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Effects of Pb^{2+} ions on Na^+ transport in the isolated skin of the toad *Pleurodema thaul*

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Abstract

The effects induced by lead ions on the short-circuit current (SCC) and on the potential difference (V) of the toad *Pleurodema thaul* skin were investigated. Pb^{2+} applied to the outer (mucosal) surface increased SCC and V and when applied to the inner (serosal) surface decreased both parameters. The stimulatory effect, but not the inhibitory action, was reversible after washout of the metal ion. The amiloride test showed that the increase was due principally to stimulation of the driving potential for Na^+ ($V\text{-}E_{Na+}$) and that inhibition was accompanied by a reduction in the $V\text{-}E_{Na+}$ and also by a significant decrease in skin resistance indicating possible disruption of membrane and/or cell integrity. The effect of noradrenaline was increased by outer and decreased by inner administration of Pb^{2+}. The results suggest that mucosal Pb^{2+} activates toad skin ion transport by stimulating the $V\text{-}E_{Na+}$ and that serosal Pb^{2+}, with easier access to membrane and cellular constituents, inactivates this mechanism, revealing greater toxicity when applied to the inner surface of the skin.

Abbreviations: SCC – short-circuit current; V – potential difference; $V\text{-}E_{Na+}$ – driving potential for Na^+; ENaC – epithelial sodium channel; R_{Na+} – active sodium resistance; RS – passive or shunt resistance; G_{Na} – active sodium conductance; GS – passive or shunt conductance; Gmax – total conductance; EC_{50} – half-maximal excitatory concentration; IC_{50} – half maximal inhibitory concentration; NA – noradrenaline.

Introduction

Lead is an environmental toxic metal to which people can be exposed through industrial applications or commercial contamination. Toxic effects are due to combinations with ligands essential for normal physiological functions. Exposure to lead has decreased markedly over the past 60 years because of appropriate environmental and preventive measures; nevertheless, acceptable blood levels of less than 0.25 μmol/l in adults may rise to over 0.5 μmol/l in children since the gastrointestinal absorption is up to 40% in the latter and only 10% of ingested lead in the former (Klaassen 2001). For children this concentration is regarded (CDC, US) as being the concentration at risk. Lead exposure, particularly in children, is mainly via food, dust and water. Acute lead poisoning, although infrequent, occurs from ingestion of acid-soluble lead compounds or inhalation of lead vapors which produce vomiting and central nervous system symptoms, such as paresthesias, pain and muscle weakness, hemolysis and kidney damage. Chronic lead poisoning leads to multisystemic damage; neuromuscular and central nervous system effects are usually due to intense exposure and gastrointestinal syndrome is the manifestation of a more insidiously developing intoxication. Chronic lead poisoning is therefore more frequent in children; the most serious manifestation is lead encephalopathy, where concentrations of lead exceeding 2.9 μmol/l of whole blood may be found. Learning is impaired and there is mental deterioration; there are also gastrointestinal, neuromuscular, hematological and renal effects. Pb^{2+} neurotoxicity has been amply documented; it is one of the most potent inhibit-

ors of voltage-gated calcium channels (Audesirk 1993; Domann *et al.* 1997; Braga *et al.* 1999a). Cytotoxic effects are due to cell membrane damage (Steffensen *et al.* 1994; Suwalsky *et al.* 2003). Activation of conductances as for instance in red cell ghosts (Kiss & Osipenko 1994) and induction of molecular changes in glutamatergic synapses are directly associated with learning deficits (Nihei & Guilarte 2001). In Helix neurons Pb^{2+} induced either an increase, or a block of Na^+ conductance (Osipenko & Kiss 1991/1992); micromolar concentrations of lead caused inhibition of Na^+/K^+-ATPase activity in rat brain synaptic plasma membrane (Carfagna *et al.* 1996). Although skin exposure of lead is rare, this study was undertaken in order to detect mechanisms causing the functional effects induced by the metal ion on the isolated toad skin, which is a model for the examination of active sodium transport (Ussing & Zerahn 1951; Larsen *et al.* 2001). In this tissue, Na^+ entering through the apical (outer or mucosal) membrane epithelial sodium channels (ENaC) (Horisberger, 2003) is actively extruded by a Na^+-K^+-ATPase in the basolateral (inner or serosal) border, which exchanges Na^+ for K^+ (Rytved *et al.* 1996; Ussing 1994). This process was examined by measurement of the transepithelial potential difference (V) and of the short-circuit current (SCC). The possible site of action of Pb^{2+} applied to either surface of the skin was investigated by analysis of the comparative effects of amiloride, Pb^{2+} and noradrenaline (NA) (Acevedo *et al.* 1981), and by use of Isaacson's (1977) amiloride test.

Materials and methods

The experiments were performed using the toad *Pleurodema thaul* (7–14 g) collected from fresh water ponds. The amphibians were kept in bins containing tap water at room temperature (18–22 °C) at least one day prior to use and fed on sow bugs (*Oniscus asellus*). They were pithed and samples of the abdominal skin were dissected, washed in toad Ringer's solution and mounted between two halves of a perspex Ussing-type chamber. A circular area of 1.0 cm^2 was exposed to 3 ml Ringer's bathing solution on each side. The composition of the solution was (mM): Na^+ 114, K^+2.5, Cl^- 117.5, Ca^{2+} 2.0, $HC0_3^-$ 2.3 and glucose 11 (pH 7.4). The bathing medium was oxygenated by means of an Elite 800 aerator (R.C. Hagen). The SCC was monitored with non-polarizable Ag/AgCl electrodes placed at 15 mm distance from the skin and connected to a voltage-clamp circuit (G. Métraux Electronique) set to keep the V across the skin at zero mV. The V was measured with calomel-agar electrodes at intervals of 2 min for 4 s. Both parameters were displayed on a 2-channel Cole-Parmer recorder. Thirty min after steady readings had been obtained, $Pb(NO_3)_2$ was applied in the solution bathing either the outer or the inner surface of the skin in the final concentrations specified in the text. Amiloride (a gift from Merck, Sharp and Dohme), was applied to the solution bathing the outer surface of the skin (final concentration 8 μM). Noradrenaline (Sigma) was applied to the inner surface (final concentration 10 μM). The use of Isaacson's amiloride test was based on the representation of the sodium transport mechanism by an equivalent electrical circuit, each component of which can be evaluated: the electromotive force of the sodium transporting mechanism ($V\text{-}E_{Na+}$), the resistance in series with this force, representing the path through which the actively transported sodium ion must pass (R_{Na+}), and the resistance of the shunt representing the leak paths in parallel (cellular or extracellular) for passively transported ions (RS). An agent which induces changes only in R_{Na+} [($V\text{-}E_{Na+}$) and RS remaining constant)] will result in changes in V and SCC which permit the algebraic evaluation of all three parameters within the circuit. The changes should be fairly large, of rapid onset, to ensure that concomitant spontaneous changes in the several parameters remain insignificant. The equation using resistance values is based on calculations involving double reciprocal plots which lead to exaggeration of minor errors and therefore the following equation using conductance values was used: $SCC/V = SCC/\ V\text{-}E_{Na+} + GS$. The equation was solved graphically for skins with and without Pb^{2+}. In each case points were obtained with and without amiloride treatment; in four preliminary experiments two or three successive increments of amiloride were employed, to confirm a linear relationship between tissue conductance and SCC. Such increments were not given to skins exposed to lead, because metal ions might exert continuing effects on circuit parameters during the periods of time required to administer the several doses of amiloride. Results were expressed as means ± SE. Student's paired *t* test was used for statistical analysis.

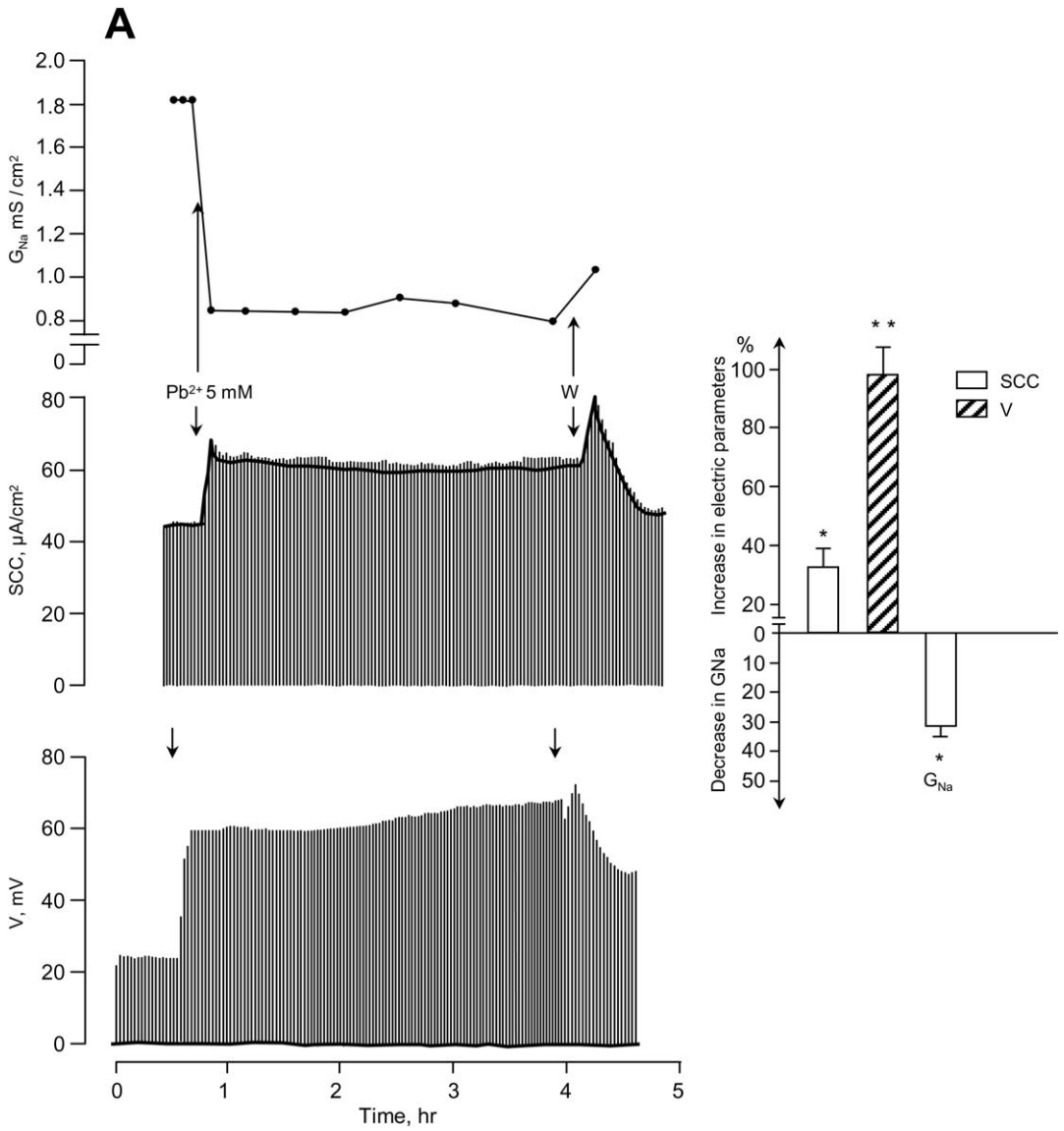


Figure 1. Effect of lead (Pb^{2+} 5 mM) on the short-circuit current (SCC), potential difference (V) and on sodium conductance (G_{Na+}) when added to the outer (A) or inner (B) surface of the isolated toad skin. Left: representative experiment. W = washout. Right: Each bar represents means ± S.E. of 7 experiments. The values of SCC, V and G_{Na+} (expressed as percentages) were taken 90 min after the application of lead.

Results

The electrical response of the toad skin to Pb^{2+} ions applied in either the outer or the inner bathing solution was a concentration-dependent change in V and in SCC consistent with an effect on Na^+ transport. Pb^{2+} (5 mM, Figure 1A) in the mucosal (outer) side of the skin increased SCC by 34 ± 4.7% and decreased sodium conductance (G_{Na+}) by 30 ± 3.4%, values taken 90 min after application of the metal; the increase in SCC may be interpreted as an increase in Na^+ absorption (Nielsen 1997). The increment in the V (98 ± 4%) notably exceeded that of the SCC (Figure 1A), an observation whose explanation could be sought in events initiated by the application of mucosal Pb^{2+}, such as increased activity of inwardly rectifying K^+ channels in the basolateral membrane (Nagel & Katz 2003). The effect of 10 mM Pb^{2+} applied to the outer surface was similar to that of 5 mM Pb^{2+} The duration of the response was about 75.0 ± 9.5 min (n = 16); recovery was obtained in only 50% of the experi-

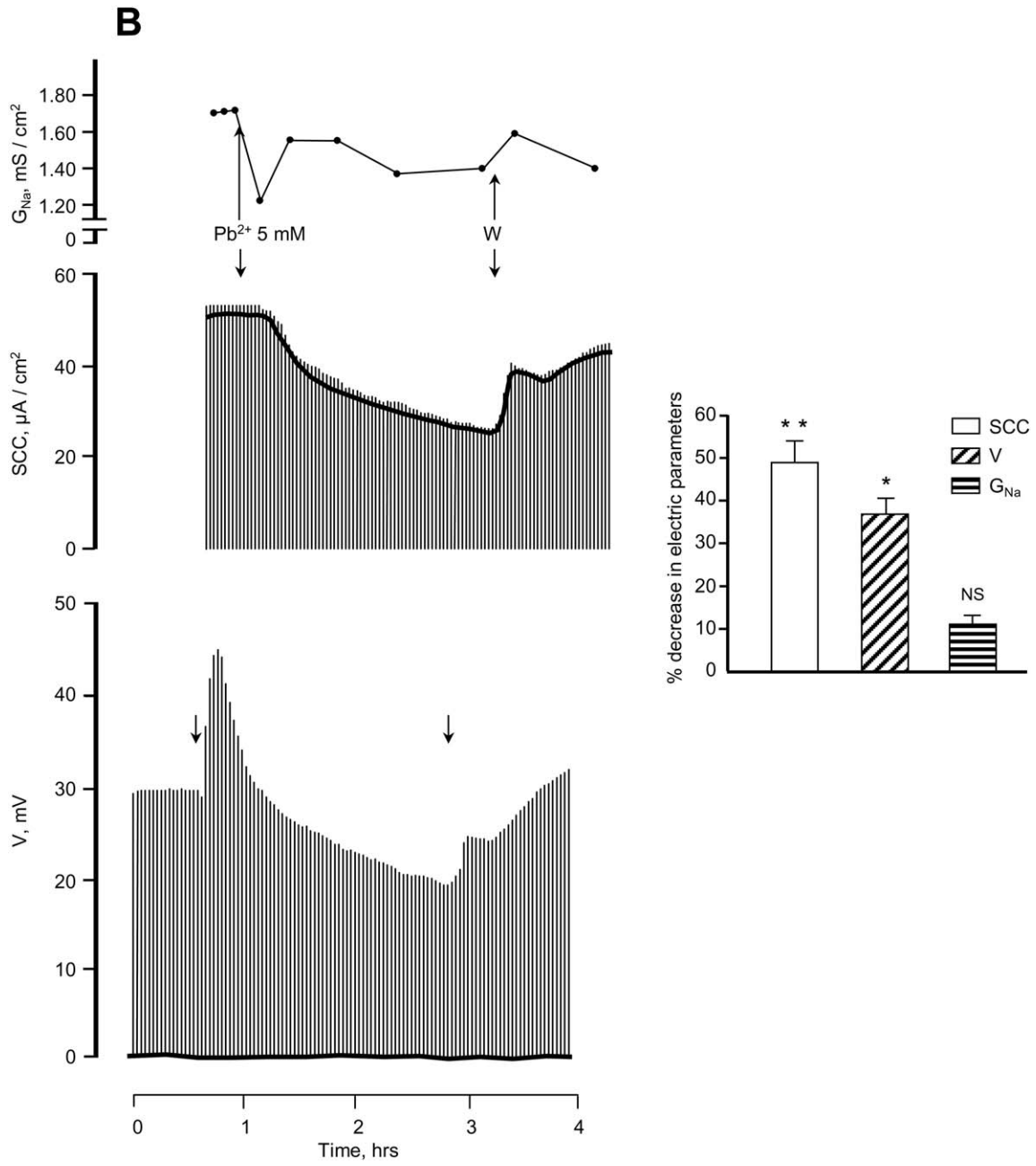


Figure 1. Continued.

ments after washout. In contrast, Pb^{2+} applied from the serosal (inner, Figure 1-B) side led to a notable decrease in the skin parameters. 5 mM Pb^{2+} induced a rapid decline (50%) in both parameters, which lasted 121.0 ± 16.0 min ($n = 7$) and was only partially or was not reversible after repeated washout of the skin. 10 mm Pb^{2+} induced a 77% decrease in both electric parameters and a non-significant (10%) decrease in resistance in 7 experiments. The decline in V and in SCC, interpreted as an inhibition of Na^+ transport, was also found in Helix neurons (Osipenko 1991/2) where 150 μM Pb^{2+} blocked the Na^+ channel. For most experiments, the latency period for the response to Pb^{2+} applied in either side of the skin was 1.5 ± 0.13 min ($n = 42$). Figures 2-A and 2-B illustrate the concentration- dependent effects of lead applied

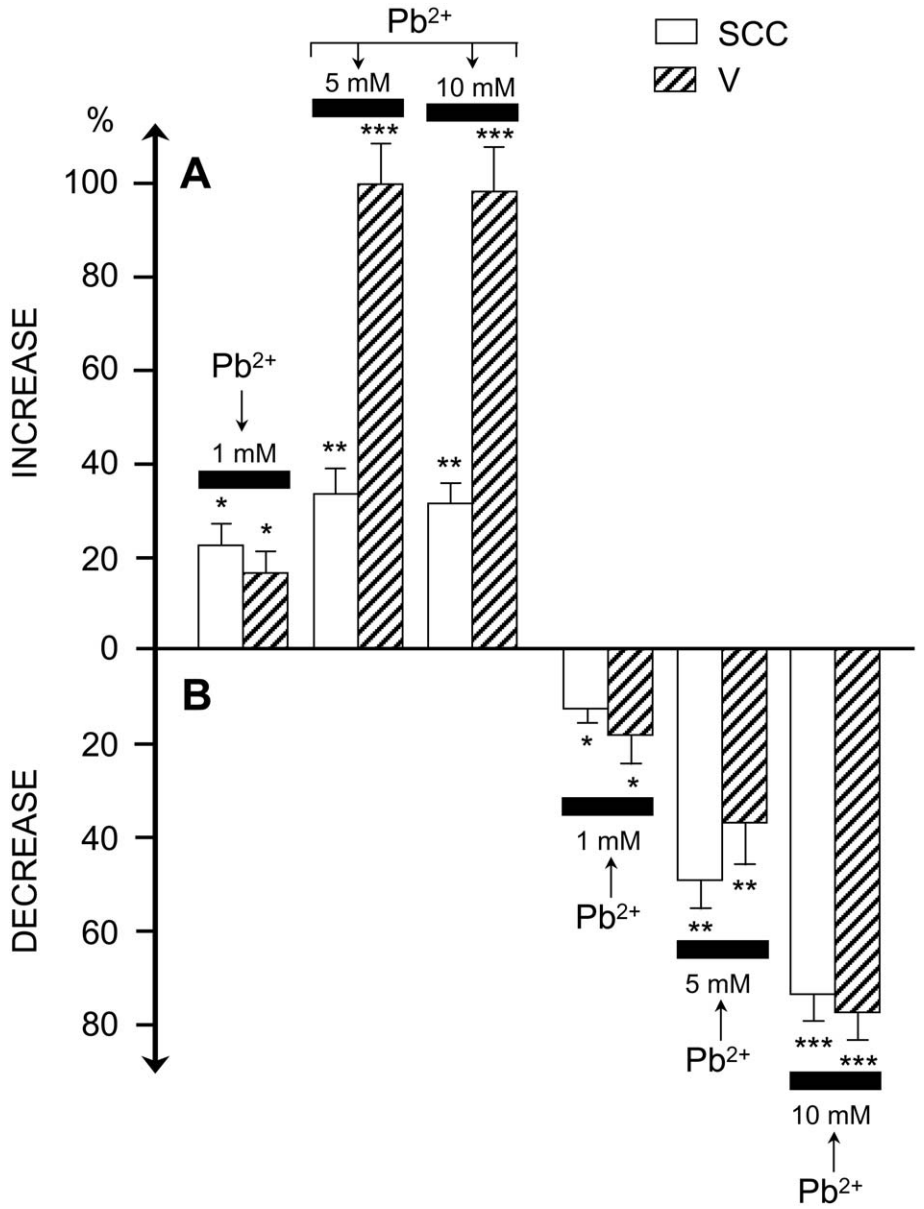


Figure 2. The response of the toad skin electrical properties to Pb^{2+} increasing concentrations applied in the outer (A) and in the inner (B) bathing solutions. Short.circuit current (SCC) and potential difference (V) values for untreated skins were 53.9 $\pm$ 3.2 μA/cm^2 and 40.6 $\pm$ 2.4 mV respectively. Results obtained in the presence of Pb^{2+} (1, 5 and 10 mM) are expressed as the percentage change of these control values. Each bar showing lead effects represents means $\pm$ S.E.; $n = 7$. A) Pb^{2+} applied to the outside surface. B) Pb^{2+} applied to the inside surface. It is noteworthy that Pb^{2+} applied to the outer surface of the skin was always stimulatory whereas inhibition was predominant when Pb^{2+} was applied to the inner surface. Significance by Student's paired t test: $*P < 0.05$; $** < 0.01$; $*** < 0.001$; NS = not significant.

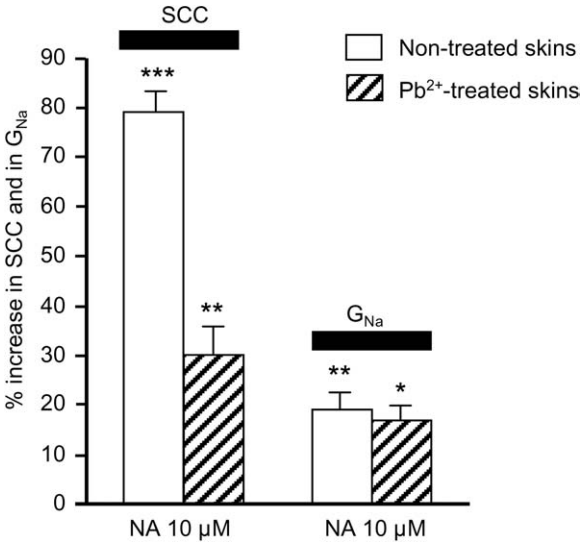


Figure 3. Effects of 10 μM noradrenaline (NA), 2 min after administration, added to the inner surface of untreated and lead pretreated (during 30–45 min) toad skins; $n = 14$. SCC = short-circuit current; G_{Na+} = sodium conductance. Significance by Student's paired t test: $*P < 0.05$; $** < 0.01$; $*** < 0.001$.

to both surfaces. EC_{50} (SCC) for the outer surface was about 2 mM and IC_{50} for the inner surface was about 4.4 mM. Although conductance increased at the lowest (1.0 mM, outer surface) Pb^{2+} concentration used, higher concentrations (5 and 10 mM) caused a significant decrease in conductance. As stated above, the possible sites of action of Pb^{2+} were examined by analysis of the comparative effects of amiloride, Pb^{2+} and noradrenaline on the skin, and by determination of the parameters of Isaacson's amiloride test.

Effect of noradrenaline (NA) before and after Pb^{2+} (inner surface)

Figure 3 shows that NA (10 μM) increased SCC by 79.0 $\pm$ 4.1% and G_{Na+} by 19.5 $\pm$ 3.4% in 14 skins. The effect of NA was inhibited by pretreating the skins for 30 min with 5 mM Pb^{2+}; SCC increased by only 30.5 $\pm$ 4.0% and G_{Na+} increased 18.5 $\pm$ 2.6%. This effect is not surprising; Chandra *et al.* (1984) showed that Pb^{2+} at concentrations higher than 0.1 mM inhibited NA uptake in rat brain synaptosomes.

Comparative effects of amiloride and lead (outer surface) and NA (inner surface)

Amiloride blocks apical Na^+ channels when added to the outer surface of the frog skin (Flonta *et al.* 1998). Figure 4-A shows that 5 mM Pb^{2+} applied to the outer surface of the toad skin after amiloride ($n = 6$), caused a 70% reversible decrease in SCC and G_{Na+}, an effect that might be interpreted as indicating the possibility of amiloride displacement of Pb^{2+} to a different binding site on the Na^+ channel, and lead thus enhances the blocking effect of amiloride. After washout of Pb^{2+}, the addition of noradrenaline (inner surface) after amiloride led to a 110.0 $\pm$ 4.1% increase in SCC and a 58.0 $\pm$ 3.5% increase in G_{Na+}, revealing a significantly ($P < 0.001$) greater effect than when administered to untreated skins.

Comparative effects of amiloride (outer surface), lead and NA (inner surface)

Addition of 5 mM Pb^{2+} after amiloride (Figure 4-B) was followed by a transient and reversible decrease (57.5 $\pm$ 3.7, $n = 6$) in SCC and G_{Na+}, which could be explained by a direct effect on the sodium pump or by interference with apical sodium entry (Lebowitz *et al.* 2003). Figure 4-B also shows that after Pb^{2+} was washed out, the addition of NA after amiloride increased SCC by 54 $\pm$ 6.6% and G_{Na+} by 33.5 $\pm$ 3.2%.

Determination of the parameters of the amiloride test

The site of action of Pb^{2+} applied to either surface of the skin was investigated by the amiloride test. Amiloride-sensitive Na^+ channels are control elements for the regulation of Na^+ transport into cells and across epithelia. They belong to the degenerin ENaC superfamily (Kellenberger & Schild 2002) and are not homologous with the voltage-gated Na^+ channel (Benos & Stanton 1999). The relationship between conductance and SCC was determined before and after addition of 8 μM amiloride to the mucosal surface, and also in the presence of 5 mM Pb^{2+}. Values for V-E_{Na+}, G_{Na+} and GS were calculated for skins with and without Pb^{2+}. In each experiments two points were found, with and without amiloride. The values were found by solving Isaacson's equation graphically from the numerical estimates of SCC and V before and after the addition of lead in the presence of amiloride. The final concentration of amiloride sufficed to lower the SCC to a desired level of half or less of the initial value, in order to avoid errors such as small inexactitudes in reading the electrical parameters. Pb^{2+} was used in a concentration sufficient to ensure a SCC

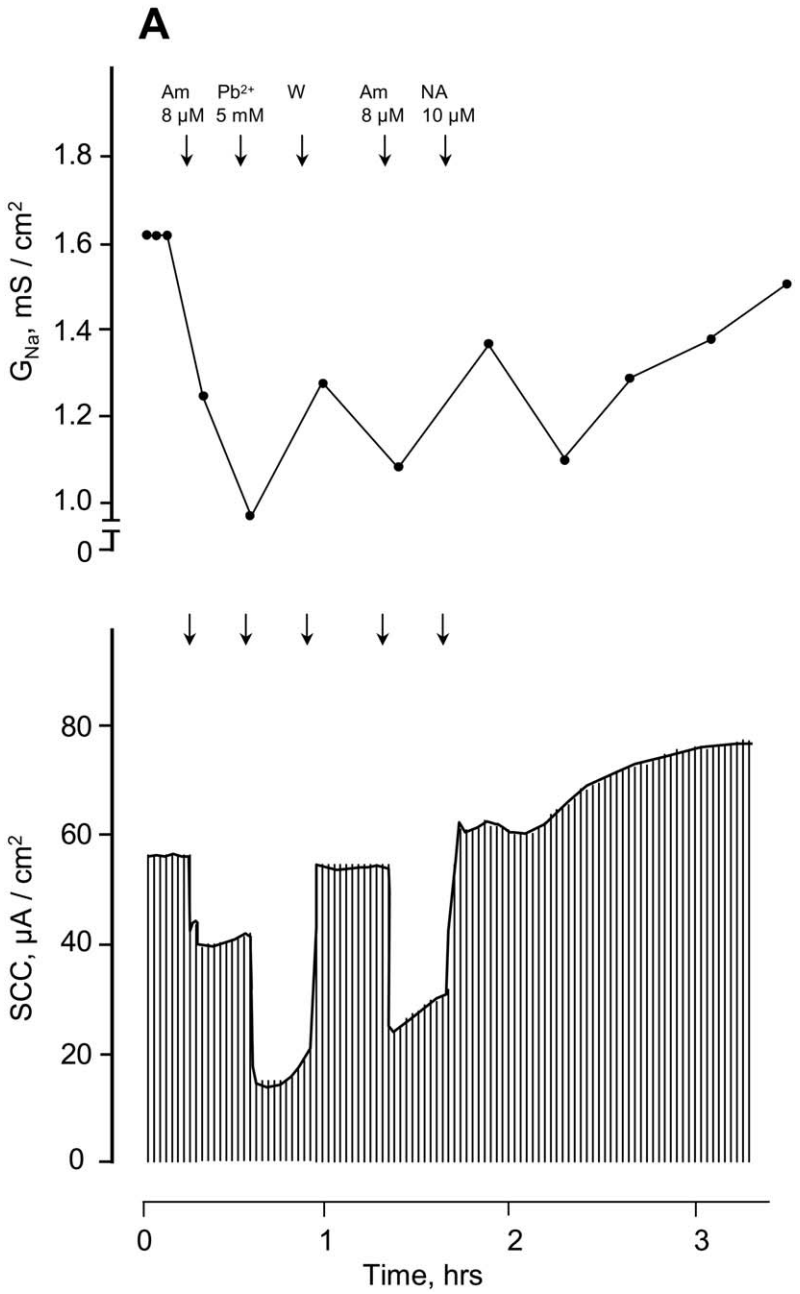
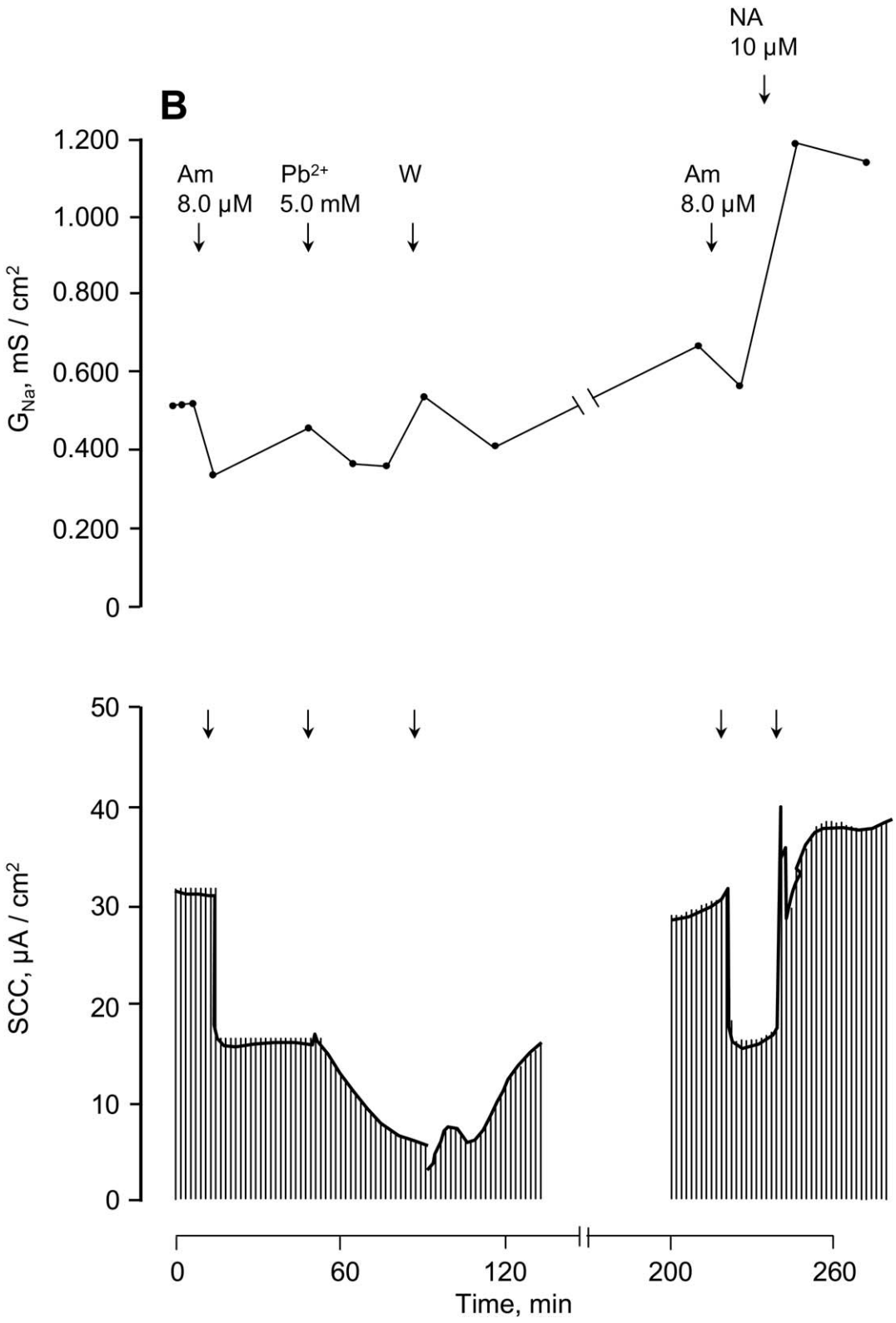


Figure 4. Single experiment (one of five) showing the comparative effects of amiloride (A, 8 μM), lead (Pb^{2+}, 5 mM), and noradrenaline (NA, 10 mM) on the short-circuit current (SCC) and on the sodium conductance (G_{Na+}) of the isolated toad skin. A. Amiloride and lead added to the outer surface; NA added to the inner surface. W = washout. B. Amiloride (outer surface); lead and NA (inner surface).



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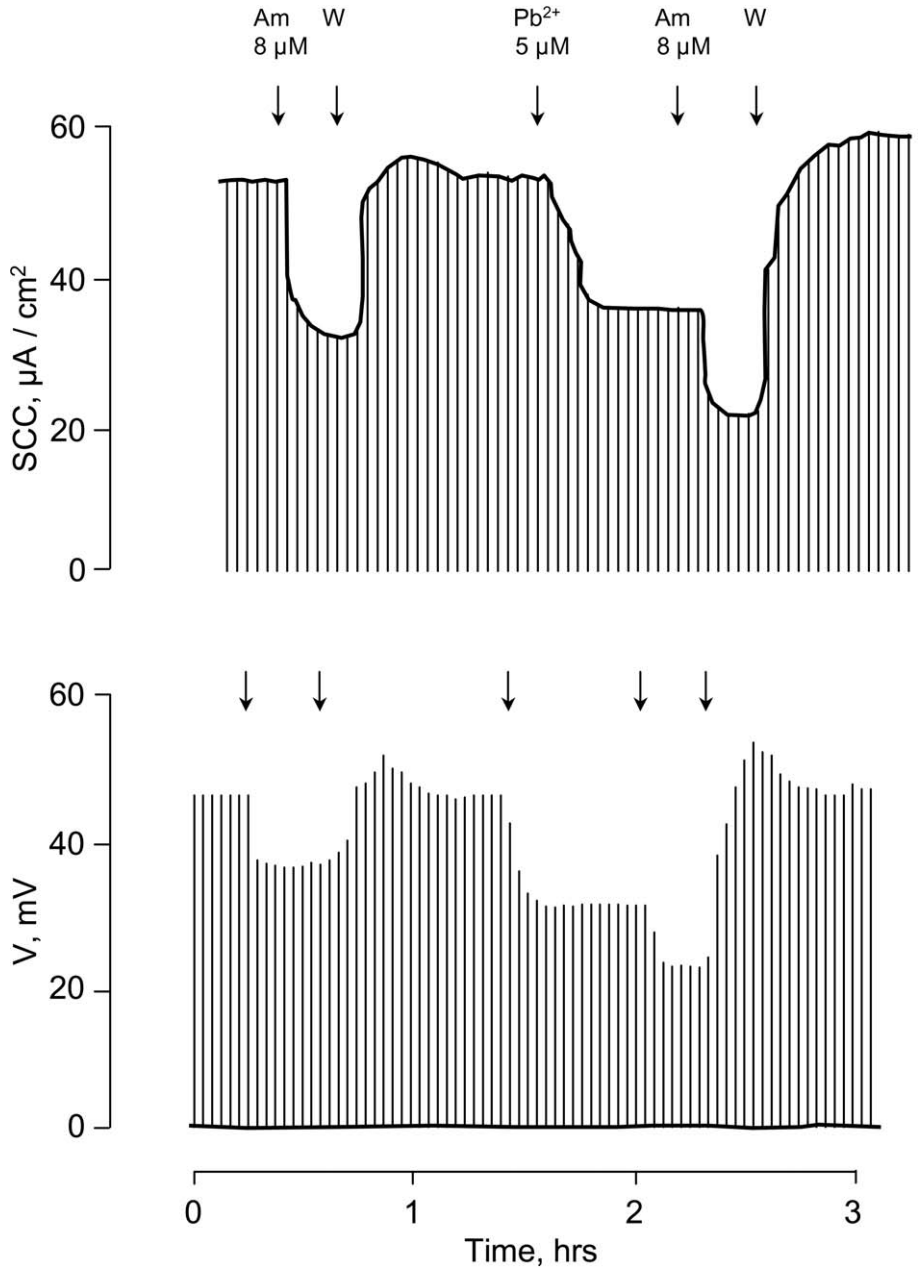

Figure 5. Changes in the electric properties of the isolated toad skin after addition of 8 μM amiloride (A) to the outer surface, before and after exposure of the inner surface to 5 mM Pb^{2+}. SCC = short-circuit current; V = potential difference; W = washout. Values of SCC and V before and after the effect of Pb^{2+} were used to solve Isaacson's amiloride test (see Materials and methods).

descent similar to that of amiloride, since larger concentrations of the metal (serosal surface) were often followed by a rapid descent of current to zero, thus preventing the completion of the test. Figure 5 represents the changes in SCC and in V on addition of amiloride before and after exposure to Pb^{2+}. Figure 6 shows that the slope of the line represents 1/ V-E_{Na+} and the intercept with the ordinate indicates conductance when SCC is null. The value of this conductance represents the passive or shunt conductance (GS). The results presented in Table 1 show that Pb^{2+} in the mucosal surface significantly (26%) increased the driving force for sodium reabsorption (V-E_{Na+}) and decreased conductance, in accordance with a possible feedback

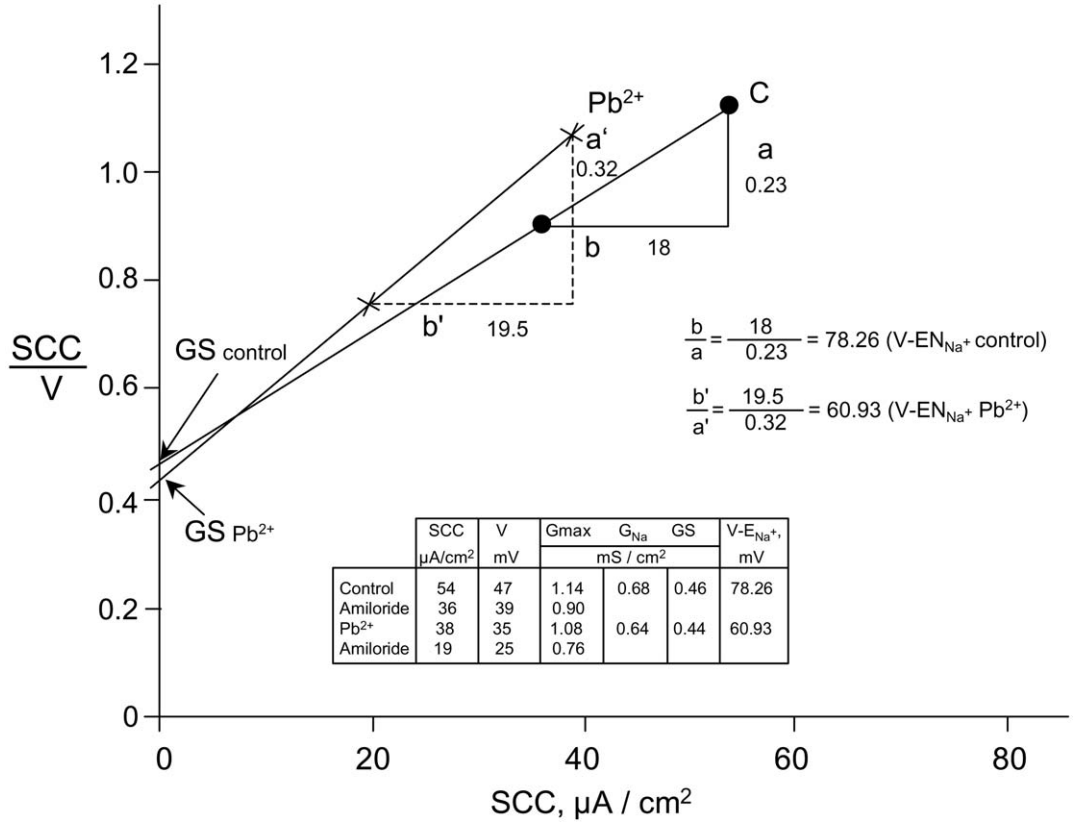

Figure 6. Relation between conductance (SCC/V) and SCC in a toad skin before and after addition of 8 μM amiloride to the outer surface (line C) and relation between conductance and SCC when 5 mM Pb^{2+} (inner surface) followed by amiloride (outer surface) were added (line Pb^{2+}). The values shown for E_{Na+} (sodium potential) and GS (passive conductance) have been derived from the slope and intercept, respectively, of the lines drawn through the points obtained; G_{Na+} (sodium conductance) has been derived from the difference between Gmax and GS.

Table 1. Effects of 5 mM Pb^{2+}, applied in either the outer or the inner surface, on the potential difference (V), short-circuit current (SCC), sodium potential (V-E_{Na+}), total conductance (Gmax), sodium conductance (G_{Na+}) and passive conductance (GS) of toad skins.

Parameter	Outer surface of the skin		Inner surface of the skin	
	Control	Pb^{2+}	Control	Pb^{2+}
V, mV	38.6 ± 6.0	60.8 ± 6.4**	46.5 ± 7.0	24.2 ± 3.8**
SCC, μA/cm^2	50.0 ± 5.8	61.9 ± 6.3*	57.5 ± 6.0	27.3 ± 4.1**
V-E_{Na+}, mV	98.0 ± 13.0	123.6 ± 10.0***	90.3 ± 12.0	51.8 ± 6.0**
Gmax, mS/cm^2	1.36 ± 0.11	1.02 ± 0.03**	1.3 ± 0.10	1.25 ± 0.11^{NS}
G_{Na+}, mS/cm^2	0.42 ± 0.05	0.39 ± 0.06^{NS}	0.64 ± 0.08	0.40 ± 0.07**
GS, mS/cm^2	0.94 ± 0.08	0.62 ± 0.06**	0.66 ± 0.07	0.85 ± 0.10*
n	9	9	5	5

Values are means ± S.E.: n = number of experiments. In contrast with control values, $P* < 0.01$; ** < 0.02; *** < 0.001; NS = not significant (Student's paired t test).

mechanism of the sodium pump. On the contrary, Pb^{2+} in the serosal surface decreased V-E_{Na+} by 35% and increased shunt (GS) conductance by 28%.

Discussion

The present study demonstrates that Pb^{2+} applied in either the outer or the inner surface has two separate effects on ion transport across the toad skin: stimulation and inhibition, respectively, of the active transcellular Na^+ transport (Nielsen 1997). The mechanism of action of this cytotoxic metal is not established although it is known to interact with a wide variety of molecules involved in signal transduction. The main hypotheses that attempt to elucidate its molecular mechanisms of action are as follows: a) interaction with membrane phospholipids; b) action on lipid-protein interfaces affecting ion channel activity; c) direct interaction with ion channels and/or enzymes or pumps; d) effects on intracellular signalling cascades. Recent work on the effects of lead on model and native membranes (Suwalsky *et al.* 2003) presents evidence of deformation of the lipid-protein interface in a model membrane as well as disturbance of ion channel activity in the erythrocyte membrane, in which transmission electron microscopy studies showed that lead particles formed masses that adhered to the external and internal membrane surfaces; all three mechanisms point to possible perturbation of ion channel and enzyme activities. It has been shown (Nagel & Katz 2003) that active Na^+ transport across epithelia depends on a) apical membrane permeability; b) rheogenic activity of Na^+-K^+-ATPase and c) basolateral K^+ conductance. Stimulation of transcellular Na^+ transport by mucosal Pb^{2+} is mainly controlled by the transepithelial potential difference defined as the sum of the apical and basolateral membrane potentials (Horisberger 2003), which modifies the driving force for apical Na^+ uptake (Bjerregaard & Nielsen 1987) and increases the activity of the basolateral membrane Na^+ pump.

Lead, as well as other metal ions such as Ag^+, Cu^{2+}, Hg^{2+}, Cd^{2+} and Al^{3+}, increases membrane permeability to sodium (Kiss & Osipenko 1994). The concentrations used in the work are very high; our results show that only the smallest concentration used (1 mM) increased apical permeability; at larger concentrations (5 and 10 mM) the increase in SCC was associated with a decrease in conductance. It has been demonstrated that when G_{Na+} decreases, inwardly rectifying K^+ channels are predominant, and basolateral conductance rapidly and notably increases, overcompensating the decrease in apical membrane permeability, and increasing the transepithelial V which drives ion transport (Nagel & Katz 2003). At the same time the activity of the Na^+ pump is incremented. We also found that GS decreased significantly, thus preventing further increase in SCC (Horisberger 2003). The increase in resistance reveals that mucosal Pb^{2+} does not affect the integrity of the epithelium; this might be explained by its high complexing ability, especially with phosphate groups on cell membranes, which reduces the cytotoxic potency of the metal, making it unable to reach vulnerable intracellular targets, at concentrations from 50 μM and higher (Steffensen *et al.* 1994). The inhibitory effect of Pb^{2+} applied to the serosal surface of the skin might be explained by a possibly easier access of the metal ion to cellular structures, since there is no cornified layer covering the basolateral membrane; as, for instance, the uptake of metal ions such as cadmium from the inner side is far larger than the uptake from the outer side (Takada & Hayashi 1980). It is known that many drugs block Na^+ channels from the cytoplasmic end (Zamponi & French 1994).

Although the concentrations used in the work are high, it should be borne in mind that the epithelial skin membrane is a very efficient barrier which impedes diffusion of drugs to the ion channels, a difficulty which is not encountered in structures such as excised peripheral nerve membrane patches used in the study of Na^+ channel blocking concentrations (Braga *et al.* 1999a). Physiological differences in skins from different species of amphibians have been underscored (Castillo & Orce 1997) and there is a high degree of functional polymorphism in biotransforming enzymes which contribute to variability in response to chemical toxicity (Lindeman *et al.* 2002). Toad skin contains three types of cells below the stratum corneum: the principal cells (stratum granulosum), the mitochondria-rich cells and the glandular cells form a syncytial compartment. Under short-circuit conditions Na^+ mainly passes the apical membrane of the skin via Na^+ specific channels in the principal cells (Nielsen 1997). The junction between the stratum corneum and the stratum granulosum may act as an outside barrier (Takada & Hayashi 1980), whereas the inside or serosal barrier is weaker.

In an attempt to further examine the site or sites of action of lead on the toad skin, its effects were compared with those of noradrenaline (NA). NA (Tom-

linson & Wood 1978) increases the SCC of the toad skin by stimulating the sodium pump, as also in other structures such as brain synaptic plasma membrane (Abashidze *et al.* 2001) and rat cardiac myocytes (Stimers & Dobretshov 1998). Catecholamine stimulation of SCC is also partly due to increased Cl^- apical efflux. If lead is acting at sites similar to those of NA, then NA applied after lead, should produce similar responses. However, this is not the case (Figure 2): the decreased response to NA in lead-pretreated (serosal side) skins could be explained by a decrease in sodium conductance (G_{Na+}) of the apical membrane produced by Pb^{2+} and by the finding (Chandra *et al.* 1984) that lead at concentrations larger than 0.1 mM inhibited NA uptake in rat brain synaptosomes. That the decrease in G_{Na+} is indeed an important factor was demonstrated when the inhibitory effect of amiloride, which reversibly blocks apical sodium channels in frog skin (Flonta *et al.* 1998), was followed by additional inhibition of the electric parameters by Pb^{2+}. It may be considered that lead binds to a site different from amiloride, which is known to bind to cysteine groups on ENaC. Thus, Rana skin has a tryptophan at the outer mouth of the ion pore which could be the binding site for Pb^{2+} (Babini *et al.* 2003). The decrease in SCC and G_{Na+} induced by addition of Pb^{2+} to the inner surface of the skin after an initial decrease induced by apical amiloride could also be due to a blocking effect of the metal on a residue such as lysine in the intracellular terminus of α- and β-ENaC which regulate the number of channels in the membrane and thereby determine sodium entry (Benos & Stanton 1999). Figure 4B also shows that after washout of apically administered Pb^{2+}, subsequent application of amiloride was followed by a significantly ($P < 0.01$, $n = 6$) greater increase in SCC and G_{Na+} on addition of NA. It has been shown that at the NA concentration used, the SCC response became insensitive to amiloride (Castillo & Orce 1997) and is probably dependent not only on stimulation of the Na,K pump but also on glandular secretory response of Cl^-. Cl^- currents of amphibian skin are also generated by epithelial mitochondria-rich cells (Larsen *et al.* 2001). Previous investigations have shown that the increment in SCC produced by NA was the same in the absence or presence of amiloride and were interpreted to mean that the catecholamine could open up new Na^+ channels not blocked by the amiloride present (Tomlinson & Wood 1978). The amiloride test showed that serosally applied Pb^{2+} not only decreased the sodium potential, but also increased passive conductance, which points to disturbance of membrane integrity; this may be due to Pb^{2+} enhanced opening of interendothelial tight junctions. The test was effected in only five experiments because it was often not possible to determine resistance in tissues where the driving forces changed during the transepithelial voltage perturbations (Van Driessche 1991). A recent review (Glynn 2002) gives evidence that the sodium pump is thermodynamically reversible because artificially steep transmembrane ion gradients make it run backward, a finding in accordance with results of three experiments (not shown) in which V-E_{Na+} showed negative values. However, the significant ($P < 0.01$, $n = 5$) decrease in the response to NA (Figure 4-B) after amiloride, in spite of Pb^{2+} washout, needs explanation. Neurotransmitter release from hippocampal neurons by Pb^{2+} is persistent in spite of washout (Braga *et al.* 1999b) and the amiloride test showed that Pb^{2+} applied in the inner surface of the skin significantly decreased V-E_{Na+}: this, added to the fact that the metal administered to the serosal surface also decreases K^+ permeability of the basolateral membrane, could mean that the consequent decrease in the activity of the Na^+, K^+ pump would reduce the stimulatory effect of NA. In contrast, mucosal application of Pb^{2+} significantly increased V-E_{Na+} while reducing skin conductance. This is in apparent contradiction with the inhibitory effect of Pb^{2+} on Na^+-K^+-ATPase demonstrated by Carfagna *et al.* (1996); but considering that the enzyme extrudes three Na^+ in exchange for two K^+, Apell (1998) found that binding of the third Na^+ is a trigger for a structural rearrangement of the ATP-binding moiety of the enzyme, which enables its phosphorylation by Pb^{2+} and increases the kinetic properties of the ion pump. Furthermore, transmission electron microscopy studies on erythrocytes (Suwalsky *et al.* 2003) showed fine lead particles adhering to the external and internal erythrocyte membrane, thus decreasing the quantity of Pb^{2+} which reaches the cytosol; and only Pb^{2+}-activated protein kinase C (PKC) is able to carry out enzyme phosphorylation. Higher concentrations of Pb^{2+} which are able to enter the cytoplasm from the serosal surface, on the contrary, are known to inhibit PKC activity (Tomsig & Suszkiw 1995). In conclusion, these results suggest that Pb^{2+} toxicity is the consequence of multisite effects, with the following differential actions: a) applied to the outer surface of the skin the metal ion probably partially inactivates epithelial sodium channels while stimulating sodium transport due to increased basolateral membrane conductance; b)

applied to the inner surface could inactivate apical channels and the sodium pump, and possibly disturbs membrane and/or cell integrity.

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