Identification of Thr\(^29\) as a Critical Phosphorylation Site That Activates the Human Proton Channel Hvcn1 in Leukocytes*

Received for publication, November 5, 2009, and in revised form, December 15, 2009

Boris Musset,†† Melissa Capasso,† Vladimir V. Cherny,‡ Deri Morgan,§ Mandep Bhamrah, ‡ Martin J. S. Dyer,‡ and Thomas E. DeCoursey*‡‡

From the †Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, Illinois 60612 and the ‡Toxicology Unit, Medical Research Council (MRC), University of Leicester, Leicester LE1 9HN, United Kingdom

Voltage-gated proton channels and NADPH oxidase function cooperatively in phagocytes during the respiratory burst, when reactive oxygen species are produced to kill microbial invaders. Agents that activate NADPH oxidase also enhance proton channel gating profoundly, facilitating its roles in charge compensation and pH\(_i\) regulation. The “enhanced gating mode” appears to reflect protein kinase C (PKC) phosphorylation. Here we examine two candidates for PKC-\(\delta\) phosphorylation sites in the human voltage-gated proton channel, H\(_{\text{v}}\)\(_1\) (Hvcn1), Thr\(^29\) and Ser\(^97\), both in the intracellular N terminus. Channel phosphorylation was reduced in single mutants S97A or T29A, and further in the double mutant T29A/S97A, by an \textit{in vitro} kinase assay with PKC-\(\delta\). Enhanced gating was evaluated by expressing wild-type (WT) or mutant H\(_{\text{v}}\)\(_1\) channels in LK35.2 cells, a B cell hybridoma. Stimulation by phorbol myristate acetate enhanced WT channel gating, and this effect was reversed by treatment with the PKC inhibitor GF109203X. The single mutant T29A or double mutant T29A/S97A failed to respond to phorbol myristate acetate or GF109203X. In contrast, the S97A mutant responded like cells transfected with WT H\(_{\text{v}}\)\(_1\). We conclude that under these conditions, direct phosphorylation of the proton channel molecule at Thr\(^29\) is primarily responsible for the enhancement of proton channel gating. This phosphorylation is crucial to activation of the proton conductance during the respiratory burst in phagocytes.

Voltage-gated proton channels enable sustained superoxide anion (O\(_2^•\)) production by NADPH oxidase during the respiratory burst in phagocytes. NADPH oxidase generates O\(_2^•\) by transferring electrons from NADPH in the cytosol across the membrane to reduce extracellular or phagosomal O\(_2\) to O\(_2^•\). O\(_2^•\) is the precursor to other reactive oxygen species that contribute to killing microbial invaders. The consequences of NADPH oxidase activity, in particular decreased internal pH (pH\(_i\)) and membrane depolarization, are counteracted by H\(^+\) efflux through open voltage-gated proton channels (1–6). The activities of NADPH oxidase and voltage-gated proton channels are coordinated in several ways. The depolarization and pH\(_i\) decrease resulting from NADPH oxidase activity both directly promote proton channel opening. In addition, interventions that activate NADPH oxidase profoundly enhance the gating properties of proton channels (3, 7). This “enhanced gating mode” consists of four changes in proton channel properties, each of which increases the likelihood of channel opening under any given set of conditions. The channels open faster (smaller activation time constant, \(\tau_{\text{act}}\)) and close more slowly (larger deactivation time constant, \(\tau_{\text{tail}}\)), display increased maximum proton conductance (\(g_{\text{max}}\)) and manifest a 40-mV hyperpolarizing shift of the entire proton conductance-voltage relationship (\(g_{\text{V}}\)). The enhanced gating mode improves the efficiency of NADPH oxidase by minimizing the depolarization required to open enough proton channels to fully compensate the electrical consequences of NADPH oxidase activity (i.e. the electron current) (5). Depolarization directly inhibits NADPH oxidase (4, 8).

The enhanced gating mode is induced by PMA, an activator of PKC, and is prevented and at least partially reversed by the PKC inhibitor GFX (9, 10). Although these results suggest regulation by PKC phosphorylation, they do not clarify whether the target of PKC is an accessory protein or the channel itself. This study identifies phosphorylation sites on the human proton channel molecule and determines their involvement in converting the proton channel to the enhanced gating mode. We find that a single residue, Thr\(^29\), in the intracellular N-terminal domain appears to be responsible for inducing enhanced gating. Evidently, enhanced gating reflects a phosphorylated state of the proton channel and does not require accessory proteins.

EXPERIMENTAL PROCEDURES

Plasmids and Retroviral Infection—Myc-tagged Hvcn1 was cloned by PCR in green fluorescent protein-bicistronic MigRI retroviral vector. Hvcn1 T29A/S97A, T29A, T29D, S97A, and S97D mutants were generated by site-directed mutagenesis of wild-type Hvcn1 sequence in MigRI vector using the Stratagene (La Jolla, CA) QuickChange technology. Sequences of primers used for mutagenesis are available upon request.

Lentiviral particles were prepared as follows. Phoenix \(\alpha\) packaging cell line was transfected with empty vector con-
REPORT: Thr<sup>29</sup> Phosphorylation Activates Human Proton Channels

trol and Hvcn1 MigRI plasmids by Ca<sup>2+</sup> phosphate transfection. Viral supernatants were collected after 24, 36, and 48 h and frozen at −80 °C until use. LK35.2 cells were infected by spinoculation at 2300 rpm for 90 min in the presence of 4 μg/ml Polybrene (Sigma Aldrich, Dorset, UK) three times over a period of 2 days. At day 3, highly green fluorescent-protein-positive cells were sorted on a FACSVantage with CellQuest software (Becton Dickinson, Oxford, UK) and used for patch clamp studies.

In Vitro Kinase Assay—HEK-293 cells were transfected with Myc-tagged Hven1, Hven1 T29A/S97A, Hven1 T29A, and Hven1 S97A MigRI constructs by Ca<sup>2+</sup> phosphate. 24 h after transfection, cells were harvested and lysed (1% Triton X-100, 20 mM Hepes, 137 mM NaCl, 2.5 mM β-glycerophosphate, 1 mM Na<sub>2</sub>SO<sub>4</sub>, 2 mM EDTA, protease inhibitor mixture (Sigma Aldrich)) prior to immunoprecipitation with anti-Myc antibody (Cell Signaling Technology) conjugated to protein G-Sepharose beads. Beads were then incubated in kinase assay buffer (20 mM Hepes, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 0.05 mM dithiothreitol, 0.2 mg/ml phosphatidylinositol, 2.5 mM β-glycerophosphate, 1 mM PMA, 40 mM PKC-δ (Cell Signaling Technology), 10 μCi of [γ<sup>32</sup>P]ATP (GE Healthcare)) for 20 min at 30 °C, prior to being suspended in 2× Laemmli buffer. Four-fifths of samples were loaded on an SDS-PAGE that was then dried in a gel dryer prior to exposure to x-ray film; one-fifth of samples was loaded on a separate SDS-PAGE and was assessed in an in vitro kinase assay with recombinant PKC-δ. Fig. 1A shows that phosphorylation was reduced significantly when compared with WT H<sub>H1</sub> for either single mutant S97A or T29A. Phosphorylation was further reduced in the double mutant T29A/S97A, which supports the conclusion that both sites are phosphorylated by PKC-δ. The τ<sub>act</sub> was obtained by fitting the rising current with a single exponential to obtain the τ<sub>tail</sub>. The values obtained for τ<sub>act</sub> increased progressively over several minutes. On average, τ<sub>act</sub> increased 2.4-fold during test pulses (Table 1). After application of a voltage clamp family and measurement of the reversal potential, V<sub>rev</sub>, test pulses were again applied. Then GFX was added to the bath, and the enhanced proton current was progressively reduced toward its amplitude before stimulation.

In each experimental condition, we applied families of depolarizing pulses, as illustrated in Fig. 2A–C, to characterize the response in greater detail. Fig. 2D shows that PMA increased g<sub>t</sub>, whereas LK35.2 cells transfected with Hven1 expressed large proton currents (Fig. 2A). The experimental approach is illustrated in Fig. 2F. Test pulses were applied, and then PMA was introduced, after which the proton current amplitude I<sub>t</sub> increased progressively over several minutes. On average, I<sub>t</sub> increased 2.4-fold during test pulses (Table 1). After application of a voltage clamp family and measurement of the reversal potential, V<sub>rev</sub>, test pulses were again applied. Then GFX was added to the bath, and the enhanced proton current was progressively reduced toward its amplitude before stimulation.
and shifted the \( g_{fr} \) vs. \( V \) relationship negatively. The mean shift of the \( g_{fr} \) vs. \( V \) relationship was \(-11 \text{ mV}\) (Table 1). GFX reversed this shift substantially. The rate of channel opening increased after PMA stimulation; the time constant of \( H_{9270} \) change in \( V \) (mean \( \pm S.E., n = 17 \)). \( V_{\text{threshold}} \) in T29D expressing cells was \(-0.7 \pm 4.4 \text{ mV}\) (mean \( \pm S.E., n = 7 \)), a small shift in the expected direction but not significantly different. The effects of PMA and GFX on individual cells are clearly evident because each cell serves as its own control. In contrast, detecting a small difference in properties between populations of unstimulated cells may not be feasible. Alternatively, phosphorylation mimics \((X \rightarrow \text{Glu or } X \rightarrow \text{Asp})\) are not always distinguishable from simple alanine mutants \((X \rightarrow \text{Ala})\) (16).

The lack of response of Thr\(^{29}\) mutants suggests that the entire phosphorylation response is ascribable to phosphorylation of Thr\(^{29}\).

However, we compared the responses in Ser\(^{97}\) mutants statistically to test the possibility that Ser\(^{97}\) might contribute measurably to the response (Table 1). Although the enhancement of proton channel gating by PMA and its reversal by GFX tended to be larger in WT controls than in Ser\(^{97}\) mutants, the difference failed to reach statistical significance for any parameter (whether S97A and S97D were compared with WT separately or together). However, the probability that all eight parameters would exhibit smaller responses in S97X than WT is \(< 0.01\). Thus, we cannot rule out that Ser\(^{97}\) may play a minor role or affect other functions of the channel. However, it is clear that phosphorylation of Thr\(^{29}\) is the major contributor to the enhanced gating of proton channels.

**DISCUSSION**

Voltage-gated proton channels are necessary for a normal phagocyte respiratory burst; NADPH oxidase activity by neutrophils, eosinophils, monocytes, macrophages, B lymphocytes and PLB-985 cells is greatly reduced when proton currents are inhibited by Zn\(^{2+}\) or other polyvalent metal cations (4, 9, 17–22) or when the proton channel is removed genetically (23–

**FIGURE 1. Identification of candidate phosphorylation sites in the human proton channel.** A, location of the two putative PKC-\(\delta\) phosphorylation sites in the sequence of \( H_{1} \). Both predicted PKC-\(\delta\) sites are in the N terminus, with Ser\(^{97}\) near the membrane boundary. Boxed residues indicate helical transmembrane regions (37). B, phosphorylation of \( H_{1} \), WT, T29A, S97A, and T29A/S97A mutants in the presence of recombinant PKC-\(\delta\) and \([\gamma-\text{P}]\text{ATP}\) in an \textit{in vitro} kinase assay. PKC-\(\delta\) was activated with 1 \( \mu\text{M} \) PMA. The \( \text{myc immunoblot} \) indicates loading. EV indicates empty vector control. The graph on the right-hand side represents densitometric analysis of the \({}^{32}\text{P}\) band versus loading control of 3–4 separate experiments (\( p < 0.05 \) for each mutant versus WT by Student’s \( t \) test). Error bars indicate S.E.
REPORT: Thr29 Phosphorylation Activates Human Proton Channels

Proton channels and NADPH oxidase are both activated during phagocytosis, but their interaction is complex, and the mechanism remains controversial (15). The idea that the proton channel is part of the NADPH oxidase complex (26) and is thus active de facto during NADPH oxidase activity (3) appears untenable in light of the demonstration of NADPH oxidase-generated electron current in phagocytes from Hven1-deficient mice (6). Another view is that these two molecules are activated independently but by similar pathways (10).

The suspicion that voltage-gated proton channels in leukocytes are activated by PKC phosphorylation (10, 27, 28) is strongly supported by the present results. Furthermore, our data indicate that the channel protein itself is phosphorylated. The in vitro kinase assay detected phosphorylation of both Ser97 and Thr29. However, in our experimental system, Thr29 appeared to be responsible for the entire measurable response of proton channels. T29A or T29D mutants did not respond to PMA, nor did the double mutant T29A/S97A. In contrast, S97A or S97D mutants did respond to PMA, although their response may have been slightly weaker than that of WT channels (Table 1). These results do not resolve the physiological significance of phosphorylation of Ser97. That Ser97 was phosphorylated, yet S97A and S97D exhibited no deficit, is not without precedent. Many proteins have multiple phosphorylation sites at which only a fraction exert specific functional effects. For example, cardiac troponin T has four phosphorylation sites, of which only one exhibited functional defects upon mutation (16). Another protein with multiple phosphorylation sites is p47phox, whose phosphorylation is an absolute prerequisite for NADPH oxidase assembly and function. Individual mutation of 10 phosphorylated serines revealed just one mutation, S379A, that abolished activation, five Ser → Ala mutations that inhibited activation by ~50%, and three that had no effect (29).

In future studies, we will attempt to identify the PKC isoform that phosphorylates the proton channel. By criteria determined by Fujii et al. (13), Thr29 is a likely PKC-δ site, but ScanSite identifies Thr29 as a possible target for CAMP-dependent protein kinase, PKC-μ, Akt kinase, or calmodulin kinase. Ser97 is also identified by criteria determined by Fujii et al. (13) as a likely PKC-δ site, but ScanSite identifies Ser97 as a possible target for PKC-α, PKC-β, or PKC-γ. PKC-δ appears to be important in responses of human neutrophils (9, 30–32) and murine B lymphocytes (33, 34).

Proton channels studied in perforated-patch conditions in human neutrophils, eosinophils, monocytes, and to a lesser extent, in basophils, respond dramatically to PMA (7, 12, 14, 35). In contrast, proton channels in other cells, such as rat alve-

TABLE 1

|                      | WT                  | S97A or S97D          | CGD neutrophils | Neutrophils |
|----------------------|---------------------|-----------------------|----------------|-------------|
| I_p(A) PMA/c         | 2.39 ± 0.51 (10)    | 1.42 ± 0.13 (12)      | 2.55           | 3.3         |
| I_p(A)/PMA/GFX       | 2.19 ± 0.37 (12)    | 1.22 ± 0.11 (8)       |                |             |
| t_50 A/PMA           | 2.12 ± 0.53 (10)    | 1.62 ± 0.15 (12)      | 1.94           | 3.7         |
| t_50 G/FX/PMA        | 1.58 ± 0.22 (12)    | 1.21 ± 0.21 (8)       | 1.15           | 5.5         |
| t_50 PMA/c           | 1.54 ± 0.17 (13)    | 1.14 ± 0.06 (8)       | 1.15           | 5.5         |
| g_pV shift PMA (mV)  | −10.7 ± 1.9 (7)     | −7.8 ± 1.6 (8)        | −13.3          | −38.8       |
| g_pV shift GFX (mV)  | 9.4 ± 1.6 (8)       | 6.7 ± 1.2 (7)         |                |             |
olar epithelial cells (7) or HEK-293 or COS-7 cells transfected with H$_{v}$1 (12), do not respond at all. Hypothetical explanations for this variable response in different tissues include (a) different proton channel isoforms, (b) different signaling pathways, and (c) regulation of the channel by a separate molecule whose phosphorylation modulates proton channel behavior (15). Our results do not support the existence of a separate regulatory molecule (other than NADPH oxidase; see next paragraph).

Proton channels expressed in the B lymphocytic cell line LK35.2 were found to respond qualitatively like those in primary leukocytes, but the response was substantially less profound (Table 1). Each of the four main responses is weaker in LK35.2 cells than in neutrophils, but the most obvious differences are the lack of profound slowing of channel closing ($\tau_{\text{tail}}$) and the smaller hyperpolarizing shift of the $g_{\text{irr}}V$ relationship. The requirements for a full phagocyte-like proton channel response are unclear. Circumstantial evidence suggests that a crucial interaction between these molecules resembles that in other cells lacking NADPH oxidase activity, (36). The responses of proton channels in LK35.2 cells were essentially identical to the responses in CGD cells (Table 1) and in PLB-985 cells lacking gp91$^{phox}$ (36). We did not detect electron current in PMA-stimulated LK35.2 cells, consistent with low levels of NADPH oxidase activity (23). Human basophils also lack NADPH oxidase and exhibit PMA and GFX responses smaller than those of neutrophils or eosinophils and specifically lack a slowing of $\tau_{\text{tail}}$ (35). Thus, it is clear that a proton channel response is possible without NADPH oxidase, but the response differs in characteristic ways from that in cells with NADPH oxidase activity. The alternative postulate, that proton channels are preferentially phosphorylated by specific PKC isoforms that are more active in phagocytes, does not explain the meager response of CGD neutrophils (Table 1).

In summary, enhanced gating of H$_{v}$1 is primarily the result of phosphorylation at Thr$^{29}$ by PKC. The magnitude and specific characteristics of the PMA response in LK35.2 cells closely resembled that in other cells lacking NADPH oxidase activity, suggesting that a crucial interaction between these molecules occurs in phagocytes. The identification of the location of the key phosphorylation site that activates phagocyte proton channels provides a rational target for modulation of innate immune responses.

REFERENCES

1. Henderson, L. M., Chappell, J. B., and Jones, O. T. (1987) Biochem. J. 246, 325–329
2. DeCoursey, T. E., and Cherny, V. V. (1993) Biophys. J. 65, 1590–1598
3. Bánfi, B., Schrenzel, J., Nüssle, O., Lew, D. P., Ligeti, E., Krause, K. H., and Demaurex, N. (1999) J. Exp. Med. 190, 183–194
4. DeCoursey, T. E., Morgan, D., and Cherny, V. V. (2003) Nature 422, 531–534
5. Murphy, R., and DeCoursey, T. E. (2006) Biochim. Biophys. Acta 1757, 996–1011
6. Morgan, D., Capasso, M., Musset, B., Cherny, V. V., Rios, E., Dyer, M. J., and DeCoursey, T. E. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 18022–18027
7. DeCoursey, T. E., Cherny, V. V., Zhou, W., and Thomas, L. L. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 6885–6889
8. Petheo, G. L., and Demaurex, N. (2005) Biochim. J. 388, 485–491
9. Bankers-Fulbright, J. L., Kita, H., Gleich, G. J., and O’Grady, S. M. (2001) J. Cell. Physiol. 189, 306–315
10. Morgan, D., Cherny, V. V., Finnegan, A., Bollinger, J., Gelb, M. H., and DeCoursey, T. E. (2007) J. Physiol. 579, 327–344
11. Morgan, D., Cherny, V. V., Murphy, R., Xu, W., Thomas, L. L., and DeCoursey, T. E. (2008) J. Physiol. 586, 2477–2486
12. Fuji, Z., Zhu, G., Liu, Y., Hallam, I., Chen, L., Herrero, J., and Shaw, S. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 13744–13749
13. DeCoursey, T. E., Cherny, V. V., Dyer, M. J., Xu, W., and Thomas, L. L. (2001) J. Physiol. 535, 767–781
14. Musset, B., Cherny, V. V., Morgan, D., and DeCoursey, T. E. (2009) FEBS Lett. 583, 7–12
15. Sumandee, M. P., Pyle, W. G., Kobayashi, T., de Tombe, P. M., and Solaro, R. J. (2003) J. Biol. Chem. 278, 35135–35144
16. Chvapil, M., Stankova, L., Bernhard, D. S., Weldy, P. L., Carlson, E. C., and Campbell, J. B. (1977) Infect. Immun. 16, 367–373
17. Henderson, J. M., Chappell, J. B., and Jones, O. T. (1988) Biochem. J. 255, 285–290
18. Simchowitz, L., Foy, M. A., and Crago, E. J., Jr. (1990) J. Biol. Chem. 265, 13449–13456
19. Lowenthal, A., and Levy, R. (1999) J. Biol. Chem. 274, 21603–21608
20. Freem, I. K., Cherny, V. V., Morgan, D., Rada, B., Davis, A. P., Czirják, G., Enyedi, P., England, S. K., Moreland, J. G., Ligeti, E., Nauseef, W. M., and DeCoursey, T. E. (2006) J. Gen. Physiol. 127, 659–672
21. Musset, B., Cherny, V. V., and DeCoursey, T. E. (2009) Biophys. J. 96, 667a–668a
22. Capasso, M., Bhamrah, M. K., Henley, T., Boyd, R. S., Langlais, C., Cain, K., Dinsdale, D., Pulford, K., Khan, M., Musset, B., Cherny, V. V., Morgan, D., Gascoyne, R. D., Vigorito, E., DeCoursey, T. E., MacLennan, I. C., and Dyer, M. J. (2010) Nat. Immunol., in press
23. Ramsey, I. S., Ruchti, E., Kaczmarek, J. S., and Clapham, D. E. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 7642–7647
24. Okochi, Y., Sasaki, M., Iwasaki, H., and Okamura, Y. (2009) Biochem. Biophys. Res. Commun. 382, 274–279
25. Henderson, L. M., Banting, G., and Chappell, J. B. (1995) J. Biol. Chem. 270, 5909–5916
26. Nanda, A., and Grinstein, S. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 10816–10820
27. Kapus, A., Sza„zi, K., and Ligeti, E. (1992) Biochem. J. 284, 5909–5916
28. El-Benna, J., Dang, P. M., Gougerot-Pocidalo, M. A., Marie, J. C., and Clapham, D. E. (2009) J. Biol. Chem. 284, 7642–7647
29. Zhao, X., Xu, B., Bhattacharjee, A., Oldfield, C. M., Wientjes, B. F., Feldman, G. M., and Cathcart, M. K. (2005) J. Leukoc. Biol. 77, 414–420
30. Cheng, N., He, R., Tian, J., Dinauer, M. C., and Ye, R. D. (2004) J. Immunol. 179, 7720–7728
31. Mecklenbräuer, L., Saijo, K., Zheng, N. Y., Leitges, M., and Tarakhovsky, A. (2002) Nature 416, 786–806
32. Miyamoto, A., Nakayama, K., Imaki, H., Hirose, S., Jiang, Y., Abe, M., Tsukiyama, T., Nakamura, H., Ohno, S., Hatakeyama, S., and Nakayama, K. I. (2003) J. Immunol. 170, 7462–7470
33. Zhao, X., Xu, B., Bhattacharjee, A., Oldfield, C. M., Wientjes, B. F., Feldman, G. M., and Cathcart, M. K. (2005) J. Leukoc. Biol. 77, 414–420
34. Cheng, N., He, R., Tian, J., Dinauer, M. C., and Ye, R. D. (2004) J. Immunol. 179, 7720–7728
35. Mecklenbräuer, L., Saijo, K., Zheng, N. Y., Leitges, M., and Tarakhovsky, A. (2002) Nature 416, 786–806