Sodium-dependent Inhibition of the Epithelial Sodium Channel by an Arginyl-specific Reagent*

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Effects of the arginyl- and lysyl-specific reagent phenylglyoxal (PGO) on the epithelial Na+ channel were evaluated by measuring the amiloride-blockable 22Na+ fluxes in membrane vesicles derived from the toad bladder epithelium. Incubating whole cells or isolated membranes with PGO readily and irreversibly blocked the channel-mediated tracer flux. Na+ ions present during the interaction of membranes with PGO could protect channels from inactivation by PGO. This effect required the presence of Na+ at the luminal side of the membrane and was characterized by an IC50 of 79 mM Na+. Amiloride, too, could desensitize channels to PGO, but its effect was significant only when whole cells were interacted with the protein-modifying reagent. The data are compatible with a model in which the conductive path of the channel contains a functional arginine, possibly forming a salt bridge with a carboxylic group, which is involved in Na+ translocation and amiloride binding. It was also shown that the augmentation of transport induced by incubating whole cells in Ca2+-free solution (Garty, H., and Asher, C. (1985) J. Biol. Chem. 260, 8330–8335) involves the activation or recruitment of channels that are not vulnerable to PGO prior to incubation.

The apical membrane of several tight epithelia contains Na+-specific channels characterized primarily by their high affinity to the diuretic amiloride (Km < 1 mM). These channels mediate the entry of Na+ from the luminal fluid (filtrate, urine, perspiration, or feces) into the epithelial cells in the first stage of active transepithelial Na+ transport. Their basic properties and regulatory mechanisms have been extensively studied by a variety of methods in model epithelia such as frog skin, toad bladder, and toad kidney cultured cells (1–4). Yet, a number of fundamental issues regarding the mechanism of Na+ translocation, amiloride binding, and intracellular regulation of channels are unresolved. It is not known for instance whether amiloride blocks transport by directly plugging the conductive path or by interacting with a separate regulatory site and inducing a conformational change which closes the channel (3, 4). Also unclear are the molecular details of the interaction between Na+ ions and the channel. Two Na+-binding sites have been postulated. One is the translocation site (or the "selectivity filter") which is located in the conductive path and is responsible for the Na+ specificity of this transporter (5). The other is a regulatory site on the luminal face of the membrane. Binding of Na+ to this site closes the channel and causes a decrease in the membrane permeability at high external Na+ activities (self-inhibition) (2, 4).

Recently, Na+ channels were investigated in membrane vesicles derived from the toad bladder epithelium by means of electrical potential-driven tracer uptake (6–9). An intriguing observation in this work is that Na+ activity in isolated membranes strongly depended on the temperature and Ca2+ activity of the medium in which cells were incubated prior to their homogenization (7, 8). The data suggested that lowering cell Ca2+ triggers a process which increases the luminal Na+ permeability by inducing a sustained change of a membrane component.

This paper reports that the arginyl- and lysyl-specific reagent PGO1 can readily block the Na+ channel and that this blockage is sensitive to the presence of either Na+ or amiloride. This observation enabled us to explore some of the above issues and conclude that: 1) the channel has an essential arginine, located near its outer mouth; 2) this residue is involved in Na+ translocation and possibly in amiloride binding; and 3) incubating cells in Ca2+-free solutions causes an increase in the accessibility of channels to PGO.

**EXPERIMENTAL PROCEDURES**

Cell Incubation and Membrane Preparation—Specimens of Bufo marinus toads (either sex, Mexican origin obtained through Lemberger, Oshkosh, WI) were doubly pithed and deblooded by transventricular procision with approximately 500 ml of NaCl:Ringer's solution consisting of 110.0 mM NaCl, 1.0 mM CaCl2, 0.5 mM MgCl2, and 3.5 mM KH2PO4 + K2HPO4 at pH 7.5. The urinary bladders were excised and rinsed well in ice-cold medium composed of 90 mM KCl, 45 mM sucrose, 5.0 mM MgCl2, 10 mM EGTA, and 10.0 mM Tris-HCl (pH 7.8). Unless otherwise indicated, all subsequent operations were done using this solution. The epithelium was scraped off the underlying connective tissue with a glass slide, and cells were dispersed by rapidly drawing them in and out of a Pasteur pipette several times. Cells were washed twice at 0 °C and then incubated for at least 30 min at 25 °C. This incubation was essential for maximizing the channel activity in vesicles (7, 8). In a few experiments, the incubation was also used to interact channels with PGO. It was terminated by washing cells in ice-cold medium and breaking them with a Polytron-type homogenizer (Kinematics GmbH, Luzern, Switzerland; 6–8 bursts at setting 6). In a few experiments, the final washing, homogenization, and vesicle isolation were done using a modified solution containing 90 mM NaCl instead of KCl or containing 250 mM NaCl and no KCl nor sucrose. Cell homogenates were centrifuged at 1,000 × g for 5 min to sediment unbroken cells, nuclei, and debris; and the supernatants were then centrifuged at 27,000 × g for 1 h. The membrane pellets were suspended in a minimal volume of the homogenizing medium (1–2 mg of protein/ml) and either used within 2–4 h or stored

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The abbreviations used are: PGO, phenylglyoxal; EGTA, [ethylene bis(oxycarbonylmethyl)iminodiacetic acid.
in liquid N₂ and used over a period of several weeks.

**Incubation with PGO and Assay of Channel Activity**—Aliquots of membrane vesicles (50–100 μl) were incubated with 10⁻³ to 10⁻⁷ M PGO for various periods of time at 25°C. The arginine-specific reagent was always added from a freshly made 10-fold concentrated solution dissolved in a 1:1 mixture of homogenizing medium (dimethyl sulfoxide) and 0.9% NaCl. Immediately afterwards, the vesicles were assayed for ²²Na⁺ uptake. The first stage of this assay involved a 20-fold dilution of the membranes in isotonic sucrose solution (see below) and was assumed to terminate the PGO reaction. The channel activity in vesicles was evaluated by measuring the potential-driven, amiloride-sensitive ²²Na⁺ uptake as described in detail previously (6-9). The negative-inside potential was imposed using either a Na⁺ or K⁺ gradient (in the presence of valinomycin). As shown in Ref. 9, both gradients (and also any K⁺:Na⁺ mixture that maintains the same alkali cation gradient) provide comparable driving forces for ²²Na⁺ uptake. The transport assay consisted of three stages: (a) establishing a 60-μl volume of isotonic sucrose solution (175 mM in vesicles prepared to contain 90 mM KCl or NaCl and 440 mM in vesicles prepared to contain 250 mM NaCl). The eluent pH was adjusted to 6.1 with a small volume of Tris base, and 5 μl valinomycin was added (to KCl-containing membranes only). Two 60-μl aliquots of the polarized vesicles were then mixed with two 60-μl volumes of isotonic sucrose solution containing 22NaCl (final concentration = 4 μCi/ml; 0.2-0.4 μM Na⁺) and either amiloride (final concentration = 1.5 μM) or water diluent. Samples of 150 μl were removed from the radioactive suspensions after 1.5 and 3.5 min and eluted through Dowex columns into counting vials with 1.5 ml of sucrose. ²²Na⁺ uptake was linear over the assay period; and under control conditions, most of it could be blocked by the diuretic. The channel activity was defined as the difference in initial rates in the presence and absence of amiloride. It was expressed as picomoles of ²²Na⁺ milligram of protein⁻¹ minute⁻¹ or in relative units (e.g. percent of the amiloride-sensitive flux inhibited by PGO). The intravesicular content (90 mM KCl, 90 or 250 mM NaCl) had little effect on the amiloride-sensitive flux under control conditions (see also Ref. 9). Protein was determined according to Bradford (10) using γ-globulin as a standard.

**Binding of [¹⁴C]PGO to Membranes**—Membrane vesicles suspended either in KCl or NaCl homogenizing medium were incubated for 15 min with [¹⁴C]PGO (1.5 μCi, 18 mCi/mmol). The reaction was terminated by a 50-fold dilution in ice-cold homogenizing medium containing 10 mM unlabeled PGO and was buffered to pH 5.5 (to slow down dissociation of [¹⁴C]PGO from proteins (11)). The membrane pellets, washed twice in 0.9% saline, were re-dissolved in 20% trichloroacetic acid, and assayed for ¹³C radioactivity and protein content. The background radioactivity, estimated by assaying a sample that received [¹⁴C]PGO immediately before centrifugation, was <5% of the membrane-associated radioactivity.

**Materials**—PGO, valinomycin, Trizma (Tris base), and EGTA were obtained from Sigma. Dowex beads (50WX8, 50–100 mesh) were from Fluka AG Chemische Fabrik (Buchs, Switzerland). ¹¹¹NaCl (carrier-free; 1.13 mCi/ml) and [¹⁴C]PGO (135 μCi/mg) were from Amersham Radiochemicals (Buckinghamshire, United Kingdom). Amiloride was a gift from Merck Sharp and Dohme GmbH (Munich, Federal Republic of Germany).

**RESULTS**

PGO is known to interact specifically with the guanidino group of arginine and, to a lesser degree, with the ε-amino group of lysine and α-amino groups (11, 12). Incubating toad bladder membrane vesicles with 10⁻³ M PGO for 15 min caused almost complete abolishment of their amiloride-sensitive conductance, but only slightly decreased the amiloride-insensitive tracer uptake (Table I). PGO also decreased the short-circuit current across intact bladders mounted in a Ussing-type chamber (data not shown). The simplest interpretation of the above observation is that the channel has an essential arginyl or lysyl residue whose modification by PGO blocks the conductive path. Since the inhibition of the amiloride-blockable uptake was not accompanied by an increase of the amiloride-insensitive uptake, the above data cannot be accounted for by a PGO-induced desensitization of channels to amiloride.

In principle, abolishment of ²²Na⁺ uptake may also represent nonspecific PGO effects such as disruption of vesicles or depolarization of the membrane potential that drives the tracer flux. These possibilities were excluded by measuring effects of PGO added after ²²Na⁺ had been accumulated in vesicles and equilibrated with the transmembrane potential. Blocking channels at this stage should have little effect on the intravesicular radioactivity. Disrupting particles or permeabilizing them to other ions should induce a substantial tracer efflux (13). In three separate experiments, it was found that 10⁻³ M PGO, added after ²²Na⁺ had been accumulated in vesicles (at t = 15 min), had no effect on the intravesicular radioactivity during the next 10 min. Thus, under the conditions of this study, PGO directly blocks channels and does not rupture or depolarize vesicles.

Inhibition of the initial rate of channel-mediated ²²Na⁺ uptake by PGO was time- and dose-dependent (Fig. 1). The abolishment of transport was characterized by a IC₅₀ of 2.5 X 10⁻⁴ M PGO and t₅₀ of less than 1 min. Both the fast inhibition and the relatively low doses of PGO needed to induce it indicate that the positively charged residue whose modification blocks the channel is a very reactive one. Such
The protective effect is best understood by assuming that the isotonic sucrose solution. Under these conditions, PGO in that contained either KCl or NaCl but were suspended in a residue is likely to be an arginyl and not lysyl or an α-amino group (11, 12).

The above measurements were all carried out in Na+-free KCl solution. A considerably reduced response to PGO was noted if vesicles were suspended in NaCl rather than KCl (Fig. 2). Whereas the interaction of membranes with $5 \times 10^{-4}$ M PGO in 90 mM KCl blocked 87 ± 7% of the channels, a similar treatment of vesicles suspended in 90 mM NaCl inhibited only 30 ± 5% of the amiloride-sensitive uptake. At higher concentrations (e.g., $5 \times 10^{-3}$ M PGO), the channels could be fully blocked in NaCl solutions as well (data not shown). This protective effect is best understood by assuming that the binding of Na+ to a specific site either directly masks the reactive residue from PGO or induces a conformational change which makes it inaccessible to the glyoxal. To examine the sidedness of this effect, PGO was interacted with vesicles that contained either KCl or NaCl but were suspended in isotonic sucrose solution. Under these conditions, PGO induced a similar, nearly complete inhibition of channels irrespective of the intravesicular composition (Fig. 2, right two bars, 83 ± 7 and 78 ± 12%, respectively). Thus, desensitization of channels to PGO requires the presence of Na+ in the extravesicular compartment only. Since the vesicles used in this study are mostly right-side-out oriented (9, 14), this would mean that Na+ has to interact with the luminal face of the channel in order to prevent its blockage by PGO. The fact that a similar degree of inhibition was obtained for external KCl and sucrose solutions indicates that the above interaction is Na+-specific and that K+ does not influence the binding of PGO to channels.

To demonstrate that the above phenomenon does not reflect a general effect of Na+ ions on the modification of proteins by this reagent, we have measured the overall incorporation of $^{14}$C from [7-$^{14}$C]PGO into membrane proteins in

$^5$ The Na+ and K+ gradients established by suspending NaCl- or KCl-loaded vesicles in sucrose do not dissipate in a time scale of minutes. This is evident from the fact that at steady state, $^{22}$Na+ leaks from such vesicles very slowly (6, 8).

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Effects of amiloride on PGO-channel interaction

For membrane vesicles, four aliquots of membrane vesicles were incubated for 15 min with $5 \times 10^{-4}$ M PGO, 100 $\mu$M amiloride, PGO + amiloride, and diluent. The vesicles were then diluted 50-fold, pelleted at 27,000 x g for 1 h (to remove amiloride), suspended in 100 $\mu$l of fresh homogenizing medium, and assayed for $^{22}$Na uptake. For intact cells, cells were activated in 45-min incubation in homogenizing medium at 25°C and then divided into four aliquots. They were incubated for an additional 30 min at 25°C in the presence of 10$^{-3}$ M PGO, 100 $\mu$M amiloride, PGO + amiloride, and diluent. The reaction was terminated by pelleting cells and suspending them in fresh, ice-cold, homogenizing medium. Vesicles were then prepared and assayed for $^{22}$Na uptake as described under "Experimental Procedures." Data are expressed as percent of $^{22}$Na uptake inhibited by the various treatments (means ± S.E. of four experiments). The average amiloride-sensitive and -insensitive fluxes under control conditions were 15.8 ± 5.6 and 3.11 ± 0.4 pmol of $^{22}$Na -mg of protein$^{-1}$-min$^{-1}$, respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibition of $^{22}$Na uptake in vesicles</th>
<th>Amiloride-blockable</th>
<th>Amiloride-insensitive</th>
</tr>
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<tbody>
<tr>
<td>Membrane vesicles</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PGO</td>
<td>94.2 ± 4.1</td>
<td>20.0 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>PGO plus amiloride</td>
<td>92.2 ± 4.3</td>
<td>17.8 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>Amiloride</td>
<td>19.7 ± 7.4</td>
<td>3.5 ± 5.9</td>
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<tr>
<td>Intact cells</td>
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</tr>
<tr>
<td>PGO</td>
<td>91.5 ± 3.9</td>
<td>11.8 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>PGO plus amiloride</td>
<td>21.2 ± 6.6</td>
<td>−9.7 ± 7.3</td>
<td></td>
</tr>
<tr>
<td>Amiloride</td>
<td>5.0 ± 3.6</td>
<td>−7.5 ± 6.3</td>
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</table>

### DISCUSSION

This paper describes the effects of the arginyl- and lysyl-modifying reagent PGO on the channel-mediated $^{22}$Na fluxes in toad bladder membrane vesicles. Incubation of either whole cells or isolated membranes with 10$^{-3}$ M PGO for 30 min caused a nearly complete inhibition of their amiloride-sensitive conductance. It was shown that this inhibition is not induced by a nonspecific disruption of vesicles nor does it represent desensitization of channels to amiloride (used as a marker of the channel-mediated flux). Thus, it appears that the reaction of PGO with a functional residue in the channel protein irreversibly blocks the conductive path. Compared to other proteins and peptides whose activity is impaired by glyoxals (11, 12), the inhibition of channels is fast ($t_{1/2} < 1$ min) and requires low doses of PGO ($IC_{50} \sim 2.5 \times 10^{-4}$ M). Both the time course and dose response depicted in Fig. 1 indicate that the positively charged residue whose modification by PGO blocks the channel is very reactive. Such a residue is likely to be the guanido group of arginine and not the normal group of lysine or the amino terminus of the channel protein.

An interesting feature of the above PGO-channel interaction is its Na$^+$ dependence. The presence of the "channel substrate" in the luminal (but not intravesicular) phase of the membrane could at least partially protect channels from inactivation by PGO. Na$^+$ had no effect on the overall binding of PGO to membrane proteins. Thus, it appears that Na$^+$ binding to a specific site on the channel either directly masks the functional residue from PGO or induces a conformational change that causes such masking.

The site whose occupation by Na$^+$ desensitizes channels to PGO can be one of two Na$^+$-specific sites postulated before. The first is the selectivity filter which is located in the conductive path and is responsible for the ionic specificity of this transporter (5). The other is a regulatory site on the luminal face of the channel (2, 4). Its occupancy by Na$^+$ closes the channel and is responsible for the inhibition of transport at high luminal Na$^+$ activities (self-inhibition). Distinction between these possibilities was made by measuring the Na$^+$-dose-response relationships. Protection of channels from PGO was characterized by an $IC_{50}$ of 79 mM Na$^+$. This value is clearly higher than the $K_w$ for Na$^+$ self-inhibition in toad bladder (6.6-16 mM (16, 17)). It is in better agreement with the Na$^+$ activity at which the single channel conductance is half-maximal: 75 mM in rat cortical collecting tubules (15), >60 mM in frog skin (18), and 17-47 mM in A6 cells (19). Thus, it appears that Na$^+$ ions have to occupy the translocation site in order to desensitize channels to PGO. In light of this interpretation, it is interesting to note that the above protection cannot be induced by 90 mM Na$^+$ present only in the intravesicular (cytoplasmic) compartment.

$^{3}$At least a small decrease in channel activity is expected due to the inactivation of channels that are conductive already before the incubation at 25°C. However, because the channel activity in cells that were not activated at 25°C is often very small (~10% of the maximal value), such a decrease is within experimental error.
Na\(^{+}\)-dependent Inhibition of the Na\(^{+}\) Channel

The finding that PGO directly blocks the Na\(^{+}\) channel is in agreement with a previous observation that trypsin, a L-lysyl and L-arginyl protease, irreversibly inhibits the short-circuit current across the intact epithelium (20). The trypsin-induced inhibition of channels could be prevented by the presence of amiloride during the proteolysis. Amiloride was found to protect channels from PGO as well, but this effect was significant only in whole cells and not in isolated membranes (Table II). The reason for the different sensitivities to the channel blocker in the two preparations is not clear. One possibility is that the protective action of amiloride is mediated by a process that takes place in whole cells only, e.g., changes in the cytoplasmic Na\(^{+}\), Ca\(^{2+}\), or H\(^{+}\) activities caused by an amiloride-induced blocking of the channel, the Na\(^{+}\)/Ca\(^{2+}\) exchanger, or the Na\(^{+}\)/H\(^{+}\) antiporter. Such an indirect effect of the blocker is, however, ruled out by the fact that cells were interacted with PGO and amiloride in Na\(^{+}\)-free solution. It is also possible, although not very likely, that at the concentration used (30 \(\mu\)M), amiloride has some nonspecific effect which influences the PGO-channel interaction in whole cells but not in isolated membranes. What seems to us the most reasonable explanation for the above preparation dependence is that homogenizing cells and forming vesicles induce some structural change in the apical surface which makes channels highly sensitive to PGO even in the presence of amiloride. Another indication of the existence of such a structural change is the fact that luminal pH has different effects on the channel conductance in whole cells and isolated membranes (21). By assuming that, like Na\(^{+}\), amiloride protects channels from PGO by directly affecting the modified residue, it is very tempting to postulate that the channel blocker and the transported ion bind at the same site.

The fact that PGO rapidly and irreversibly inhibits channels in intact cells was also used to analyze the Ca\(^{2+}\)-dependant augmentation of transport in intact cells. It was found that treating cells with PGO for 5 min at the beginning of the incubation with EGTA has no apparent effect on the channel activity measured after an additional 40 min of incubation in its absence (Fig. 4). The apparent insensitivity of the activation process to PGO pretreatment does not reflect dissociation of PGO from the channel since a similar treatment carried out after a 45-min incubation at 25 \(\circ\)C produced a sustained inhibition of transport. Thus, these channels that become conductive during the incubation of cells in Ca\(^{2+}\)-free solution are not vulnerable to the protein-modifying reagent before the incubation. This finding can be accounted for by assuming either an EGTA-induced translocation of channels from a cytoplasmic pool to the apical surface or a conformational change in dormant apical channels which involves a shift in the position of this residue. The data of Fig. 4 seem to exclude other less interesting explanations for the Ca\(^{2+}\)-dependent modulation of transport, such as effects of the pretreatment on the size, yield, or tightness of apical vesicles formed by cell homogenization.

The finding that modification of a positively charged residue by PGO can be prevented by the presence of a cation (Na\(^{+}\) or amiloride) is surprising since the two should, of course, repel. One possibility is that occupation of the channel by Na\(^{+}\) or amiloride has a long-range allosteric effect on the modified residue. Another alternative is that the functional arginyl or lysyl is located on the binding site(s) of these cations, but its charge is neutralized by an interaction with a negatively charged residue, e.g., carboxyl. This hypothesis is supported by the finding that the Na\(^{+}\) channel has at least one essential carboxylic residue whose modification by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline is amiloride-sensitive (22). Salt bridges between positively and negatively charged residues were postulated to construct the conductive path of the voltage gated Na\(^{+}\) channel (23) and are thought to be involved in the translocation of ions by other transporters such as the Na\(^{+}\)/H\(^{+}\) exchanger (24), the red cell anion transporter (25), and the Na\(^{+}/K\(^{+}\)-ATPase (26). Inhibition of the Na\(^{+}/H\(^{+}\) exchanger by carboxyl- and imidazole-specific reagents was at least partly prevented by amiloride and Na\(^{+}\) or Li\(^{+}\) ions (24). Thus, the possible involvement of an arginyl-carboxyl salt bridge in translocating Na\(^{+}\) ions through the amiloride-blockable channel is not surprising and may hint at a feature common to the Na\(^{+}\)-binding site of different transporters.

REFERENCES