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Voltage-gated proton channels in microglia

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Abstract

Microglia, macrophages that reside in the brain, can express at least 12 different ion channels, including voltage-gated proton channels. The properties of H^+ currents in microglia are similar to those in other phagocytes. Proton currents are elicited by depolarizing the membrane potential, but activation also depends strongly on both intracellular pH (pH_i) and extracellular pH (pH_o). Increasing pH_o or lowering pH_i promotes H^+ channel opening by shifting the activation threshold to more negative potentials. H^+ channels in microglia open only when the pH gradient is outward, so they carry only outward current in the steady state. Time-dependent activation of H^+ currents is slow, with a time constant roughly 1 s at room temperature. Microglial H^+ currents are inhibited by inorganic polyvalent cations, which reduce H^+ current amplitude and shift the voltage dependence of activation to more positive potentials. Cytoskeletal disruptive agents modulate H^+ currents in microglia. Cytochalasin D or colchicine decrease the current density and slow the activation of H^+ currents. Similar changes of H^+ currents, possibly due to cytoskeletal reorganization, occur in microglia during the transformation from ameboid to ramified morphology. Phagocytes, including microglia, undergo a respiratory burst, in which NADPH oxidase releases bactericidal superoxide anions into the phagosome and stoichiometrically releases protons into the cell, tending to depolarize and acidify the cell. H^+ currents may help regulate both the membrane potential and pH_i during the respiratory burst. By compensating for the efflux of electrons and counteracting intracellular acidification, H^+ channels help maintain superoxide anion production.

Contents

Abbreviations: ACM, astrocyte conditioned media; ADP, adenosine diphosphate; ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CGD, chronic granulomatous disease; CNS, central nervous system; CRAC, Ca^{2+}-release-activated Ca^{2+} [channels]; E_H, Nernst potential for H^+; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; fMLP, *N*-formyl-Met-Leu-Phe chemotactic peptide; GABA-A, gamma aminobutyric acid; g_H, H^+ chord conductance; $g_{H,max}$, maximum or limiting value of g_H; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBC, hydrogen-bonded chain; ICAM-1, intercellular adhesion molecule-1; IFN-γ, gamma-interferon; I_H, H^+ current amplitude; $I_{H,max}$, maximum H^+ current amplitude; $InsP_3$, inositol triphosphate; IR, inward rectifier; $[K^+]_o$, extracellular K^+ concentration; LFA-1, leukocyte function-associated antigen-1; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; O_2^-, superoxide anion; ΔpH, PH gradient ($pH_o - pH_i$); pH_i, intracellular pH; pH_o, extracellular pH; PKC, protein kinase C; PMA, phorbol myristate acetate; P_{rel}, relative permeability; TEA, tetraethylammonium; TNF-α, tumor necrosis factor; $V_{threshold}$, threshold level of depolarization required to activate H^+ currents; V_{rev}, reversal potential.

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1. Introduction

Microglial cells are the resident macrophages of the brain. Microglia and peripheral macrophages share many phenotypic markers and are capable of performing similar functions. In the normal adult brain microglia appear to be in a functionally resting state, in which they are highly ramified, extending processes far into surrounding brain tissue, acting as antennae. A variety of stimuli, including neuronal injury, trauma, ischemia, inflammation, infection, and several neurological diseases lead to microglial activation. Activation of microglia appears to proceed through a series of steps that include changes in morphology and expression of surface antigens, release of cytokines and cytotoxins, antigen presentation, and phagocytosis. A number of excellent reviews describing the properties and functions of microglia in the normal and pathological brain have been published recently (Raivich et al., 1999; Stoll and Jander, 1999; Streit et al., 1999), therefore we do not describe these topics in detail here.

Electrophysiological properties of microglial cells have been studied intensively during the past decade. It has been demonstrated that microglia are capable of expressing a wide variety of ion channels. Under certain conditions, microglial cells express channels that selectively conduct K^+, H^+, Na^+, Ca^{2+}, or Cl^-. Changes in the functional state of the microglial cells cause changes in the expression levels of some ion channels, including inward rectifier and delayed rectifier K^+ channels. Properties, expression, regulation and functional roles of the 12 different ion channels discovered so far in microglia have been reviewed recently (Eder, 1998).

The goal of the present review is to describe the properties and functions of voltage-gated proton chan-

nels in microglia. Because only a handful of papers have been published specifically on this narrow topic, the scope of the review will be somewhat more general. The properties of H^+ channels are similar in most phagocytes, and are at least qualitatively similar in most respects in all cells and species. There are several distinct varieties of H^+ channels, however, and their distinguishing features may have significant functional consequences.

2. pH changes in the brain

Within the central nervous system (CNS), neuronal activity is accompanied by substantial changes in extracellular pH. Changes in pH occur under pathological situations, such as ischemia and epilepsy, but also in the normally functioning brain. Because activation of proton channels depends strongly on voltage, pH_i, and pH_o, activity-dependent pH changes of the external milieu will influence the activity of microglial proton channels. We therefore first give an overview of the pH changes in the brain and their possible influence on neuronal and microglial activity.

2.1. Activity-dependent pH changes in the brain

In many CNS regions, a rapid extracellular alkalinization is seen at the onset of neuronal activity. Such activity-dependent alkalosis of the extracellular space was detected in cortex (Urbanics et al., 1978), in the cerebellum (Kraig et al., 1983; Chesler and Chan, 1988), in some spinal cord preparations (Endres et al., 1986; Jendelova and Sykova, 1991), and in the hippocampus (Jarolimek et al., 1989; Walz, 1989; Chen and Chesler, 1992; Chen and Chesler, 1992a,b; Kaila et al., 1992). Alkaline pH shifts have a rapid onset and can sometimes be maintained for as long as the stimuli are applied (Chesler and Kaila, 1992). In many cases, alkaline pH shifts are transient and are followed by a more slowly developing extracellular acidification (see below).

Ligand-gated channels play a major role in the generation of activity-induced alkaline transients. Activation of GABA-A receptors leads to the efflux of bicarbonate through anion channels resulting in a consequent rise in pH_o (Kaila and Voipio, 1987; Chen and Chesler, 1990; Kaila et al., 1990; Chen and Chesler, 1992a). A bicarbonate-independent alkaline shift can be elicited by activation of ionotropic glutamate receptors (Chesler and Chan, 1988; Chen and Chesler, 1992a) or by direct electrical stimulation of a neuronal population (Chen and Chesler, 1992b; Grichtchenko and Chesler, 1996; Paalasmaa and Kaila, 1996; Tong and Chesler, 1999). It has been suggested that the glutamate-induced extracellular alkalinization is caused by fluxes of H^+/OH^- equivalents down their electrochemical gradient through the glutamate receptor-coupled ion channels of both neurons and glial cells (Chen and Chesler, 1992b; Deitmer and Munsch, 1992). Since the glutamate-induced alkalinization was suppressed upon removal of extracellular Ca^{2+}, it has been proposed by others (Paalasmaa et al., 1994; Smith et al., 1994) that this alkalinization results from the activation of a cellular Ca^{2+}/H^+ ATPase. Activation of the Ca^{2+}/H^+ ATPase leads to a decrease in the concentration of extracellular H^+ due to the outward transport of intracellular Ca^{2+} in exchange for extracellular H^+ (Schwiening et al., 1993).

In some CNS regions, e.g. in the optic nerve (Davis et al., 1987), in the spinal cord (Sykova and Svoboda, 1990), and in the cortex (Siesjö et al., 1985; Chesler and Kraig, 1987), neuronal activity induces an early acid shift. Activity-induced alkaline transients are also often followed by a slow, persistent acid shift. Moreover, prolonged acidification of the extracellular space has been detected during repetitive stimulation, epileptiform activity, spreading depression, and ischemia (Chesler and Kaila, 1992).

Several potential mechanisms may underlie acidification, including lactic acid efflux, Na^+/H^+ exchange, $Na^+/HCO_3^-/Cl^-$-coupled transport, or electrogenic Na^+/HCO_3^- cotransport (Chesler, 1990). In hippocampal slices, the late stimulus-elicited acidification is caused by accumulation of interstitial CO_2 (Voipio and Kaila, 1993). Since the capacity of the tissue to acidify the extracellular space increased over the first postnatal weeks in correlation with the proliferation of glia, it has been proposed that acid secretion by glial cells is also an important source for the extracellular acidification (Chesler and Kaila, 1992; Deitmer and Rose, 1996).

2.2. Influence of extracellular pH changes on brain function

Physiological alterations in pH_o can induce a variety of changes in brain functions, including alterations in the neuronal excitability, changes in the resting membrane potential, and induction or inhibition of ionic currents in neurons and glial cells. Since many enzymes are sensitive to small shifts in the concentration of protons (e.g. NADPH oxidase, Section 2.3.3.), most cellular processes are modulated by changes in pH_i. Changes in the steady-state pH_o have a strong influence on neuronal behavior. Thus, alkaline shifts lead to an increase in excitability, while acid shifts cause the opposite. Alkalinization, seen at the onset of neuronal activity, may transiently remove the proton block of NMDA receptors, enabling or enhancing spreading depression and neuronal injury (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Vyklicky et al., 1990). In contrast, acid pH has been regarded as neuroprotec-

tive due to proton inhibition of NMDA receptors. However, rapid acidification may cause the activation of proton-gated ion channels (Korkushko and Kryshtal, 1984; Grantyn and Lux, 1988; Bevan and Yeats, 1991) and would thus contribute to membrane depolarization, subsequent Ca^{2+} accumulation and neurodegeneration. Extracellular pH changes have also been shown to influence the bursting behavior of neurons (Church and McLennan, 1989; de Curtis et al., 1998), presumably due to an enhancement or inhibition of voltage-activated conductances (Tombaugh and Somjen, 1996). Dependence on extracellular pH has been reported for a wide variety of transmitter receptors and voltage-gated ion channels (for reviews see Green and Andersen, 1991; Chesler and Kaila, 1992). Proton-induced inhibition of voltage-gated ion channels may occur due to direct binding of protons to specific sites on the channel. Protons can induce surface charge screening effects (Hille, 1968), decrease the permeability of ion channels, and modulate their kinetics (Hille, 1992).

2.3. Influence of extracellular and intracellular pH changes on microglial function

2.3.1. Effects of pH on microglial function

Whereas intensive studies have been performed in order to investigate the influence of alkaline or acid shifts in the extracellular and intracellular pH on neuronal activity, only little is known about their influence on microglial activity. It has been demonstrated that changes in pH_o influence the organization of the microglial cytoskeleton and modulate microglial motility (Faff and Nolte, 2000). In an acidic environment, basal motility and C5a-induced chemotaxis of microglia were decreased. These effects were paralleled by rearrangement of the actin cytoskeleton. Since similar observations have been made upon decreases in pH_i without affecting pH_o, it had been proposed that changes in pH_i were responsible for the observed cytoskeletal reorganization and inhibition of microglial motility during extracellular acidification (Faff and Nolte, 2000). As in many other cell types, in microglia pH_i follows changes in pH_o. An alkaline shift of pH_i was found to result in elevation of $[Ca^{2+}]_i$ (Minelli et al., 2000).

2.3.2. Effects of pH on ion channels in microglia

An increase in the concentration of intracellular protons causes inhibition of microglial inward rectifier K^+ channels (Eder et al., 1995a). In recordings using intracellular solutions with pH of less than 7.0, inward rectifier K^+ currents decreased rapidly and finally disappeared within a few minutes after establishing the whole-cell configuration. In contrast, changes in pH_o did not influence inward rectifier currents. In microglia, inward rectifier K^+ channels are believed to help set the resting membrane potential (Fischer et al., 1995; Visentin et al., 1995; Eder, 1998), similar to their role in other macrophages (Gallin and Sheehy, 1985; McKinney and Gallin, 1990; Gallin, 1991; DeCoursey and Grinstein, 1999). It is reasonable to expect that intracellular acidification would lead to membrane depolarization of microglial cells due to the proton-induced inhibition of inward rectifier channels.

Delayed rectifier outward K^+ channels in microglia can be modulated by both intracellular and extracellular protons. Intracellular acidification reduced the amplitudes of delayed rectifier currents, while intracellular alkalinization enhanced whole-cell delayed rectifier currents. Neither kinetics nor voltage sensitivity of delayed rectifier currents were affected by changes in pH_i from 5.8 to 7.8 (Eder and Heinemann, 1996). In contrast, a shift of the steady-state activation and inactivation curves of the delayed rectifier currents in depolarizing direction was observed during acidification of the external milieu, while extracellular alkalinization showed the opposite effect (Eder and Heinemann, 1996). In addition to this surface charge screening effect, time-dependent inactivation of microglial delayed rectifier currents was slower when superfusing cells with acid solutions than with alkaline ones (Eder and Heinemann, 1996), presumably due to specific interaction of protons with the channel (Deutsch and Lee, 1989). Modulation of delayed rectifier channels by intra- and extracellular pH changes may have significant functional implications, since delayed rectifier channels in microglia and other immune cells play an important role in the maintenance of a large driving force for Ca^{2+} influx through Ca^{2+}-release-activated Ca^{2+} (CRAC) channels (Lewis and Cahalan, 1995). Functional CRAC channels seem to be required for several macrophage activities (DeCoursey and Grinstein, 1999). Because untreated microglia do not express delayed rectifier K^+ channels (Eder, 1998), it is unlikely that they play a role in maintaining the membrane potential in these cells. In activated microglia that express both delayed rectifier and inward rectifier K^+ channels, delayed rectifier channels might serve this function when there is sufficient depolarizing force to overcome the capacity of inward rectifier channels to keep the membrane potential near E_K. The depolarizing shift of delayed rectifier channel gating produced by low pH_o has been reported to depolarize the membrane potential of microglia activated by LPS (Chung et al., 1998).

2.3.3. Effects of pH on NADPH oxidase function

NADPH oxidase is of central importance in the killing of bacteria and other invaders by phagocytes. This enzyme functions best at pH 7.0–7.5 (Rossi, 1986; Clark, 1990). Superoxide anion release is strongly and progressively inhibited by interventions that lower pH_i below 7.5 (Simchowitz, 1985; Rotstein et al., 1987),

with almost complete inhibition at pH_i 6.0 (Simchowitz, 1985). The oxidase is also inhibited by decreases in pH_o (Gabig et al., 1979; Liberek et al., 1993). Because pH_i tends to follow pH_o the effects of an acidic environment may be mediated by the consequent decrease in pH_i (Simchowitz, 1985; Liberek et al., 1993). One of the main functions proposed for voltage-gated proton channels in phagocytes is to ensure pH homeostasis during the respiratory burst, which represents a severe challenge to the pH regulatory mechanisms of the cell.

3. Regulation of the intracellular pH of microglia

In the present paper, voltage-gated H^+ channels of microglia are described in detail. In addition to H^+ channels, several other H^+ transport systems that have been detected in microglia might be involved in pH_i regulation of the cells under physiological and pathophysiological conditions. Faff and colleagues (1996) reported that cultured microglial cells can recover from acidification due to the activity of an amiloride- and EIPA-sensitive Na^+/H^+ exchanger under HCO_3^- free conditions. In peripheral macrophages, Na^+/H^+ exchange in addition to a plasmalemmal vacuolar-type H^+-ATPase (V-ATPase) participate in pH_i recovery from intracellular acid loads (Bidani et al., 1994; McKinney and Moran, 1995). Whether V-ATPase activity is important for pH regulation and determination of basal pH_i in microglia as reported for other macrophages needs to be experimentally demonstrated.

A H^+/K^+-ATPase that is activated at increased concentrations of extracellular K^+ and H^+ has also been detected in microglia (Shirihai et al., 1998). Since increases in the concentration of extracellular K^+ and decreases in extracellular pH are hallmarks of injured brain tissue, this transporter may play an important role in pH_i regulation in microglia during pathological conditions.

In the presence of HCO_3^- a more alkaline resting pH_i than under HCO_3^- free conditions was determined in cultured mouse microglia. Two mechanisms, namely Na^+/HCO_3^- cotransport and Na^+-dependent Cl^-/HCO_3^- exchange seem to be responsible for the observed HCO_3^- induced intracellular alkalinization (Faff et al., 1996).

Increasing pH_i in microglia leads to an increase in $[Ca^{2+}]_i$ (Minelli et al., 2000). Intriguingly, this response is inhibited by removal of extracellular Ca^{2+} if pH_i is increased by raising pH_o but not by an NH_4^+ prepulse (Minelli et al., 2000). These data provide indirect evidence that a Ca^{2+}/H^+ exchanger may exist in microglia.

So far all H^+ transporters have been studied in cultured microglial cells. Further experiments are required to demonstrate the existence of these transporters in microglia of the normal and pathological brain as well as the contribution of each of those transport systems in microglial pH_i regulation under various conditions.

4. H^+ channels in microglia

4.1. Cells/species expressing H^+ channels

Voltage-gated proton channels were first described by Thomas and Meech in snail neurons almost two decades ago (Thomas and Meech, 1982). These studies were elegant (Fig. 1): pH_o and pH_i were monitored using pH electrodes, and membrane current was measured under voltage clamp during acid injection and other manipulations (Thomas and Meech, 1982; Meech and Thomas, 1987; Thomas, 1988). Shortly afterwards, voltage gated proton channels were observed in *Ambystoma* (axolotl or salamander) oocytes (Barish and Baud, 1984). The possibility that voltage gated proton channels might exist in mammalian cells was proposed by Henderson and colleagues (Henderson et al., 1987) on the basis of pH measurements in human neutrophils. The first direct voltage-clamp evidence for voltage gated proton channels in mammalian cells was in rat alveolar epithelial cells (DeCoursey, 1991). Henderson's prediction that voltage gated proton channels exist in human neutrophils was confirmed by patch-clamp in 1993 (DeCoursey and Cherny, 1993). Proton currents were first described in microglia in 1995 (Eder et al., 1995a). H^+ currents have now been observed in cultured murine (Eder et al., 1995a; Klee et al., 1998, 1999), rat (Visentin et al., 1995), and human (McLarnon et al., 1997) microglia, as well as in two microglia-derived cell lines, BV-2 and MLS-9.

The cells in which voltage gated proton channels have been reported are listed in Table 1, and include a dozen species, and two dozen cells and cell lines. Several new cells that express these channels are reported each year, so the table is growing. In surveying cells as potential expression systems for voltage gated proton channels, it has been difficult to find cells that definitely do not express this conductance. Cell lines such as CHO or HEK that are commonly used as expression systems for ion channels, have been found to express native voltage gated proton channels, although at low density (Cherny et al., 1997; Table 1). Amphibian oocytes, another popular expression system, also have robust native H^+ currents (Barish and Baud, 1984; Humez et al., 1995). The level of expression spans three orders of magnitude in different cell types. The greatest expression level in a native cell is in human eosinophils, where the H^+ current density can be >200 pA/pF (Gordienko et al., 1996; Schrenzel et al., 1996). Currents of this magnitude are so large that even with

highly buffered pipette solutions (100 mM buffer), the H^+ efflux during large depolarizations depletes intracellular buffer and raises pH_i, producing a characteristic 'droop' of the current (see also Section 4.3.4).

4.2. How are H^+ channels studied?

Two main approaches have been used to study voltage gated proton channels: electrical recording using voltage-clamp technique and measurement of pH changes using pH electrodes or pH sensitive fluorescent dyes. Both approaches are important and each has advantages and limitations that must be recognized.

4.2.1. Voltage-clamp

For the study of small cells like microglia, the patch-clamp technique (Hamill et al., 1981) is invaluable. Conventional microelectrode puncture experiments are not feasible, because the leak inevitably caused by electrode penetration of the cell membrane conducts large non-specific currents that obscure the physiological membrane conductances. The essential feature of the patch-clamp technique is the formation of an electrically tight seal between the patch pipette and the cell membrane. A good seal does not allow even small ions to pass between the pipette and the membrane, and allows recording the current flowing through a single ion channel in the patch of membrane spanning the pipette tip. The whole-cell configuration allows recording from all the channels in the entire cell membrane. There are several limitations, however. In the whole-cell configuration, the cytoplasm is dialyzed as a result of its continuity with the pipette solution. This is at once an advantage and a limitation. The advantage of being able to control the intracellular solution by diffusion from the pipette is offset by the loss of cytoplasmic constituents, which may interrupt second messenger pathways and alter the behavior of ion channels. This particular limitation can be overcome by use of the permeabilized-patch configuration. Including pore-forming molecules, such as ATP (Lindau and Fernandez, 1986), nystatin (Horn and Marty, 1988), or amphotericin B (Rae et al., 1991) in the pipette solution results in eventual incorporation of these molecules into the patch membrane, where they allow passage of ionic current (carried by small ions) but prevent the loss of larger cytoplasmic molecules such as proteins and enzymes. The value of this approach is demonstrated by the fact that the response of voltage gated proton channels in neutrophils to PMA is lost in conventional

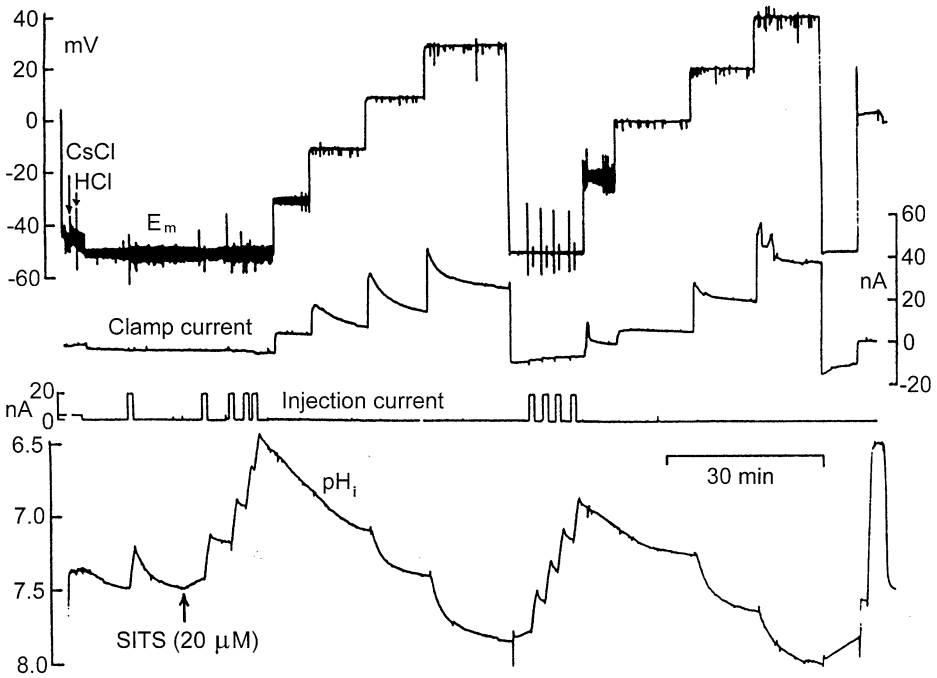

Fig. 1. Simultaneous records of membrane potential (E_m), voltage-clamp current, HCl injection current, and pH_i (from top to bottom) that enabled the discovery of voltage-gated proton channels in snail neurons (*Helix aspersa*) by Thomas and Meech (1982). Several HCl injections were made to lower pH_i to <6.5, then the membrane was depolarized stepwise. At -10 mV and at more positive voltages a decaying outward current is observed. The recovery of pH_i (bottom trace) from the acid load clearly is faster at more positive voltages, consistent with the outward current being carried by H^+. The outward H^+ current decays because the driving force ($V-E_H$) decreases as pH_i increases (due to the continuous H^+ efflux). H^+ currents in small cells like microglia decay two orders of magnitude faster because of the much smaller cell volume than in snail neurons. The neuron was pretreated with CsCl to inhibit K^+ currents and SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) to inhibit other endogenous pH regulatory mechanisms. Taken from Fig. 3 of Thomas and Meech (1982).

Table 1
I_H density in cells reported to have H^+ channels[c]

Cell type	Species	$I_{H,max}$ (pA/pF) (pH_i)	Reference
Neuron	Helix aspersa	4.5 (~6.8)	Meech and Thomas, 1987
	Lynmaea stagnalis	14.6 (5.9)	Byerly and Suen, 1989
Oocyte	Ambystoma	8.4 (~7.2)	Barish and Baud, 1984
	Rana esculenta	~32	Humez et al., 1995
Epithelium			
Alveolar	Rat	27.3 (5.5)	DeCoursey, 1991
Lung A549	Human	~2 (5.5)	DeCoursey and Cherny, 1994b
Prostate PC-3	Human	~4 (5.5)	DeCoursey and Cherny, 1994b
HEK-293	Human	~1 (6.5)	[b]
Renal proximal tubule	Rana pipiens	<75 (6.5)	Gu and Sackin, 1995
CHO ovary	Hamster	1.6 (5.5)	Cherny et al., 1997
Skeletal muscle			
Myocyte	Human	~10 (5.5)	Bernheim et al., 1993
Lymphocyte			
Jurkat E6-1	Human	36 (6.0)	Schilling et al., 2000a
Macrophage			
Peritoneal	Mouse	~30 (6.0)	Kapus et al., 1993a
Monocyte-derived	Human	1.1 (6.0)	Holevinsky et al., 1994
Alveolar	Rat	~2 (7.5)	[b]
Osteoclasts	Rabbit	6.7 (6.0)	Nordström et al., 1995
Osteoclasts	Chicken	[a]? (7.3)	Weidema, 1995
THP-1 monocyte	Human	22 (5.5)	DeCoursey and Cherny, 1996a
Granulocyte			
Neutrophil	Human	17 (6)	DeCoursey and Cherny, 1993
Eosinophil	Human	~200 (6.0)	Gordienko et al., 1996
Basophil	Human	~100 (5.5)	Cherny et al., 1999
Mast cell	Mouse	9.6 (5.5)	Kuno et al., 1997
HL-60	Human	133 (5.5)	Demaurex et al., 1993
K-562	Human	~5 (6.0)	DeCoursey and Cherny, 1994b
Microglia			
Microglia	Mouse	42 (6.0)	Eder et al., 1995a
Microglia	Human	[a]? (7.3)	McLarnon et al., 1997
Microglia	Rat	[a]~66 (7.2)	Visentin et al., 1995
BV-2 microglia	Rat	~20 (5.5)	[b]
MLS-9 microglia	Rat	0.22 (5.5)	[b]

[a] Identity of the conductance not established with certainty.
[b] Unpublished data of V.V. Cherny and T.E. DeCoursey, and for MLS-9 cells P.S. Pennefather.
[c] This table includes only cells in which the existence of H^+ channels was established by direct voltage-clamp studies. Not included are cells in which the existence of H^+ channels was established by indirect measurements such as pH changes. $I_{H,max}$ is the largest H^+ current measured in a given cell (normalized to capacity, which reflects surface area), usually at ~150 mV positive to V_{rev}; $g_{H,max}$ values were converted to current at $V_{rev}+150$ mV. In studies where typical values or cell size was not specified, estimates were made from data in figures, etc. and are preceded by a tilde (~), as are values from surveys including a small number of cells. All values are at room temperature (20–25°C).

whole-cell recording, but is preserved by use of the permeabilized patch configuration (DeCoursey et al., 2000). In order to use the permeabilized-patch approach to study voltage-gated proton channels, it is necessary to take additional measures to control pH_i, because buffer molecules are too large to pass from the pipette to the cell through the nystatin or amphotericin B pores. The classical weak base gradient approach, in which pH_i is controlled by establishing a gradient of a weak base (or acid) by virtue of the much higher permeability of the neutral form of the molecule (Jacobs, 1920; McLaughlin and Dilger, 1980; Roos and Boron, 1981; Boron, 1983), has been used successfully for this purpose (Grinstein et al., 1994; DeCoursey et al., 2000).

4.2.2. pH measurements

The operation of voltage gated proton channels in intact cells can be deduced by carefully designed measurements of intracellular and extracellular pH. Thomas and Meech (1982) used pH sensitive electrodes in their pioneering study of voltage gated proton currents in snail neurons (Fig. 1). Combined with voltage-clamp and HCl injection, this elegant study

demonstrated clearly the existence of a conductive H^+ flux pathway. Many recent studies take advantage of the existence of fluorescent pH sensitive dyes to monitor pH_i. When combined with voltage-clamp, these studies can provide relatively unambiguous information relating H^+ currents to cell functions. The main drawback of the use of pH measurements in isolation to study H^+ fluxes is that it can be difficult to establish which transport molecule is involved in a particular response. The pH can change as a result of any of a number of different mechanisms that result in flux of acid-equivalents. Cells have a wide variety of such mechanisms, including Na^+/H^+-antiport, K^+/H^+ exchange, H^+-ATPases, HCO_3^- transporters such as HCO_3^-/Cl^- exchange and $NaHCO_3/HCl$ exchange (Roos and Boron, 1981), Cl^-/OH^- exchange (Sun et al., 1996), as well as H^+ channels. pH changes can also result from the flux of the neutral form of weak acids or bases across the membrane (McLaughlin and Dilger, 1980; Roos and Boron, 1981; Boron, 1983; Cherny et al., 1990). Some of these transport mechanisms require ATP, some are electroneutral, some require counterions, and most cells have a multiplicity of transporters. Unambiguous identification of the transport mechanism is thus difficult and requires careful elimination of all other possibilities.

A second limitation of the use of pH sensing fluorescent dyes is that the spatial resolution is limited. Living cells may have substantial variation in local pH. In large cells, intracellular pH gradients can be detected using pH sensing microelectrodes (Vanheel et al., 1988) or pH sensing dyes (Gonda et al., 1999; Stewart et al., 1999). For example, apical-to-basal pH_i differences as high as 0.84 Units were observed in the epithelial HT29 cell line (Maouyo et al., 2000) and stable 1 Unit gradients were seen in cardiac myocytes (Spitzer et al., 2000). However, local pH in the 'reaction layer' adjacent to the membrane cannot be resolved using this approach. The reaction layer, a small, operationally-defined space near the membrane during H^+ flux in which proton-buffer reactions are not at equilibrium, has a thickness comparable with the plasma membrane (< 10 nm under 'typical' conditions) (Delahay, 1954; Neher, 1986; Kasianowicz et al., 1987; Mathias et al., 1990; DeCoursey, 1991). Local pH near the membrane is likely to be quite different from the bulk pH whenever there is membrane transport of acid equivalents. Membrane transporters that are sensitive to pH, including proton channels, can sense only the local pH.

4.3. Properties of H^+ channels

The H^+ channels in microglial cells exhibit many properties in common with proton channels in other cells. Therefore, we describe the general properties that are characteristic of all H^+ channels, including their selectivity, pH dependence of gating, and pharmacological properties. For properties that differ among H^+ channels, such as gating kinetics, we compare the properties of microglial channels with those in other cells.

4.3.1. Selectivity

Selectivity refers to the extent to which a channel discriminates among permeating ions. This property is studied by measuring the reversal potential, V_{rev} (Fig. 2), and varying the ionic composition of the solutions on either side of the membrane. Traditionally, the relative permeability of a channel for one ion (Y) compared with another ion (X) of like charge is calculated using the Goldman-Hodgkin-Katz (Goldman, 1943; Hodgkin and Katz, 1949; Hille, 1992) voltage equation:

$$V_{rev} = \frac{RT}{zF} \ln \frac{[X]_o + P_{rel}[Y]_o}{[X]_i + P_{rel}[Y]_i} \quad (1)$$

where $[X]_o$ and $[X]_i$ indicate are the extracellular and intracellular concentrations of cation X, P_{rel} is the

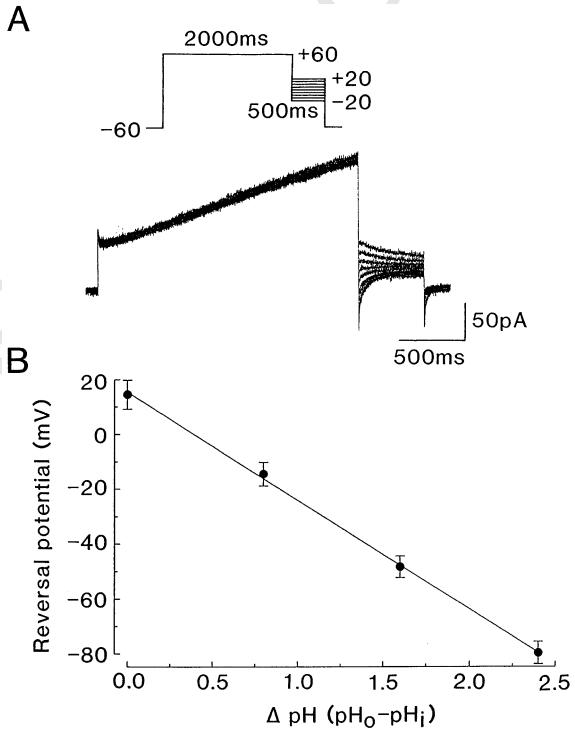

Fig. 2. (A) The determination of reversal potential, V_{rev}, is illustrated in a murine microglial cell. A depolarizing prepulse opens many channels, and then when the potential is repolarized to various voltages (diagram), 'tail currents' are observed, which decay as H^+ channels close. The initial tail current is outward or inward depending on the relation between the potential and V_{rev}; at V_{rev} there is no net current. (B) Mean values of V_{rev} at several ΔpH are plotted, along with a line which shows a slope of 40 mV/Unit. The Nernst potential for H^+, has a slope ~ 58 mV/Unit. The discrepancy between V_{rev} and E_H is likely due to imperfect control of the local pH near the membrane, and some deviation is seen in all studies of voltage gated proton channels. Taken from Fig. 6 of Eder et al. (1995a).

relative permeability defined as P_Y/P_X, z is the valence of the ion, and R, T, and F have their usual thermodynamic meanings. The Goldman–Hodgkin–Katz equation is based on assumptions that are not generally true, and for H^+ channels this equation produces nearly meaningless values of the absolute permeability of H^+ (DeCoursey & Cherny, 1997). The main problem is that the Goldman–Hodgkin–Katz equation assumes that each ion permeates independently of other ions, and hence predicts the conductance to be directly proportional to permeant ion concentration. As discussed below (Section 4.3.3), the H^+ conductance increases anomalously gradually as pH is lowered, and thus the *absolute* permeability does not scale according to H^+ concentration. Nevertheless, Eq. (1) still provides a useful and explicitly defined estimate of *relative* permeability. The reversal potential, V_{rev}, is measured in ion substitution experiments (Fig. 2), and Eq. (1) is used to calculate P_{rel}. By this method, the voltage-gated H^+ channel is found to be at least 10^6–10^8 more permeable to H^+ than to any other ion (Demaurex et al., 1993; Kapus et al., 1993a; DeCoursey and Cherny, 1994a,b; Cherny et al., 1995; Gordienko et al., 1996; DeCoursey and Cherny, 1997). In fact, because no change in V_{rev} is detected when either cations or anions in the bathing solution are replaced, there is no evidence that any other ion can permeate at all. Ordinary ion channels typically have relative permeabilities of 100:1, so $P_{rel} > 10^6$ is unheard of. This extreme selectivity is a strong argument that the permeation mechanism of H^+ channels differs drastically from the traditional water-filled pore concept that applies to most other ion channels (see Section 4.3.3).

4.3.2. Temperature dependence

Voltage gated proton channels are extremely sensitive to temperature, with a stronger temperature dependence than almost any other ion channel (Kuno et al., 1997; DeCoursey and Cherny, 1998). H^+ currents are larger and turn on faster at higher temperatures. The Q_{10} of the H^+ current amplitude is >2 (Byerly and Suen, 1989; DeCoursey and Cherny, 1998). The rates of H^+ channel opening and closing have Q_{10} 6–9 for various mammalian cells including microglia (DeCoursey and Cherny, 1998). For example, a 1°C increase in temperature during fever would enhance the activation of H^+ current by 34%.

4.3.3. Permeation mechanism

Most ion channels are water-filled pores that provide a low resistance hydrophilic pathway that enables ions to traverse the hydrophobic interior of the cell membrane. John Nagle and colleagues proposed a very different type of conduction pathway for protons. A continuous chain of molecules or chemical groups hydrogen-bonded together and spanning a cell membrane (a hydrogen-bonded-chain or HBC) could comprise an efficient pathway for protons to cross the membrane (Nagle and Morowitz, 1978; Nagle and Tristram-Nagle, 1983). Protons hop along a HBC much like the way they hop from one water to the next in bulk solution, by a Grotthuss mechanism (de Grotthuss, 1806). An intriguing aspect of HBC conduction is that it requires two distinct steps, the hopping of a proton across the chain, and then an obligatory structural rearrangement of the hydrogen bonds that reorients the chain to permit entry of a subsequent proton into the chain. In fact, the proton-hopping step carries only about two-thirds of the charge, with the HBC reorientation step moving the remaining one-third charge (Scheiner and Nagle, 1983). An important goal of future structure-function studies of voltage-gated proton channels will be to determine the amino acids and other elements (such as intercalated water molecules) that comprise the permeation pathway.

Several unique features of voltage-gated proton channels can be explained most easily if the pathway for protons is postulated to be a hydrogen-bonded-chain (HBC), rather than a water-filled pore. First, the H^+ channel is extremely selective. How could a simple hole in a membrane protein discriminate so perfectly between a hydronium ion (H_3O^+) and a K^+ ion, which have identical net charge and similar radii? On the other hand, if protons permeate via a HBC mechanism, this could account for the extremely high selectivity. Second, several properties of H^+ conduction through gramicidin channels, which are known to be water-filled pores (Myers and Haydon, 1972; Levitt et al., 1978), differ from the corresponding properties of voltage-gated H^+ channels. The deuterium isotope effect (DeCoursey and Cherny, 1997) and the temperature dependence of the conductance (Byerly and Suen, 1989; Kuno et al., 1997; DeCoursey and Cherny, 1998) are substantially greater for voltage-gated H^+ channels than for H^+ permeation through gramicidin (Akeson and Deamer, 1991). In light of the complex, two-step hop-turn mechanism required, HBC conduction mechanisms could easily be envisioned as having stronger temperature and isotope effects than a simple water-filled pore. Furthermore, the H^+ conductance is proportional to H^+ concentration over a wide range in gramicidin, but the voltage-gated H^+ conductance is only weakly pH dependent (Cherny et al., 1995; DeCoursey and Cherny, 1995; DeCoursey, 1998; DeCoursey and Cherny, 1999a,b). Finally, the rate at which protons can permeate the water-filled gramicidin channel is truly phenomenal, up to 2×10^9 H^+/s (Cukierman et al. 1997), and may be limited only by the rate at which H^+ can diffuse to the channel entry (DeCoursey and Cherny, 1999a). In contrast the estimated voltage gated proton channel current is several fA (femtoamperes, 10^{-15} Amperes), or $\sim 10^4$ H^+/s

Table 2
Varieties of H^+ Channels[e]

	Invertebrate	Amphibian	Mammalian cells		
Type	*n* (neuron)	*o* (oocyte)	*e* (epithelial)	*p* (phagocyte)	*x* (oxidase-related)
Gated by	V[a], ΔpH	V[a], ΔpH	V[a], ΔpH	V[a], ΔpH	V[a], pH_o, pH_i, PMA, NADPH oxidase activity?
τ_{act}[b]	Fast	Medium	Slow	slower	Slow
Sigmoid activation?	no	no	Pronounced	yes	?
τ_{tail}[c]	Fast	Medium	Medium	slow	Very slow
τ_{tail}[c] components	1	1	2	1	?
$[Ca^{2+}]_i$ enhances I_H?	no	?	Slight	yes/no	?
Cells expressing	snail neurons	frog and newt oocytes	Alveolar epithelium	microglia, neutrophils, Mϕ[d], HL-60, THP-1, eosinophils, CHO, mast cells, basophils	Eosinophils, neutrophils

[a] V, voltage (depolarization).
[b] τ_{act}, activation time constant (channel opening rate).
[c] τ_{tail}, tail current time constant (channel closing rate).
[d] Mφ, macrophage.
[e] Table adapted and extended from (DeCoursey, 1998). It is not yet clear whether types *p* and *x* are distinct molecules or different functional modes of the same channel.

(DeCoursey and Cherny, 1993), and seems very likely to be limited by the rate at which permeation occurs rather than by diffusion of H^+ to the channel mouth (DeCoursey and Cherny, 1994b, 1996b, 1997, 1998, 1999a,b; Cherny et al., 1995).

4.3.4. Gating kinetics

Gating kinetics refers to the rates at which ion channels open and close. Practically all ion channels have at least two fundamental conformations, called 'open' and 'closed'. Open channels conduct ionic current at a virtually constant rate; closed channels do not conduct detectable current. Channels jump back and forth between the open and closed states in a random, or stochastic, manner, and the time required for these gating transitions is too brief to have been resolved clearly. Thus the opening rate under specific conditions is not really the time it takes a closed channel to open, but rather a measure of the average time spent by each channel in the closed state before it randomly opens. Strictly speaking the *rate* is the inverse of this time interval. The closing rate is the inverse of the time each channel on average stays open before it closes. For an ensemble, or large population, of channels, macroscopic currents provide the opening and closing rates in the form of the time course with which the total current increases or decreases as many channels open or close. In voltage dependent ion channels, by definition, either the opening or closing rates or both are voltage dependent. A depolarization-activated channel such as the voltage gated proton channel opens upon depolarization because at positive membrane potentials the opening rate is greater than the closing rate.

Voltage gated proton currents turn on upon depolarization with either an exponential or sigmoid time course. In cases where the time course is sigmoid, the current waveform can be approximated by a delay followed by an exponential rise. The degree of sigmoidicity seems to vary in different cells (DeCoursey, 1998; Table 2), and thus in cells in which the time course appears to be exponential there may simply be a small delay in relation to the exponentially-rising phase of current. The sigmoidicity increases when the membrane is held at more negative voltages preceding a given test pulse -the 'Cole-Moore' effect (Cole and Moore, 1960) — indicating that there are at least two closed states with a voltage dependent transition between them (DeCoursey and Cherny, 1994b). The closing time-course is generally monoexponential in phagocytes (Kapus et al., 1994; DeCoursey and Cherny, 1996a), including microglia (Klee et al., 1999).

Many ion channels exhibit 'inactivation,' which means that the current is not sustained indefinitely during a prolonged depolarizing voltage pulse. Typically the current rises to a peak, and then decays as the channels enter a non-conducting state that differs from the normal closed state. Inactivated channels are refractory to a second stimulus, and must first recover from inactivation before they can reopen. As originally defined by Hodgkin and Huxley (1952), the inactivation process is slower than the activation process. Voltage gated proton channels do not inactivate. Under most experimental conditions, the H^+ current decays during prolonged depolarization (Thomas and Meech, 1982; Meech and Thomas, 1987; Thomas, 1988; DeCoursey,

1991; Kapus et al., 1993a; Demaurex et al., 1993; DeCoursey and Cherny, 1993, 1994b; Humez et al., 1995; Gordienko et al., 1996; Schrenzel et al., 1996). However, this decay is the result of depletion of intracellular protonated buffer and the consequent increase in pH_i resulting directly from the massive H^+ efflux. This has been demonstrated by direct impalement of neurons with pH sensitive electrodes (Thomas and Meech, 1982; Meech and Thomas, 1987), by measurements of shifts in V_{rev} (DeCoursey, 1991; DeCoursey and Cherny, 1994b; Humez et al., 1995; Gordienko et al., 1996), and by pH sensitive fluorescent dyes (Demaurex et al., 1993; Kapus et al., 1993a; Schrenzel et al., 1996).

4.3.5. pH dependence of gating

A key property that distinguishes voltage gated proton channels from other ion channels, and which also provides the basis for the functional role of these channels, is the exquisite regulation of their gating by pH. Voltage gated proton channels open preferentially at positive voltages. Unlike most other voltage-gated ion channels however, voltage gated proton channels do not have absolute voltage dependence. Instead, the position of the voltage-activation curve is highly sensitive to both pH_o and pH_i. In a systematic study, it was found that the voltage dependence was determined by the pH gradient, ΔpH (defined as $pH_o - pH_i$), rather than by pH_o or pH_i alone (Cherny et al., 1995). Thus, changing ΔpH by one pH Unit, whether accomplished by changing pH_o or pH_i or both, shifted the voltage-activation curve by 40 mV. Lowering pH_i or increasing pH_o shifts the curve toward more negative voltages, tending to open H^+ channels at any given voltage. Fig. 3 illustrates the negative shift of the voltage range in which H^+ current is activated in murine microglia as pH_o is increased progressively from pH_o 5.8 to 8.2. One important result of the ΔpH dependence of H^+ channel gating is that all H^+ channels except type *x* (see below) open only when there is an outward electrochemical proton gradient. The effects of pH on other types of ion channels are qualitatively similar to the effects of H^+ channels, but generally are much less pronounced. Lowering pH_o shifts the voltage dependence of gating in the positive direction and decreases the maximum conductance, but usually to a smaller extent than for H^+ channels (Hille, 1968; Drouin and The, 1969; Mozhayeva and Naumov, 1970; Woodhull, 1973). The effects of changes in pH_i on other channels are usually much smaller than are effects of changes in pH_o and in some cases there is no detectable shift in voltage-dependent gating (Wanke et al., 1979; Deutsch and Lee, 1989; DeCoursey, 1995; Eder and Heinemann, 1996). Because the main function of voltage-gated proton channels is to extrude metabolically-produced acid, their profound sensitivity of pH_i is of central importance.

Voltage gated proton channels can be opened by any combination of lowering pH_i, increasing pH_o, or depolarizing the membrane potential. The threshold voltage, $V_{threshold}$, defined as the minimum level of depolarization needed to begin to activate H^+ channels at a given ΔpH, can be predicted by a simple formula:

$$V_{threshold} = 20\ mV - 40\ \Delta pH. \quad (2)$$

The applicability of this relationship is shown in Fig. 4. Over a wide range of pH_o and pH_i, $V_{threshold}$ is invariably positive to V_{rev} — the dotted line shows equality of $V_{threshold}$ and V_{rev}. The slope of the lines through the data is significantly lower, 40 mV/Unit. This ΔpH sensitivity has led most investigators to conclude that the general function of voltage gated proton channels must be to extrude excess metabolic acid. It has been observed (DeCoursey and Cherny, 1994b) that acid extrusion via voltage gated proton channels is accomplished at no metabolic cost to the cell, other than the cost of synthesizing the channel molecules. In contrast, H^+-ATPases and antiporters such as the Na^+

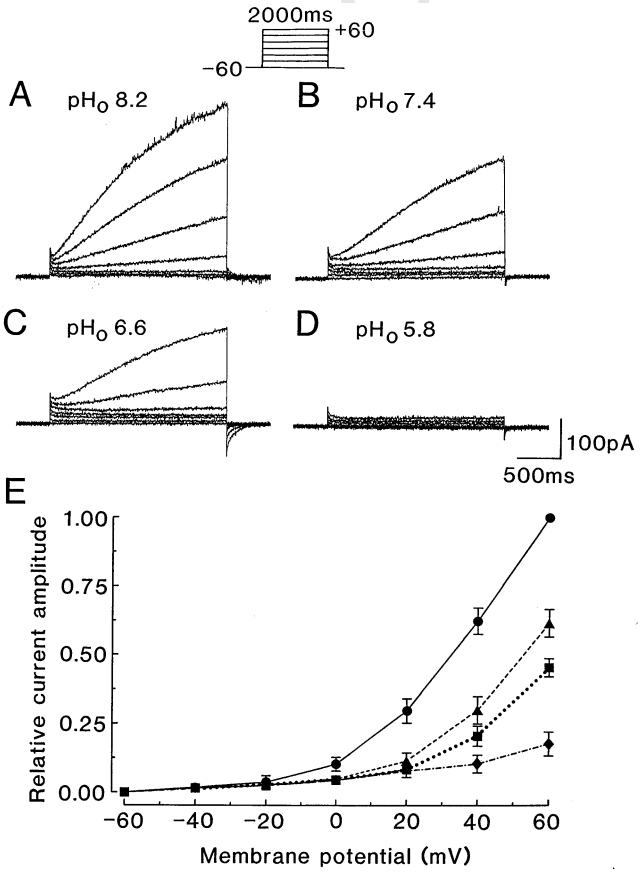

Fig. 3. Families of H^+ currents in the same microglial cell at several pH_o. Identical families of voltage pulses were applied in each solution (see inset). At higher pH_o, H^+ channels open at more negative voltages; conversely, low pH_o inhibits the current. In (E) the currents at the end of the pulses in the four solutions (A–D) are plotted: A, pH_o 8.2 (●); B, pH_o 7.4 (▲); C, pH_o 6.6 (■), D; pH_o 5.8 (◆). Taken from Fig. 5 of Eder et al. (1995a).

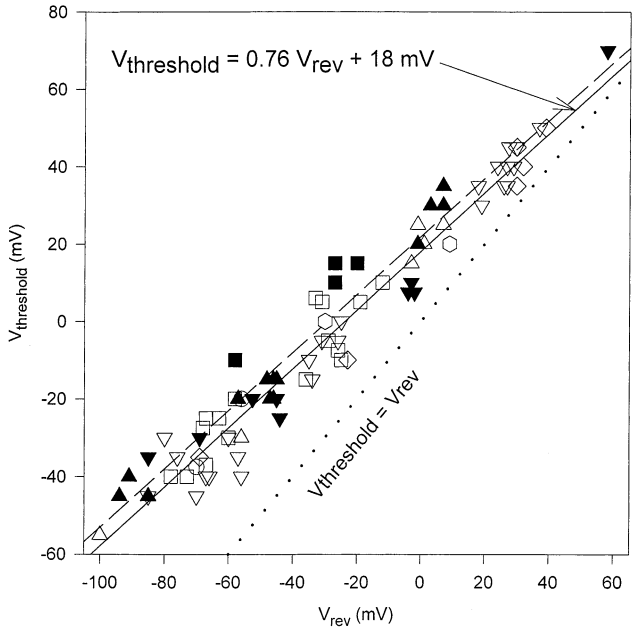

Fig. 4. The threshold voltage for activating H^+ currents, defined as the voltage at which clearly time-dependent outward current was detected, $V_{threshold}$, is plotted as a function of V_{rev} measured in the same cell and the same solution. The dotted line shows equality of the two parameters; all of the data fall above this line, indicating that $V_{threshold}$ is always positive to V_{rev}. Open symbols indicate measurements with H_2O in the bath, filled symbols with D_2O. Bath solutions included pH_o ranging 6.5–10.0 and pD_o 7.0–10.0. Pipette solutions (pH_i) are indicated by the shape of the symbol: pD 7.0 (Δ), pD 8.0 (∇), pD 9.0 (*hexagons*), pH 7.5 (◇), pH 5.5 (○), 50 mM NH_4^+ (□) The lines show the results of linear regression of the H_2O data (*solid line*), $r = 0.963$, slope = 0.760, y-intercept = 18.1 mV. The D_2O data (*dashed line*) were described by $r = 0.926$, slope = 0.750, intercept = 22.0 mV. Data in alveolar epithelial cells, from Fig. 11 of DeCoursey and Cherny, 1997.

/H^+-antiporter consume metabolic energy in the form of ATP either directly or indirectly by dissipating the Na^+ gradient. Furthermore, in comparison with other acid-regulating membrane transporters, when activated maximally, voltage gated proton channels extrude acid at ~100 times higher rate than any other transporter (DeCoursey and Cherny, 1994b).

How does the regulation of gating by ΔpH work? Cherny et al. (1995) proposed a simple general model to account for the ΔpH dependence of the voltage gated proton channels. The features of this model are illustrated by three cartoons in Fig. 5, each of which represents a physical mechanism that could operate to produce the observed ΔpH dependence. The channel is postulated to have protonation sites that are accessible to the external solution, protonation of which stabilizes the closed conformation of the channel. Deprotonation allows a conformational change that switches the accessibility of the protonation sites to the internal solution. Finally, protonation of the sites from the internal solution stabilizes the open conformation of the channel. Two key assumptions are required for the model to work. First, the protonation sites are accessible only to the external or internal solution at any given time, never both simultaneously. Second, the conformational change that switches the accessibility of the channel can occur only when the sites are deprotonated. This model reproduces the main features of the regulation of H^+ channel gating by pH_o and pH_i (Cherny et al., 1995). Identifying the molecular correlates of these regulatory protonation sites will be an important goal of future structure-function studies of H^+ channel molecules.

4.3.6. Pharmacology

Voltage gated proton channels are unusual in having no high affinity blockers. Most ion channels are sensitive to specific proteins found in toxins or venoms — in fact, the toxicity of many of these substances is a direct result of their effects on ion channels. Two main classes of inhibitors exist for voltage gated proton channels: weak organic bases and polyvalent cations. The inhibition by weak organic bases (4-aminopyridine, TEA^+, D600, verapamil, amiloride, rimantadine, amantadine) (Byerly et al., 1984; Meech and Thomas, 1987; Bernheim et al., 1993; DeCoursey and Cherny, 1994a,b) may reflect direct interaction of these molecules with the channel protein, such as occurs with other ion channels. However, because in most cases relatively high concentrations are required, it is conceivable that some or all of the inhibition may be the result of local pH changes near the membrane due to the flux of neutral form of these molecules. Influx of neutral form of a weak base will lower the pH at the outer surface of the membrane and raise pH near the inner face of the membrane. Both changes would reduce H^+ currents by decreasing the driving force and by shifting the voltage-activation curve in the positive direction. Further studies are needed to evaluate this hypothesis, which is discussed further elsewhere (DeCoursey and Cherny, 1994b).

Microglial H^+ currents are inhibited during extracellular application of 1 mM 4-aminopyridine or TEA^+ and by several inorganic polyvalent cations at micromolar concentrations with the following order of potency: $Zn^{2+} > La^{3+} > Ni^{2+} > Cd^{2+} > Co^{2+} > Ba^{2+}$ (Eder et al., 1995a). The effects of polyvalent cations on H^+ currents resemble qualitatively their effects on many other ion channels. As illustrated in Fig. 6, the rate of channel activation is slowed, and the voltage dependence of channel opening is shifted in the positive direction. At first glance it appears reasonable to describe these effects as voltage dependent block, because the fractional reduction of H^+ current differs at different voltages. However, several types of evidence make it clear that Zn^{2+} does not enter the channel and block at a site within the membrane electrical field. There is no effect on the shape of the instantaneous current-voltage relationship, block/unblock kinetics are incom-

patible with the observed effects of Zn^{2+}, and the voltage dependent effects all are consistent with the idea that Zn^{2+} binds to an external site where it alters the transmembrane voltage sensed by the voltage-sensor of the H^+ channel (Cherny and DeCoursey, 1999). Inhibition by polyvalent metal cations is extremely sensitive to pH_o: the apparent efficacy of $ZnCl_2$ was 10-fold lower at pH_o 6 than at pH_o 7, and 100-fold lower at pH_o 5 than at pH_o 6 (Cherny and DeCoursey, 1999). This pH_o dependence suggests that the external Zn^{2+} receptor on voltage-gated proton channels is comprised of multiple protonatable groups which together coordinate the Zn^{2+} atom. The active form appears to be the divalent cation: i.e. Zn^{2+} not $ZnOH^+$ (Cherny and DeCoursey, 1999).

In practical terms, any evaluation of the potency of metal cations for inhibiting H^+ current is complicated by the strong pH dependence. Furthermore, many biological buffers bind Zn^{2+} at least weakly (Table 1 of Cherny and DeCoursey, 1999). These factors must be taken into consideration in order to compare potency among studies done under different conditions. Studied under comparable conditions, Zn^{2+} inhibits H^+ currents more potently than it inhibits other channels such as Ca^{2+} currents (Mahaut-Smith, 1989) and delayed rectifier K^+ currents (Spires and Begenisich, 1994; Eder et al., 1995b; Cherny and DeCoursey, 1999).

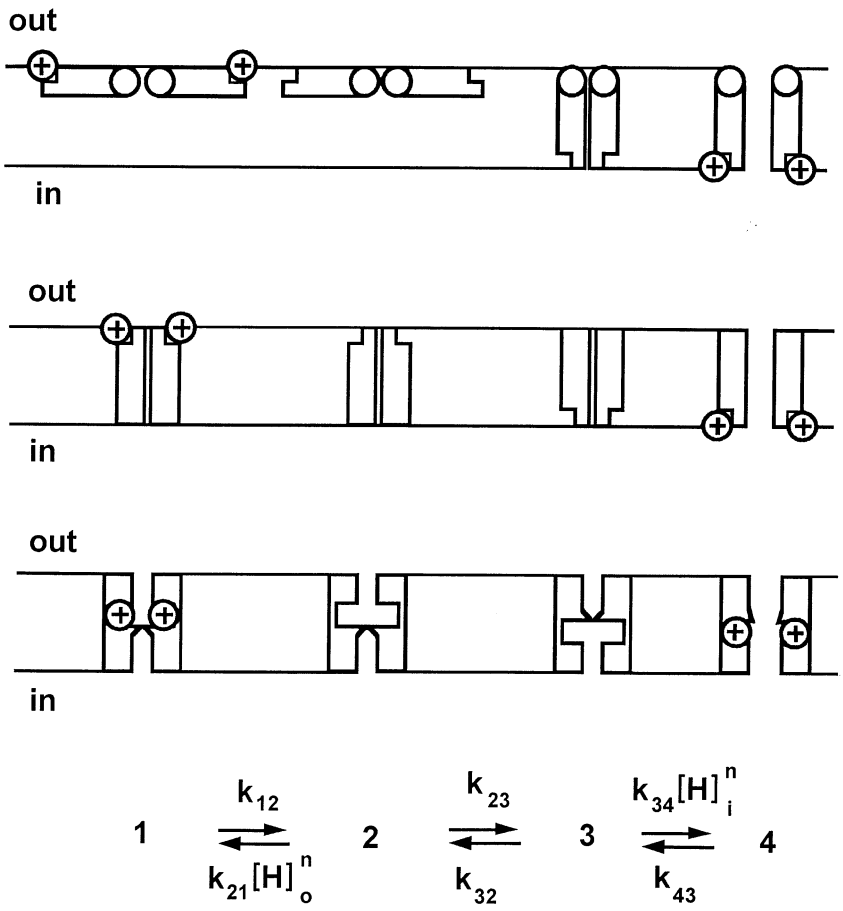

Fig. 5. Model of the regulation of H^+ channel gating by the pH gradient, ΔpH. The state diagram at the bottom defines the kinetic model; the three cartoons illustrate possible physical manifestations of this model. The closed channel conformation (state 1) is stabilized by protonation of an external site, and the open conformation (state 4) is stabilized by protonation of an internal site. These hypothetical allosteric regulatory protonation sites might be the same or distinct. Only the external or internal site is accessible to protonation at any given time, not both simultaneously. A conformational change (the transition between states 2 and 3) exposes the protonation site to the internal solution. The formation of a conducting H^+ channel requires a conformational change in each channel protomer which can occur only when the regulatory site is deprotonated. The open channel probability therefore increases at high pH_o or low pH_i. The model defined by the state diagram can be envisioned physically as (*top*) a 'butterfly' in which the protonation site on each channel protomer or 'wing' moves across the membrane, (*middle*) distinct external and internal sites which when protonated, allosterically prevent protonation at the opposite site, (*lower*) a protonation site in a proton well whose accessibility depends on a small conformational change, or other variants not illustrated. The voltage dependence of H^+ channel gating could arise either from voltage-dependent binding/unbinding of protons to the regulatory protonation site, or from a voltage dependent conformational change, or some combination of the two. We assigned all of the voltage-dependence to proton binding, so that the regulatory sites behave like 'proton wells'. The gating of voltage gated proton channels in rat alveolar epithelial cells was described by the following parameters: $d_{in} = d_{out} = 0.71$, $K_w = 10$, $m = 0$, $n = 1.5$, $k_{12} = 1000\ s^{-1}$, $k_{32} = 10^6\ s^{-1}$, $k_{43,fast} = 3\ s^{-1}$, $k_{43,slow} = 0.05\ s^{-1}$, $pK_{in} = pK_{out} = 8.5$. Taken from Fig. 10 in Cherny et al. (1995).

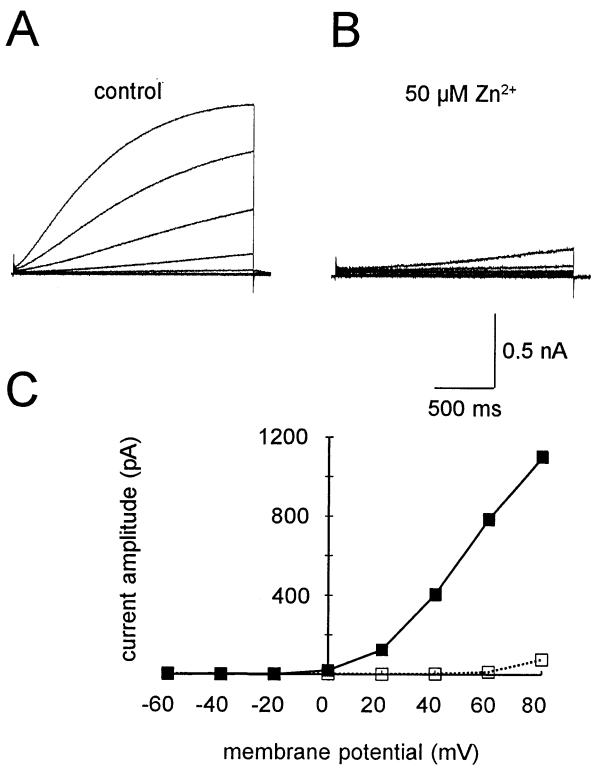

Fig. 6. Identical families of pulses were applied in the absence (A) or presence (B) of 50 μM $ZnCl_2$ in a microglial cell. Note the profound slowing of the turn-on of the H^+ current. The currents at the end of the pulses in A and B are plotted in C. Note that the $V_{threshold}$ is shifted by ~60 mV toward more positive voltages. Taken from Fig. 7 of Eder et al. (1995a).

The effects of $ZnCl_2$ and $CdCl_2$ are weaker and qualitatively different for internal than for external application. Intracellularly applied Zn^{2+} reduces the H^+ current weakly even at relatively high concentrations (≥ 0.17 mM free Zn^{2+}) and slows deactivation without effect on activation (Cherny and DeCoursey, 1999). Effects of both external and internal metals are qualitatively consistent with their binding to the hypothetical regulatory protonation sites whereby the voltage dependence of gating is established by the ΔpH.

4.4. Physiological modulation of H^+ channels

Physiological modulation mechanisms can be separated into two general categories: changes in the total number of active channels in the cell membrane and changes in the properties of the channels. Up- or down-regulation of the g_H presumably requires regulation of the synthesis or breakdown of the channel molecule and thus occurs on a time scale of hours or days. It could also occur on a more rapid time scale by insertion of channels into the membrane during exocytosis of secretory vesicles, or by removal of channels through endocytosis. Alteration of the properties of pre-existing channels typically would take place on a rapid time scale, and could occur directly or via second messenger pathways.

Changes in H^+ channel expression have been detected upon deactivation or activation of microglia. Direct modulation of H^+ channels has not studied extensively yet in microglia. Therefore, the influence of modulatory agents, such as arachidonic acid or PMA, on microglial H^+ currents can currently only be hypothesized by analogy with data that have been obtained in other cells.

Following common parlance, we describe modes of microglial behavior as untreated, resting, activated, or deactivated. These terms are useful, but unquestionably also represent an oversimplification and they may be misleading. For example, ramified (and presumably resting) microglia in culture are not by any means inert. They display high levels of pinocytotic activity, suggesting that microglia may normally function to cleanse brain fluids (Booth and Thomas, 1991). Deactivated microglia, nominally having reverted from an activated to a resting state (Merrill and Zimmerman, 1991; Loughlin et al., 1993; Suzumura et al., 1993), nevertheless differ from unstimulated resting microglia. For example, the patterns of K^+ channel expression are different in deactivated microglia compared with either unstimulated or activated cells (Schilling et al., 2000b). Activation can be defined in many ways, because a variety of disparate stimuli result in a large array of responses, ranging from morphological to functional. The capacity to respond differentially to different stimuli seems logical and appropriate in light of the many roles of microglia. For example, microglia clean up necrotic cellular debris, phagocytose invading bacteria and kill them by undergoing a respiratory burst, and release an armamentarium of cytokines such as chemotactic signals to peripheral macrophages. As in other phagocytes, the respiratory burst in microglia can be elicited by various agonists. In addition, many substances such as TNF-α, IFN-γ, or GM-CSF 'prime' macrophages, such that the response to a given agonist is greatly enhanced by pretreatment with priming agents (Klebanoff, 1999). One of the most widely used activators of microglia and other macrophages is bacterial LPS. LPS elicits little or no respiratory burst in neutrophils, but greatly enhances the release of superoxide anion induced by fMLP, PMA, or immune complexes (Guthrie et al., 1984; DeLeo et al., 1998). Other important chemokines such as TNF-α or IFN-γ also prime microglia, with TNF-α itself eliciting some superoxide anion release, but IFN-γ eliciting no response (Chao et al., 1995). Some authors refer to priming of macrophages as 'activation.' In summary, although we use general terms to describe microglial behavior patterns, we emphasize the arbitrariness of this nomenclature.

4.4.1. Regulation by astrocytic factors, lipopolysaccharide and cytoskeletal disruptive agents

Treatment of microglial cells with astrocyte-conditioned medium (ACM) leads to deactivation that is characterized by a variety of changes in the immunophenotypical, morphological and electrophysiological properties. The striking morphological transformation from amoeboid to ramified is illustrated in Fig. 7. ACM-induced deactivation of microglia also results in downregulation of surface antigens such as LFA-1, ICAM-1 and MHC class II molecules, and upregulation of delayed rectifier K^+ channels (Eder et al., 1999).

The expression of microglial proton channels is also regulated by factors released from astrocytes. Twenty-four hours after exposure to ACM, the microglial proton current density was about 50% smaller than that in untreated microglial cells. In addition, proton currents of ACM-treated microglial cells activated significantly more slowly than proton currents of untreated microglia (Klee et al., 1999). Intriguingly, proton currents changed in a similar way in microglia after exposure for 24 h to lipopolysaccharide (LPS) (Klee et al., 1999), which shifts microglia and other macrophages into an activated functional state (Hauschildt and Kleine, 1995). Moreover, after differentiation of THP-1 monocytes into macrophage-like cells (Auwerx, 1991), voltage gated proton currents were decreased by $\sim 50\%$ and activation during depolarization was twice slower (DeCoursey and Cherny, 1996a). Thus, several physiological mediators produce similar changes in the amplitude and properties of H^+ currents. It is not clear whether these rather subtle modifications of H^+ currents have functional consequences, or if they simply reflect a non-specific response to cytoarchitectural stress that is common to these cellular responses, as discussed next.

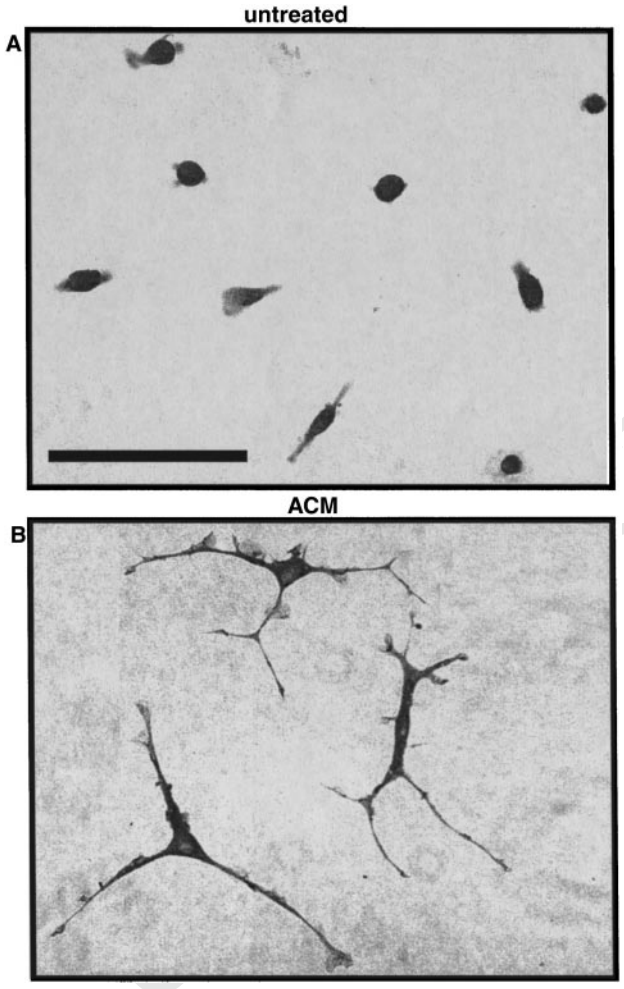

Fig. 7. The morphological changes during ACM-induced deactivation of microglia. Untreated cultured murine microglia are amoeboid cells with few processes (A). After treatment with ACM (B) the microglia become markedly ramified. Adapted from Fig. 1 of Eder et al. (1999).

It appears that the expression and properties of voltage gated proton channels can be regulated by cytoskeletal interactions. Cytoskeletal reorganization may be responsible for the alterations in H^+ currents observed after treatment of microglia with ACM or LPS. As illustrated in Fig. 8, exposure of microglial cells to several cytoskeletal disruptive agents produced the same kinds of changes in H^+ currents as just discussed for ACM or LPS treatment, namely smaller H^+ current density and slower time-dependent activation (Klee et al., 1998, 1999). These effects were observed after 24 h treatment of murine microglia with the cytoskeletal disruptive agents cytochalasin D or colchicine. In contrast, stabilization of the cytoskeleton by phalloidin or taxol did not have any significant effect on microglial H^+ currents. Since acute changes in microglial H^+ currents were not observed during short-term treatment with cytoskeletal disruptive agents (Klee et al., 1998), the incorporation of new channels in the membrane presumably was inhibited due to disruption of the cytoskeleton without direct modulation of the channels. The slowing of H^+ current activation is evidently an indirect response to metabolic changes occurring after treatment of the cells.

It has been reported that cytochalasin D inhibits neutrophil spreading and the concomitant activation of NADPH oxidase (Demaurex et al., 1996). These observations together provide intriguing hints of a relationship between NADPH oxidase activity and the voltage gated proton channel in phagocytes (see also below).

4.4.2. Modulation by arachidonic acid

During the respiratory burst, phagocytes, including microglia, release arachidonic acid, which itself is a bioactive fatty acid that enhances phagocyte responses. In addition, neurons and astrocytes are capable of releasing arachidonic acid. Arachidonic acid is released by neuronal and glial cells following binding of neurotransmitters to receptors, e.g. glutamate, acetylcholine,

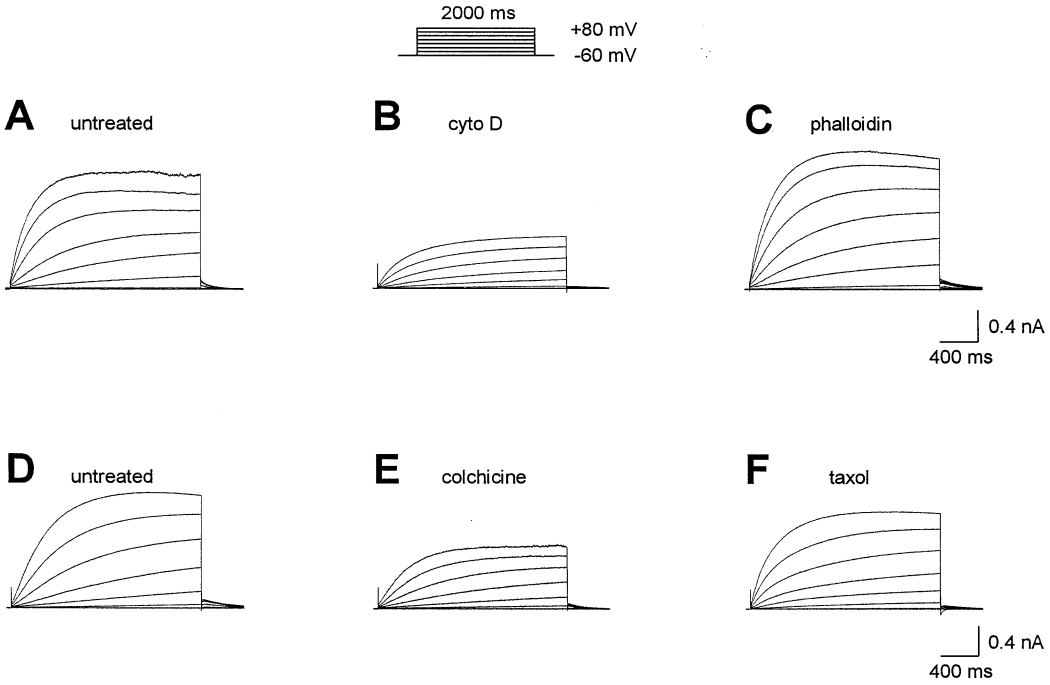

Fig. 8. Families of H^+ currents in microglial cells before (A, D) or after treatment for 1 day with 2 μM cytochalasin D (B), 20 μM phalloidin (C), 1 μM colchicine (E), or 0.5 μM taxol (F). All families recorded during the same pulse sequence (inset). Taken from Klee et al. (1998).

serotonin, adrenaline, or ATP. Several pathological conditions, such as ischemia, hypoglycemia, epilepsy, or hypoxia are associated with elevated concentrations of free arachidonic acid (reviewed by Katsuki and Okuda, 1995). Henderson and colleagues have shown that arachidonic acid is a strong stimulus for both the respiratory burst-associated H^+ conductance (Henderson and Chappell, 1992) and for NADPH oxidase, concluding that it was necessary to trigger this response (Henderson et al., 1993). Subsequent studies have supported this role, suggesting that PKC activates the g_H indirectly, by stimulating phospholipase A_2, which leads to release of arachidonic acid (Kapus et al., 1993b; Suszták et al., 1997; Dana et al., 1998; Lowenthal and Levy, 1999). As illustrated in Fig. 9, voltage-clamp studies show that arachidonic acid directly enhances the g_H in phagocytes (DeCoursey and Cherny, 1993; Kapus et al., 1994; Gordienko et al., 1996; Schrenzel et al., 1996; Suszták et al., 1997; Henderson and Meech, 1999). Two mechanisms are involved (DeCoursey and Cherny, 1993; Kapus et al., 1994; Gordienko et al., 1996; Henderson and Meech, 1999). First, the maximum H^+ conductance is increased. Secondly, the voltage-activation curve is shifted in the negative direction. The result of this shift is that more H^+ channels will be open at any given membrane potential.

4.4.3. Phosphorylation

There is indirect evidence, based on H^+ fluxes deduced from pH measurements, that phosphorylation up-regulates H^+ channels. Certainly phosphorylation of the cytosolic components of NADPH oxidase is a key trigger for assembly of the functional oxidize for the respiratory burst (Babior, 1999). Although the classic potent agonist for the respiratory burst is PMA (phorbol myristate acetate), the role of PKC (protein kinase C) in activating the g_H is ambiguous. There is some evidence that tyrosine kinase but not PKC is involved in activating the g_H (Nanda and Grinstein, 1995). As just discussed, phosphorylation of phospholipase A_2 may result indirectly in activation of the g_H

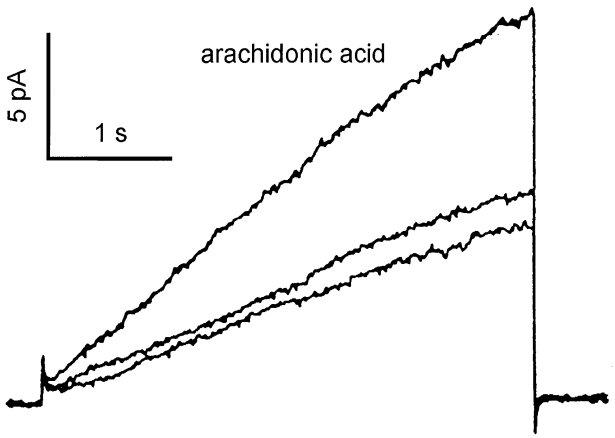

Fig. 9. Whole-cell H^+ currents at +100 mV in a human neutrophil before, during and after exposure to 50 μM arachidonic acid. The current was reversibly enhanced by arachidonic acid. The pH was 7.0//6.0. Adapted from DeCoursey and Cherny (1993).

(Kapus et al., 1993b; Suszták et al., 1997; Dana et al., 1998; Lowenthal and Levy, 1999). However, the enhancement of H^+ currents by arachidonic acid in whole-cell studies (DeCoursey and Cherny, 1993; Kapus et al., 1994; Gordienko et al., 1996; Henderson and Meech, 1999) is generally less pronounced, and differs qualitatively from the enhancement seen by PMA in permeabilized patch experiments (DeCoursey et al., 2000). Whether this difference reflects the involvement of different regulatory pathways or simply different experimental conditions remains to be determined.

In spite of the importance of phosphorylation in the signaling events that surround the respiratory burst, there is little direct evidence that H^+ channels themselves are modified by phosphorylation. Including ATP in the pipette solution enhanced the $g_{H,max}$ of mast cells studied in whole-cell configuration by two-fold, although H^+ currents were detected with ATP-free pipette solutions (Kuno et al., 1997). However, the enhancement by ATP is not due to phosphorylation, because in excised inside-out patches (from alveolar epithelial cells) H^+ currents were enhanced similarly by ATP, ADP, or simply phosphate (unpublished data of V.V. Cherny and T.E. DeCoursey). On the other hand, H^+ currents in phagocytes are unaffected by PMA when studied in whole-cell configuration, but respond dramatically when studied in permeabilized patch configuration (DeCoursey et al., 2000). This result indicates that a diffusible intracellular second messenger is involved in activating the g_H during the respiratory burst. The precise signaling pathways are currently under investigation.

4.5. Physiological functions of H^+ channels

4.5.1. General principles

The efflux of H^+ through open H^+ channels has two obvious effects: it changes local pH and removes positive charge from the cell. Extrusion of protons from the cell increases pH_i and decreases pH_o. The removal of positive charge hyperpolarizes the membrane potential. In the case of NADPH oxidase, H^+ efflux compensates for the positive charge introduced into the cell by this electrogenic enzyme (Henderson et al., 1987), thus preventing changes in membrane potential. Because the gating of H^+ channels is tightly regulated by a combination of voltage, pH_o and pH_i it is important to emphasize that the channel can detect only the voltage across the membrane in which it is located, and can detect and respond to only the local pH.

4.5.2. Specific functions

Direct evidence exists for activation of the g_H in four specific physiological situations: during the respiratory burst of phagocytes (Section 4.5.3, Fig. 10), in recovery from an acute acid load in neutrophils (Nanda et al., 1992; Kapus et al., 1993b), osteoclasts (Nordström et al., 1995) and mast cells (Kuno et al., 1997), during spreading of human neutrophils (Demaurex et al., 1996), and in facilitating $InsP_3$-dependent Ca^{2+} oscillations in frog oocytes (Humez et al., 1996). It has also been proposed that voltage gated proton channels may participate in the intracellular alkalinization after fertilization of oocytes (Barish and Baud, 1984; Baud and Barish, 1984), in acting as the oxygen sensor to mediate hypoxic pulmonary vasoconstriction (Jones and Morice, 1999), and in alveolar epithelial cells, in CO_2 extrusion by the lung (DeCoursey, 2000). H^+ currents in snail neurons (Byerly et al., 1984), and possibly in human skeletal myotubes (Bernheim et al., 1993), activate rapidly enough to extrude significant quantities of protons during an action potential, and specifically may extrude protons that entered the cell via Ca^{2+}/H^+ exchange triggered by Ca^{2+} influx during action potentials (Schwiening et al., 1993).

Experimental data demonstrating a role for H^+ channels in microglia are not yet available. Intensive studies on the functional importance of H^+ channels have been performed in other phagocytes, including macrophages, eosinophils, and neutrophils. From data obtained in these cells it can be proposed that H^+ channels in microglia play an important role in the regulation of the intracellular pH and the membrane potential under physiological and several pathophysiological situations. The importance of functional H^+ channels during the respiratory burst of phagocytes has been well documented, although the details of this involvement appear complex and have not been worked out completely (Section 4.5.3). Moreover, H^+ currents may help maintain both the membrane potential and pH_i during changes in the extracellular pH that occur in response to neuronal activity as well as during ischemia, epilepsy, or other pathological situations (Section 2.1). It can also be assumed that in microglia strong membrane depolarization, for example induced by increases in $[K^+]_o$ during spreading depression or following ion fluxes through ATP receptor channels, leads to the activation of voltage-gated H^+ channels and a subsequent membrane repolarization. Outward H^+ currents may contribute to the repolarization of the cell membrane in situations that shifts the membrane potential above the H^+ current activation threshold.

4.5.3. Respiratory burst in microglia and other phagocytes

The best-known and best-established functional role for voltage gated proton channels is in phagocytes. Henderson and colleagues discovered that electrogenic H^+ extrusion occurred in human neutrophils during the 'respiratory burst,' the rapid consumption of oxygen that occurs during phagocytosis as the enzyme NADPH oxidase is activated and converts oxygen to

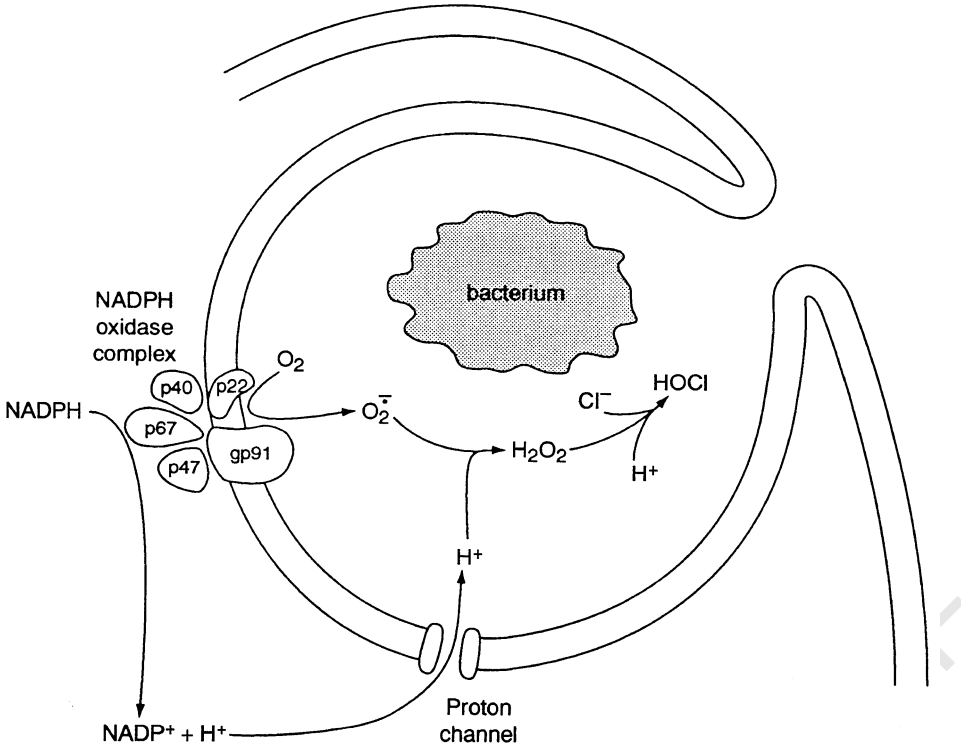

Fig. 10. Role of H^+ channels in the 'respiratory burst' of phagocytes. After engulfing a bacterium into the phagocytic vacuole, the normally quiescent enzyme NADPH oxidase assembles in the phagosome membrane from five major and several other components from the membrane and cytosol. Reactive oxygen species generated by this enzyme are essential to the killing process. Superoxide anion, O_2^-, dismutates spontaneously to form hydrogen peroxide, H_2O_2. Myeloperoxidase then combines H_2O_2 with Cl^- and H^+ to form hypochlorous acid, HOCl, the active ingredient in Chlorox™ bleach as well as being a major bactericidal compound in phagocytes. The stoichiometries of the reactions shown are simplified. During O_2^- production, protons are released into the cell. To prevent accumulation of H^+ in the cell, which would depolarize V_m and lower pH_i, H^+-selective ion channels open in the cell membrane allowing passive H^+ extrusion. The H^+ channel activated during the respiratory burst is shown here as a distinct molecule from the oxidase complex, but it has been proposed that it is contained within the $gp91^{phox}$ subunit (Henderson et al., 1995, 1997; Henderson and Meech, 1999). Taken from DeCoursey and Grinstein (1999).

produce superoxide anion (Henderson et al., 1987, 1988a,b). They proposed that this H^+ efflux might be mediated by a voltage gated proton channel analogous to that known at that time to exist in snail neurons. Fig. 10 illustrates the proposed role of voltage gated proton channels in phagocytes. NADPH oxidase is electrogenic (Henderson et al., 1987; Schrenzel et al., 1998), releasing one H^+ into the cell for each superoxide anion released into the phagosome (Borregaard et al., 1984; Test and Weiss, 1984). During the respiratory burst (NADPH oxidase activity) there is measurable cytoplasmic acid production which is exacerbated when voltage gated proton channels are inhibited by $CdCl_2$ or $ZnCl_2$ (Henderson et al., 1988b; Nanda et al., 1992; Kapus et al., 1992; Demaurex et al., 1996; Lowenthal and Levy, 1999). Thus voltage gated proton channels in the plasma membrane most likely serve to extrude acid during the respiratory burst. The importance of this role in phagocytes is demonstrated by the inhibition of superoxide anion release by H^+ channel block by Cd^{2+} or Zn^{2+} (Henderson et al., 1988b; Lowenthal and Levy, 1999).

It seems likely that voltage gated proton channels play a similar role in microglia. Microglia generate superoxide anion when stimulated with phorbol esters or opsonized zymosan (Colton and Gilbert, 1987). The release of superoxide anion is greatly enhanced by pretreatment (priming) with γ-interferon, tumor necrosis factor-α (Chao et al., 1995), or amyloid β protein (Van Muiswinkel et al., 1996). In fact, β-amyloid peptides were found to stimulate superoxide anion release from microglia directly and without priming, at a rate ~10% of that elicited by PMA (Bianca et al., 1999). This result supports the idea that β-amyloid protein, a hallmark component of the plaques seen in the brain in Alzheimer's disease, may cause damage indirectly by triggering microglia to produce excessive reactive oxygen species. Moreover, in response to brain injury, ischemia, or inflammation, activated microglial cells generate superoxide anion and subsequently other oxygen free radicals (reviewed in Colton and Gilbert, 1993; Love, 1999). The potential for stimulated microglia to generate oxygen radicals during the respiratory burst may also have implications in several degenerative neu-

rological diseases where activated microglia are found in association with the lesions (McGeer and McGeer, 1998; Schubert and Rudolphi, 1998; Kalaria, 1999).

This picture has been complicated by recent evidence for an additional variety or mode of operation of voltage gated proton channel in human eosinophils (Bánfi et al., 1999) and human neutrophils (DeCoursey et al., 2000). The novel type *x* H^+ channel (Table 2) conducts small inward H^+ currents under some conditions. One would expect a priori that inward H^+ currents would be detrimental to the well being of a cell. However, it is not certain that inward H^+ currents occur under physiological conditions, and furthermore, it is possible that inward H^+ currents may serve some function under specialized conditions. Bánfi et al. (1999) speculated that inward H^+ flux due to type *x* channel activation during the respiratory burst might contribute to pH regulation of secretory organelles, or might be a negative-feedback mechanism to turn off the respiratory burst in situations where pH_o was abnormally low.

An important question that must be answered to support a role for H^+ channels in dissipating the acid load during the respiratory burst is one of kinetic competence. Are there enough H^+ channels to extrude the acid as fast as it is produced during the respiratory

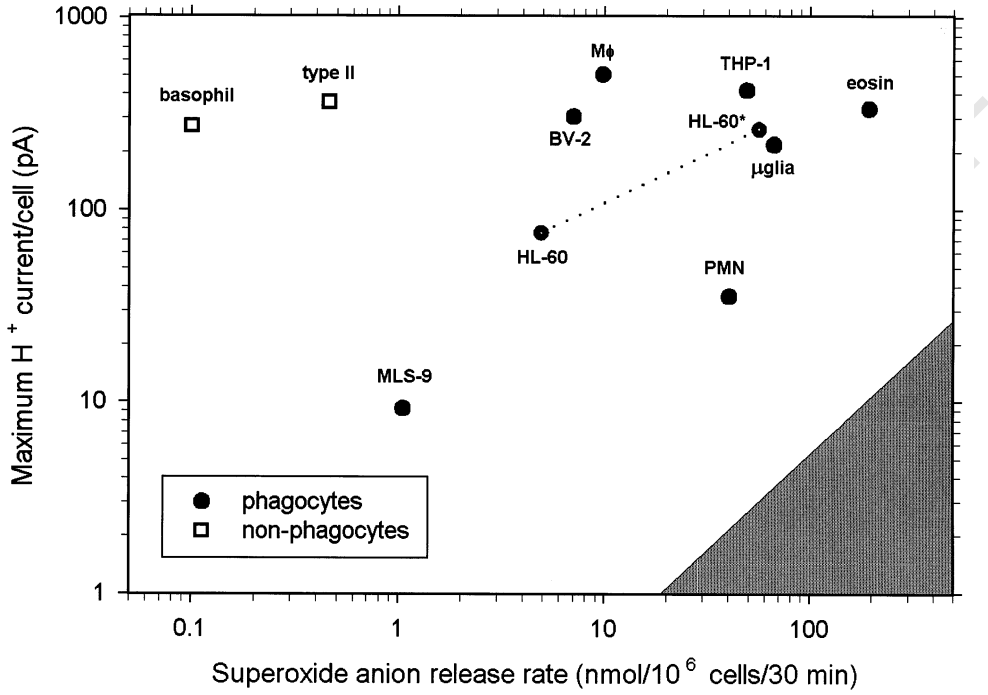

Fig. 11. Comparison of the density of voltage gated proton current and the ability of various phagocytic cells (●) and non-phagocytic cells (□) to produce superoxide anion in response to PMA (or LPS for THP-1 cells). The dashed line indicates the H^+ current required to extrude all of the acid at the rate it is produced during the respiratory burst; the shaded area shows H^+ current amplitudes that would be inadequate to serve this purpose. The intent here is to give a general impression of the relative magnitudes of these two cellular processes; these data were collected by many different groups, in a wide variety of conditions, and although we attempted to 'standardize' the results, they are not all directly comparable. The 'standard' conditions are nmoles $O_2^-/10^6$ cells collected over 30 min, and maximum I_H measured in the whole-cell configuration at pH_i 5.5 and pH_o 7.0. However, superoxide anion release was measured in different ways, and the attempt to standardize the results to a 30 min sample period ignores non-linearity in the temporal response. Similarly, some measurements of I_H were at different pH_i — in cases where multiple values were given, data at the lowest pH_i studied was used. Two values connected by a dotted line are given for HL-60 cells before and after being induced to differentiate by DMSO (Qu et al., 1994; personal communication from S. Grinstein). Other sources of data for this figure: I_H: (DeCoursey, 1991; Kapus et al., 1993a; DeCoursey and Cherny, 1993; Eder et al., 1995a; Schrenzel et al., 1996; Gordienko et al., 1996; Cherny et al., 1999; unpublished data of V.V. Cherny and T.E. DeCoursey; O_2^-: Petreccia et al., 1987; Swallow et al., 1990; Chao et al., 1995; Leibbrandt and Koropatnick, 1994; van Klaveren et al., 1997; unpublished measurements by T. Iastrebova, V.V. Cherny and T.E. DeCoursey). Cell types as indicated in the figure are: basophil = human basophil, type II = rat alveolar type II epithelial cell, MLS-9 = rat microglial cell line, BV-2 = rat microglial cell line, Mϕ = mouse macrophage, PMN = human neutrophil, THP-1 = human monocytic cell line, μglia = mouse microglia, eosin = human eosinophil. There is uncertainty about the rate of O_2^- release by non-phagocytes because the rates are very low and because of possible contamination by other types of cells (especially problematic is contamination by phagocytes). Several cells that express voltage-gated proton channels but do not produce superoxide anion (snail neurons, amphibian oocytes, kidney epithelial cells, mast cells) are not included on the graph. In contrast with Table 1, which expresses the density of H^+ channels in the membrane, the values plotted here are for the total current in the whole cell membrane, and thus reflect cell size as well as current density. For example, eosinophils are small cells with ten times denser H^+ channel expression than THP-1 cells, but because THP-1 cells have ten times larger membrane surface area, their whole-cell H^+ current is similar.

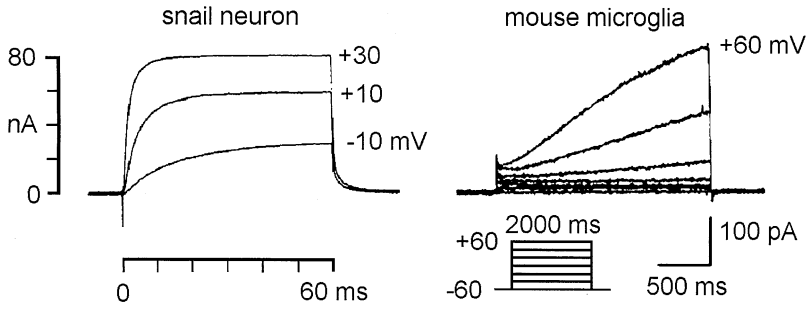

Fig. 12. Families of H^+ currents in a snail neuron (left panel) at pH_o 7.4 and pH_i 5.9 (Byerly et al., 1984) and in a murine microglial cell (right panel) at pH_o 7.4 and pH_i 5.8 (Fig. 5B of Eder et al., 1995a). Note the different time scales. The measurements were at similar pH and both were at room temperature (left, 20–25°C, right, 20–23°C). Modified from Byerly et al. (1984) and Eder et al. (1995a).

burst? Fig. 11 shows the relationship between the rate of superoxide anion release and the maximum H^+ current that can be elicited in a number of different types of cells. The dashed line shows the whole-cell H^+ current necessary to extrude all of the acid produced during superoxide release (assuming 1 H^+ is released into the cell for each O_2^- produced). In most cases, the maximum H^+ current is 10–100 times or more larger than that required, and thus only 1–10% of the maximum possible current need be activated to serve this function. In fact, the safety factor is likely more favorable than this, for two reasons. First, superoxide anion release was measured at 37°C, whereas the H^+ currents were measured at room temperature. H^+ currents are highly temperature sensitive (Byerly and Suen, 1989; Kuno et al., 1997; DeCoursey and Cherny, 1998), and I_H at body temperature would be at least 3–6 times larger than estimates at room temperature. Secondly, the H^+ currents in Fig. 11 were all recorded in the conventional whole-cell configuration. When studied using the permeabilized patch approach, the maximum H^+ current doubles during the respiratory burst, whether by activation of type *x* channels, or by up-regulation of the pre-existing type *p* channels (DeCoursey et al., 2000).

In keeping with the role of voltage gated proton channels in charge compensation during the respiratory burst, there seems to be a general if loose correlation between the ability of phagocytes (●, Fig. 11) to produce superoxide anion, and the density of voltage gated proton channels expression. Essentially all cells known to generate superoxide anion, that have been studied electrophysiologically, express voltage gated proton channels (Table 1), and as shown in Fig. 11, all cells have enough H^+ channels for this task. For non-phagocytes (□), the H^+ currents are far larger than needed to compensate for the tiny amounts of superoxide anion produced, and most likely this reflects that the H^+ channels in these cells serve a different function.

4.6. Taxonomy of H^+ channels

4.6.1. Varieties of H^+ channels

Although many properties of H^+ currents are similar in all cell preparations, at least five different types of voltage gated proton channels can be distinguished, based on behavior. The distinguishing properties are summarized in Table 2. One of the most obvious is the rate of channel opening during a depolarizing pulse. Fig. 12 illustrates families of voltage-clamp currents in snail neurons (left panel) where H^+ channels were first described (Thomas and Meech, 1982). The channels open rapidly, within a few milliseconds after depolarization. In contrast, the H^+ currents in murine microglia (right panel, Fig. 12) turn on more than 100 times more slowly. In general H^+ currents in mammalian cells activate slowly. However, H^+ currents have a very strong temperature dependence (see Section 4.3.2); thus the kinetics at body temperature would be 20–40 times faster than when measured at 20°C.

There are a number of distinct differences in gating kinetics among the varieties of H^+ channels. Some of these differences, such as the sigmoidicity of activation, or the number of components of tail current decay, may seem to be subtle and of little obvious functional consequence. However, they indicate that not all voltage gated proton channels are the same. The extent to which some of these differences reflect structural variations rather than effects of physiological modulation remains a problem for future study.

H^+ channels may differ with respect to their sensitivity to $[Ca^{2+}]_i$. The comparison of Ca^{2+} sensitivity has been studied by a variety of approaches, and definitive statements are difficult to make. Several reports indicate that voltage gated proton currents are enhanced by elevations of $[Ca^{2+}]_i$. The extent of this regulation ranges from a requirement for elevated $[Ca^{2+}]_i$ to activate H^+ channels in macrophages (Holevinsky et al., 1994) or type *x* channels in eosinophils (Bánfi et al., 1999), to relatively subtle enhancement (Schrenzel et al., 1996; Gordienko et al., 1996). Gordienko et al. (1996)

found no effect of varying $[Ca^{2+}]_i$ from 10 to 100 nM, but significant enhancement at 1 μM. In contrast, the voltage gated proton channel in neurons is insensitive to $[Ca^{2+}]_i$ up to 10 μM (Byerly et al., 1984). In cases where it has some effect, it is unclear whether $[Ca^{2+}]_i$ affects H^+ channels directly or acts indirectly via intermediaries, such as Ca^{2+}-dependent kinases. Studies need to be done using excised patches of membrane. Dependence of microglial proton currents on intracellular Ca^{2+} has not been investigated yet.

Based on the properties of the H^+ currents that are summarized in Table 2, it can be concluded that voltage-gated proton channels of microglial cells closely resemble type *p* channels, i.e. those found in other phagocytes including peripheral macrophages. The properties and expression levels of other types of ion channels are similar in microglia and other macrophages (Eder, 1998; DeCoursey and Grinstein, 1999).

In addition to type *p* channels that are present in unstimulated phagocytes studied in whole-cell configuration, type *x* channel behavior appears in human eosinophils and neutrophils under conditions where the NADPH oxidase is active (Bánfi et al., 1999; DeCoursey et al., 2000). It is possible that type *x* channels are distinct molecules (Bánfi et al., 1999). Alternatively, during the respiratory burst type *p* channels may become altered and convert to the type *x* phenotype (DeCoursey et al., 2000). Whether type *x* channel behavior also occurs in microglia needs to be investigated. The regulation of type *x* channel gating is unique. All other varieties are exquisitely sensitive to the pH gradient, ΔpH, with the result that they open only when there is an outward electrochemical gradient (Kapus et al., 1993a; DeCoursey and Cherny, 1994b; Cherny et al., 1995; DeCoursey and Cherny, 1997). The functional implication is that these channels are cleverly designed to extrude acid from cells, while preventing acid influx. In contrast, type *x* channels appear to be sensitive to pH_i only in the acid range, with little shift of the voltage-activation curve at $pH_i > 7.1$ (based on inspection of the data of Bánfi et al., 1999). As a result, at high pH_i the threshold voltage, $V_{threshold}$, is negative to E_H and these channels conduct inward currents in the voltage range between $V_{threshold}$ and E_H. Type *x* channels (or type *p* channels after conversion to type *x*) are also unique in being activated by PMA-type *e* H^+ channels in alveolar epithelium do not respond to PMA applied under identical conditions (DeCoursey et al., 2000).

4.6.2. Are they really channels?

The stock answer to the question 'what is an ion channel?' is 'a water-filled pore that spans the cell membrane and allows ions to cross the otherwise nearly impermeant membrane.' The ability of water molecules to permeate ion channels is not explicitly required, but the belief is so widespread that the channel's hydrophilic interior is key to the ability of charged ions to permeate, that 'water-filled' has become part of the de facto definition. We do not believe that voltage gated proton channels are water-filled pores (see Section 4.3.3), and thus the question arises whether they should be considered ion channels. Three properties distinguish ion channels from other membrane ion transporters. First, channels are completely passive entities that simply allow flux down the electrochemical gradient. They do not consume ATP, unlike pumps, nor do they couple transport of one ion to another, and thus are distinguishable from antiporters and symporters. Secondly, the ion channel molecule, unlike transport molecules such as carriers, does not undergo a significant conformation change during the permeation of each ion. It is inevitable that the presence of an ionic charge at any specific location inside a channel will drastically alter the local structure, but these electrostatic interactions do not extract much energetic cost, because the temperature dependence of permeation through ion channels is almost invariably close to that of ion diffusion in bulk solution. Finally, all ion channels are gated-they can open and close. A channel without a gating mechanism would simply be a pernicious hole in the membrane that would dissipate ionic gradients and kill the cell. Voltage gated proton channels meet the four main criteria that define ion channels: (1) they facilitate H^+ permeation across the membrane; (2) the H^+ flux is entirely passive; (3) they do not require ATP, nor do they couple H^+ flux with that of any other ion; and (4) they are gated. The final point has not been established directly, because the single channel current amplitude is smaller than can be measured by present techniques. However, when ion channels open and close stochastically, they generate electrical noise that can be analyzed to provide estimates of the unitary conductance (Hille, 1992). Distinct excess H^+ current noise (well above background noise levels) can be detected in human neutrophils (DeCoursey and Cherny, 1993). This is strong evidence that channels underlie H^+ currents. A carrier could produce similar noise if it turned on and off stochastically, but to our knowledge no such gating mechanism has been described for carriers. Voltage gated proton channels exhibit all of the fundamental characteristics of ion channels. They differ in the mechanism of permeation, and are at the extreme low end of the range of single-channel conductance (next section), but these are fine points that should not disqualify them from being members of this family.

4.6.3. Comparison of H^+ channels with other ion channels

Although as just discussed, we feel that voltage gated proton channels are most appropriately called 'ion channels,' they differ in five main respects from most other ion channels (DeCoursey and Cherny, 1999a).

4.6.3.1. The single-channel conductance is very small. Direct measurement of single-channel proton current has not been achieved, and may not be possible using existing approaches. Indirect estimates can be made using current fluctuation (noise) analysis. There is distinct excess H^+ current noise at voltages above the threshold for activating the g_H in human neutrophils (DeCoursey and Cherny, 1993). Calculated from noise, the single-channel H^+ current appears to be on the order of a few fA, roughly 3 orders of magnitude smaller than for typical ion channels.

4.6.3.2. The proton selectivity of the channels is almost perfect. Most ion channels exhibit moderate to high selectivity, allowing their favorite ions to pass $\sim$10–100 more readily than other ions. In contrast, voltage-gated H^+ channels appear to be perfectly selective. There is no detectable permeability to any other ion, as assessed by measurements of reversal potentials in various ionic media, which reveal a relative permeability of at least 10^6–10^8 for H^+ compared with other cations (Demaurex et al., 1993; Kapus et al., 1993a; DeCoursey and Cherny, 1994a; Cherny et al., 1995; DeCoursey and Cherny, 1997). This degree of selectivity is unheard of for traditional ion channels, and appears most reasonably explained by a special mechanism of H^+ conduction.

4.6.3.3. Nothing blocks H^+ channels. Many ion channels have characteristic peptide inhibitors — toxins and venoms are good sources — which bind specifically to the entry or vestibule of the channel and prevent ion conduction. In contrast, no known inhibitor binds to the channel and physically occludes the permeation pathway. Perhaps specific inhibitors will be found, but their absence may reflect the nature of the HBC conduction pathway in which the entrance to the channel may consist of a superficial protonation site rather than a commodious vestibule.

4.6.3.4. No multiple occupancy. Many ion channels exhibit behaviors that generally have been interpreted to mean that more than one permeant ion can be present inside the channel at the same time. Because of the fundamental difference between ionic diffusion through a water-filled pore and proton hopping across a HBC, we consider it unlikely that proton channels can sustain multiple occupancy, especially in the physiological pH range (DeCoursey and Cherny, 1999a).

4.6.3.5. H^+ channels do not inactivate. During a sustained voltage stimulus, many voltage-gated ion channels first open, then inactivate. As mentioned above (Section 4.3.4), voltage-gated proton channels do not inactivate in any preparation even during depolarizations lasting minutes (DeCoursey and Cherny, 1993). This property seems teleologically reasonable, because if the main function of these channels is to extrude metabolic acid during periods of high metabolic activity, it is desirable for the g_H to remain active as long as the acid load persists (DeCoursey and Cherny, 1993).

4.6.4. Molecular identification of H^+ channels

No voltage gated proton channel has been identified on a molecular level with complete certainty. The proposed function of voltage gated proton channels in phagocytes of facilitating the respiratory burst has led logically to the proposal that the H^+ channel is actually part of the NADPH oxidase complex. Henderson and colleagues (Henderson et al., 1995, 1997; Henderson and Meech, 1999) identified the gp91^{phox} glycoprotein, one of the membrane-bound components of NADPH oxidase, as comprising a proton channel. Most existing evidence is compatible with gp91^{phox} being either a proton channel or an accessory protein that modulates the function of proton channels. If gp91^{phox} is indeed a proton channel, then it most likely is the type *x* channel, which is activated during the respiratory burst in phagocytes. However, as shown in Fig. 13B, there was no correlation between the amplitude of electron current, which reflects the electrogenic activity of NADPH oxidase (Schrenzel et al., 1998), and the amplitude of the g_H activated by PMA, when compared directly in individual human neutrophils (DeCoursey et al., 2000). This observation can be reconciled with the idea that a component of the oxidase is a proton channel only if the plasma membrane contains a large excess of dysfunctional flavocytochrome b_{558} (containing gp91^{phox} and p22^{phox}) or if most functional oxidase components in the membrane do not function as H^+ channels. Perhaps 'rogue' gp91^{phox} monomers present in the membrane outside the NADPH oxidase complex might act as H^+ channels. This idea is compatible with the apparent H^+ channel activity of gp91^{phox} expressed in CHO cells that lack the other oxidase components (Henderson and Meech, 1999). However, in human neutrophils, gp91^{phox} and p22^{phox} monomers that are not assembled into the flavocytochrome b_{558} heterodimer are rapidly degraded (DeLeo et al., 2000). Furthermore, in several rare forms of chronic granulomatous disease (CGD) in which gp91^{phox} is expressed at normal levels, activation of the g_H by PMA was reduced dramatically (Nanda et al., 1994). Finally, human T lymphocytes have an arachidonic acid-activatable, Zn^{2+}-sensitive electrogenic H^+ efflux pathway, but do not have detectable cytochrome b_{558} (Káldi et al., 1996). We look forward

to the resolution of this intriguing mystery in the future.

It was reported recently that Mox-1, a homologue of gp91^{phox} that functions as a NADPH oxidase and generates superoxide anion (Suh et al., 1999), functions as a voltage gated proton channel when expressed in HEK cells (Bánfi et al., 2000). The properties reported for the gene product, termed NOH-1 or NOX-1, are consistent with type *e* or *p* channels, but are similar enough to the properties of H^+ channels expressed constitutively in HEK cells that it remains possible that Mox-1 simply up-regulates the expression of endogenous H^+ channels. This role might thus be analogous to that proposed for gp91^{phox}, as an accessory protein that is not itself a proton channel (DeCoursey et al., 2000). Although progress has been made, the molecular identities of the voltage gated proton channels in Table 2 remain obscure.

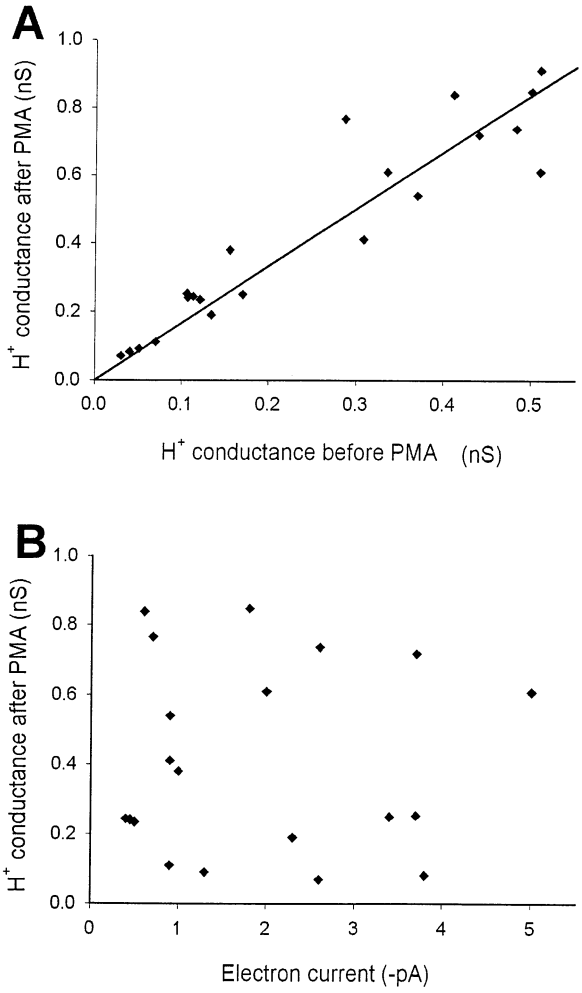

Fig. 13. Clues to the identity of the H^+ channels activated during the respiratory burst in human neutrophils. Both H^+ currents and electron currents, which reflect the electrogenic pumping of electrons across the cell membrane by NADPH oxidase, were recorded simultaneously in neutrophils studied in permeabilized-patch configuration. (A) The amplitude of the maximum g_H, $g_{H,max}$, before and after treatment with PMA was tightly correlated in human neutrophils. This result suggests that PMA modulates pre-existing H^+ channels, rather than inducing a new type of channel. (B) In contrast, there was no correlation between the amplitudes of electron currents and H^+ currents in individual human neutrophils. As discussed further in the text, this result is hard to reconcile with the notion that a component of the oxidase forms the H^+ channels activated during the respiratory burst. Taken from DeCoursey et al. (2000).

5. Conclusions

Microglia express a panoply of ion channels similar to other macrophages, including voltage-gated proton channels. H^+ channels in microglia have properties similar to those in other phagocytes. Although no functional role for H^+ channels has yet been demonstrated explicitly in microglia, it seems likely that the functions are analogous to those of H^+ channels in other phagocytes. Voltage-gated proton channels in all cells enable recovery from an acute acid load. All cells that are known to generate superoxide anion and that have been studied electrophysiologically, including microglia, express voltage gated proton channels in sufficient numbers to extrude all of the acid produced during the respiratory burst. In light of the activity-dependent pH changes that occur in the brain, it is possible that H^+ channels may play additional, more specialized roles in microglia. Important goals of future research will be to determine the general and specialized functions H^+ channels play in microglia, to elucidate the regulatory pathways that govern H^+ channel expression and properties, to identify the channel molecule and to explore structure-function relationships. Specific goals of structure-function studies will be to identify the external and internal protonation sites that govern the voltage-dependence of gating, and to identify the structural elements that comprise the permeation pathway. Eventually, pharmacological or genetic intervention directed at H^+ channels in microglia could provide a mechanism for regulating the behaviors of microglia that contribute to Alzheimer's disease, AIDS-related dementia, multiple sclerosis, Parkinson's disease, amytrophic lateral sclerosis, and acute injury processes associated with stroke.

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