Functional implications of calcium permeability of the channel formed by pannexin 1

Fabien Vanden Abeele, Gabriel Bidaux, Dmitri Gordienko, Benjamin Beck, Yuri V. Panchin, Ancha V. Baranova, Dmitry V. Ivanov, Roman Skryma, and Natalia Prevarskaya

Institut National de la Santé et de la Recherche Médicale, U800, Equipe labellisée par la Ligue Contre le Cancer, Université des Sciences et Technologies de Lille, Villeneuve d’Ascq Cedex, F-59655 France

Although human pannexins (PanX) are homologous to gap junction molecules, their physiological function in vertebrates remains poorly understood. Our results demonstrate that overexpression of PanX1 results in the formation of Ca\(^{2+}\)-permeable gap junction channels between adjacent cells, thus, allowing direct intercellular Ca\(^{2+}\) diffusion and facilitating intercellular Ca\(^{2+}\) wave propagation. More intriguingly, our results strongly suggest that PanX1 may also form Ca\(^{2+}\)-permeable channels in the endoplasmic reticulum (ER). These channels contribute to the ER Ca\(^{2+}\) leak and thereby affect the ER Ca\(^{2+}\) load. Because leakage remains the most enigmatic of those processes involved in intracellular calcium homeostasis, and the molecular nature of the leak channels is as yet unknown, the results of this work provide new insight into calcium signaling mechanisms. These results imply that for vertebrates, a new protein family, referred to as pannexins, may not simply duplicate the connexin function but may also provide additional pathways for intra- and intercellular calcium signaling and homeostasis.

Introduction

Intercellular channels, which cluster together to form gap junctions, are involved in various physiological functions (e.g., adaptation of retinas to the dark, conduction of excitation in the heart, and suppression of cell proliferation in cancer tissues). For vertebrates, gap junctions are formed by connexins, a multigene family of which 20 members have been identified in humans (Willecke et al., 2002). A new family of gap junction molecules, which are unrelated to connexins, has been identified in insects and nematodes and named innexins (Phelan et al., 1998).

We have recently demonstrated the presence of innexin homologues in various taxonomic groups, including vertebrates (Panchin et al., 2000; Panchin, 2005). Given the ubiquitous distribution of this protein family in the animal kingdom, we termed these proteins “pannexins” (PROSITE accession number PS51013; www.expasy.org/prosite). Three genes, pannexin-1 (PanX1), -2 (PanX2), and -3 (PanX3), have been cloned from the human and mouse genome, and the pattern of their expression in various tissues has been studied (Panchin et al., 2000; Bruzzone et al., 2003; Baranova et al., 2004; Panchin, 2005). It has been found that the human PanX1, which encodes mRNAs, are ubiquitously, although differentially, expressed in normal tissues. Human PanX2 is a brain-specific gene (Bruzzone et al., 2003; Baranova et al., 2004; Panchin, 2005). It has been found that the human PanX1, which encodes mRNAs, are ubiquitously, although differentially, expressed in normal tissues. Human PanX2 is a brain-specific gene (Bruzzone et al., 2003; Baranova et al., 2004). Recently, it was demonstrated that in paired oocytes rodent PanX1, alone and in combination with PanX2, induced the formation of intercellular channels (Bruzzone et al., 2003). When expressed in a single Xenopus laevis oocyte, PanX1 hemichannels were shown to be functional in plasma membrane (Bruzzone et al., 2003; Bao et al., 2004). Pannexin membrane channels are mechanosensitive conduits for ATP (Bao et al., 2004). This type of nonjunctional function has been previously reported for connexins (for review see Stout et al., 2004). However, it is not clear if pannexins simply duplicate connexin functions or play some special physiological role.

F. Vanden Abeele, G. Bidaux, and D. Gordienko contributed equally to this paper.

Correspondence to Natalia Prevarskaya: natacha.prevarskaya@univ-lille1.fr

Y.V. Panchin’s present addresses are Institute for Information Transmission Problems, Russian Academy of Sciences, Moscow 101447, Russia; and the A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia.

D.V. Ivanov’s present address is Dept. of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL 33136.

D. Gordienko’s present address is Dept. of Basic Medical Sciences, St. George’s University of London, London SW17 0RE, England, UK.

A.V. Baranova’s present addresses are Dept. of Molecular and Microbiology, Georges Mason University, Manassas, VA 20110; and the Russian Academy of Medical Science, 123098 Moscow, Russia.

Abbreviations used in this paper: BODIPY, boron dipyrromethene difluoride; HEK, human embryonic kidney; ODN, oligodeoxynucleotide; RyR, ryanodine receptor; SERCA, sarcoplasmic/ER Ca\(^{2+}\)-ATPase; TG, thapsigargin.
In this work, we investigated the pannexin function in human cell lines transiently or stably transfected with pannexin (human PanX1). Our results demonstrate that overexpression of PanX1 enables the formation of Ca\(^{2+}\)-permeable gap junction channels between adjacent cells, thus, allowing direct intercellular Ca\(^{2+}\) diffusion and facilitating intercellular Ca\(^{2+}\) wave propagation. Furthermore, we obtained evidence that strongly indicate that, in addition to the gap junction function, PanX1 overexpression increases the Ca\(^{2+}\) permeability of the ER membrane and thereby affects intraluminal ER Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{L}}\)). PanX1 overexpression dramatically reduces the intraluminal Ca\(^{2+}\) fluorescent indicator, Mag-fura-2. Endogenous PanX1 depletion by antisense and siRNA strategy in human prostate cancer cells increased the Ca\(^{2+}\) content of the ER. Therefore, it seems likely that pannexins, which are structurally similar to gap junction–forming molecules, may also be involved in intracellular calcium homeostasis via the formation of the ER Ca\(^{2+}\)-leak channels. These results give new insight into the mechanisms of the basal ER Ca\(^{2+}\) leak, which has remained poorly understood until now.

Thus, the results of our study imply that where vertebrates are concerned, the pannexin family of gap junction proteins not only facilitates an intercellular Ca\(^{2+}\) movement but also represents one of the mechanisms responsible for ER Ca\(^{2+}\) leak.

**Results**

In this study, we used human prostate cancer epithelial LNCaP cells (Vanden Abeele et al., 2002) and human embryonic kidney (HEK)-293 cells, which are classically used for heterologous transfection, as the experimental models.

**Pannexin cloning, endogenous expression in prostate cell lines, and localization**

There are three distinct PanX genes: mammalian PanX1 mRNA is ubiquitously present in various tissues; PanX2 is a brain-specific gene; a low level of PanX3 has also been detected in the brain, and EST data suggest that PanX3 is expressed in osteoblasts and synovial fibroblasts (Panchin et al., 2000; Bruzzone et al., 2003; Baranova et al., 2004; Panchin, 2005). There is no indication of PanX2 or PanX3 expression in the prostate. The expression of PanX1 in the prostate has been shown by Northern blot of human tissues (Baranova et al., 2004). EST database inspection (http://cgap.nci.nih.gov) revealed five PanX1-related sequences from prostate cDNA libraries supporting this Northern blot data. Using RT-PCR, we have demonstrated the presence of PanX1 mRNA in LNCaP and HEK-293 cells used in our experiments (Fig. 1). The PanX1 fragment (encompassing what is believed to be the pore region of PanX1), was amplified by sequence-specific primers from 30 ng cDNA at 30 PCR cycles. Under control conditions, when RT was not added to the reaction, no product was amplified, even at 40 cycles. Because no antibodies to PanX1 are available, detection of the product expression at protein level is problematic.

To study the localization and the function of PanX1, we transiently overexpressed the PanX1-EGFP–fused protein in LNCaP and HEK-293 cells. PanX1 localization was verified using fluorescence confocal microscopy. 2 d after transfection, chimeras were found to be exclusively intracellular and revealed a pattern consistent with that of the ER network (Fig. 2 A). 3 d after transfection, gathered cells displayed very thin and bright fluorescence at the regions of intercellular junctions furthest from intracellular staining. Fig. 2 B shows two groups of
PanX1-EGFP–transfected LNCaP cells, and magnification of their tight cell–cell junctions revealed a dotted pattern similar to those of standard gap junctions. On day four, most of the cells overexpressed PanX1 in their entire plasma membrane, as shown in Fig. 2 C. Such intracellular and junctional localization of PanX1 is expected for a putative gap junction protein and suggests that our transient overexpression model reproduced the physiological pattern of endogenous pannexin distribution. Nevertheless, the abundance of PanX1 in the plasma membrane on the fourth day of the expression was probably caused by a protein overload of the membrane. Finally, to encounter the troubles caused by the low rate of transfection, we developed a stable cell line of LNCaP overexpressing PanX1-EGFP, referred to hereafter as “LNCaP-PanX1.”

Both transiently and stably transfected cells had similar PanX1 distribution patterns. This is further illustrated by Fig. 3, showing fluorescence confocal images of LNCaP-PanX1 in which the spatial arrangement of the ER network was visualized using specific ER marker boron dipyrromethene difluoride (BODIPY) 558/568 brefeldin A (Deng et al., 1995; Oh-hashi et al., 2003). Interconnected tubules of the ER are clearly seen in EGFP and BODIPY brefeldin A fluorescence confocal images (Fig. 3). The overlay of the images allowed clear distinction between subplasmalemmal ER (which revealed both EGFP and BODIPY brefeldin A fluorescent images) and plasma membrane (which shows EGFP fluorescence only), as illustrated in Fig. 3 (A and B, bottom). Thus, staining of LNCaP-PanX1 with BODIPY brefeldin A confirmed localization of PanX1 in the ER in these cells also.

**Pannexin’s intracellular leak-channel function**

Because PanX1 is localized in the ER in both LNCaP and HEK-293 cells, we investigated whether PanX1 is involved in intracellular calcium homeostasis at ER level. To estimate the ER Ca²⁺ load and leak under PanX1 overexpression, we monitored changes in the cytosolic Ca²⁺ concentration ([Ca²⁺]c) after the inhibition of sarcoplasmic/ER Ca²⁺-ATPase (SERCA) with thapsigargin (TG). In these experiments, LNCaP and HEK-293 cells were loaded with the Ca²⁺ indicator fura-2. The application of...
TG in Ca\(^{2+}\)-free external solution to control LNCaP cells unmasked a Ca\(^{2+}\) leak from the ER by inhibiting ER Ca\(^{2+}\) reuptake, which resulted in an increase in [Ca\(^{2+}\)]\(_i\) (Fig. 4 A). In PanX1-transfected cells, the amplitude of the TG-induced [Ca\(^{2+}\)] transient was dramatically smaller than that of control cells. This is summarized in the histograms in Fig. 4 B, where the amplitudes of TG-induced [Ca\(^{2+}\)] transients in control and under PanX1 overexpression in LNCaP and HEK-293 cells are compared. In both cell lines, the amplitude of the TG-induced [Ca\(^{2+}\)] transients was dramatically reduced in transfected cells; from 690 ± 68 nM (n = 65) to 218 ± 22 nM (n = 43) in LNCaP cells and from 590 ± 52 nM (n = 61) to 200 ± 40 nM (n = 32) in HEK-293 cells.

[Ca\(^{2+}\)]\(_i\) was measured using the low-affinity Ca\(^{2+}\) indicator Mag-fura-2-AM, as previously described (Vanden Abeele et al., 2002). Imaging experiments with Mag-fura-2-AM were conducted on cells permeabilized by mild digitonin treatment. In PanX1-transfected LNCaP cells, [Ca\(^{2+}\)]\(_i\) was found to be reduced by 80% (70 ± 30 μM; n = 12) in comparison to control cells (350 ± 55 μM; n = 15; Fig. 4, C and D). At the end of the experiment, a high dose (1 μM) of a calcium ionophore ionomycin was added to assess the total calcium store content. Similarly, in HEK-293 cells, overexpression of PanX1 resulted in a reduction of [Ca\(^{2+}\)]\(_i\) by 73%; [Ca\(^{2+}\)]\(_i\) was 300 ± 40 μM (n = 36) in control and 80 ± 29 μM (n = 16) in transfected cells.

It has been previously demonstrated by Kumar et al. (1995) that overexpression of Cx32 (B1) in BHK cells may result in accumulation of assembled channels in the ER. To test whether the effect of pannexin overexpression on the ER Ca\(^{2+}\) leak could be mimicked by an overexpression of connexins, we analyzed the effect of connxin-32 protein (Cx32) and connxin-43 protein (Cx43) overexpression on the ER Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and compared these results with those obtained with PanX1 overexpression. In these experiments, Cx32 and EGFP-fused Cx43 proteins were overexpressed in both LNCaP and HEK-293 cells, and [Ca\(^{2+}\)]\(_i\) was measured using the low-affinity Ca\(^{2+}\) indicator Mag-fura-2. In contrast to overexpression of PanX1, overexpression of Cx32 or Cx43 did not result in reduction of [Ca\(^{2+}\)]\(_i\) in either cell type (Fig. 4 D).

In another series of experiments, the ER Ca\(^{2+}\)-leak rate was assessed. Because [Ca\(^{2+}\)]\(_i\) was found to be, on average, 80% lower in cells transiently transfected with PanX1, the kinetics of the ER Ca\(^{2+}\) leak could not be evaluated by monitoring the time course of TG-induced decline in [Ca\(^{2+}\)]\(_i\), in these cells (Fig. 4, C and D). Therefore, we conducted this series of the experiments on LNCaP-PanX1 cells (stably transfected with PanX1), which showed only 20% reduction in [Ca\(^{2+}\)]\(_i\) in comparison with control cells (Fig. 5 A, top). To assess the kinetics of the ER Ca\(^{2+}\) leak, we monitored the time course of TG-induced decline in [Ca\(^{2+}\)]\(_i\) in digitonin-permeabilized LNCaP control cells (Fig. 5 A) and LNCaP-PanX1 cells with Mag-fura-2 (Fig. 5 C). From these measurements the rate of the ER Ca\(^{2+}\) leak (d. ratio/dt) was calculated (Fig. 5, B and D). LNCaP-PanX1 cells revealed a higher rate of Ca\(^{2+}\) leak than control cells, suggesting that PanX1 diminished ER Ca\(^{2+}\) content through an increase in passive Ca\(^{2+}\) leak from the ER. The validity of this approach has been justified in recent publications (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000).

To evaluate a possible contribution of other Ca\(^{2+}\)-permeable ER channels, such as InsP\(_3\) receptors (IP\(_3\)Rs) and ryanodine receptors (RyRs), to the ER Ca\(^{2+}\) leak in cells overexpressing pannexins, the rate of the ER Ca\(^{2+}\) leak in PanX1-transfected cells was measured in a solution supplemented with 100 μM ryanodine (to inhibit RyRs) and 500 μg/ml heparin (to inhibit IP\(_3\)R) using a similar experimental approach. Inhibition of IP\(_3\)R and RyRs did not affect the rate of the ER Ca\(^{2+}\) leak (Fig. 5 E),

**Figure 4.** PanX1 overexpression is associated with a reduction in the amount of Ca\(^{2+}\) that can be released from intracellular stores and the Ca\(^{2+}\) concentration within the ER. (A) Two representative [Ca\(^{2+}\)]\(_i\) traces evoked by external application of 1 μM TG in control (solid line) and PanX1-transfected (dashed line) LNCaP cells. (B) Amplitudes of TG-induced [Ca\(^{2+}\)] transients in control (filled bars) and under PanX1 overexpression in LNCaP and HEK-293 cells (open bars). Mean ± the SEM. (C) Resting [Ca\(^{2+}\)], in control (solid line) and PanX1-transfected (dashed line) LNCaP cells. Please note that in both cases the calcium store was then depleted with 1 μM ionomycin to assess the total calcium store content. (D) Resting [Ca\(^{2+}\)], (cumulative data; mean ± the SEM,) for control (filled bars), PanX1/EGFP-transfected (open bars), Cx43/EGFP-transfected (light gray bars), and Cx32-transfected (gray bars) LNCaP and HEK-293 cells.
leaving PanX1 as the only molecule yet to be identified as being responsible for the ER Ca\(^{2+}\) leak.

Furthermore, we performed a series of experiments to determine whether the overexpression of pannexins affects the level of Bcl-2, Bax, and Bak, which have been suggested as other potential Ca\(^{2+}\)-leak channels (Camello et al., 2002; Oakes et al., 2005). The dot-blot experiments revealed that levels of Bcl-2, Bax, and Bak proteins were the same in the PanX1-transfected LNCaP cells as in control LNCaP cell (expressing EGFP only), thus, excluding a possible contribution of these molecules to the enhanced Ca\(^{2+}\) leak also observed in LNCaP-PanX1 (Fig. 5 F).

On the other hand, immunodetection experiments revealed that the EGFP detection level in LNCaP cells expressing PanX1-EGFP or EGFP only was a hundred times higher than that of the Bcl-2 family proteins. It is therefore unlikely that drastic change in Ca\(^{2+}\) handling by the ER in LNCaP overexpressing PanX1 can be attributed to the expression of the Bcl-2 family proteins.

The question remains, however, as to whether endogenous PanX1 is actually involved in ER Ca\(^{2+}\) leak. To test this, we compared the ER Ca\(^{2+}\) content and kinetics of Ca\(^{2+}\) leak from the ER in control LNCaP cells and in LNCaP cells subjected to a depletion of endogenous PanX1 protein using antisense and siRNA technology (Khvorova et al., 2003). As shown in Fig. 6 A, siRNA-PanX1 reduced the endogenous PanX1 mRNA expression in HEK-293 cells. Western-blot revealed that siRNA-PanX1 also suppressed the PanX1 protein expression in LNCaP cells transfected with PanX1 (Fig. 6 B). Semiquantification revealed a decrease of 42.38 ± 7.06 for 5 nM siRNA-PanX1 transfection (statistically different with control, P < 0.001) and 3.91 ± 1.50 for 100 nM (statistically different with both control and 5 nM siRNA, P < 0.001), respectively. Given that siRNA treatment can cause effects unrelated to the depletion of the specific protein of interest, we tested the effect of the treatment with two different siRNA (directed against PanX1 and PanX1-unrelated murine TRPC6 protein) on ER Ca\(^{2+}\) content in PanX1-transfected cells. As shown in Fig. 6 C, only siRNA-PanX1 partially restored ER Ca\(^{2+}\) content in the LNCaP-PanX1 cell line, whereas siRNA had no effect against mTRPC6. The likely explanations for the fact that siRNA-PanX1 restored the ER Ca\(^{2+}\) content only partially are that the siRNA transfection rate was ~80% and the silencing efficiency of the siRNA was ~90%.

The inhibition of PanX1 expression with specific siRNA increased [Ca\(^{2+}\)]\(_L\). In LNCaP cells, [Ca\(^{2+}\)]\(_L\) measured with Mag-fura-2 was found to be on average 40% higher in
PanX1-depleted LNCaP cells (495 ± 30 μM; n = 32) than in control (340 ± 50 μM; n = 25). To reveal the effect of depletion of endogenous PanX1 on the ER Ca\(^{2+}\) leak, we calculated the rates of [Ca\(^{2+}\)]\(_L\) decline in response to TG application (measured with Mag-fura-2). PanX1-depleted LNCaP cells showed a significant decrease in the rate of ER Ca\(^{2+}\) leak, suggesting a contribution of endogenous PanX1 to the ER Ca\(^{2+}\) leak (Fig. 6 D).

In parallel experiments, we depleted endogenous PanX1 by the use of phosphorothioate anti-sense oligodeoxynucleotides (ODNs) and discovered a similar effect on ER Ca\(^{2+}\) leak (not depicted).

Because ER Ca\(^{2+}\) content is determined by the balance between Ca\(^{2+}\) leak and reuptake, the possible effect of PanX1 overexpression on SERCA activity was tested (Fig. 6 E). The ER Ca\(^{2+}\) uptake was investigated in digitonin-permeabilized cells using Mag-fura-2-AM, as previously described. The application of 100 μM IP\(_3\) triggered a rapid drop in [Ca\(^{2+}\)]\(_L\), caused by IP\(_3\)-induced Ca\(^{2+}\) release (Fig. 6 E). After IP\(_3\) washout, the recovery of [Ca\(^{2+}\)]\(_L\) reflected reuptake of Ca\(^{2+}\) into the ER (Fig. 6 E).

The rate of the ER Ca\(^{2+}\) reuptake (d. ratio/dt) was identical in control and PanX1 mRNA–depleted LNCaP cells (Fig. 6 F). Similarly, overexpression of PanX1 in either LNCaP or HEK-293 cells had no effect on Ca\(^{2+}\) reuptake assessed in the same way (unpublished data). Therefore, we concluded that PanX1 overexpression has no direct inhibitory effect on SERCA.

Altogether, our results strongly suggest that PanX1 may form Ca\(^{2+}\)-permeable channels in the ER membrane.
the line stably transfected with PanX1-EGFP (LNCaP-PanX1) (Fig. 7, A and C), from the line stably transfected with EGFP only (“LNCaP-EGFP”; Fig. 7 D), and from the control line (Fig. 7, B and E). In contrast to uniform EGFP fluorescence, which was observed when EGFP was solely expressed (Fig. 7 D), the fluorescence signal from EGFP coupled to PanX1 had distinct nonuniform cellular distribution, reflecting the expression of PanX1 in the ER and in the plasmalemma (Figs. 2, 3, 7 A, and C). The gap junction plaques formed by PanX1 clusters were clearly seen in the images taken with increased magnification (Fig. 2 B). It should be emphasized that regions of junction between neighboring cells were the regions of the plasmalemma, where PanX1 was first expressed after its expression in the ER membrane.

Intercellular Ca2+ wave propagation may generally involve a release of paracrine agents (such as ATP), which then activate receptors in neighboring cells triggering Ca2+-permeable membrane ion channels and/or Ca2+ release from the ER mediated by IP3 receptors (IP3Rs) and/or RyRs. Ca2+ entering the cell through gap junction channels can also activate RyRs, via a Ca2+-induced Ca2+ release mechanism. To prevent the contribution of these mechanisms under our experimental conditions and to unmask changes in [Ca2+]i, results were obtained from Ca2+-entry through gap junction channels, the cells were bathed in a Ca2+-free solution supplemented with 300 μM EGTA (to prevent Ca2+ entry from extracellular media), 0.5 μM TG (to inhibit SERCA and deplete the ER), 100 μM ryanodine (to inhibit RyRs), 100 μM 2-APB (to inhibit IP3Rs), and 5 μM U-73122 (to inhibit phospholipase C and prevent IP3 formation). This experimental approach effectively blocked the paracrine pathway, which may otherwise have contributed to intercellular Ca2+ wave propagation. Indeed, no [Ca2+]i transients were observed in LNCaP cells in response to application of 50 μM ATP after a 5-min incubation of the cells in this experimental media (n = 7). Upon dialysis of the cell with Ca2+-containing solutions, the Ca2+ movement rate from the dialyzed cell to the adjacent one was significantly greater in the LNCaP-PanX1 cells (Fig. 7 A; n = 8; and Fig. 7 C; n = 34) than in the LNCaP-EGFP cells (Fig. 7 D; n = 14) or in the control LNCaP cells (Fig. 7 B; n = 12; and Fig. 7 E; n = 13). This is emphasized by the time course plots of the normalized fluo-4 (Fig. 7, A and B) or rhod-2 (Fig. 7, C and E) fluorescence and is summarized further in the histograms (Fig. 7 F), where a relative increase in rhod-2 fluorescence detected in the adjacent cell 8 min after the beginning of dialysis is presented as a percentage of that observed at this moment in the dialyzed cell. The relative increase in rhod-2 fluorescence in the adjacent cell caused solely by Ca2+ movement from the dialyzed cell was significantly (P < 0.0001) higher in LNCaP-PanX1 cells (56.4 ± 3.1%; n = 34) than in LNCaP-EGFP cells (5.5 ± 1.8%; n = 14) or in control LNCaP cells (5.4 ± 1.5%; n = 13). It should be emphasized that it is extremely unlikely that intercellular Ca2+ movement under the experimental protocol used was mediated by hemichannels (but not by gap junction channels formed by PanX1) because no measurable Ca2+ entry from the patch pipette into the cell was detected in cell-attached configuration.

Finally, we examined whether the effects of overexpression of PanX1 in LNCaP-PanX1 cell line observed in our experiments may, in fact, be caused by modification of native connexin expression. Although only Cx32 mRNA has been previously demonstrated to be expressed at a low level in LNCaP cell line (Tate et al., 2006), it has been reported that overexpression of Cx32 or Cx43 may lead to functional gap junctions in this androgeno-dependent cell line (Mehta et al., 1999; Govindarajan et al., 2002). Therefore, we checked the expression of both Cx32 and Cx43 in a LNCaP-PanX1 cell line, LNCaP cells transiently transfected with PanX1, a control LNCaP cell line, and LNCaP cells transiently transfected with either Cx32 or Cx43. In agreement with what has previously been reported (Tate et al., 2006), we found no evidence for the expression of native Cx34 in any LNCaP cell types used in our experiments (unpublished data). Using Western blot analysis we did, however, detect a low but stable level of endogenous Cx32 protein in LNCaP cells (Fig. 8), which is in agreement with previously reported observations of Cx32 mRNA in this cell type (Tate et al., 2006). As expected, a higher level of Cx32 protein was detected in LNCaP cell after overexpression of Cx32 (Fig. 8). It should be emphasized, however, that the PanX-EGFP expression level was about 100 times higher than that of endogenous Cx32. Furthermore, immunodetection failed to reveal any native Cx34-forming gap junctions in either the LNCaP-PanX1 cell line or in control LNCaP cells (unpublished data).

In conclusion, our results strongly suggest that in our experimental model, Ca2+-permeable gap junction channels were formed by PanX1, but not by connexin proteins.

**Discussion**

Our study demonstrates for the first time that human PanX1, a protein encoded by a member of a new family of genes with yet unknown physiological role or roles, is a Ca2+-permeable ion channel that is localized on both the ER and plasma membrane and participates in two physiologically important processes: the ER Ca2+ leak and intercellular Ca2+ movement.

Intercellular gap junction channels provide the primary pathway for communication between cells, which is crucial for coordination of tissue metabolism and sensitivity to extracellular stimuli. Where vertebrates are concerned, the integral membrane proteins forming intercellular channels are referred to as connexins. In insects and nematodes, this function has been attributed to proteins named innexins (Phelan et al., 1998). By homology with these invertebrate gap junction proteins, we predicted that another protein family, pannexins, might also form gap junctions (Panchin et al., 2000). Connexin channels have been demonstrated to clus-ter in maculae known as gap junctions and to allow cell–cell diffusion of ions (predominantly monovalent cations; for re-view see Nicholson et al., 2000) and small molecules. Presently, it is commonly agreed that connexins provide two major pathways for intercellular calcium signaling. The first one, an “intracellular” pathway, involves the passage of a Ca2+-mobilizing messenger, such as IP3, through gap-junctional connexins (Leybaert et al., 1998; Fry et al., 2001). The second one, an “extracellular” pathway, involves the
Figure 7. **PanX1 facilitates intercellular Ca\(^{2+}\) movement in LNCaP cells.** One of the two adjacent LNCaP cells from the line stably transfected with PanX1 coupled to EGFP (A and C), from the line stably transfected with EGFP only (D) and from the control line (B and E) was dialyzed through the patch pipette with solution containing either 1 \(\mu\)M Ca\(^{2+}\) (A and B) or 100 \(\mu\)M Ca\(^{2+}\) (C–F), while changes in the fluorescence of the Ca\(^{2+}\)-sensitive indicator fluo-4 (A and B) or rhod-2 (C–F), preloaded into the cells as AM ethers (see Materials and methods), were monitored in the dialyzed and adjacent cells using x–y confocal imaging. The cell membrane potential was clamped at \(-50\) mV. The pipette solution was composed of 145 mM KCl, 10 mM Hepes, and 5 mM glucose and was supplemented either with 125 \(\mu\)M MgCl\(_2\), 3.8 mM HEDTA, and 0.3 mM CaCl\(_2\) (to give a free Ca\(^{2+}\) concentration of 1 \(\mu\)M) or with 2 mM MgCl\(_2\) and 100 \(\mu\)M CaCl\(_2\); pH was adjusted to 7.4 with KOH. The cells were bathed in a solution composed of 140 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 0.3 mM Na\(_2\)HPO\(_4\), 0.4 KH\(_2\)PO\(_4\), 4 mM NaHCO\(_3\), 5 mM glucose, and 10 mM Hepes; pH adjusted to 7.4 with NaOH. Breakthrough (whole-cell configuration) was commenced during the x–y time series imaging protocol comprising 100–200 frames each and acquired 6–10 s apart. Note the fluorescence signal from GFP coupled to PanX1 that appears as a bright outline of the cell membranes in transfected LNCaP cells (image galleries in A and C). The plots below the image galleries illustrate the time course of the normalized fluorescence averaged within a total confocal optical slice of the dialyzed (red dotted line) and adjacent (blue dotted line) cells. The numbered arrows on the plots show the moments when the images presented above were captured. Please note a decline in the fluo-4 fluorescence in a dialyzed cell (A and B) reflecting a washout of fluo-4 into the patch pipette (containing a dye-free solution).
release of a purinergic messenger, such as ATP, through connexin hemichannels, and a subsequent activation of P2Y receptors in a paracrine way (Frame and de Feijter, 1997; Guthrie et al., 1999; Klepeis et al., 2001; Braet et al., 2003).

Although Ca\textsuperscript{2+} ions are recognized as second messengers within individual cells, their role as diffusible messengers in intercellular signaling has largely been overlooked because elevated [Ca\textsuperscript{2+}], has been shown to reduce gap-junctional conductance in several systems, including insects and vertebrates (Rose and Loewenstein, 1975; Spray and Bennett, 1985; Verselis et al., 2000). Indeed, it is now generally agreed that even if a small amount of Ca\textsuperscript{2+} can diffuse across gap junctions, it probably does not play a significant role in intercellular calcium signaling through connexin channels, which is mediated mainly by IP\textsubscript{3} or other small signaling molecules (Churchill and Louis 1998; Niessen et al., 2000; Clair et al., 2001). This view is also supported by the recent observation that, in articular chondrocytes, intercellular calcium waves evoked by mechanical stimulation were abolished by incubation with TG and the phospholipase C inhibitor U73122 (D’Andrea et al., 2000). In contrast, in PanX1-transfected LNCaP cells an abrupt increase in [Ca\textsuperscript{2+}]\textsubscript{i} evoked by dialysis of one cell through the patch pipette with solution containing high Ca\textsuperscript{2+} concentration (such an elevation of [Ca\textsuperscript{2+}]\textsubscript{i}) is expected to uncouple gap junction channels formed by connexins) caused an elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in adjacent cells, which was consistent with cell–cell Ca\textsuperscript{2+} diffusion via gap junction channels formed by pannexin. Indeed, intercellular Ca\textsuperscript{2+} movement was observed under conditions where the contribution of both the intracellular (or IP\textsubscript{3}-dependent) and the extracellular pathway was eliminated by the inhibition of PLC and IP\textsubscript{3}Rs, depletion of ER, and removal of Ca\textsuperscript{2+} from extracellular medium. Furthermore, the inability of LNCaP cells to respond to external ATP application (unpublished data) also argues against any possible involvement of an ATP-dependent extracellular pathway. Thus, our results suggest that pannexin proteins may form Ca\textsuperscript{2+}-permeable channels providing a pathway for intercellular Ca\textsuperscript{2+} diffusion.

Even more intriguing is our finding that pannexins may also function as “leak channels” in the ER membrane. Indeed, the ER is the major calcium store (Berridge and Irvine, 1989; Pozzan et al., 1994), and the Ca\textsuperscript{2+}-filling status of the ER controls many physiological processes, ranging from gene expression to apoptosis and proliferation (Bao et al., 2004). Under resting conditions, steady-state [Ca\textsuperscript{2+}]\textsubscript{i}, is determined by the dynamic equilibrium of two components; an active Ca\textsuperscript{2+} uptake mediated by ATP-dependent Ca\textsuperscript{2+} pumps of the SERCA family and passive Ca\textsuperscript{2+} efflux via leak channels. Even though this pump–leak cycle appears to be a common property of Ca\textsuperscript{2+}-storing organelles, little is known about the molecular nature of the Ca\textsuperscript{2+}-leak pathway. Several mechanisms involving quite different proteins have been previously suggested to explain the basal Ca\textsuperscript{2+} leak from ER (for review see Camello et al., 2002), namely: (a) reverse Ca\textsuperscript{2+} flux through the pumps (Toyoshima and Nomura, 2002), (b) Ca\textsuperscript{2+} leak in neutral complexes with small molecules by translocon channels (Lomax et al., 2002; Van Coppenolle et al., 2004), (c) the fluxes of Ca\textsuperscript{2+} through “natural” ionophores, such as bile acids (Combettes et al., 1988; Zimniak et al., 1991), (d) an antiapoptotic protein Bcl-2–mediated Ca\textsuperscript{2+} leak (Pinton et al., 2000; Vandenberg et al., 2002; Bassik et al., 2004), and (e) IP3R- or RYR-mediated Ca\textsuperscript{2+} leak (Oakes et al., 2005). However, as concluded by Camello et al. (2002), “the drawing of these mechanisms is only a fantasy map of the leak terra incognita and discovery of the exact mechanisms of calcium leak remains a challenge to scientists working in the calcium signaling field.” The results of our study strongly suggest that the heterologous expression of PanX1 in LNCaP and HEK cells dramatically reduces the ER Ca\textsuperscript{2+} content and alters the Ca\textsuperscript{2+} permeability of the ER membrane, which is consistent with an ion leak-channel function of PanX1 in the ER.

This is not evident in the case of rhod-2 (C-E) which, being positively charged, is kept within the cell by several negatively charged molecules (and organelles). To unmask [Ca\textsuperscript{2+}] changes in adjacent cell caused solely by Ca\textsuperscript{2+} diffusion from the dialyzed cell, the cells were bathed in Ca\textsuperscript{2+}-free solution supplemented with 300 \textmu M EGTA (to prevent Ca\textsuperscript{2+} entry from extracellular media), 0.5 \textmu M TG (to inhibit SERCA and deplete the ER), 100 \textmu M ryanodine (to inhibit RyRs), 100 \textmu M 2-APB (to inhibit IP\textsubscript{3}Rs), and 5 \textmu M U-73122 (to inhibit phospholipase C and prevent IP\textsubscript{3} formation). The results are summarized in the histograms (F), where a relative increase in rhod-2 fluorescence detected in the adjacent cell 8 min after the beginning of dialysis is presented as a percentage of that observed at this moment in the dialyzed cell. Note that the increase in rhod2 fluorescence in the adjacent was significantly (p < 0.0001) higher in PanX1-EGFP-transfected cells than in cells transfected with EGFP only or in control cells.
membrane. To estimate the potential role of pannexins in endogenous ER basal Ca\(^{2+}\) leak, we used the siRNA and antisense hybrid depletion strategy for the endogenous PanX1 protein. Interestingly, in PanX1-depleted cells, the ER Ca\(^{2+}\) content was found to be ~40% higher than in control cells. Moreover, the rate of the ER Ca\(^{2+}\) leak (unmasked by inhibition of SERCA-mediated Ca\(^{2+}\) uptake with TG) was substantially reduced in PanX1-depleted cells, thus, suggesting an important contribution of endogenous PanX1 to the global ER basal Ca\(^{2+}\) leak. One may speculate that reduced resting concentration of calcium in the ER associated with the PanX1 overexpression could be caused by the following: (a) the modified level of the BCL-2 family of proteins with ER localization and known to play an important role in the regulation of the calcium leak from the ER, (b) the increased level of the antiapoptotic protein BCL-2 (Pinton et al., 2000; Vanden Abeele et al., 2002; Bassik et al., 2004), and/or (c) the deficiency for two “multidomain” pro-apoptotic proteins Bax and Bak (Scorrano et al., 2003; Oakes et al., 2005). Our results show that the levels of these proteins expression were not changed by PanX1 overexpression, thereby suggesting that the PanX1 may mediate Ca\(^{2+}\) leak by itself, independently of other potential ER leak modulators. In this respect, it would be interesting to investigate whether the function of a pannexin, such as the ER Ca\(^{2+}\)-leak channel, is specific to vertebrates, or if, in fact, some invertebrate innexins (which are pannexin homologous) share this function.

In conclusion, this study directly demonstrates the involvement of PanX1 in intra- and intercellular Ca\(^{2+}\) signaling, thus, illustrating the multifunctional role of a single molecule.

**Materials and methods**

**Plasmid construction**

PanX1 cDNA encompassing the entire coding region was synthesized by PCR amplification of the cDNA clone (Panchin et al., 2000) using two gene-specific primers. A sense primer, containing a HindIII site at its 5'-end (PanX1F: 5'-TGAAGCTTGGCAGGCTGCTCGCAAC-3'), and an anti-sense primer, containing an EcoRI site at its 5'-end (PanX1R: 5'-TGGTAATTTCCAGAAGTCTTGGCGGC-3'), were used. 500 ng of the pEGFP-N1 vector (CLONTECH Laboratories, Inc.) and PCR product were digested by HindIII and EcoRI. The digested vector and the PCR products were gel purified, ligated together, and cloned. The cDNA insert was sequenced to verify its identity and absence of mutation. Cx43-EGFP was a gift from B. Rose (Gene Porter 2 reagent, following the manufacturer’s instructions. The blots were then exposed to X-Omat AR films (Eastman Kodak Company).

**Western blot assay**

LNCaP or PanX cells (vehicle or siRNA-transfected) were harvested and pelletized in PBS and then sonicated in ice-cold buffer, pH 7.2, containing the following: 10 mM PO4-Na2/K buffer, 150 mM NaCl, 1 g/100 ml sodium deoxycholate, 1% Triton X-100, 1% NP-40, a mixture of protease inhibitors (Sigma-Aldrich), and a phosphatase inhibitor (sodium orthovanadate; Sigma-Aldrich). Samples were electrophoretically analyzed on a 10% polyacrylamide gel using the SDS-PAGE technique. The proteins were then transferred for 1 h (50 mA, 25 V) onto a nitrocellulose membrane using a semi-dry electrophoretic system (Bio-Rad Laboratories). The membrane was then cut into thin, equally sized strips and processed for Western blot. The strips were blocked in 5% TNT-milk (15 mM Tris buffer, pH 8.0, 140 mM NaCl, 0.05% Tween 20, and 5% non-fat dry milk) for 30 min at room temperature, washed three times in TNT, soaked in primary antibody (anti-GFP (CHEMICON International, Inc.), anti-actin (M-1925P; Neomarkers), anti-Cx32 (CHEMICON International, Inc.), or anti-Cx43 (CHEMICON International, Inc.), and then diluted 1:500, 1:500, 1:200, and 1:200, respectively, in TNT-milk for 1 h at room temperature. After three washes in TNT, the strips were transferred into the IgG horseradish peroxidase–linked secondary antibodies (CHEMICON International, Inc.), and diluted in TNT-milk for 1 h at room temperature. After three washes in TNT, the bands were visualized using an X-Omat AR film (Eastman Kodak Company).

**Construction of a stable LNCaP cell line expressing PanX1-GFP**

Stable cell lines expressing PanX1-GFP protein were constructed by transfection with 2 μg of pEGFP-N1/PanX1 plasmid in a 6-well plate for 6 h using a Gene Porter 2 reagent, following the manufacturer’s recommended protocol. The cells in culture were then maintained under selected pressures with 700 μg/ml G418 for 4 wk. Colonies expressing GFP were identified under fluorescence microscope, subcloned, and maintained under the selected pressure for at least 3 wk.

**siRNA and ODN assays**

LNCaP, LNCaP-PanX, or HEK cells were transfected overnight by either 5′-UUUGGCUCCGUGGUCCAGACGUCAU (5′-end) (1362–1380) and 5′-UAUUGCCCGAGACCCUCU (5′-end) (1593–1611). The expected DNA length of the PCR product generated by these primers was 465 bp (NM_013568, National Center for Biotechnology Information database). PCR was performed on the RT-generated cDNA using random hexamer primers (Perkin Elmer) and MuLV reverse transcriptase (Perkin Elmer) in a 20-μl final volume, followed by PCR. The PCR primers used to amplify pannexin cDNAs were designed with Gene Runner 3.05 (Hastings Software). Primers for the human pannexin synthesized by Life Technologies were as follows: forward 5′-CCACATGATGACGACTCTTG-3′ (963–984) and reverse 5′-AGACACTTCGTATGGCCTC-3′ (1403–1427). The expected DNA length of the PCR product generated by these primers was 658 bp (NM_013568, National Center for Biotechnology Information database).

**Analysis of PanX1 expression (RT-PCR)**

Total RNA was isolated from different cell lines using the guanidium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). After a DNase I (Life Technologies) treatment to eliminate genomic DNA, 2 μg of total RNA was reverse transcribed into cDNA at 42°C using random hexamer primers (Perkin Elmer) and MuLV reverse transcriptase (Perkin Elmer) in a 20-μl final volume, followed by PCR. The PCR primers used to amplify pannexin cDNAs were designed with Gene Runner 3.05 (Hastings Software). Primers for the human pannexin synthesized by Life Technologies were as follows: forward 5′-CCACATGATGACGACTCTTG-3′ (963–984) and reverse 5′-AGACACTTCGTATGGCCTC-3′ (1403–1427). The expected DNA length of the PCR product generated by these primers was 465 bp (NM_013568, National Center for Biotechnology Information database).

**Preparation of PanX1-GFP transfected cells for confocal analysis**

LNCaP cells were transfected at 60% confluence, as described in Transient transfection protocol. The cells in culture were then maintained under selected pressures with 700 μg/ml G418 for 4 wk. Colonies expressing GFP were identified under fluorescence microscope, subcloned, and maintained under the selected pressure for at least 3 wk.
cells were fixed with 4% formaldehyde-1X PBS for 15 min. After two washes in PBS, the slides were mounted with Mowiol.

Fluorescence measurements of [Ca²⁺]ᵢ and [Ca²⁺].

Fluorescence imaging was performed using fura-2-AM loaded cells. LNCaP cells were loaded for 45 min at room temperature with 2 μM fura-2-AM prepared in HBSS and, subsequently, washed three times with the same dye-free solution. The coverslip was then transferred into a perfusion chamber on a microscope [IX70; Olympus equipped for fluorescence].

Fluorescence was alternatively excited at 340 and 380 nm with a monochromator [Polychrome IV; TILL Photonics] and was captured after filtration through a long-pass filter (510 nm) by a 5 MHz charge-coupled device camera [MicroMax; Princeton Instruments]. Acquisition and analysis were performed with the Metafluor 4.5 software [Universal Imaging Corp.].

The intracellular calcium concentration was derived from the ratio of the fluorescence intensities for each of the excitation wavelengths [F340/F380] and from the equation of Grynkiewicz et al. (1985). All recordings were performed at room temperature. The cells were continuously perfused with the HBSS solution, and chemicals were added via the perfusion system. The flow rate of the whole-chamber perfusion system was set to 1 ml/min, and the chamber volume was 500 μl.

[Ca²⁺]ᵢ was monitored using Mag-fura-2 as previously described (Vanden Abeele et al., 2002).

Confocal microscopy

The HEK-293 and LNCaP cells were grown on coverslips and transfected with pannexin-GFP-N1. Fluorescence imaging was performed using a confocal scanner (488 nm excitation for GFP; LSM 510; Carl Zeiss MicroImaging, Inc.) based on an Axiovert 200 M motorized inverted microscope [20× objective (plan-Apochromat 63×; Imaging, Inc.)] with a plan-Apochromat 63× objective (488 nm excitation for GFP; LSM 510; Carl Zeiss MicroImaging, Inc.) based on an Axiovert 200 M motorized inverted microscope (488 nm excitation for GFP; LSM 510; Carl Zeiss MicroImaging, Inc.)

The ER was stained by 10–30 min incubation of the cells at room temperature in the solution containing 5 mM MgCl₂, 3.8 mM HEDTA, and 0.3 mM CaCl₂; pH 7.4 with KOH, while changes in the fluorescence of the Ca²⁺-sensitive indicator were monitored in the dialyzed and adjacent cells using x-y confocal imaging. To unmask Ca²⁺ entry from extracellular solution, Ca²⁺-free solution was perfused into the pipette solution (145 mM KCl, 10 mM Hepes, 5 mM glucose, 2 mM MgCl₂, and 100 μM CaCl₂; pH 7.4 with KOH), while changes in the fluorescence of the Ca²⁺-sensitive indicator were monitored in the dialyzed and adjacent cells using x-y confocal imaging.

Rhod-2 fluorescence was excited by the 543-nm line of a 5-mW HeNe ion laser and the emitted fluorescence was captured at 560 nm. Fluo-4 and GFP fluorescence were excited by the 488-nm line of a 20 mW argon ion laser and the fluorescence emitted was detected at wavelengths >560 nm. To visualize the fine spatial pattern of the BODIPY 558/568 fluorescence, the fluorescent signal was collected from the confocal optical slice below 0.5 μm with x-y frame size of 2048 × 2048 pixels, and the final images were obtained as a result of averaging of four sequential images taken in multitrack (line-by-line acquisition) configuration of the confocal scanner followed by low-pass filtering (7 × 7 pixels; LSM 510 software) to improve the signal-to-noise ratio.

Chemicals

All chemicals were obtained from Sigma-Aldrich, except for fura-2-AM and TG, which were purchased from Calbiochem, and Fluo-4-AM, Rhod-2, and BODIPY 558/568 rhodamine A, which were obtained from Invitrogen.

Data analysis and statistics

Each experiment was repeated several times. The data were analyzed using Origin 5.0 (Microcal) software. Results were expressed as the mean ± the SEM where appropriate. The t-test was used for statistical comparison of the differences, and P < 0.05 was considered significant.

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Functional implications of calcium permeability of the channel formed by pannexin 1

Fabien Vanden Abeele, Gabriel Bidaux, Dmitri Gordienko, Benjamin Beck, Yuri V. Panchin, Ancha V. Baranova, Dmitry V. Ivanov, Roman Skryma, and Natalia Prevarskaya

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The PanX1 panel in the original version of Fig. 6 A was a partial duplicate of the PanX1 panel in Fig. 1. The authors have indicated that this was due to a clerical error during figure preparation. The original version of Fig. 1 and its legend also did not indicate that intervening lanes of the gel image had been removed for presentation purposes. Corrected versions of Fig. 1 and Fig. 6 A and their respective figure legends are shown below.

**Figure 1.** Pannexin1 (PanX1) mRNA is ubiquitously expressed in prostate cell lines. A 2% agarose gel showing the expression of the PanX transcripts in human prostate cell lines LNCaP, PNT1A, DU-145, and PC-3, as well as in HEK-293 cells. A no-template control (H2O) was also run with the PCR samples, where the cDNA was replaced with water. White lines indicate the removal of intervening lanes for presentation purposes.

**Figure 6.** (A) siRNA-PanX1 reduced the endogenous PanX1 mRNA expression in HEK-293 cells. Semiquantitative RT-PCR (PanX1, 36 cycles; β-actin, 27 cycles) showing a decrease in the expression of the PanX1 transcripts in HEK-293 cells transfected for 2 d with 100 nM of siRNA-PanX1. Note that this reduction was not observed when the cells were incubated with vehicle only. The β-actin mRNA expression was used to control the RNA rate in each sample.

The html and pdf versions of this article have been corrected. The errors remain only in the print version.