Genetic Demonstration That the Plasma Membrane Maxianion Channel and Voltage-dependent Anion Channels Are Unrelated Proteins*

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The maxianion channel is widely expressed in many cell types, where it fulfills a general physiological function as an ATP-conductive gate for cell-to-cell purinergic signaling. Establishing the molecular identity of this channel is crucial to understanding the mechanisms of regulated ATP release. A mitochondrial porin (voltage-dependent anion channel (VDAC)) located in the plasma membrane has long been considered as the molecule underlying the maxianion channel activity, based upon similarities in the biophysical properties of these two channels and the purported presence of VDAC protein in the plasma membrane. We have deleted each of the three genes encoding the VDAC isoforms individually and collectively and demonstrate that maxianion channel (~400 picosiemens) activity in VDAC-deficient mouse fibroblasts is unaltered. The channel activity is similar in VDAC1/VDAC3-double-deficient and in double-deficient cells with the VDAC2 protein depleted by RNA interference. VDAC deletion slightly down-regulated, but never abolished, the swelling-induced ATP release. The lack of correlation between VDAC protein expression and maxianion channel activity strongly argues against the long held hypothesis of plasmalemmal VDAC being the maxianion channel.

Purinergic signaling is a widespread phenomenon of general biological significance, and mechanisms accounting for ATP release from cells remain a contentious issue (1). We have recently identified a maxianion channel as a nanoscopic pore (2) well suited to function as the ATP-releasing pathway (3, 4). This pore accounts for the swelling-induced ATP release from neonatal rat cardiomyocytes (8).

See Ref. 17. We thoroughly tested this hypothesis by gene deletion and gene silencing. In mammals, three isoforms of mitochondrial porin, VDAC1, VDAC2, and VDAC3, have been cloned (13, 28–35). The deletion of vdac genes was shown to result in various physiological dysfunctions (36). These include male infertility (37), disrupted fear conditioning and spatial learning (38), enhanced apoptosis (39), and growth retardation and increased fatigue (36). If the maxianion channel is a pl-VDAC, then deletion and/or silencing of the VDAC genes would be expected to eliminate the channel activity and abolish the maxianion channel-mediated ATP release. Here we present the results of such a study.

EXPERIMENTAL PROCEDURES

Generation of VDAC-deficient Mouse Fibroblasts—The gene targeting strategies for the vdac1, vdac2, and vdac3 genes have been described previously (40). VDAC1-deficient mouse embryonic fibroblasts (MEFs) were isolated from day 11.5 vdac1−/− embryos, derived from the cross of vdac1−/− mice. Mouse embryos derived from the cross of vdac3−/− females and vdac3−/− males were harvested at day 11.5, and vdac3−/− MEFs were genotyped by polymerase chain reaction (36). vdac2−/− embryos die in midgestation, but MEFs derived from embryos harvested before then are viable. vdac2−/− embryonic stem cell clones were microinjected into blastocysts obtained from C57BL/6 female mice. MEFs derived from 8.5-day chimeric embryos were treated with 0.3 mg/ml G418. Deficiency of VDAC2 following G418 selection was confirmed by Western blot analysis.

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2 The abbreviations used are: VDAC, voltage-dependent anion channel; pl-VDAC, plasmalemmal VDAC; MEF, mouse embryonic fibroblast(s); MAF, mouse adult fibroblast(s); dKO, double knockout; WT, wild type; RNAi, RNA interference; pS, picosiemens.
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firmed by both PCR and immunoblotting with anti-VDAC2 antibodies. Double-deficient (dKO) mouse adult fibroblasts (MAFs) were isolated from skin biopsies of adult double-knockout (vdac1−/−/vdac3−/−) mice generated by crossing vdac1−/−/vdac3−/− females to vdac1+/-/vdac3−/− males. Cultured fibroblasts were maintained in Dulbecco’s modified Eagle’s high glucose plus pyruvate medium (In VitroGen) containing 10% fetal bovine serum and penicillin (100 units/ml) plus streptomycin (100 units/ml) in a humidified atmosphere containing 5% CO2. VDAC protein expression was verified by Western blotting using affinity-purified anti-VDAC1 rabbit polyclonal antibodies and anti-VDAC2 and anti-VDAC3 chicken polyclonal antibodies (41).

vdac2 gene silencing was performed by RNA interference in dKO fibroblasts. The following oligonucleotides, targeting three different regions of the mouse vdac2 gene, were used to generate the hairpin-encoding inserts for the three RNAi constructs: 5’-TTTTCTCGAGCCGGCTGCTGTGAGATTCTCTCTTTTCAAGAGA-3’ (RNAi-1 construct); 5’-TTTTCTGACCAGGCCTTTGAGTCGCTACAGGTTCAAGAGA-3’ (RNAi-2 construct); 5’-TTTTCTGACCAGGCTGAGCAATTTTGAGGACCTTCCAAAAATGGGACAGAATTGGAGATCCTTCTGAGA-3’ (RNAi-3 construct). The underlined sequences are 19-nucleotide target sequences corresponding to the regions of murine vdac2. The oligonucleotides were annealed, extended with the Klenow fragment of DNA polymerase, and cloned into a pBluescript II KS+ vector (Stratagene, La Jolla, CA) containing the mouse RNAase P H1 promoter and a puromycin resistance gene. Fugene 6 (Roche Applied Science)-mediated transfection of fibroblasts with KOH or acetic acid, respectively). For ATP permeability measurements, the bath solution contained 1000 mM KCl, 2 mM MgCl2, and 5 mM HEPES (pH 7.4, adjusted with KOH). The hypotonic solution for ATP release measurements contained 1000 mM KCl, 2 mM MgCl2, 5 mM HEPES, 6 mM HEPES, 5 mM glucose (pH 7.4; 290 mosmol/kg H2O). For selectivity measurements, 135 mM NaCl in Ringer solution was replaced with 135 mM of tetraethylammonium or sodium glutamate. The pipette solution for inside-out experiments was either normal Ringer solution or solution containing 100 mM KCl, 2 mM MgCl2, and 5 mM HEPES (pH 7.4, adjusted with KOH). The pipette solution for outside-out experiments contained 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM Na-HEPES, 6 mM HEPES, 5 mM glucose (pH 7.4; 290 mosmol/kg H2O). For selectivity measurements, 135 mM NaCl in Ringer solution was replaced with 135 mM of tetraethylammonium or sodium glutamate. The pipette solution for inside-out experiments was either normal Ringer solution or solution containing 100 mM KCl, 2 mM MgCl2, and 5 mM HEPES (pH 7.4, adjusted with KOH). The pipette solution for outside-out experiments contained 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM Na-HEPES, 6 mM HEPES, 5 mM glucose (pH 7.4, 290 mosmol/kg H2O, 72% hypotonicity).

GdCl3 was stored as a 50 mM stock solution in water and added directly to Ringer solution immediately before each experiment. 5-Ni-
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**RESULTS**

**Single Maxianion Channels in Wild-type Mouse Fibroblasts**—In the cell-attached (on-cell) configuration, no single channel events were observed from wild-type mouse embryonic fibroblasts (WT-MEFs). However, after excision of the patch membrane, single channel events with a large amplitude could be readily recorded (Fig. 1A). The channels exhibited voltage-dependent inactivation at both positive and negative potentials greater than ±20 mV. The unitary current-to-voltage (I-V) relationship was linear in the range of ±50 mV, with a reversal potential of about 0 mV in symmetrical conditions with Ringer solution in the bath and pipette and a slope conductance of 400.3 ± 3.8 pS (Fig. 1B). The single channel amplitude was insensitive to the replacement of NaCl with tetraethylammonium-Cl. However, the inward currents greatly decreased, and the reversal potential shifted to a negative value of −33.7 ± 1.6 mV when NaCl in the bath solution was replaced with sodium glutamate (Fig. 1B). This result indicates that the large conductance channel in WT-MEFs is anion-selective with a permeability ratio of glutamate/Cl− of 0.23 ± 0.02. This value is close to P$_{\text{glutamate}}$/P$_{\text{Cl}}$ = 0.22 obtained for the maxianion channel in mammary C127 cells (4).

In order to test the ATP permeability of the maxianion channels in WT-MEFs, we recorded the I-V curves in response to ramp pulse from −50 to +50 mV. The ramp I-V relationship was linear in symmetric ionic conditions with Ringer solution both in the pipette and in the bath, with reversal potential at 0 mV. When all anions in the bath were replaced with 100 mM ATP$^4^-$, large outward currents (carried by Cl$^-$ from the pipette solution) as well as small inward currents (presumably carried by ATP$^{3^-}$ from the bath solution (see Ref. 5) were consistently observed. The average reversal potential shifted to −18.2 ± 1.2 mV ($n$ = 15 from five different patches), giving $P_{\text{ATP}}/P_{\text{Cl}} = 0.083 ± 0.005$.

The maxianion channel in WT-MEFs was insensitive to Gd$^{3^+}$ ions (50 μM) added from the intracellular side of excised patches ($n$ = 5; data not shown) but completely blocked ($n$ = 6) when the drug was applied from the extracellular side in the outside-out configuration (Fig. 1D). Another well known modulator of the maxianion channel, arachidonic acid (20 μM), potently inhibited the channel activity when added from the cytosolic side ($n$ = 7; Fig. 1D).

The biophysical properties of the WT-MEF maxianion channel, such as the single channel conductance, voltage-dependent inactivation, ATP permeability, and sensitivity to Gd$^{3^+}$ and arachidonate, are very

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**FIGURE 1.** Single maxianion channel currents in inside-out patches excised from wild-type mouse fibroblasts. A, representative current traces recorded at different voltages as indicated on the left of each trace. The pipette was filled with normal Ringer solution, and the maxianion channels were activated by excising the patch into the normal Ringer solution. The arrowheads on the right indicate the zero current level. B, unitary current-to-voltage (I-V) relationship for maxianion channel events recorded in the inside-out mode with Ringer solution in the bath (open circles) or when 135 mM NaCl in Ringer solution was replaced with 135 mM tetraethylammonium (TEA)-Cl (filled triangles) or sodium glutamate (filled circles). Open squares, filled squares, and filled diamonds correspond to I-V relationships obtained with 100 mM KCl in the pipette and 1000 mM KCl, 1000 mM KBr, and 1000 mM potassium acetate in the bath solution, respectively (these solutions additionally contained 2 mM MgCl$_2$, and 5 mM HEPES, pH 7.4). Each data point represents the mean ± S.E. of 5–22 measurements from five different patches. The solid line for symmetrical conditions is a linear fit with a slope conductance of 400.3 ± 3.8 pS. The solid lines for asymmetrical conditions are polynomial fits with the reversal potentials indicated under “Experimental Procedures.” C, representative ramp I-V records from a patch exposed to standard Ringer solution and after replacing the Ringer solution in the bath with 100 mM Na$_4$ATP solution. The pipette solution was standard Ringer. The current recorded with ATP in the bath reversed at ±17 mV. D, effect of Gd$^{3^+}$ (50 μM) on the maxianion channel activity in the outside-out mode and of arachidonic acid (20 μM) in the inside-out mode. The applied pulse protocol is shown at the top.
similar to those observed previously in C127 cells (5, 6), kidney macula densa cells (7), and cardiomyocytes (8).

In a separate set of experiments, we determined the ionic selectivity of WT-MEF maxianion channels in conditions close to those commonly used for mitochondrial VDAC proteins reconstituted in lipid bilayers. When 10-fold KCl gradient (100 mM in the pipette versus 1000 mM in the bath) was applied to the maxianion channel-containing patches, the reversal potential was 40.6 ± 1.5 mV (Fig. 1B). This value is much greater than the value of 10.2 mV reported for the mitochondrial porin under the same conditions (45). The calculated permeability ratio $P_{Cl}/P_{K} = 13.5 ± 2.3$ also largely exceeded the $P_{Cl}/P_{K}$ of 1.7–1.9 for mitochondrial porins from different sources (45, 46). Replacing 1000 mM KCl in the bath with equimolar KBr or potassium acetate resulted in the reversal potentials of 41.2 ± 1.5 and 28.6 ± 0.5 mV, respectively (Fig. 1B). The calculated permeability ratio $P_{Cl}/P_{Cl} = 1.01 ± 0.06$ was close to that for mitochondrial VDAC (1), as derived from the partitioned ionic conductances in Ref. 47. However, $P_{act}/P_{Cl}$ of 0.58 ± 0.01 was greater than $P_{act}/P_{Cl} = 0.41$ for mitochondrial VDAC (47) under similar experimental conditions (both values were calculated after accounting for incomplete dissociation of potassium acetate at 1000 mM; see Ref. 47).

Effect of Deletion of VDAC Genes on the Occurrence of Maxianion Channels in Mouse Fibroblasts—The hypothesis that the maxianion channel is a plasmalemmal subtype of VDAC, as suggested by Buettnet al. (14), was based on the proposed existence of an alternative first exon in the murine $vDAC-1$ gene. The hypothetical alternative exon encodes a leader peptide at the N terminus that targets the protein to the plasma membrane through the Golgi apparatus. Therefore, deleting the $vDAC$ gene would interrupt this process and consequently abolish plasmalemmal VDAC expression and maxianion channel activity. We have generated MEFs lacking the $vDAC1$ gene and assayed the expression of VDAC1 protein using specific antibodies. Western blotting demonstrated a complete absence of VDAC1 protein in these cells (Fig. 2A, a). However, when the same cells were assayed electrophysiologically, they exhibited maxianion channel activity indistinguishable from WT-MEFs, including activation by patch excision, voltage-dependent gating, linear current-voltage relationship, and unaltered single channel conductance and $K^{+}$ to $Cl^{-}$ selectivity (Fig. 2B). The channel occurrence in $vDAC1^{-/-}$ fibroblasts was only slightly less (~60% of channel incidence) than in wild-type cells (~70%).

The very existence of maxianion channels in $vDAC1^{-/-}$ cells decisively refutes the “maxianion channel = pl-VDAC” hypothesis as formulated for the $vDAC1$ gene. However, the hypothesis can still be modified by replacing VDAC1 protein with one of the two other isoforms (VDAC2 and VDAC3) and hypothesizing the presence of an alternative leader peptide. To test this possibility, we first generated the cells lacking the $vDAC3$ gene and double-deficient cells lacking both the $vDAC1$ and $vDAC3$ genes. The $vDAC3^{-/-}$ cells do not express VDAC3 protein,
channel activity is unrelated to the expression of VDAC1, VDAC2, and VDAC3. Therefore tested the pl-VDAC protein as a maxianion channel is the VDAC2 isoform. We depleted cells, and the number of maxianion channels per patch was not approximately the same occurrence in about 70% of membrane patches. The solid line for symmetrical conditions is a linear fit with the reversal potential of 38.3 ± 1.1 mV. C, maxianion channel activity in macropatches excised from wild-type fibroblasts (WT-MEF and WT-MAF) from dKO cells and from dKO cells with the vdac2 gene silenced by RNAi-1, RNAi-2, and RNAi-3. The mean numbers of channels per patch are not significantly different at p < 0.05.

**Effect of Deletion of VDAC Genes on Swelling-induced ATP Release**—Our previous studies strongly suggest that the maxianion channel serves as a pathway for ATP release induced by osmotic cell swelling and other stimuli from several different types of cells (3). Therefore, we assayed the release of ATP induced by 72% hypotonicity from VDAC-deficient cells. WT-MEFs responded to this stimulation with ATP release pathway (3, 4). Arachidonic acid had a significant inhibitory effect on ATP release from WT-MEFs was very similar to that observed in our earlier studies with mammary C127 cells (5, 6) and neonatal cardiac myocytes (8), where maxianion channels play a key role in the release of ATP. Fibroblasts deleted for *vdac1*, *vdac2*, and *vdac3* genes displayed slightly reduced mass release of ATP compared with the wild-type cells (Fig. 4C). However, the observed difference did not reach statistical significance.
significance when analyzed by the analysis of variance test. Thus, none of these genes alone can be entirely responsible for the osmotic ATP release from these cells. When VDAC1/VDAC3-double-deficient cells were tested with and without VDAC2 protein depletion by RNA interference, the swelling-induced ATP release levels were not statistically different within this group (dKO, dKO-RNAi-1, dKO-RNAi-2, and dKO-RNAi-3), suggesting that the decreased VDAC2 protein expression in dKO fibroblasts (Fig. 3A) did not result in the comparable decrease in ATP release.

It should be noted, however, that the ATP release from dKO cells themselves was significantly lower than that from WT-MEFs when compared by unpaired t test (Fig. 4D). This result is probably due to impaired ATP production by mitochondria lacking an important ATP transport pathway in their mitochondrial outer membrane (36). To verify this possibility, we measured the total ATP content in the cell lysates. The total ATP content of dKO cells was only 78.5 ± 4.5% (n = 9) of that for wild-type adult fibroblast cells (significant at p < 0.05). Thus, we believe that reduced ATP release from dKO cells and its clones derived by RNAi is mainly due to the decreased ATP gradient caused by reduced mitochondrial ATP production and not due to reduced activity of an ATP-releasing maxianion channel, which was normal in these cells (Fig. 3C).

**DISCUSSION**

VDAC, or mitochondrial porin, resides in the outer membrane of mitochondria and when reconstituted in lipid bilayers displays large single channel conductance and bell-shaped voltage-dependent inactivation with maximal open probability at around 0 mV (10–12). These biophysical properties are similar to those observed for maxianion channels in patch clamp experiments (3, 4, 9). Probing the mitochondrial porin by the nonelectrolyte partitioning method yielded a value for the pore radius of 1.5 nm for the fully open state of the channel in lipid bilayers (49). This figure is very close to the cut-off size of around 1.3 nm obtained in our experiments with the maxianion channel (2). Using the asymmetric PEG application method, Carneiro et al. (50, 51) described an asymmetrical pore for mitochondrial VDAC with a radius of 1.16 and 1.42 nm for its cis- and trans-entrances, respectively (cis designates the side of the bilayer into which the protein was added). This asymmetry parallels the asymmetry of the maxianion channel demonstrated in experiments using a one-sided application of PEG: 1.16 and 1.42 nm for radii of intracellular and extracellular vestibules (2). Electron microscopic images have demonstrated that mitochondrial porin has an inner radius of 1.4 nm (52), which is very close to the value obtained by polymer size exclusion for VDAC in lipid bilayers (44–46) and for the maxianion channel in our patch-clamp study (2). Thus, the structural
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features of mitochondrial porin and the ATP-conductive maxianion channel do converge. Maxianion channels also resemble (5) mitochondrial porins with respect to their open channel block by ATP (53) and ATP conductivity (53–55).

The hypothesis that maxianion channels observed in patch-clamp experiments in different cells represent a plasmalemmanly expressed VDAC (pl-VDAC) protein (13–18) has received great attention and is currently considered to be an established concept in the field. A large number of research groups have indeed detected the presence of VDAC protein in the plasma membrane of various cells (13–26), and several genetic mechanisms for targeting the same protein to such different locations as mitochondrial outer membrane and plasmalemma have been postulated (14, 27).

From the present study, we conclude that the maxianion channel activity in mouse fibroblasts does not correlate with the presence of any one of the three individual isoforms of VDAC, as established in single cell knockout experiments. Moreover, simultaneous deletion of vdac1 and vdac3 genes and vdac2 gene silencing in VDAC1/VDAC3-double-deficient cells did not notably affect the maxianion channel activity, unequivocally establishing that the maxianion channel protein is not encoded by any of the vdac genes. This result contradicts the long held hypothesis that the maxianion channel represents a plasmalemma VDAC protein (13–18). Our data, however, do not exclude the possibility that VDAC protein(s) could be, in fact, targeted to the plasma membrane (e.g. by the mechanism proposed by Buettner et al. (14) or via mRNA untranslated regions, as speculated by Bathori et al. (27)). If VDAC retargeting does occur, the plasmalemmal VDAC proteins may perform some other functions, such as being a receptor for plasminogen kringle 5 (56) or a trans-plasma membrane NADH-ferricyanide reductase (57), activities that are unrelated to the maxianion channel activity. The mass release of ATP could be modulated (17) indirectly by VDAC protein expression, possibly due to an altered rate of ATP production by mitochondria, or by affecting other ATP-releasing pathways. Another conclusion from these studies is that mitochondrial outer membrane permeability can be effectively a blated, yet the cell remains viable. This may reflect the limited dependence of fibroblasts on oxidative metabolism, as underscored by the ability to create rho 0 cells by mitochondrial DNA depletion (58).

It should be noted that, although both proteins transport ATP, the similarities in single channel properties between VDAC and the maxianion channel are rather superficial, and closer inspection reveals crucial differences. Specifically, the single channel conductance of the maxianion channel saturates at 617 pS ($K_d = 77$ nM) and 640 pS ($K_d = 319$ nM) with increases in the chloride concentration in skeletal muscle “sarcobains” (59) and L6 myoblasts (60), respectively. In contrast, the VDAC single channel conductance may reach levels of over 10 nS at high salt concentrations without any saturation (61), suggesting a principally different mechanism of ionic transport in these two pores. The same inference can be drawn by comparing the $K^+$ to Cl$^-$ selectivity of these two channels. Under the same 10-fold KC1 gradient, the maxianion channel generated a reversal potential of about 40 mV, whereas no less than $-10$ mV was observed for mitochondrial VDAC (45), indicating that the maxianion channel is much more selective for chloride over potassium. Although the overall ranking of anionic permeability was similar for both channels (Br$^-$ >> Cl$^-$ >> acetate), the numeric value of the permeability ratio was notably different for acetate. The permeability ratio for glutamate to Cl$^-$ of 0.23 for the WT-MEF maxianion channels was also different from the value of $P_{\text{glutamate}}/P_{\text{Cl}^-} = 0.4$ reported for mitochondrial porin (62).

Voltage-dependent gating is considered to be a common property for the two channels. Mitochondrial VDAC is known to retain $\sim40\%$ of its initial conductance in the so-called “closed” state, which is cation-selective (10, 12, 46). Like many other channels, the maxianion channel also displays subconductance states. However, normally it closes completely at high positive and negative voltages (e.g. see Fig. 1C). Voltage-dependent modulation of ionic selectivity has never been reported for maxianion channels, supporting our conclusion that the maxianion channel and mitochondrial VDAC are unrelated proteins.

The "maxianion channel = pl-VDAC" hypothesis has been stimulating. We believe, however, that a fresh start to molecular identification of the maxianion channel, an important gateway of purinergic signaling, should be undertaken. Particular attention to other candidates, such as connexin hemichannels or orthologs of the twy gene found in the flightless locus of Drosophila (63) is warranted.

Acknowledgments—We thank K. Shigemoto and M. Ohara for technical support as well as T. Okayasu for secretarial help.

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