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Functional Expression of p64, an Intracellular Chloride Channel Protein

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Abstract. p64 is a protein identified as a chloride channel by biochemical purification from kidney microsomes. We expressed p64 in HeLa cells using a recombinant vaccinia virus/T7 RNA polymerase driven system. Total cell membranes were prepared from infected/transfected cells and fused to a planar lipid bilayer. A novel chloride channel activity was found in cells expressing p64 and not in control cells. The p64-associated activity shows strong anion over cation selectivity. Single channels show prominent outward rectification with single channel conductance at positive potentials of 42 pS. The chloride channel activity is activated by treatment of the membranes with alkaline phosphatase and inhibited by DNDS and by TS-TM calix(4)arene. Whole membrane anion permeability was determined by a chloride efflux assay, revealing that membranes from cells expressing p64 showed a small but highly significant increase in chloride permeability, consistent with expression of a novel chloride channel activity.

Key words: Chloride channel — p64 — Lipid bilayer — Vaccinia — Outwardly rectifying chloride channel

Introduction

Chloride channels play a variety of roles in cellular physiology. Plasma membrane chloride channels are important in transepithelial transport, in setting the membrane potential in skeletal muscle cells, and in the defense against hypotonic swelling [9]. In addition to the well known roles of plasma membrane channels, chloride channels in intracellular membranes also play a number of critical roles [1]. Particularly well documented are the importance of chloride channels in the acidification of intracellular organelles [2,3,13] and in exocytosis of certain regulated secretory vesicles [5,6,22].

Over the past several years, a number of genes responsible for some of the plasma membrane chloride channels have been identified [9]. However, the proteins responsible for intracellular channels have been less well defined. p64 is a protein originally identified as purifying with chloride channel activity from bovine kidney microsomes [10]. cDNA encoding p64 has been cloned [12]. Antisera raised against a p64 fusion protein expressed in bacteria were found to selectively remove reconstitutable chloride channel activity from solubilized kidney microsomes, thus demonstrating that p64 is physically associated with this activity [12].

p64 cDNA encodes a 438 amino acid protein which, based on hydropathy analysis, has been proposed to have two transmembrane segments with both N and C terminal hydrophilic domains exposed on the cytoplasmic face of the membrane [12]. These presumed cytoplasmic domains of the protein contain consensus phosphorylation sites for protein kinases A and C as well as casein kinase. Immunolocalization studies suggest that native p64 is expressed exclusively in intracellular membranes [15,16]. When exogenous p64 is expressed in *Xenopus* oocytes or mammalian cells, it is excluded from the plasma membrane [12,16]. Thus p64 is thought to be a component of a chloride channel which normally resides in intracellular membranes.

P64 is a member of a closely related gene family. Recently, two homologues of p64 have been implicated in specific chloride channel activities. A 27 kD human homologue of p64 has been proposed to function as a chloride channel in the nuclear membrane [23]. A 62 kD protein which is antigenically related to p64 has been identified as the chloride channel in osteoclast ruffled border [17].

A key point in determining whether p64 is in fact a channel is to determine whether expression of p64 results in the appearance of new channel activity. However, be-

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cause p64 is normally an intracellular protein excluded from the plasma membrane, expressed p64 has been inaccessible to the usual whole cell ion flux or electrophysiologic approaches commonly used to assess channel expression.

In this study, we have taken a novel approach to functional expression of a channel protein. We expressed p64 at high levels in HeLa cells using a vaccinia/T7 RNA polymerase-driven system. Using a lipid bilayer method to study channels in unfractionated whole cell membranes, we demonstrate and partially characterize a novel anion channel activity associated with expression of p64 in these membranes.

Materials and Methods

Cells and Clones

HeLa cells and recombinant vaccinia stock encoding T7 RNA polymerase, were provided by Dr. Andrey Shaw, Washington University, St. Louis MO. Plasmid NHΔP [12], containing the entire p64 coding region downstream of a T7 promoter, was used to drive expression of p64. pBluescript KS-(Stratagene, La Jolla, CA) which is the parent plasmid for NHΔP served as the control plasmid.

Preparation of Membranes

Vaccinia-T7 driven expression was carried out essentially as described [4,20]. In brief, HeLa cells were plated in 3.5 cm dishes at 5×10^5 cells/dish (about 50% confluence) in DMEM (Dulbecco's modified Eagle's medium) with 10% newborn calf serum. The next day, cells were rinsed with serum-free medium. Cells were infected with vaccinia in 0.75 ml of DMEM at a multiplicity of infection of about 10 and incubated at 37°C for 30 min. 5 μg plasmid DNA and 15 μl of Lipofectace (Gibco-BRL, Bethesda, MD) were combined in 0.75 ml of DMEM and added to the infected cells. The infected/transfected cells were then incubated at 37°C in 5% CO_2 for 10 hr.

Cells were rinsed with PBS and then scraped into 0.5 ml ice-cold buffer containing (in mM): 250 sucrose, 10 imidazole, 1 DTT (dithiothreitol), 1 EDTA (ethylene diamine tetra-acetic acid), 0.1 PMSF (phenylmethysulfonyl fluride) (pH 7.0). Cells were homogenized with twenty strokes in a tight fitting Potter homogenizer, passed through a 25-gauge needle 10 times, and then centrifugated at 4,000 rpm at 4°C in an Eppendorf microfuge to remove unbroken cells and debris. The resulting whole cell lysate was centrifuged at 40,000 rpm for 1 hr in a Beckman 70.1Ti rotor (100,000 × *G*), yielding a soluble fraction supernatant and a membrane fraction pellet. The pellet was resuspended in 200 μl of homogenization buffer and used directly in the bilayer experiments.

Western blotting was carried out by standard methods [7], using affinity-purified rabbit anti-p64 antibodies [16] and the Supersignal detection method from Pierce Biochemical (Rockford, IL).

Metabolic Labeling

Infected/transfected cells were rinsed with phosphate-free medium and then incubated in phosphate-free medium supplemented with 500 μCi/ml ^{32}P orthophosphate for 1 hr at 37°C. Cells were then stimulated with vehicle or agonist for 10 min at 37°C. Cells were then quickly rinsed with ice-cold PBS. Cells were solubilized in (mM) 200 NaCl, 1% Triton-X100, 1 EDTA, 25 Tris pH 8.0, 0.1 PMSF at 4°C for 15 min. Insoluble material was removed by centrifugation in an Eppendorf microcentrifuge at 15,000 RPM for 10 min. 5 μl of anti-p64 anti-sera were added to each and incubated at 4°C overnight. 20 μl of 50% suspension of protein A agarose was added to each and incubated 1 hr. The agarose beads were collected by centrifugation and washed four times with solubilization buffer before separation by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by autoradiography.

To test alkaline phosphatase activity on p64, cells were labeled with ^{32}P as above. Cells were collected in (mM) 250 sucrose, 10 Tris pH 8.0, 1 EDTA and homogenized in a Potter homogenizer as described in the preceding section. The homogenate was centrifuged at 4,000 RPM for 10 min and the supernatant divided into three equal parts. Two samples were kept on ice. To one sample, $MgCl_2$ was added to 3 mM and alkaline phosphate (Promega, Madison WI) added to 10 u/ml followed by incubation at room temperature for 20 min. EDTA was then added to 3 mM. All three samples were then solubilized by addition of Triton-X100 to 1% final concentration. The samples were then processed for immunoprecipitation as above. The control consisted of nonphosphatase treated sample precipitated with pre-immune será.

Lipid Bilayer Methods

Bilayers were prepared using soybean asolectin (cat #P5638, Sigma, St. Louis, MO) that was extracted with chloroform, the solvent evaporated and then stored under nitrogen. Lipid was dissolved in decane at 30 mg/ml. Two microliters of lipid solutions was applied to the 0.25 mm orifice in the bilayer chamber and solvent allowed to evaporate. The cuvette and chamber were filled with the indicated solutions and connected to a BCA-525 bilayer clamp and headstage (Warner Instruments, Hamden, CT) with 3M KCl-agar bridges and silver chloride-coated silver wire. The lipid was spread over the orifice with a polished glass rod and the bilayer was allowed to thin to 0.4 $\mu F/cm^2$. Bilayers were formed and thinned in 140 mM KCl in the cis chamber and 140 mM CsCl in the trans chamber, both solutions buffered with 10 mM HEPES pH 7.0. After thinning, channels were allowed to fuse into the bilayer by adding 0.1 to 1.0 μg (protein) of HeLa cell membranes to the cis chamber and recording for channel activity. In some experiments, alkaline phosphatase was added to the cis chamber at a concentration of 17 u/ml. After activity was observed, the vesicle containing media was replaced with fresh solution and the channels studied as described. Solutions in the cis cuvette were changed as indicated. All solutions were buffered to pH 7 with 10 mM HEPES. Recordings were done under voltage-clamp conditions and data were collected using Axotape or Axoscope software and analyzed using Pclamp6 software (Axon Instruments) and Origin5. Open and closed current levels for single channel data were determined by all points histograms. Standard errors were derived from histogram statistics. Data were archived on videotape using a Neurocorder DR-484 (Neuro Data Instruments, NY).

Chloride Efflux Assay

Membranes from cells expressing p64 or from control cells were prepared and suspended as above. Membrane suspension was solubilized by addition of one tenth volume 14% N-octyl glucoside followed by centrifugation at 100,000 × *G* for one hour to remove nonsolubilized material. Protein concentration of the supernatant was determined. 100 μg of solubilized protein was brought to 1 ml in (mM) 250 sucrose,

10 imidazole, 1 EDTA, 1.4% N-OG (N-octyl glucoside) and combined with 0.1 ml of 100 mg/ml asolectin, 90 mg/ml N-OG. The mixture was dialyzed for a total of two days against three changes of 200 mM KCl, 2 mM HEPES pH 7.

Chloride efflux assays were performed by monitoring the release of chloride from vesicles into isosmotic sucrose using a chloride selective electrode (essentially as described by Preston et al. [14]). 200 μl of the reconstituted vesicle suspension was passed over a 3-ml spin column prepared from Bio-gel P6 (Bio-Rad) equilibrated in 400 mM sucrose. The sample was chased through with 300 μl of 400 mM sucrose. The 500 μl eluate from the spin column was immediately added to 2 ml of 400 mM sucrose and the extravesicular chloride concentration monitored with an Accumet chloride selective electrode (Fisher Scientific) connected to a pH meter. The output of the pH meter in millivolts was collected at 0.25-sec intervals on a personal computer using a digidata A-D converter and Axotape software (Axon Instruments). After a steady state was reached, 2.5 μl of 1 mM valinomycin in ethanol was added (1 μM final) and recording continued. Triton-X100 was then added to 0.1% to release all remaining intravesicular chloride. The output of the meter is linear with the log of chloride concentration in the range between 5×10^{-5} to 10^{-1} M. A standard curve was generated with each set of experiments and the raw data converted to chloride concentration. The difference between the final total chloride after addition of Triton and the extravesicular chloride at the time of addition of valinomycin is taken as the initial intravesicular chloride. Fraction of intravesicular chloride released after addition of valinomycin is plotted against time. The initial rate of fractional chloride release is determined by linear regression from the first 10 sec after addition of valinomycin.

Abbreviations

The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; Tris, tris-(hydroxymethyl) aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; CFTR, cystic fibrosis transmembrane conductance regulator; ORCC, outwardly rectifying chloride channel; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; N-OG, N-octyl glucoside.

Results

Expression of p64 in HeLa Cells

HeLa cells were infected with recombinant vaccinia encoding the T7 RNA polymerase and then transfected with control plasmid DNA or DNA encoding p64 downstream of a T7 promoter. Expression of p64 was documented by western blot as shown in Fig. 1. Whole cell lysates (Fig. 1*A*), soluble fractions (*B*), and membrane pellets (*C*) from control cells ('−' lanes) or p64 transfected cells ('+' lanes) were probed with affinity-purified anti-p64 antibodies. Control infected/transfected cells have no detectable p64 while p64 is abundantly expressed in cells that were transfected with p64 cDNA. In cells expressing p64, the protein is present both in the soluble fraction and in the membrane pellet. Although p64 is abundantly expressed and easily detected by western blot, it does not become a major fraction of total cell protein. The p64 band is not detectable above the normal pattern of proteins in crude extracts or membrane fractions when gels are stained for total protein.

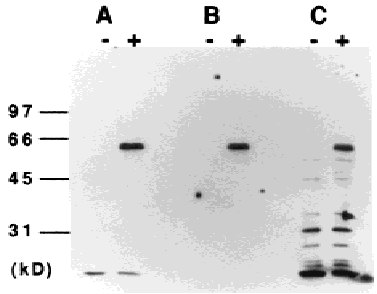

Fig. 1. Vaccinia/T7-driven expression of p64 in HeLa cells. Thirty micrograms of whole cell lysates (*A*), soluble protein (*B*), and membrane fractions (*C*) from recombinant vaccinia infected cells transfected with control plasmid (−lanes) or with p64-encoding plasmid (+lanes) were separated on a 10% polyacrylamide gel, blotted, and probed with affinity-purified antibody to p64. Positions of molecular weight standards are marked.

Chloride Channel Activity Associated with Expression of p64

Membrane suspensions of control or p64-expressing cells were introduced to the cis chamber of the bilayer apparatus and the bilayer was monitored for evidence of channel activity. At the dilution of membranes we used, spontaneous channel activity in control membranes was rare. However, with prolonged recording, potassium-selective channels were observed in all membrane preparations. To avoid confusion between potassium and chloride selective channels, many recordings were initiated with 140 mM KCl in the cis chamber and 140 mM CsCl in the trans chamber. With these solutions, chloride selective channels will show a reversal potential of 0 mV while potassium selective channels will reverse at strongly negative potential. Under these conditions, a single type of chloride selective channel activity was routinely encountered in membranes from p64 expressing cells (quantification presented below). Single channel recordings from this channel are shown in Fig. 2.

Data collected at a series of holding potentials from single channels observed in four independent experiments was analyzed to construct the current voltage relationship shown in Fig. 2*B*. The single channel conductance is Ohmic at positive holding potentials, showing a slope conductance of 42 ± 2 pS. At negative holding potentials, single channels show rectification. The *I/V* relationship for channel in symmetric 140 mM KCl (*not shown*) is indistinguishible from that derived with the KCl cis, CsCl trans conditions shown here. Current-

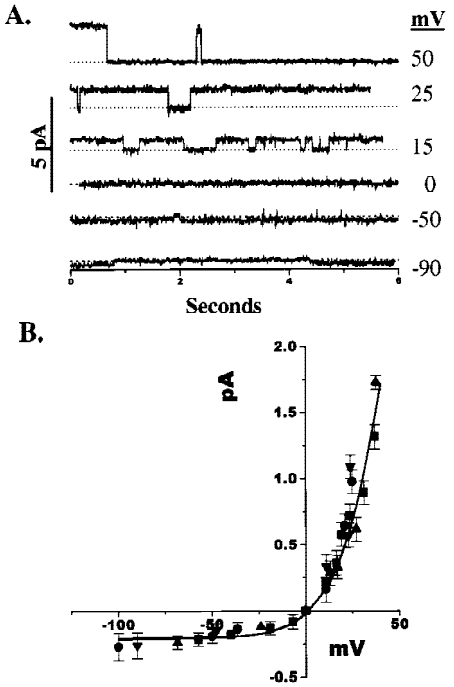

Fig. 2. Chloride channel activity associated with expression of p64. Bilayer recordings were made as described using membranes from cells expressing p64. (*A*) Single channel transitions of the p64-associated activity at a series of holding potentials as marked. The zero current level for each tracing is marked with the dotted line. Solutions (in mM): 140 KCl, 10 HEPES pH 7 cis; 140 CsCl, 10 HEPES pH 7 trans. (*B*) Voltage dependence of single channel chloride currents averaged from 4 independent p64-expressing HeLa cell membrane preparations. Data from each experiment is represented by a different symbol. The error bars represent the standard error of mean derived from the determination of transition amplitudes. The linear region at positive potentials gives a slope conductance of 42 ± 2 pS. The calculated slope conductance at negative potentials is less than 5 pS.

voltage plots of channels in asymmetric KCl solutions were used to determine the reversal potential determined. The derived Cl to K selectivity is greater than 20:1.

The chloride channel activity in the bovine microsomal membranes from which p64 was originally purified was activated by phosphatase [11]. To see if channel activity from exogenous p64 expressed in HeLa cells could be regulated by dephosphorylation, alkaline phosphatase was added to the cis chamber during membrane fusion. We routinely found marked increase in activity of the rectifying Cl-selective channel in response to alkaline phosphatase in the p64-containing membranes but not from the control membranes. We have been unable to effect channel activity by treatment with catalytic subunit of protein kinase A and Mg-ATP in the bilayer chamber.

To determine objectively whether the chloride channel was in fact related to expression of p64, a standard approach was taken to assess presence of channel. Membrane preparations were fused into lipid bilayers in asymmetric KCl and held at zero potential so that the polarity of current would indicate whether an observed channel was anion or cation selective. After introduction of membranes into the cis chamber, the bilayer was monitored for 10 min for appearance of channel. If no channel was encountered, alkaline phosphatase was introduced into the cis chamber at 17 u/ml. The bilayer was again monitored for 10 min. Representative traces from this protocol are shown in Fig. 3. In this experiment, the trans chamber contained 0.75 M KCl, 10 mM HEPES; the cis chamber contained 0.15 M KCl, 10 mM HEPES. Under these conditions, chloride channel activity at 0 mV holding potential will produce a positive current with a predicted reversal potential of −41 mV. Membranes from p64 expressing cells were added to the cis chamber at the beginning of the trace shown in panel A. At intervals, the bilayer was briefly clamped to plus or minus 50 mV as reflected in capacitance spikes in the recording. There was no spontaneous activity in >400 sec of continuous recording. Alkaline phosphatase was added to the cis chamber and observation of the bilayer was continued (panel *B*). About 6 min after addition of alkaline phosphatase, a large positive current suddenly appeared, presumably representing fusion of a vesicle with many active channels. The current-voltage relationship derived from this macro current is shown in panel *D*. The reversal potential is −42 mV, consistent with a chloride selective permeability. Panel *C* shows a representative tracing using membranes from control cells not expressing p64. No channel activity appears in 500 sec of recording after addition of alkaline phosphatase.

In 6 independent paired sets of p64 and control membranes, 2 preparations of membranes from p64-expressing HeLa cells demonstrated spontaneous channel activity and 4 displayed activity only after alkaline phosphatase treatment. None of the 6 preparations of control membranes showed chloride channel activity either spontaneously or following alkaline phosphatase treatment.

The bilayer from the alkaline phosphatase treated samples shown in Fig. 3*B* was broken and reformed until single channel activity was obtained. A representative recording from this activity is shown in Fig. 3*E* and the derived *I/V* relationship shown in 3*F*. The single channel activity in alkaline phosphatase membranes shows conductance and rectification properties identical to the activity seen in untreated membranes (*see* Fig. 2) but the alkaline phosphatase activated channels seem to show more rapid openings and closings.

In another series of studies, the experimenter operating the bilayer was blinded to the identity of control and p64 expressing membranes. In 4 out of 4 independent trials using the protocol described above, the membranes from p64-expressing cells were correctly identified based on the presence or absence of the characteristic p64-associated channel.

To summarize data from all experiments, we have

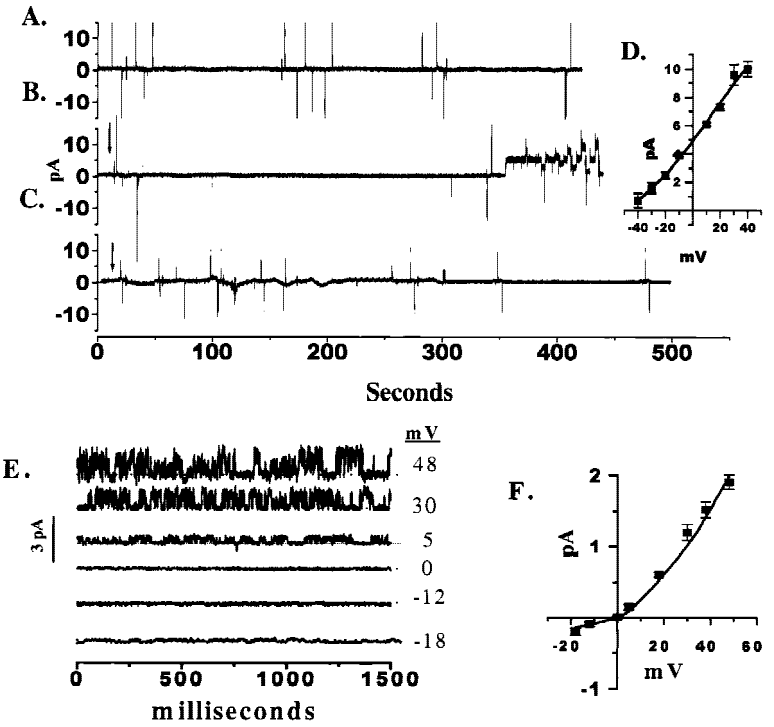

Fig. 3. Activation of p64 associated activity by alkaline phosphatase. Membrane vesicles were introduced into the cis chamber of the bilayer apparatus and current recorded at 0 mV holding potential. Solutions: 0.15 M KCl, 10 mM HEPES pH 7 cis; 0.75 M KCl, 10 mM HEPES pH 7 trans. (*A*) 5 μl of membrane suspension from cells overexpressing p64 were added at the beginning of the tracing. At intervals, + or −50 mV command potentials were applied and these are reflected in capacitance spikes in the current record. No channel activity appeared during this recording. (*B*) Continuation of the trace shown in A with addition of alkaline phosphatase to 17 u/ml at point marked with the arrow. At the end of this trace, command voltages of +/− 10, 20, 30, and 40 mV were applied to test the voltage dependence of the current (plotted in panel *D*). (*C*) 5 μl of membranes vesicles from control cells were studied as in panels *A* and *B*. 500 seconds of recording are shown after addition of alkaline phosphatase (arrow). (*D*) *I/V* relationship derived from the macro current from panel *C*. The reversal potentials is −42 mV. (*E*) Single channel activity from an alkaline-phosphatase-activated bilayer. Recordings were made at a series of holding potentials as in Fig. 2 with (mM): 140 KCl, 10 HEPES pH 7 cis; 140 CsCl, 10 HEPES pH 7 trans. The zero current level for each trace is marked with the dotted line. (*E*) *I/V* relationship derived from the data shown in panel *D*. The slope conductance at positive potentials is 40 pS.

performed 102 trials of membranes derived from more than 20 independent sets of HeLa cells overexpressing p64. In 26 of these trials (26%), the characteristic p64 associated channel was observed to be spontaneously active, the activity appearing at 10.2 ± 9.9 min after addition of vesicles to the cis bilayer chamber. In 44 of the 76 trials in which no spontaneous activity was observed, alkaline phosphatase was introduced into the cis chamber. In 28 of those 44 trials (65%), activity appeared after addition of alkaline phosphatase, the activity appearing 5.2 ± 2.6 min after the addition of phosphatase. Combining data from those membranes that showed spontaneous activity and those that received alkaline phosphatase, we found the p64 associated activity in 73% of the trials. In contrast, we have carried out 33 trials using membranes from 20 independent preparations from cells that were infected with vaccinia but transfected with the parent plasmid vector rather than the plasmid encoding p64. In none of these trials was the characteristic p64 associated channel seen, either spontaneously or after treatment with alkaline phosphatase. In all 20 membrane preparations we observed potassium channel activity in at least one trial.

P64 is Phosphorylated in vivo

The response of channel activity to alkaline phosphatase suggests that the channel is phosphorylated when expressed in HeLa cells. To assess in vivo phosphorylation of p64, control vaccinia-infected HeLa cells or cells expressing p64 were labeled by growth for 1 hr in phosphate-free media supplemented with ^{32}P. Cells were solubilized, immunoprecipitated with anti-p64 antisera, separated by SDS-PAGE and detected by autoradiography. As shown in Fig. 4, p64 is phosphorylated at baseline in vivo. Treatment of the cells for 10 min with forskolin, PMA or calcium ionophore each failed to alter significantly the amount of radioactive phosphate incorporated in p64.

To see if the in vivo phosphorylation of p64 is sensitive to dephosphorylation by alkaline phosphatase in vitro, cells expressing p64 were labeled with inorganic phosphate as above. Cells were homogenized and treated with alkaline phosphatase before solubilization and immunoprecipitation with anti-p64 antibodies. As shown in Fig. 4*C*, the radioactivity associated with p64 is sensitive to alkaline phosphatase treatment in vitro.

Effect of Inhibitors on p64 Channel Activity

In some experiments, a large number of channels simultaneously fused into the bilayer, resulting in large currents in which individual channel transitions could not be discerned. We used such preparations to assess effect of inhibitors on channel activity as shown in Fig. 5. Figure

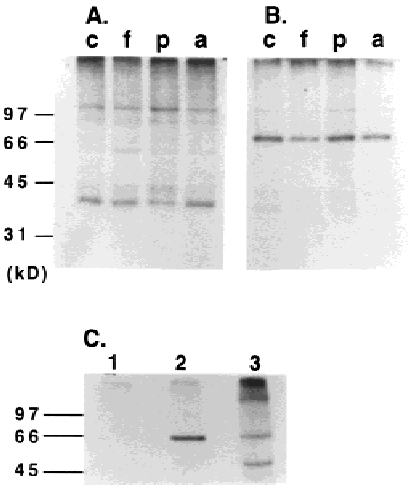

Fig. 4. Phosphorylation of p64 in vivo. (*A* and *B*) Infected/transfected HeLa cells expressing control DNA (panel A) or p64 (panel *B*) were labeled with inorganic ^{32}P for one hour. At the end of the labelling period, cells were treated for 15 min with vehicle (c), 10 μM forskolin (f), 0.1 μM PMA (p) or 1 μM A23187 (a) before solubilization and immunoprecipitation with p64 antisera. (*C*) HeLa cells expressing p64 were labeled with ^{32}P as above and then homogenized. Homegenate was solubilized and immunoprecipitated with pre-immune sera (lane 1) or with p64 antisera either without (lane 2) or with (lane 3) prior treatment with alkaline phosphatase.

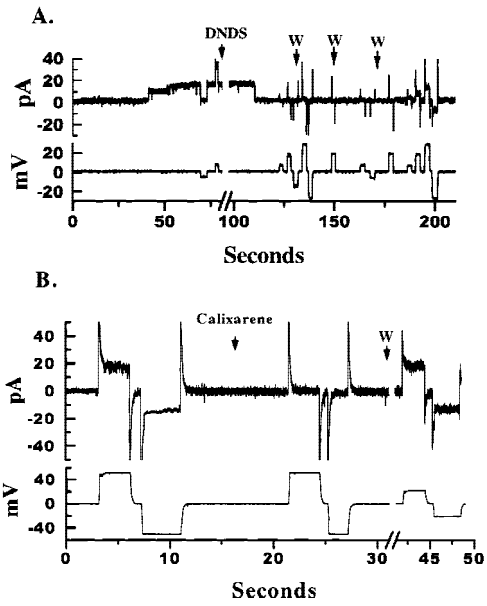

Fig. 5. Pharmacologic Inhibition of p64-associated channel activity. Simultaneous recording of current (above) and holding potential (below) are shown. (*A*) DNDS. Bilayers were formed and p64-expressing HeLa cell membranes were introduced into the cis chamber (Solutions in mM): 140 K-gluconate, 10 HEPES pH 7.0 cis; 140 CsCl, 10 HEPES pH 7.0 trans). At about 40 and 60 sec, fusions occurred with appearance of multichannel current. DNDS was introduced into the cis chamber to 200 μM at 110 sec and the bilayer rechallenged with a series of holding potentials. No current is evident. The cis solution was then exchanged twice (arrows marked W) with 140 mM KCl, 10 mM HEPES pH 7 to remove DNDS, and the bilayer was rechallenged with a series of holding potentials, revealing reappearance of the conductance. (*B*) TS-TM calix(4)arene. Vesicles from p64-expressing HeLa cells were introduced into the cis chamber, resulting in outwardly rectifying multichannel macro current. (Solutions: 140 mM KCl, 10 mM HEPES pH 7 both cis and trans). TS-TM calix(4)arene was added to the cis chamber at a final concentration of 3 nM. Subsequent +/− 50 mV challenges resulted in capacitance spikes but no steady state current. The cis chamber was washed several times (arrow marked W) and the outwardly rectifying current returned.

5*A* shows a continuous recording in a bilayer formed between 140 mM K-gluconate, 10 mM HEPES cis; and 140 mM CsCl, 10 mM HEPES trans. Membranes from p64-expressing cells were introduced into the cis chamber. There are two spontaneous insertions at about 45 and 60 sec resulting in a large outward chloride current which varies with voltage as expected. DNDS was introduced into the cis chamber as marked and the activity disappeared. The cis solution was then replaced twice with 140 mM KCl and the activity reappeared. This observation has been repeated 3 times. Thus, DNDS reversibly inhibits the p64-associated chloride channel.

TS-TM calix(4)arene is a recently described high affinity inhibitor of plasma membrane outwardly rectifying chloride channels [21]. Figure 5*B* shows a multichannel current in membranes from p64-expressing cells with 140 mM KCl, 10 mM HEPES in both chambers. The macro current is outwardly rectifying. TS-TM calix(4)arene was added to the cis chamber at 3 nM final concentration and the macro current disappeared. The cis chamber was washed with several exchanges of 140 mM KCL 10 mM HEPES and the outwardly rectifying current returned. We have repeated this observation 3 times. Thus, like DNDS, TS-TM calix(4)arene reversibly inhibits the p64-associated chloride current. Neither inhibitor affected the K channel activity observed in these membrane preparations.

Expression of p64 Results in Increased Reconstitutable Chloride Permeability

The lipid bilayer results indicated that expression of p64 results in the appearance of an novel chloride channel. To determine whether expression of p64 has any effect on total chloride permeability, we turned to a population based assay rather than the single molecule bilayer system. Membranes from cells expressing p64 or from control cells were solubilized and reconstituted in phospholipid vesicles using detergent dialysis (10,12,15) such that the vesicles were loaded with 200 mM KCl. To assay chloride permeability, extravesicular chloride is rapidly removed by passage through a gel filtration spin column equilibrated in 400 mM sucrose. The column

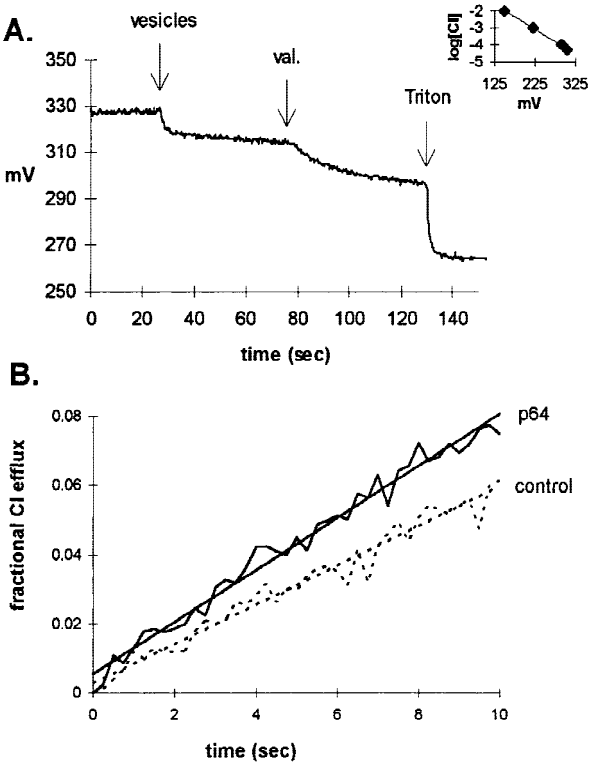

Fig. 6. Chloride efflux assay. (*A*) Representative tracing from a chloride efflux experiment. KCl loaded phospholipid vesicles were reconstituted using solubilized membrane proteins from HeLa cells expressing p64. Extravesicular chloride was removed by passing the vesicles through a spin column equilibrated in isosmolar sucrose. The vesicles were then added to 2 ml of sucrose solution whose chloride concentration was continuously monitored using an ion-selective electrode as shown in the tracing. The initial deflection is at the point of addition of vesicles with some residual extravesicular chloride. Valinomycin and Triton-X100 were added at the arrows as marked. A standard curve for chloride was generated for the electrode immediately before the experiment as shown in the inset. (*B*) Fractional chloride efflux. Data like that in panel *A* were converted from millivolts to 'fraction of total intravesicular chloride released' as described in the text. Fractional chloride release was plotted as a function of time for the first 10 sec after addition of valinomycin. These data represent and average of four replicas from a single reconstitution experiment. Lines derived from these data using linear regression are plotted. The slopes of the regression lines represent the vesicular chloride permeability.

eluate is then diluted into 400 mM sucrose and extravesicular chloride concentration continuously monitored using a chloride selective electrode. Valinomycin is introduced to initiate potential driven chloride efflux. At the end of the experiment, the vesicles are solubilized with detergent to release any remaining intravesicular chloride. A typical tracing from such an experiment is shown in Fig. 6*A*. The initial deflection is at the point of addition of vesicles with some small amount of residual extravesicular chloride. Valinomycin and detergent are added at the time points marked. The output of the electrode in mV is converted to chloride concentration using

Table. Rates of chloride efflux from reconstituted proteoliposomes

Sample	Cl efflux (fraction/sec)	*n*	Standard error
p64	7.08×10^{-3}	19	2.88×10^{-4}
Control	5.53×10^{-3}	19	2.78×10^{-4}
No protein vesicles	1.34×10^{-3}	4	2.2×10^{-4}

Proteoliposomes were reconstituted with solubilized membrane protein from cells expressing p64, from control cells, or in the absence of added protein. Initial rates of fractional chloride efflux were determined from the first 10 sec after addition of valinomycin as described in the text. $P < 0.0005$ for each combination of pairwise comparisons.

a standard curve generated with each set of experiments. To determine chloride permeability, the fraction of intravesicular chloride released per second after addition of valinomycin is plotted versus time as shown in Fig. 6*B*. An initial rate of fractional chloride efflux representing chloride permeability of the reconstituted vesicles is determined from the first 10 sec after addition of valinomycin.

We have determined the rate of chloride efflux from 19 individual assays of material derived from 6 independent expression-reconstitution experiments. In each case, reconstituted vesicles were prepared in parallel from control infected/transfected cells and from cells expressing p64. There was no difference in yield of membranes or efficiency of solubilization between control cells or cells expressing p64. The reconstitution of vesicles was also equivalent with the intravesicular volume (as reflected by the total chloride concentration after solubilization with detergent) identical between the control and p64 samples. The results are shown in the Table. The efflux rate for control membranes was $5.53 \times 10^{-3} \pm 0.00029$ sec^{-1} while the efflux rate of membranes from cells expressing p64 was $7.08 \times 10^{-3} \pm 0.00028$ sec^{-1}. As an additional control, asolectin was reconstituted into vesicles in the absence of protein. Chloride efflux from 'no protein vesicles' was $1.34 \times 10^{-3} \pm 0.00022$ sec^{-1}. The difference in fractional efflux rate between vesicles reconstituted from cells overexpressing p64 and from control cells is highly statistically significant ($P < 0.0005$) and amounts to a 28% increase over control permeability. Thus, expression of p64 results in increased reconstitutable chloride permeability. To date, we have not detected a difference in efflux rates as a result of alkaline phosphatase treatment before reconstitution. Treatment with alkaline phosphatase after reconstitution causes a significant loss of intravesicular volume in both p64 and control vesicles, making it impossible to measure efflux rates accurately. We have not yet been able to assess the effects of inhibitors on this activity due to contaminants in available inhibitor preparations which interfere with the electrode.

Discussion

We have described a novel chloride channel activity associated with expression of p64 in HeLa cells. The p64-associated activity is strongly anion selective, rectifying, phosphatase activated, and inhibited by DNDS and calixarene. This activity is detectable not only as single channels in lipid bilayers but also as increased total reconstitutable chloride permeability as measured by chloride efflux from vesicles. Our methods represent a novel approach to study intracellular channels which may be otherwise inaccessible to more commonly used methods.

Since the orientation of membrane vesicles prepared from cells is somewhat unpredictable, the polarity of the rectification demonstrated by the p64-associated channel is not immediately apparent. However, since we find predominantly one polarity of rectification, the p64 vesicles must be mostly either cytoplasmic side out or cytoplasmic side in. The response to alkaline phosphatase is a useful clue as to orientation. One would predict that a phosphorylation site important in channel regulation would be exposed on the cytoplasmic face of the membrane. Since we observed response to alkaline phosphatase when added to the cis chamber only, the cytoplasmic face of the membrane must be exposed on the cis side of the bilayer and on the outside surface of the vesicles. Thus, these vesicles must be in a cytoplasmic face out orientation. Consequently, the polarity of rectification would be outward if the channel were expressed on the plasma membrane.

Although we clearly see a new activity when p64 is expressed, this result does not necessarily mean that p64 is itself a channel. p64 could be activating an endogenous channel or complexing with an endogenously expressed subunit to form a channel. Previous immunodepletion studies showed that p64 is physically associated with chloride channel activity in solubilized kidney microsomes. Here we have described that appearance of a chloride channel activity coincident with expression of p64. Taken together, the data strongly support the hypothesis that p64 is a component of an intracellular chloride channel.

The single channel properties of the chloride channel activity associated with expression of p64 is particularly interesting since it is quite similar to the plasma membrane outwardly rectifying chloride channel (ORCC) which has been associated with the cystic fibrosis gene product, CFTR [18,19]. Like the p64-associated channel, the CFTR-associated ORCC is an outwardly rectifying channel of about 40 pS conductance inhibited by DNDS and calixarene. The protein responsible for the plasma membrane ORCC activity has not yet been identified. It seems quite unlikely that p64 itself encodes the CFTR-associated ORCC since p64 is excluded from the plasma membrane. However, one homologue of p64 from rat brain has been reported [8] and a number of p64 homologues have been reported as expressed sequence tags in Genbank. Perhaps one of the homologues of p64 is responsible for the CFTR-associated ORCC.

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