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Water Permeability Properties of the Ovarian Oocytes from *Bufo arenarum* and *Xenopus laevis:* A Comparative Study

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Abstract. The water permeability properties of ovarian oocytes from *Xenopus laevis* and *Bufo arenarum,* a toad species found in the Buenos Aires region, were studied. We report that: (i) the water osmotic permeability (P_f, cm/sec $\times 10^{-4}$) was significantly higher in *Bufo* (6°C = 12.3 ± 2.4; 18°C = 20.8 ± 4.8) than in *Xenopus* oocytes (6°C = 5.3 ± 0.3; 18°C = 6.2 ± 1.6). The corresponding water diffusion permeability values (P_d, cm/sec $\times 10^{-4}$) were: *Xenopus* = 2.3 ± 0.3 (6°C) and 4.8 ± 0.7 (18°C); *Bufo* = 2.7 ± 0.4 (6°C) and 6.0 ± 0.5 (18°C). (ii) Amphotericin B increased the P_f and P_d values. The observed $\Delta P_f/\Delta P_d$ ratio was not significantly different from the expected results ($n = 3$), after amphotericin B incorporation in both species. This means that the influence of unstirred layers and other potential artifactual compounds did not significantly affect our experimental results. (iii) Preincubation with gramicidin during 12 hr induced a clear increase in the oocyte volume. After that, a hypotonic shock only slightly increased the oocyte volume. Conversely, a hypertonic challenge induced a volume change significantly higher than the one observed in control conditions. (iv) Mercury ions did not affect the osmotic permeability in *Xenopus* oocytes but clearly inhibited, in a reversible way, the osmotic permeability in oocytes from *B. arenarum.* (v) Mercury ions did not reduce P_d values in either species. (vi) The $\Delta P_f/\Delta P_d$ values calculated from the differences observed in these parameters between both species were 11.9 ± 5.1 at 18°C and 15.5 ± 2.4 at 6°C. These numbers are similar to those previously reported in the case of membranes having water channels. From these results, we propose that water channels are present in the ovarian oocyte from *B. arenarum* but not in the ovarian oocyte from *X. laevis.*

Key words: Diffusional permeability—Image analysis—Mercury chloride—Osmotic permeability

Introduction

The existence of specific water channels in the animal cell has been widely investigated [19]. Evidence from biophysical [19] and structural [1] investigations gave strong support to the hypothesis that water channels are present in the red cell, the proximal tubule of the kidney and in the ADH-sensitive tissues [2, 10, 15, 16, 21, 26]. These views have been considerably strengthened with the cloning of specific proteins reputed to be water channels [23, 27, 29].

The oocyte from *X. laevis* has been used for the expression and cloning of a variety of membrane proteins [9, 12, 24, 25]. Among these are those originated in the red cell [22] and in the mammalian kidney [8, 29] that are considered as being water channels. The experiments using *Xenopus* oocytes were based on the low value of their endogenous water permeability; e.g., the absence of water channels. Nevertheless, it has been reported that in *Rana esculenta* the ovarian oocytes have a water permeability 20 times higher than body cavity oocytes [17].

The main purpose of this work was to compare the water permeability properties, studied from a biophysical point of view, of ovarian oocytes from *X. laevis* with *B. arenarum,* a toad species present in the Buenos Aires region. Osmotic and diffusional permeabilities were measured using hyposmotic and hyperosmotic

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gradients, as well as by changing the temperature of the medium. The effects of mercury ions and two reputed "waterphores" molecules were also studied.

From the results obtained, it is proposed that water channels are present in the ovarian oocytes from *B. arenarum* but not in the ovarian oocytes from *X. laevis*.

Materials and Methods

Oocyte Isolation

Ovarian oocytes (1.1–1.4 mm diameter) were obtained from adult *X. laevis* or *B. arenarum* females by standard procedures [4]. They were stored in Barth's buffer [in mM: 88 NaCl, 1.0 KCl, 0.8 $MgSO_4$, 0.3 $Ca(NO_3)_2$, 0.4 $CaCl_2$, 2.4 $NaHCO_3$, Tris(hydroxymethyl)-aminomethane 7.5; pH 7.6, 200 mOsM] containing penicillin (4.8 mg/ml) and streptomycin (5 mg/ml). To remove the follicular cell layer, the oocytes were incubated with 2 mg/ml of collagenase (type 1A Sigma) for 2 hr at room temperature (with continuous agitation). Afterwards, they were washed six times with Barth's buffer.

Oocyte volumes and osmotic water permeability determinations: Oocytes swelling or shrinking in response to an osmotic gradient were measured at 10 sec intervals. Hyposmotic (20 mOsM, diluted Barth's buffer) and hyperosmotic (400 mOsM, Barth's buffer plus sucrose) conditions were tested. Experiments were performed in a water-jacketed Lucite chamber with an agar bottom bed (2%). Temperature was controlled with a circulating water bath. Oocytes were viewed using an Olympus inverted microscope (IMT-2) and a video camera. The image was reproduced on a video monitor and digitalized. In a control series ($n = 7$), a computer program measured eight different oocyte diameters, found the average and calculated the volume assuming a spherical shape. Alternatively, only 2 diameters were measured (horizontal and vertical) in the same oocytes. In this last case, an ellipsoid shape was assumed. No significant differences were observed between both methods, and the second one was then routinely used.

The volume change as a function of time was considered linear during the first 80 sec after the osmotic challenge. The initial transmembrane water flux (JVo), was calculated from the slope at zero time of the V/Vo time course function and the initial oocyte volume (V_o). The apparent osmotic water permeability coefficient (P_f, cm/sec) was calculated from:

$$Pf = JV_o/Vw \cdot S \cdot (Osmo - Osmi)$$

where Vw is the partial molar volume of water (18 cm^3/mol), Osmo and Osmi the external and internal osmolarities (mol/cm^3) and S the oocyte surface area.

Unidirectional Water Fluxes and Diffusional Water Permeability

Diffusional water permeability was measured from the kinetics of tritiated water washout. Oocytes were incubated with 40 μCi/ml $^3H_20$ (Dupont, NEN) and 10 μCi/ml [^{14}C]sucrose (CEA, France) in Barth's buffer during 2 hr at the experimental temperature. Individual oocytes were then flushed with 1 ml of cold buffer on a Millipore filter to remove the external radioactivity and immediately transferred to a glass tube containing 5 ml of buffer. The tube was continuously and rapidly swirled by hand. Aliquots (150 μl) were removed at different times and counted. [^{14}C]sucrose activity (considered an extracellular marker) was deducted from the tritiated water activity at each experimental time. The diffusional water flux at each time was expressed as the fraction of the total radioactivity efflux ($(d^3H_20/\text{total } ^3H_20)/dt$). The apparent diffusional water permeability coefficient (P_d, cm/sec) was determined from:

$$P_d = [V_o\ (d^3H_20/\text{total } ^3H_20)/dt_o]/S$$

where V_o is the oocyte volume and $(d^3H_20/\text{total } ^3H_20)/dt_o$ was determined from the slope at zero time of the tritiated water efflux function.

Electron microscopy studies (*not shown*) indicated that both the *Xenopus* and *Bufo* oocyte membranes do not show important convolutions or invaginations. Some microvillae, which are similar to those observed in amphibian urinary bladder, can be mentioned. This means that the measured P_f and P_d values can be evaluated, when considering the real permeability surface, as in previous works [14, 18]. It is also evident that this factor similarly affects P_f and P_d values.

Amphotericin B, gramicidin S, beta mercapto-ethanol and $HgCl_2$ were purchased from Sigma.

Results

Volume Changes and Osmotic Permeabilities in Oocytes from *X. laevis* and *B. arenarum*

Figure 1 shows the volume changes induced by a hyposmotic or a hyperosmotic gradient in oocytes from *B. arenarum* and *X. laevis*. The experiments were run at 6°C. The volume change observed, as well as the initial rates of volume changes, were significantly higher in *B. arenarum* oocytes.

Table 1 gives the water osmotic permeability coefficients (P_f), calculated as described in Materials and Methods, in hypotonic experiments performed at 6 and 18°C. When a hypertonic challenge was imposed in oocytes from *X. laevis*, the P_f values were significantly different (cm/sec, $\times 10^{-4}$; Hypo: 5.3 ± 0.3, $n = 10$; Hyper: 0.78 ± 0.05, $n = 12$; mean diff. 5.03 ± 0.30, $P < 0.001$). This was not the case in oocytes from *B. arenarum* (cm/sec, $\times 10^{-4}$; Hypo: 12.3 ± 2.4, $n = 9$; Hyper: 11.2 ± 1.2, $n = 9$; mean diff. 1.1 ± 2.3, NS).

Incorporation of Amphotericin B

The effects of amphotericin B incorporation in oocytes from *B. arenarum* are shown in Fig. 2. The ionophore was added 10 min before the experiments and no significant changes in the oocyte volume (VO) were observed in either species during this preincubation period (*see* Table 3). Nevertheless, a rather slow swelling of the oocyte was subsequently observed, always in the absence of any change in the osmolarity of the medium. This probably reflected a sodium entry, dragging chloride and water.

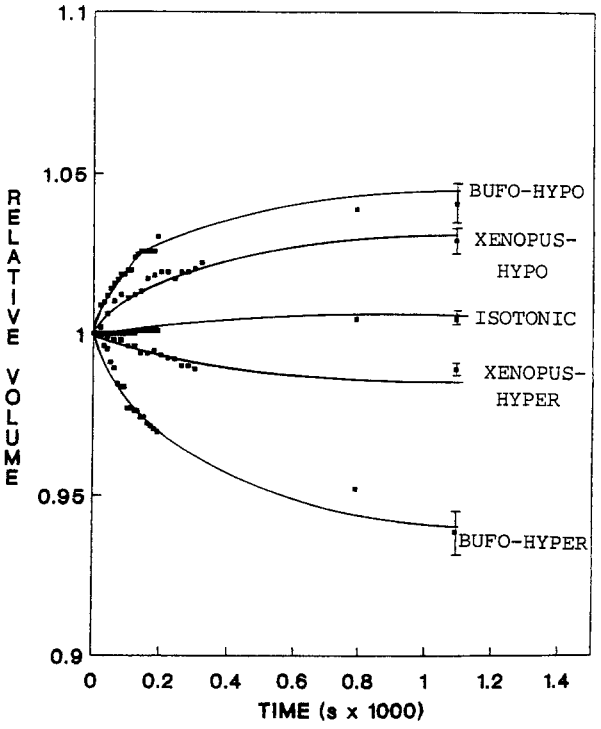

Fig. 1. Time course of oocyte relative volume (V/VO) measured in two different amphibian species: *B. arenarum* and *X. laevis*. Top curves: Hypotonic challenge (20 mOsM); Bottom curves: Hypertonic challenge (400 mOsM). Isotonic: no osmotic gradient. Experiments were performed at 6°C, and each point is the average of 9–13 separate experiments.

When a hypotonic shock was applied, the volume changes in amphotericin-pretreated oocytes were significantly higher than in control oocytes. In the case of a hypertonic gradient, the difference was less steep. It can be concluded that the final volume changes observed in amphotericin-B-pretreated oocytes probably reflect the combined effects of water and sodium movements. Conversely, the initial rate of volume change, from which the P_f values were calculated, can be reasonably attributed to water movements.

Table 2 gives the P_f values obtained (hypotonic experiments at 18°C) in *X. laevis* and *B. arenarum* oocytes, before and after the addition of amphotericin B. In this last condition, P_f values were corrected by deducting (swelling experiments) or adding (shrinking experiments) the volume changes induced by the ionophore in the absence of an osmotic gradient.

Incorporation of Gramicidin

Gramicidin incorporates slowly into biological membranes [7]. Because of this, rather long incubation periods are used. The oocyte volumes observed in both species in control conditions were similar, and did not change after 12 hr of incubation in Barth Ringer (Table 3). Nevertheless, incubation with gramicidin during the same period induced a clear increase in the oocyte volume. This probably reflected, once again, sodium, chloride and water entry into the oocyte. After 12 hr under gramicidin in an isotonic medium, no further volume change was observed.

Figure 3 shows that a hypotonic shock only slightly increased the volume of *Xenopus* oocytes pretreated with gramicidin at 6°C. The corresponding P_f value $(1.49 \pm 0.19) \times 10^{-4}$ cm/sec, $n = 9$, was almost four times lower than the control one (Table 1). Conversely, the hypertonic challenge induced a volume change significantly higher than the one observed in control conditions and the observed P_f $(7.5 \pm 0.5) \times 10^{-4}$, cm/sec, $n = 9$ was also significantly higher (control value: $0.76 \pm 0.05) \times 10^{-4}$, $n = 9$.

Similar experiments were carried out with *B. arenarum* oocytes, which are more permeable, and at a higher temperature (18°C, Fig. 4). In this case, the volume changes were significantly higher, while the asymmetry between hypo and hyper conditions remained ($P_f \times 10^{-4}$ cm/sec, Hypo-control: 20.8 ± 4.8 ($n = 13$), hypo-gramicidin: 12.7 ± 2.0 ($n = 9$); Hyper-control: 13.8 ± 3.5 ($n = 9$), hyper-gramicidin: 20.7 ± 3.8 ($n = 9$).

The Effects of Mercury Ions on the Osmotic Permeability

It has previously been reported that mercury ions do not affect the osmotic permeability in *Xenopus* oocytes [28]. We confirm these results. Nevertheless, we report here a clear inhibition induced by mercury ions on the osmotic permeability in oocytes from *B. arenarum*. This inhibitory effect was fully reversible after 10 min incubation with beta-mercapto-ethanol (5 mM) (Table 4).

Water Diffusional Permeability in Oocytes from *X. laevis* and *B. arenarum*

Figure 5 shows the tritiated water washout in *B. arenarum* oocytes at 6°C. Table 1 summarizes the water diffusional permeabilities, (P_d), calculated as described in Materials and Methods, in oocytes from both *X. laevis* and *B. arenarum*. Experiments were run at 6 and 18°C.

Incorporation of Amphotericin B

From the previous results on P_f values, which we will discuss later, amphothericin B seems to be the best candidate to test exogenous channel incorporation into amphibian oocytes. Figure 5 and Table 2 present the ef-

Table 1. Osmotic (P_f, cm/sec × 10^{-4}) and diffusional (P_d, cm/sec × 10^{-4}) water permeabilities in oocytes from *B. arenarum* and *X. laevis*

	P_f *Bufo*	P_f *Xenopus*	ΔP_f	P_d *Bufo*	P_d *Xenopus*	ΔP_d
6°C	12.3 ± 2.4 (n = 9)	5.3 ± 0.3 (n = 10)	7.0* ± 2.4	2.7 ± 0.4 (n = 6)	2.3 ± 0.3 (n = 8)	0.5 ± 0.6
18°C	20.8 ± 4.8 (n = 13)	6.2 ± 1.6 (n = 9)	14.6** ± 5.1	6.0 ± 0.5 (n = 6)	4.8 ± 0.7 (n = 12)	1.2 ± 0.8

Data are means ± SEM in swelling experiments (20 mOsM) performed at 6 and 18°C. * $P < 0.001$, ** $P < 0.01$ Student test.

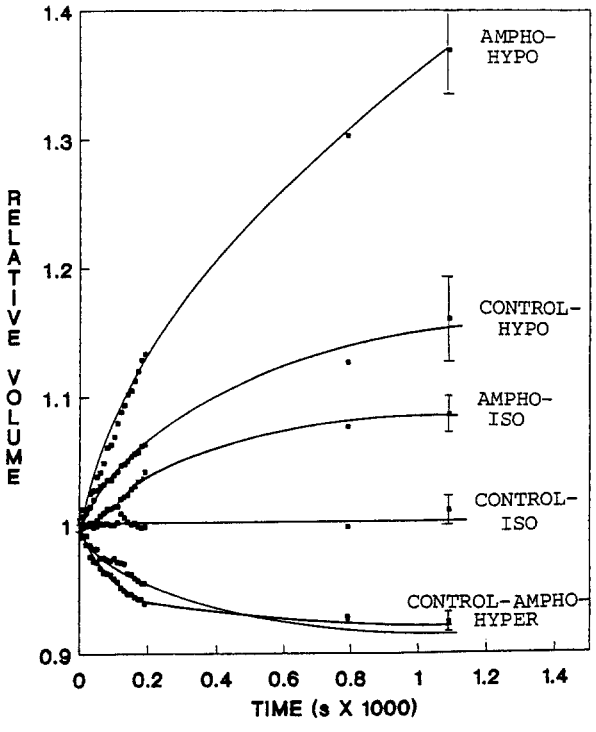

Fig. 2. Time course of oocyte relative volumes (V/VO) measured at 18°C in *B. arenarum* oocytes. Effects of amphotericin B 10 min preincubation period (100 μg/ml). Hyposmotic (20 mOsM) and hyperosmotic (400 mOsM) challenges. Each point is the average of 9–13 separate experiments.

fects of preincubation with amphothericin B on the tritiated water washout and in the diffusional water permeability of oocytes from *B. arenarum* and *X. laevis*. The experiments were run at 18°C and the ionophore clearly increased the P_d values in both cases.

The Effects of Mercury Ions on the Diffusional Water Permeability

Mercury ions did not reduce P_d values in the ADH-stimulated toad bladder [13]. We now report that pretreatment with $HgCl_2$ actually increased P_d values in *B. arenarum* oocytes, both at 6 and at 18°C (Table 5).

Discussion

The Use of Amphibian Oocytes to Express Water Channels

In pure lipid bilayers the P_f/P_d ratio equals one, after appropriated correction of the unstirred layers effects [3, 5, 7]. The incorporation of polyene or polypeptide antibiotics into the membrane increases both P_f and P_d, but now $\Delta P_f/\Delta P_d = N$; N being a number characteristic of the inserted channel. Amphotericin B incorporation (N = 3) and gramicidin incorporation (N = 5) have been previously used to test water permeability measurements in artificial [6] and natural membranes [18, 20].

Another property, associated with the presence of water channels in biological membranes is sensitivity to mercurial compounds [13, 15, 26].

To demonstrate that CHIP-28 and other related proteins are water channels, their expression in the plasma membrane of *X. laevis* oocytes was previously used. The corresponding messenger RNA or cDNA was injected [8, 23, 29], and the results obtained were as follows: (i) An enhancement of the P_f and P_d values; (ii) an increase in the observed $\Delta P_f/\Delta P_d$ ratio; (iii) the development of sensitivity to mercurial compounds and (iv) a reduction in the activation energy (Ea; [7, 11]) of the osmotic transfer.

Incorporation of Amphotericin B and Gramicidin

Table 2 and Fig. 5 show that amphothericin B clearly increased P_f and P_d in oocytes from both species, even at 18°C. These are important results, indicating that the plasma membrane remained, at both temperatures, the limiting barrier for diffusional and osmotic water transfers. Furthermore, the observed $\Delta P_f/\Delta P_d$ ratio was, in both cases (Table 2) not significantly different from the theoretical expectations (N = 3). This means that

Table 2. Effects of amphotericin B on the water permeability of *X. Laevis* and *B. arenarum* oocytes

	P_f control	P_f amphot	ΔP_f	P_d control	P_d amphot	ΔP_d	$\Delta P_f/\Delta P_d$
Xenopus	6.2 ± 1.6 (n = 9)	21.9 ± 0.2 (n = 7)	15.7* ± 4.1	4.8 ± 0.7 (n = 12)	10.9 ± 1.4 (n = 4)	6.1** ± 1.2	2.6 ± 0.8
Bufo	20.8 ± 4.8 (n = 13)	30.7 ± 6.5 (n = 9)	9.9* ± 3.1	6.0 ± 0.5 (n = 6)	10.8 ± 1.5 (n = 6)	4.8** ± 0.8	2.1 ± 0.7

P_f and P_d values in cm/sec × 10^{-4}. Experimental conditions are those described in Table 1. Amphotericin B was added 10 min before the experiments at a final concentration of 100 μg/ml. * $P < 0.01$; ** $P < 0.001$, Student test.

Table 3. Oocyte volume variations in the absence of an osmotic gradient

VO × 10^{-4} cm^3	Control	Amphotericin	Gramicidin
Xenopus	8.6 ± 0.2 (n = 22)	9.0 ± 0.2 (n = 13)	10.5 ± 0.1* (n = 19)
Bufo	8.4 ± 0.4 (n = 35)	7.9 ± 0.1 (n = 27)	11.0 ± 0.5* (n = 17)

The oocyte volumes were measured before (control) and after 10 min (amphotericin B, 100 μg/ml) or 12 hr (gramicidin, 100 μg/ml) incubation period. * $P < 0.001$ Student test.

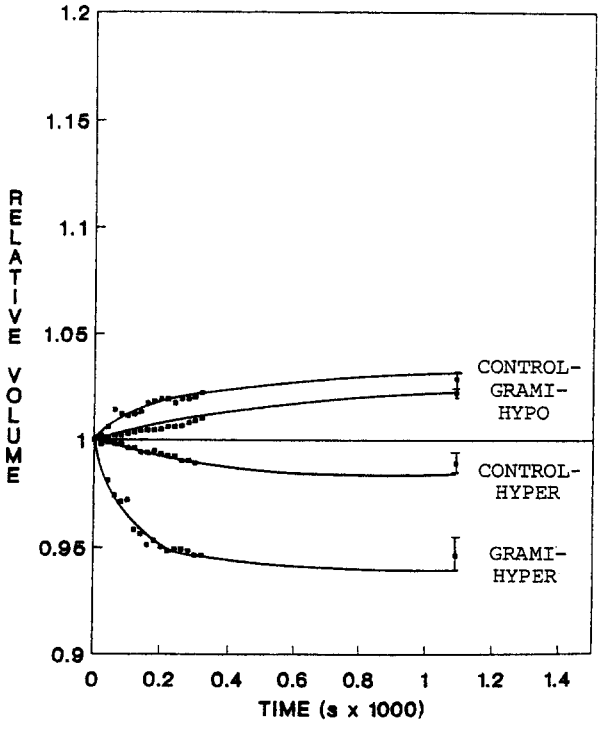

Fig. 3. Time course of oocyte relative volumes (V/VO) measured at 6°C in *X. laevis* oocytes. Effects of gramicidin S (12 hr preincubation period; 100 μg/ml). Hyposmotic (20 mOsM) and hyperosmotic (400 mOsM) challenges. Each point is the average of 9–13 separate experiments.

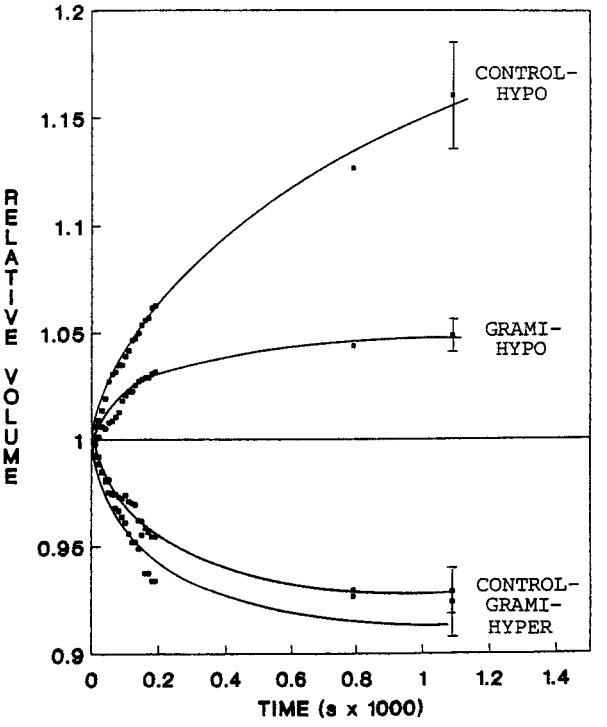

Fig. 4. Time course of oocyte relative volumes (V/VO) measured at 18°C in *B. arenarum* oocytes. Effects of gramicidin S (12 hr preincubation period 100 μg/ml). Hyposmotic (20 mOsM) and hyperosmotic (400 mOsM) challenges. Each point is the average of 9–13 separate experiments.

Table 4. Effects of mercury ions on the oocyte osmotic water permeability

	Control	$HgCl_2$	$HgCl_2$ + BME
Xenopus	6.2 ± 1.6 (n = 9)	7.5 ± 1.0 (n = 9)	
Bufo	24.2 ± 2.4 (n = 10)	7.4 ± 2.3* (n = 10)	22.5 ± 1.7 (n = 10)

(P_f values, cm/sec × 10^{-4}). The effects of $HgCl_2$ (0.3 mM) were tested in identical conditions to those described in Table 1. Beta-mercapto-ethanol (BME, 5 mM) was added 5 min after $HgCl_2$ and 10 min before the experiments. * $P < 0.02$ Student test.

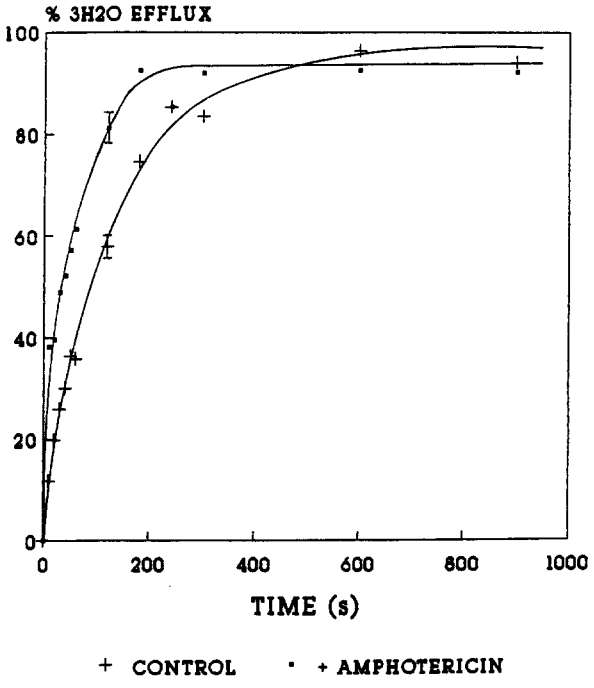

Fig. 5. Tritiated water washout in control and amphothericin-B-pretreated oocytes from *B. arenarum*.

Table 5. Effects of mercury ions on diffusional water permeability (P_d)

P_d, cm/sec × 10^{-4}	*Bufo*	*Bufo* $HgCl_2$
6°C	2.3 ± 0.3 (n = 6)	4.9 ± 0.6* (n = 6)
18°C	6.0 ± 0.5 (n = 6)	8.5 ± 1.3 (n = 6)

The effects of $HgCl_2$ (0.3 mM) were tested in identical conditions to those described in Table 1. * $P < 0.01$ Student test.

the influence of unstirred layers and other potential technical errors did not significantly affect our experimental results.

The case of gramicidin is more complex, probably because of the important volume changes induced during the incubation period. A straightforward interpretation of the observed results is to accept that swelled oocytes cannot further increase their volume in the presence of a hyposmotic shock. On the contrary, the oocyte shrinking would be easily observed.

The Effect of Mercury Ions

The reversible inhibition of the osmotic water permeability by mercurial compounds is generally accepted as a proof of the existence of water channels [13, 26, 28]. We have not observed, confirming previous results [28], an inhibitory effect of mercury ions on the osmotic permeability in *X. laevis* oocytes. Nevertheless, P_f values were clearly reduced in *B. arenarum* oocytes, and this inhibitory effect was fully reversible after incubation with beta-mercapto-ethanol. Conversely, P_d values were significantly increased. This indicated the existence of two alternative pathways for osmotic and diffusional water movements in the membrane of the oocytes from *B. arenarum*.

Water Channels in the Plasma Membrane of Oocytes from *B. arenarum*

It can be accepted, as a working hypothesis, that the difference in water permeability observed between the oocytes from *X. laevis* and *B. arenarum*, could be due to the fact that the *Bufo* oocytes have water channels not present in the *Xenopus* ones. This is supported by the observed effects of mercurial compounds, which reduced P_f values in *Bufo* oocytes, to the levels spontaneously observed in *Xenopus* oocytes. It can be then speculated that water permeability across the lipid bilayer is similar in both species. We can now calculate, from the data in Table 1 and following the same rationalism used in other cases in which water channel incorporation was studied [7, 14, 18], the $\Delta P_f/\Delta P_d$ values that result from the differences observed in these parameters between both species. The obtained values are 11.9 ± 5.1 at 18°C and 15.5 ± 2.4 at 6°C. We must be cautious when using the observed P_f and P_d values, even when the amphothericin experiments strongly suggest that unstirred layers and other potential error factors (as uncertainty on the actual membrane surface) did not significantly affect our experimental results. Nevertheless, the obtained $\Delta P_f/\Delta P_d$ values are similar to those previously accepted as the N value in the case of water channels [7, 14, 18], suggesting that the plasma membrane of the oocytes from *B. arenarum* contains water channels that are not present in the oocytes from *X. laevis*.

The final answer to the hypothesis presented here will come from molecular biology experiments. This is the subject of our present work.

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