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Functional Incorporation of P-Glycoprotein into *Xenopus* Oocyte Plasma Membrane Fails to Elicit a Swelling-Evoked Conductance

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Microinjecton of *Xenopus* oocytes with P-glycoprotein-containing membranes from multidrug resistant cells following a recently published procedure resulted in the transplantation of the protein to the plasma membrane of the oocytes and was confirmed by Western blot analysis. These oocytes showed a reduced intracellular accumulation of daunomycin, when compared to uninjected oocytes or to those injected with membrane vesicles lacking P-glycoprotein, thus indicating that the protein had been incorporated in a transport-competent form. On the other hand, transplantation of P-glycoprotein to the oocyte membrane did not significantly change either the appearance or the properties of swelling-elicited membrane conductance with respect to those determined in oocytes either uninjected or injected with membranes lacking P-glycoprotein. These results do not support a role for P-glycoprotein as a swelling-activated chloride channel.

The plasma membrane P-glycoprotein (Pgp), a member of the ATP binding cassette (ABC) transporter proteins, is thought to actively extrude a broad range of hydrophobic drugs (1) and represents the most consistent molecular alteration associated with the acquisition of the multidrug resistance phenotype (reviewed in Refs. 2-3). Valverde et al. (4) have proposed that Pgp might also act as a volume-activated chloride channel based on the observation that expression of the protein in both NIH/3T3 fibroblasts and lung epithelial cells permanently transfected with the human multidrug resistance cDNA, conferred on the cells the capability to respond to hypotonic stress. The association of Pgp with increased sensitivity to swelling-activated chloride currents has also been supported by Altenberg et al. (5) working on multidrug resistant human cancer cells, and different investigators further addressed the relationships between the transporter and chloride channel activities of Pgp by studying either the preventing effect of transportable drugs on channel activation (6-8) or the inhibition of drug efflux by cell swelling (9).

In contrast to the above hypothesis, there is an emerging body of evidence supporting a lack of correlation between expression of Pgp and swelling-activated chloride channel activities. For example, Rasola et al. (10) conclude that the size of the swelling-induced chloride current in four epithelial cell lines, is the same as that obtained in their drug-resistant counterparts expressing Pgp. Similarly, no relationship between volume-activated chloride currents and Pgp expression has been found in other cellular systems (11-17), indicating that the association between them, if any, must be complex. Actually, the controversy focuses on the proposed role for Pgp as a regulator of chloride currents activated by swelling (7, 18-22), taking into account the limited knowledge we currently have concerning the transduction mechanism by which cell swelling activates ionic conductances.

In the present study, we have incorporated a fully processed form of the membrane-bound protein Pgp into the plasma membrane of *Xenopus* oocytes using a recently described procedure (23-24), to analyze the association between its expression and swelling-activated Pgp-chloride conductances. The *Xenopus* oocyte system has been selected because of its well character-

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Abbreviations: Pgp, P-glycoprotein; Pgp^+-oocytes, oocytes microinjected with membrane vesicles containing Pgp; control oocytes, oocytes injected with membrane vesicles lacking Pgp; NR and HR-45, normal Ringer solution (220 mosm) and hypoosmotic Ringer solution (95 mosm) in which NaCl concentration was 115 and 45 mM, respectively; DNM, daunomycin; MF, microsomal membrane fraction; P1, insoluble fraction obtained after treatment of MF with sodium cholate.

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ized endogenous swelling-induced conductances (25-27), which allows discrimination between *de novo* ionic conductances resulting from transplantation of foreign proteins and background native currents. Preliminary results have been reported elsewhere (28).

MATERIALS AND METHODS

Membrane preparation. Microsomal membrane fractions (MF) from a murine L1210 cell line lacking Pgp and from a 150-fold daunomycin (DNM) resistant stable subline overexpressing Pgp (29), were obtained according to the procedure described by Escriba et al. (30). MF were treated with detergent or frozen under liquid nitrogen until use.

Preparation of Pgp-enriched membranes. To increase the relative content of Pgp in the membranes from DNM-resistant L1210 cells, MF containing 10 mg/ml of protein in 10 mM HEPES, pH 7.4, 140 mM KCl, 5 mM $MgCl_2$, 1 mM DTT 3 mM NaN_3 were incubated with the detergent sodium cholate at a protein:detergent ratio of 1:10 (w:w) during 30 min at 4°C. Upon centrifugation (200.000 g × 20 min), Pgp remained mostly associated to the insoluble fraction (P1) as demonstrated by Western analysis (see below). As control, a similar procedure was carried out to obtain the corresponding P1 fraction from L1210 cells lacking Pgp. Protein concentration was determined using the method of Bradford (31).

Western analysis of Pgp. The presence of Pgp in MF and fractions derived from sodium cholate treatment, was analyzed by resolving the protein component of the different samples on SDS-containing 7.5 % polyacrylamide gels followed by electroblotting to nitrocellulose. Immunodetection of Pgp was achieved using the monoclonal antibody C219 (32) (Centocor Diagnostics) and then with goat anti-mouse antibody conjugated to alkaline phosphatase, which allows detection of 10-50 pg of antigen (33).

Oocyte preparation and microinjection. Oocyte preparation and microinjection was carried out as previously described (24). Briefly, oocytes were isolated and kept at 15-16°C in sterile modified Barth's solution (10 mM HEPES pH 7.4, 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.40 mM $NaHCO_3$), supplemented either with gentamicin 0.1 mg/ml or penicillin 100 IU/ml and streptomycin 0.1 mg/ml. After harvesting, oocytes were treated with collagenase (0.5 mg/ml, during 50-60 minutes at room temperature) and the next day, healthy oocytes were microinjected with 100 nl of fresh or thawed MF or P1 samples from cells lacking Pgp (control oocytes) or Pgp-expressing cells (Pgp^+-oocytes).

Oocyte membrane isolation. Plasma membranes from either uninjected or injected oocytes were manually isolated following a procedure by Sadler and Maller (34), which precludes any possible contamination of the oocyte membrane with Pgp arising from the injected vesicles. Presence of Pgp in the isolated membranes was detected by Western immunoblots 24 h after microinjection.

[^{3}H]DNM accumulation. One day following injection, the intraoocyte level of DNM was determined by incubating duplicate groups of 10 oocytes with 1 μCi [^{3}H]DNM (Dupont, NEN Products) plus unlabeled DNM to obtain a final drug concentration of 0.6 μM, for 60-90 min (time reported as appropriate to analyze drug accumulation in the oocytes (9)), at 25°C in normal Ringer (NR) buffer: 5 mM HEPES pH 7.0, 115 mM NaCl, 2 mM KCl and 1.8 mM $CaCl_2$. Upon removing the extracellular medium, the cells were solubilized with 2 % SDS and counted in 5 ml of Beckman scintillant.

Electrophysiological recordings. Oocytes were placed in a small chamber (150 μl full volume) and membrane currents were recorded at room temperature (20-25°C) for 12 to 36 h after injection by using a high compliance two-electrode voltage-clamp system. Intracellular electrodes (1-4 MΩ resistance) were filled with 3 M KCl for voltage recording and 3 M potassium acetate for current injection. During recordings, oocyte membrane potential was held at −100 mV. To estimate the current to voltage (I/V) relationships, voltage pulses of 800 ms duration to −60,−20, +10, +40 and +80 mV were given while superfusing the cell at a rate of 3-9 ml/min with NR (220 mosm) or hypoosmotic Ringer's solution (HR-45; 95 mosm, in which the NaCl concentration was reduced from 115 mM to 45 mM). Membrane currents were low-pass filtered at 500 Hz and simultaneously recorded on a chart recorder and PC-computer after sampling at twice the filter frequency using the VCAN package supplied by J. Dempster (University of Strathclyde, UK). Net swelling activated currents were calculated by subtracting the records obtained at different potentials in HR-45 and NR solutions.

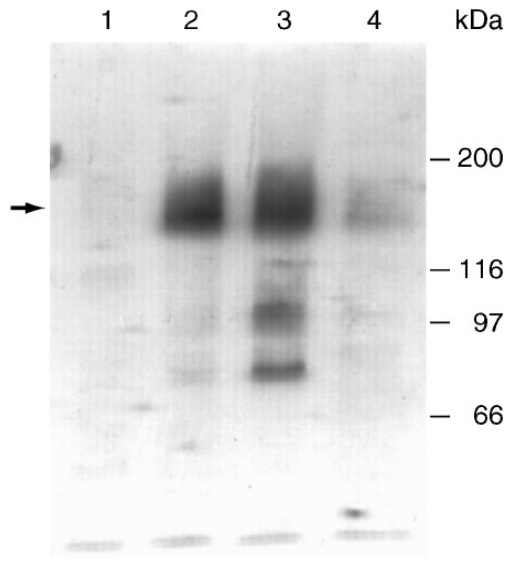

FIG. 1. P-glycoprotein in membrane fractions**.** Proteins resolved on 7.5 % SDS-polyacrylamide gel electrophoresis were analyzed by immunoblotting with the anti-Pgp monoclonal antibody C219. Lane 1, MF from DNM-sensitive L1210 cells; lane 2, MF from DNM-resistant L1210 cells; lanes 3 and 4, insoluble (P1) and soluble fractions, respectively, of cholate-treated MF from resistant cells. Lanes 1-4 contain 5 μg. The position of Pgp is denoted by an arrow. Molecular mass markers (in kDa) are shown to the right.

Statistical analysis. Data shown are mean ± S.E.M. Differences between groups were determined by analysis of variance and the group means compared by the Student-Newman-Keuls test. The Student's *t* and Z-tests were used when two group means or proportions, respectively, were compared. Differences were considered significant when *p* was less than 0.05.

RESULTS

Pgp-enrichment of membrane vesicles. MF from DNM-resistant L1210 cells overexpressing Pgp (Fig. 1, lane 2, arrow) was the starting material employed as the vesicular vehicle used to incorporate Pgp into the plasma membrane of *Xenopus* oocytes. Incubation of Pgp-containing MF with sodium cholate proceeded with solubilization of 73 ± 2 % (n = 13) of the total protein of the membranes. Western immunoblots showed that Pgp was mostly associated with the remaining insoluble fraction (P1; Fig. 1, lane 3) therefore

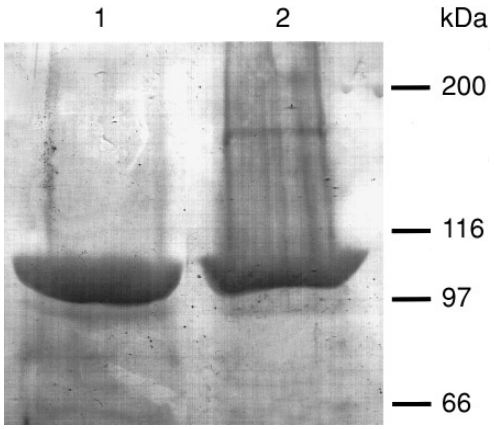

FIG. 2. Immunodetection of Pgp in the oocyte membranes**.** The protein component of manually excised plasma membrane (34) was resolved and analyzed for Pgp as in Fig. 1. Lane 1, membranes from 20 uninjected oocytes. Lane 2, membranes from 20 Pgp^+-oocytes injected with the P1 fraction. Total protein in each lane was 80 μg.

indicating that Pgp content in P1 was increased about 4-fold with respect to MF.

Functional incorporation of Pgp into the oocyte membranes. One day after microinjection of collagenase-treated oocytes with Pgp-containing vesicles (Pgp^+-oocytes), incorporation of Pgp into the oocyte plasma membrane was confirmed by Western immunoblot. While uninjected oocyte membranes showed undetectable levels of Pgp (Fig. 2, lane 1), presence of the protein displaying an apparent molecular mass close to 170 kDa (Fig. 2, lane 2), was clearly detected in membranes from Pgp^+-oocytes. The prominent protein band which appears between the 116-97 kDa molecular mass markers in both uninjected and Pgp^+-oocytes, is likely due to the unspecificity of C219 which has been reported as crossreacting with different proteins (18).

The transport activity of the transplanted Pgp was determined incubating injected oocytes with DNM, a Pgp transportable substrate, and subsequent analysis

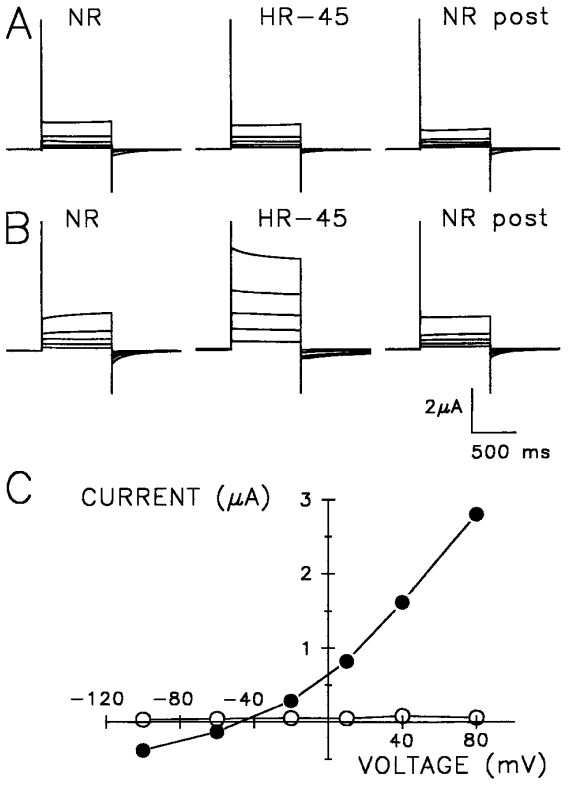

FIG. 3. Native membrane currents elicited by hypotonic medium (HR-45) in collagenase treated oocytes. (A) Lack of response to HR-45 observed in 84 % of the oocytes 48 h after collagenase treatment. Traces represent currents elicited when stepping the membrane potential from −100 mV to −60, −20, +10, +40, and + 80 mV. (B) Representative swelling response of the remaining cells when challenged by HR-45, which fully reversed when returned to NR solution (NR post). Note the current inactivation at positive potentials. The baseline current at −100 mV has been shifted arbitrarily in HR-45 recordings. (C) I/V relationship for oocytes shown in A (hollow symbol) and B (filled symbol). The net swelling-activated current was obtained by averaging the currents in NR and NR-post and subtracting, at each potential, from HR-45. Note the outward rectification and the reversal potential close to −50 mV. Upward deflections represent outward currents.

TABLE I

Accumulation of DNM in Oocytes Injected with Different Membrane Fractions

	Difference in DNM	
Injected sample	Accumulation[a]	Exported DNM (%)[b]
MF	111 ± 12 (n = 4)*	19 ± 3 (n = 4)
P1	190 ± 22 (n = 9)*,#	23 ± 4 (n = 9)

[a] Difference in DNM accumulation between control oocytes (A) and Pgp^+-oocytes (B) expressed as fmol of DNM per oocyte ± S.E.M. The number of experiments is given in parentheses.

[b] Exported DNM measured as [(A-B)/A] × 100 ± S.E.M.

* $p < 0.01$ between control and Pgp^+-oocyte groups (paired t-test).

# $p < 0.05$ between Pgp^+-oocyte groups (t-test).

of the differences in intracellular accumulation of the drug between control and Pgp^+-oocytes (Table I). Our results indicated that while accumulation of DNM in uninjected (968 ± 110 fmol/oocyte; n = 9) and control oocytes (859 ± 67 fmol/oocyte; n = 15) was indistinguishable ($p>0.05$), Pgp^+-oocytes accumulated significantly less DNM ($p<0.05$, Anova) than the previous two groups (640 ± 64 fmol/oocyte; n = 13). Interesting, a more detailed analysis considering two Pgp^+-oocytes groups, injected with MF or with P1, indicated that the difference in DNM accumulation between control and Pgp^+-oocytes significantly increased from 111 to 190 fmol/oocyte as the amount of injected Pgp increased from MF to P1 (Table I). Taking into account that the permeability of the oocytes to DNM remains apparently unaffected by the microinjection (uninjected and

TABLE II

Relative Occurrence and Properties of the Swelling-Evoked Currents in Uninjected, Control, and Pgp^+-Oocytes

			Occurrence %	Amplitude[a] nA	Reversal potential[b] mV	τ[a] ms
Uninjected[c]			16 (8 out of 50)	1019 ± 303 (n = 8)	−52 ± 4 (n = 8)	310 ± 34 (n = 8)
Injected[c]	Control	(MF + P1)[d]	12 (5 out of 40)	865 ± 327 (n = 5)	−41 ± 4 (n = 4)	473 ± 77 (n = 5)
	Pgp^+-cells	MF	8 (10 out of 120)	742 ± 207 (n = 10)	−51 ± 3 (n = 8)	442 ± 79 (n = 10)
		P1	4 (2 out of 57)	788 ± 253 (n = 2)	−51 ± 0 (n = 2)	217 ± 51 (n = 2)

[a] Current amplitude and current time inactivation constant (τ) measured at +80 mV.
[b] The reversal potential was only determined in those cells in which all the voltage pulses were given.
[c] Values reported in the table show no significant differences ($p > 0.05$) between uininjected and injected oocytes.
[d] Control cells injected with MF or P1 show no significant differences ($p > 0.05$) and were pooled.

control cells accumulated indistinguishable amounts of the drug), the previous differences in accumulation indicate the ocurrence of a drug efflux responsible for the export of 19 % and 23 % of DNM in the Pgp^+-oocytes injected with MF and P1, respectively (Table I). Together with this, the fairly consistent reduced accumulation of DNM in the Pgp^+-oocytes regardless of the microinjection with either MF (67 %; 4 out of 6 experiments) or P1 (60 %; 9 out of 15), strongly supports functional transplantation of Pgp to the oocyte membrane.

Swelling membrane conductances of the injected oocytes. The functional transplantation of Pgp to the plasma membrane of the oocytes having been confirmed, we attempted to evoke swelling-activated currents in these cells to test whether Pgp represents an osmo-dependent chloride channel. To discriminate between endogenous swelling-activated chloride channels in oocytes (25-27) and the putative chloride conductance associated with Pgp (4, 6-7), we first characterized the currents elicited in collagenase-treated oocytes when challenged by hypotonic stress. Fig. 3A shows that upon exposure to HR-45, most defolliculated oocytes were unresponsive to swelling. Only in 8 out of 50 oocytes (16 %) (Table II), could a swelling conductance be activated within 2-3 min of hypotonic exposure and was fully reversed upon returning to isotonic conditions (Fig. 3B). The swelling-elicited conductance was quite variable in amplitude (ranging 120-2805 nA at + 80 mV) and characterized by the following properties: *i*) the I/V relationships showed a clear outward rectification at negative potentials (Fig. 3C), *ii*) a current inactivation at a highly positive potential (see inactivation time constant values in Table II), *iii*) a reversal potential of − 52 ± 4 mV (n = 8) indicating as previously reported (15, 25-26), a contribution from potassium and chloride ions to this current, *iv*) the amplitude and the percentage of appearance of this current declined with time, being essentially negligible by day 3-4 after harvesting (data not shown). All these properties agree with those previously reported for endogenous swelling current in oocytes (26), which has been recently termed $I_{Clswell}$(1) or $I_{Cl(osm)}$ (27).

Taking advantage of the infrequent and well characterized endogenous swelling-activated conductance in our collagenase-treated oocytes, we studied under identical conditions, the response of the Pgp^+-oocytes to hypotonicity. Our results indicate that the presence of Pgp in the oocyte membrane did not significantly affect ($p > 0.05$) the appearance of the swelling-activated ion currents with respect to uninjected or control cells (Table II). Fig. 4A shows the lack of response exhibited by most of Pgp^+-oocytes (93 %) to hypotonic conditions. Only 12 out of 177 oocytes (considering 10 injected with MF plus 2 injected with P1, Table II), displayed a swelling-evoked conductance with similar characteristics (Fig. 4B and 4C) to those previously observed in uninjected oocytes (Fig. 3B and 3C). Table II summarizes the properties of osmo-dependent swelling currents in uninjected and injected oocytes with different membrane fractions. As stated in Table II, the characteristics of the swelling-elicited conductances were similar between uninjected, control and Pgp^+-oocytes with no significant differences between all the three groups ($p > 0.05$).

DISCUSSION

Xenopus oocyte cells have been extensively used as an expression system for many exogenous membrane proteins including Pgp (15, 35), after injection of the corresponding cDNA or mRNA (36). Recently, an innovative approach using these cells has demonstrated that they can constitute a suitable alternative for functional studies of foreign membrane proteins delivered to the oocyte membrane through vesicular carrier systems (23-24, 36-38). This latter method provides two important advantages, *i*) the transplanted protein is fully processed, thus inappropriate posttranscriptional/posttranslational events are avoided, and *ii*) functional analysis can be undertaken after a shorter

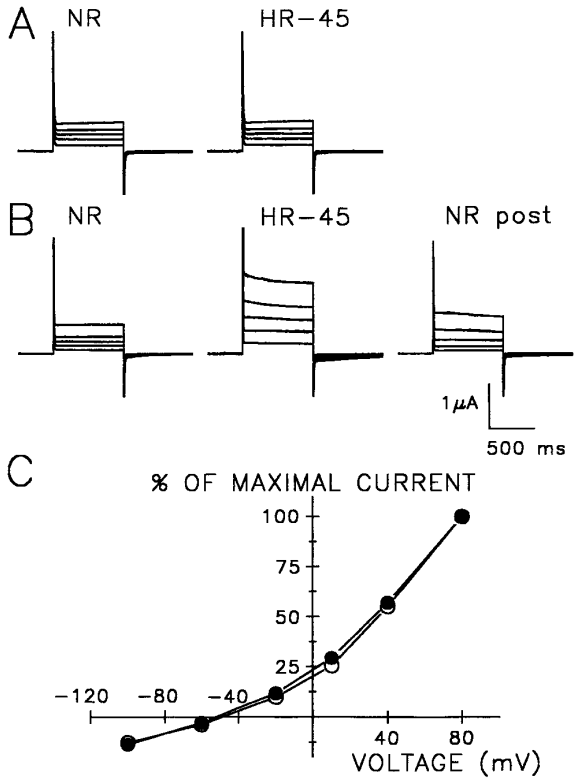

FIG. 4. Membrane conductance elicited by HR-45 in Pgp^+-oocytes. (A) Most Pgp^+-oocytes (93 %) showed no swelling-activated conductance when exposed to HR-45. Traces represent the currents obtained in response to the same voltage protocol of Fig. 3. (B) In 12 of 177 oocytes, HR-45 superfusion elicited a swelling-activated conductance similar to that found in the responsive uninjected cells (see Table II and text for details). (C) I/V curves obtained from 12 control or uninjected oocytes (hollow symbol) and from 10 Pgp^+-oocytes (filled symbol). Note the fairly similar rectification and reversal potential. Current values for different cells were normalized, 100 % being the instantaneous current value at + 80 mV (obtained by exponential fitting of the current decay). Error bars are within the symbol size.

period of time (one day after microinjection of the protein) than when injecting RNA (usually after 2-3 days (15, 35)). On this basis, *Xenopus* oocytes were injected with Pgp-containing membranes and according to the sensitivity of the detection system used in Western analysis (see Methods), at least 2×10^6 Pgp molecules (on the average) appeared to be incorporated into the plasma membrane of each oocyte. Accumulation experiments with DNM, a Pgp-transportable drug, demonstrated that the protein was transplanted in a functional form to the oocyte given, *i*) the occurrence of an active drug export in the Pgp^+-oocytes, concluded from the consistently lower level of DNM in these cells when compared to the corresponding control cells lacking Pgp, *ii*) the difference in the level of exported DNM between Pgp^+-oocytes and control cells, which significantly increased with the amount of injected Pgp, strongly suggesting that Pgp was the protein responsible for pumping the drug out of the oocytes. It should be noted, however, that in these oocytes, no linearity between the amount of injected Pgp (P1 contains ~ 4 times more Pgp than MF) and the percentage of exported DNM was observed. In this regard, the use of the detergent sodium cholate to prepare P1, could contribute to this lack of linearity by partly denaturing the membrane proteins. Also, it is conceivable that differences in the lipid composition and/or the dynamics of the vesicles bearing the protein (MF and P1 might contain a different lipid and protein composition), could modulate the efficiency of the fusion between the vesicles and the oocyte membrane and thus, the number of incorporated protein molecules.

The aim of this work was to test whether Pgp constitutes a chloride channel involved in cell volume regulation (4, 6-7). The *Xenopus* follicles express endogenous chloride osmo-dependent swelling currents (25-27), which seem to be associated with the follicular cells (27). In fact, this is in agreement with the wide range in amplitude of the swelling current determined in our uninjected oocytes, likely resulting from differences in defolliculation efficiency of the collagenase treatment. In the case of the Pgp^+-oocytes, the response to hypotonicity seems to be inconsistent with a Pgp-dependent swelling-activated chloride channel according to the following observations: *i*) The number of oocytes having a positive response to swelling was extremely low and indistinguishable from that in control or uninjected cells. If Pgp was a chloride channel, the percentage of responsive Pgp^+-oocytes, would be expected to increase with respect to the other two groups, *ii*) the properties of the swelling-evoked currents were no different from those determined in control or uninjected oocytes, including the proportion of potassium and chloride conductances to the whole current. In this context, if the transplanted Pgp molecules represented swelling-activated chloride channels, a shift to a less negative reversal potential would be expected from the additional chloride conductance.

On the other hand, it has been reported that when present, Pgp-transportable substrates may prevent the activation of Pgp-dependent swelling currents by locking the protein into a transport competent configuration (6, 39-40). Under our experimental conditions this possibility can be ruled out since the experiments related to activation of swelling currents were run in parallel to those used in accumulation assays i.e., with oocytes never exposed to the drug. Moreover, if both transporter and chloride channel functions were associated with Pgp, it would be surprising that only the former could be detected as seen in this study, taking into account that the electrophysiological methods used to measure ion channel activity are much more sensitive than those for determining the translocation activity of transporter proteins.

In summary, while the question as to whether Pgp

could constitute a channel regulator involved in the response of the cells to swelling remains open, our results do not support the notion that Pgp constitutes *per se* an osmo-dependent chloride channel.

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