharmacol 148, 463.

Contreras, E., L. Quijada, A. Germany, R. Fleckenstein and A. Hernández, 1993, Calcium channel antagonists and adenosine analogues decrease tolerance to opiate pentazocine and U 50488H, Gen. Pharmacol. 24, 1203.

Hullihan, J.P., S. Spector, T. Taniguchi and J.K.T. Wang, 1983, The binding of [$^3$H]-diazepam to guinea-pig ileal longitudinal muscle and the in vitro inhibition of contraction by benzodiazepines, Br. J. Pharmacol. 78, 321.

Krueger, B.K., 1989, Toward an understanding of structure and function of ion channels, FASEB J. 3, 1906.

Littleton, J.M. and H.J. Little, 1989, Adaptation in neuronal calcium channels as a common basis for physical dependence on central depressant drugs, in: Psychoactive Drugs. Tolerance and Sensitization, eds. A.J. Goudie and M.W. Emmett-Oglesby (Humana Press, Clifton, NJ) p. 461.

Macdonald, R.L. and M.J. McLean, 1986, Anticonvulsant drugs: mechanism of action, Adv. Neurol. 44, 713.

Norris, B., G. Núñez, G. Contreras and E. Contreras, 1991, Diazepam blocks stimulation of the nerve-skin preparation of *Caudiverbera caudivebera*, Arch. Biol. Med. Exp. 24, R80.

Rampe, D. and D.J. Triggle, 1986, Benzodiazepines and calcium channel function, Trends Pharmacol. Sci. 7, 461.

Reuveny, E., D.A. Twombly and T. Narahashi, 1993, Chlordiazepoxide block of two types of calcium channels in neuroblastoma cells, J. Pharmacol. Exp. Ther. 264, 22.

Tsien, R.W., P.T. Ellinor and W.A. Horne, 1991, Molecular diversity of voltage-dependent $Ca^{2+}$ channels, Trends Pharmacol. Sci. 12, 349.

Watabe, S., M. Yoshii, N. Ogata and T. Narahashi, 1986, Clonazepam differs from diazepam and nitrazepam in blocking two types of calcium channels, Soc. Neurosci. Abstr. 12, 1193.