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Protein kinase C activates an H^+ (equivalent) conductance in the plasma membrane of human neutrophils

(H^+ channels/pH regulation/leukocytes/H^+-ATPase/bafilomycin)

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ABSTRACT **The rate of metabolic acid generation by neutrophils increases greatly when they are activated. Intracellular acidification is prevented in part by Na^+/H^+ exchange, but a sizable component of H^+ extrusion persists in the nominal absence of Na^+ and HCO_3^-. In this report we determined the contribution to H^+ extrusion of a putative H^+ conductive pathway and its mode of activation. In unstimulated cells, H^+ conductance was found to be low and unaffected by depolarization. An experimental system was designed to minimize the metabolic acid generation and membrane potential changes associated with neutrophil activation. By using this system, β-phorbol esters were shown to increase the H^+ (equivalent) permeability of the plasma membrane. The direction of the phorbol ester-induced fluxes was dictated by the electrochemical H^+ gradient. Moreover, the parallel migration of a counterion through a rheogenic pathway was necessary for the displacement of measurable amounts of H^+ equivalents across the membrane. These findings suggest that the H^+ flux is conductive. The effect of β-phorbol esters was mimicked by diacylglycerol and mezerein and was blocked by staurosporine, whereas α-phorbol esters were ineffective. Together, these findings indicate that stimulation of protein kinase C induces the activation of an H^+ conductance in the plasma membrane of human neutrophils. Preliminary evidence for activation of a separate, bafilomycin A_1-sensitive H^+ extrusion mechanism, likely a vacuolar type H^+-ATPase, is also presented.**

Polymorphonuclear leukocytes (neutrophils) are attracted to sites of infection where they destroy invading organisms by a variety of microbicidal mechanisms, including phagocytosis, degranulation, and the production of toxic oxygen metabolites (1). Activation of neutrophils is associated with a large burst of intracellular H^+ generation, due primarily to stimulation of the NADPH oxidase and the associated acceleration of the hexose monophosphate shunt pathway. Despite this massive increase in net H^+ production, however, the cytosolic pH (pH_i) of activated cells remains at or above the resting level, even in the nominal absence of HCO_3^- (2, 3). Maintenance of pH_i in the physiological range is due, in part, to a concurrent activation of the Na^+/H^+ antiport. Indeed, if the antiport is inhibited by amiloride or by suspending the cells in Na^+-free medium, a pronounced cytosolic acidification is observed upon activation (2). However, even under these conditions, pH_i fails to reach the levels predicted by the amount of acid equivalents generated during the metabolic burst. Approximately 50 nmol of H^+ are generated in 5 min by 10^6 fully activated neutrophils (calculated from the rate of nonmitochondrial O_2 consumption of phorbol ester-stimulated cells, assuming one H^+ released per O_2 consumed). By considering the buffering power of the cytosol (≈28 mM per pH unit; ref. 4) and the cell volume (≈330 μm^3), it can be calculated that pH_i would be expected to drop by over 5 units (to pH 1.6) if the metabolically generated H^+ were to remain within the cell. However, cells stimulated in the nominal absence of Na^+ and HCO_3^- maintain their pH_i above 6.4, suggesting that alternative (Na^+- and HCO_3^--independent) H^+ extrusion mechanisms must exist in activated neutrophils. Accordingly, accelerated extracellular acidification can be recorded under these conditions.

The existence of H^+-conducting channels has been documented in snail neurones (5) and axolotl oocytes (6). In these tissues, the channels are inactive at the resting plasma membrane potential (E_m) but open when the membrane depolarizes. Such voltage-gated H^+ channels can be inhibited by Cd^{2+} and Zn^{2+} (5, 7). Conceivably, similar H^+-conducting channels may exist in mammalian neutrophils, possibly underlying all or part of the Na^+- and HCO_3^--independent acid extrusion. These putative channels may be constitutively open or may activate upon depolarization, which has been reported to occur when neutrophils are stimulated (8, 9). An electrogenic mode of action of the NADPH oxidase or changes in ionic conductance have been suggested to underlie this depolarization, but the precise mechanism remains a subject of debate (see refs. 9 and 10 for reviews). The presence of H^+ channels in neutrophils has been proposed by Henderson *et al.* (11), who observed that Cd^{2+} magnified the phorbol ester-induced depolarization in cytoplasts. These authors suggested that the putative H^+ channels may serve to compensate for the depolarization generated by the electrogenic oxidase.

The purpose of the present study was to directly assess the presence of an H^+ conductance in human neutrophils and, in particular, to establish its mechanism of activation. We present evidence that, in parallel with the stimulation of metabolic acid generation, protein kinase C (PKC) activates a conductive, H^+ (equivalent)-translocating mechanism in human neutrophils.

MATERIALS AND METHODS

Reagents and Solutions. *N*-Methyl-*N*-(2-methyl-2-propenylamino)amiloride (MMPA) was a gift from Merck Sharp & Dohme. Bafilomycin A_1 was the generous gift of K. Altendorf (Universitat Osnabrück, Osnabrück, F.R.G.). Diphenylene iodonium (DPI) was synthesized in our laboratory according to the method of Collette *et al.* (12). All other reagents were commercially available.

Na^+ medium contained 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM Hepes titrated to the required pH with concentrated NaOH. K^+ and

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Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; pH_i, cytosolic pH; di-O-C_5(3), 3,3′-dipentyloxacarbocyanine iodide; DPI, diphenylene iodonium; MMPA, *N*-methyl-*N*-(2-methyl-2-propenylamino)amiloride; E_m, plasma membrane potential; PKC, protein kinase C; TPA, 4β-phorbol 12-tetradecanoate 13-acetate; V, vacuolar; NMG, *N*-methyl-D-glucamine; pH_o, extracellular pH.

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N-methyl-D-glucamine (NMG^+) media were made by isoosmotic replacement of NaCl with KCl and the chloride salt of NMG, respectively, and titrated to the required pH with the corresponding bases. The osmolarity of all solutions was adjusted to 295 ± 5 milliosmolar with the major salt.

Methods. Neutrophils were isolated from freshly drawn human blood as described (4, 13). O_2 consumption was measured polarographically as described (4). pH_i was determined fluorimetrically using the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (4), using excitation and emission wavelengths of 495 nm and 525 nm, respectively. Calibration of pH_i vs. fluorescence intensity was performed by the method of Thomas *et al.* (14). Because the buffering power is approximately constant in the pH_i range studied (4), the rates of change of pH_i reported in the text are directly proportional to transmembrane H^+ (equivalent) fluxes.

Membrane potential was measured fluorimetrically using 0.25 μM 3,3'-dipentyloxacarbocyanine iodide [di-O-C_5(3)] and a cell density of 2×10^6 cells per ml. Excitation and emission wavelengths were 475 nm and 510 nm, respectively. Calibration was performed by correlating the fluorescence signal of cells suspended in media of varying $[K^+]$ containing 1 μM valinomycin with the calculated K^+ equilibrium potential (assuming the cytosolic $[K^+]$ = 140 mM).

Temperature was maintained at 37°C for all experiments. Traces illustrated are representative of a minimum of three similar experiments with blood from different donors. Data in the text are presented as means ± SEM of the number of experiments specified in parentheses.

RESULTS AND DISCUSSION

E_m and pH_i measurements were used to determine the H^+ conductance of resting, depolarized, and activated neutrophils. In agreement with earlier measurements (see ref. 10 for a review), we found the resting E_m of neutrophils suspended in a physiological Na^+ medium (extracellular $[K^+]$ = 3 mM) to average −78 ± 2 mV (n = 4). As shown in Fig. 1*A* (solid circles), this potential is dictated largely by the K^+ diffusion potential, as evident from the relationship between E_m and the extracellular K^+ concentration. A sizable H^+ conductive pathway is unlikely to exist in resting cells with a normally polarized membrane, as electrophoretic acid uptake would impose a continuous acid load. In accordance with this prediction, moderate alterations in the extracellular pH had minimal effect on E_m (Fig. 1*A*, solid squares).† From this data, the transference number for H^+ can be estimated to be at least 10-fold lower than that of K^+. By considering the reported values for K^+ permeability in human neutrophils (15) (which may include an electroneutral component and therefore overestimate the conductive permeability), the net conductive H^+ efflux in fully depolarized cells at physiological pH would be <0.65 milliequivalent per liter per min. This flux is considerably smaller than the phorbol ester-induced H^+ efflux recorded in cells stimulated in Na^+-free media (e.g., 10 ± 1 milliequivalents per liter per min in medium containing choline; unpublished observations). Therefore, the resting H^+ conductance is small and cannot account for the acid extrusion observed in metabolically stimulated cells.

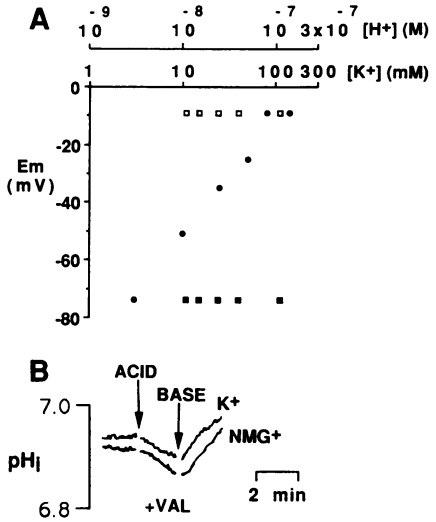

FIG. 1. H^+ conductance of resting and depolarized neutrophils. (*A*) Dependence of E_m on the extracellular $[K^+]$ (●). The osmolarity was balanced with Na^+. Dependence of E_m on the extracellular $[H^+]$ for polarized cells in Na^+ medium (■). Dependence of E_m on the extracellular $[H^+]$ for cells depolarized in K^+ medium (□). (*B*) pH_i changes of depolarized (K^+) and hyperpolarized (NMG^+) cells in response to changes in pH_o. Cell were suspended in K^+ or NMG^+ medium containing 1 μM valinomycin (VAL). Initially pH_o = 7.50; after acid, pH_o = 6.95; after base, pH_o = 7.85. E_m and pH_i were measured fluorimetrically using BCECF and di-O-C_5(3), respectively. Anti-fluorescein antibody was added in *B* to minimize the contribution of contaminating extracellular dye. Data are representative of at least three similar experiments.

The presence of voltage-gated H^+ channels was assessed in a similar manner. As shown in Fig. 1*A* (open squares), E_m remained relatively insensitive to changes in transmembrane pH gradient even after the membrane was depolarized by resuspension of the cells in a K^+-rich solution. This argues against the presence of a substantial voltage-gated H^+ conductance. This conclusion is supported by results obtained measuring H^+ (equivalent) fluxes by recording pH_i. In these experiments, Na^+- and HCO_3^--free solutions were used to preclude the major pH_i regulatory mechanisms (Na^+/H^+ exchange and Na^+-dependent and -independent HCO_3^-/Cl^- exchange). Under these conditions, a significant spontaneous cytosolic acidification occurs. For this reason, and in order to stabilize pH_i in the physiological range, somewhat alkaline media were used in this and subsequent experiments (see figure legends for details). Fig. 1*B* shows the effects on pH_i of sudden alterations of the extracellular pH in polarized and depolarized cells. Control of E_m was obtained by suspension of the cells in media of defined K^+ concentration containing valinomycin, a conductive K^+-selective ionophore. This procedure also ensured that the flux of H^+ equivalents was not limited by the endogenous counterion permeability. It is noteworthy that pH_i stabilized at comparable levels in polarized and depolarized cells (6.81 ± 0.05 and 6.82 ± 0.06, respectively; n = 4), arguing against the presence of a significant voltage-sensitive H^+ conductance. In agreement with this notion, the rate of change of pH_i was comparable in hyperpolarized and depolarized cells when the extracellular pH was made acidic (pH 6.95) and then alkaline (pH 7.85) (Fig. 1*B*). In four experiments the rates of acidification averaged 0.055 ± 0.004 and 0.049 ± 0.002 pH unit per min in NMG^+ and K^+ media, respectively. The respective rates of alkalinization were 0.076 ± 0.008 and 0.077 ± 0.014 pH unit per min. Taken together, the above data suggest that the H^+ conductance of resting neutrophils is low and unaffected by depolarization. As can be seen from Fig. 1, there does appear to be a measurable permeability to H^+ equivalents, since pH_i is affected by changes in external pH. The insensitivity of this permeability to E_m, however, suggests that it is largely nonconductive.

Because a sizable H^+ conductance does not appear to be constitutively active or voltage-gated in neutrophils, we considered the possibility that stimulation of the cells induces an increase in conductive H^+ permeability. For these experiments, cells were challenged with phorbol 12-tetradecanoate

†It is conceivable that the extracellular pH (pH_o)-induced changes in the conductance to other ions fortuitously offset E_m changes due to an H^+ conductance. Though unlikely, this possibility cannot be presently ruled out.

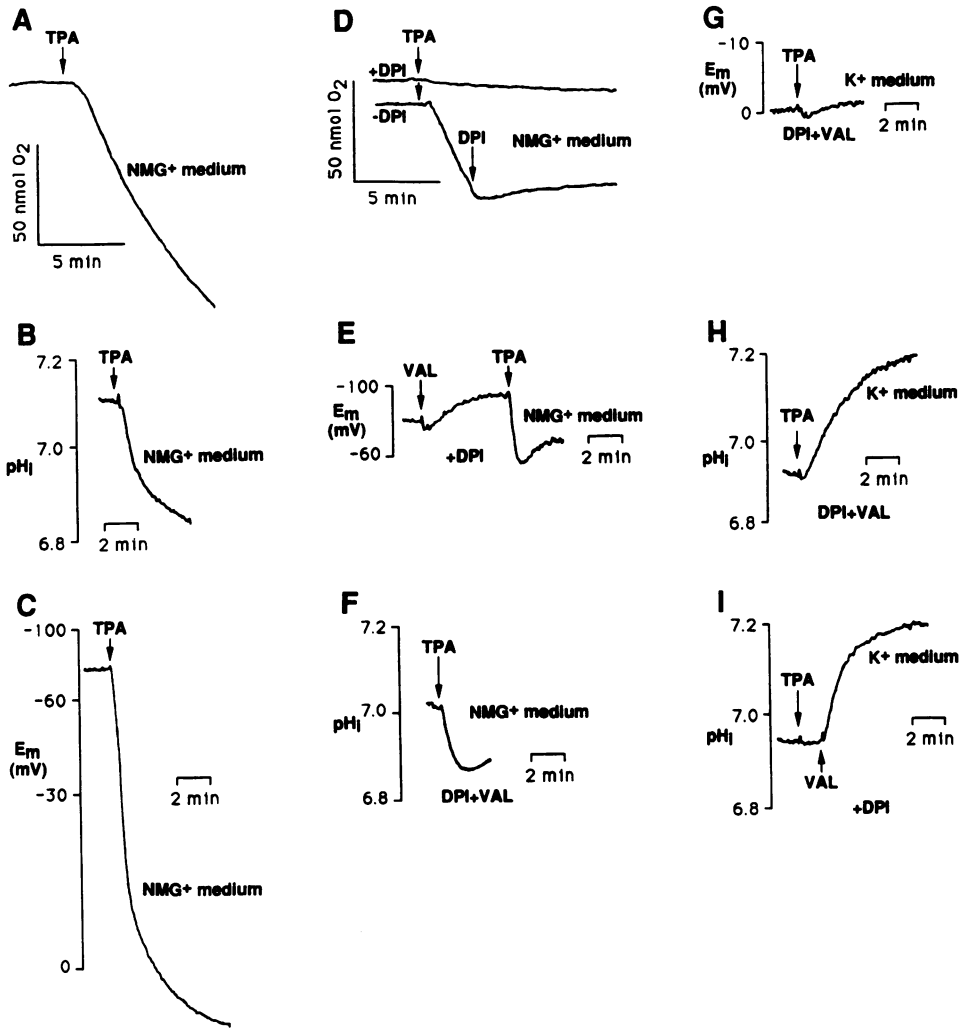

FIG. 2. Oxygen consumption and pH_i and E_m determinations in TPA-activated neutrophils. Cells were suspended in either NMG^+ medium (*A–F*) or K^+ medium (*G–I*) at pH 7.7. O_2 consumption (*A* and *D*) was measured using an O_2 electrode. pH_i (*B*, *F*, *H*, and *I*) and E_m (*C*, *E*, and *G*) were measured fluorimetrically using BCECF and di-O-C_5(3), respectively. When indicated, 50 nM TPA was added. In *E–I* and in the top trace of *D*, DPI (5 μM) was present in the medium from the outset. Valinomycin (VAL; 1 μM) was present from the outset in *F–H*. Elsewhere, DPI and valinomycin were added where indicated. Traces are representative of a minimum of three similar experiments.

13-acetate (TPA), a potent activator of PKC, which induces a large, sustained activation. As discussed above, activation of neutrophils is accompanied by a burst of O_2 utilization by the superoxide-generating NADPH oxidase (Fig. 2*A*). Oxidation of NADPH during the respiratory burst and the associated activation of the hexose monophosphate shunt result in the intracellular generation of a large amount of H^+ equivalents. In media devoid of Na^+ and HCO_3^-, this is manifested as a pronounced cytosolic acidification (Fig. 2*B*). Concomitantly, a large depolarization of the plasma membrane is observed‡ (Fig. 2*C*). Such ongoing, large changes in pH_i and E_m make it difficult to assess the appearance of a stimulus-induced H^+ conductance. To circumvent these difficulties, we designed an experimental paradigm that minimized H^+ production and stabilized E_m during stimulation. The first condition was achieved by inhibiting the respiratory burst using DPI, a competitive antagonist of the flavoprotein component of the NADPH oxidase (18). As shown in Fig. 2*D*, addition of 5 μM DPI essentially eliminated the O_2 consumption burst elicited by TPA. The inhibition ($\geq$95%) was observed whether DPI was added before or after the phorbol ester. Concomitantly, the intracellular acidification induced by TPA in Na^+-free media was also minimized (see Fig. 2*I*). To the extent that the oxidase is responsible for the E_m change (see ref. 10 for a review), treatment with DPI would also be expected to reduce the depolarization. To further stabilize E_m, the cells were additionally treated with valinomycin. When neutrophils were suspended in NMG^+ medium with DPI, treatment with the ionophore significantly hyperpolarized the plasma membrane (Fig. 2*E*). Subsequent stimulation with TPA produced a comparatively small and transient depolarization of E_m, which remained more negative than −55 mV at all times. Thus, the combined treatment with DPI and valinomycin minimized acid production and E_m changes, providing adequate conditions to evaluate the appearance of a stimulated H^+ conductance.

As shown in Fig. 2*F*, when cells suspended in NMG^+ medium containing DPI and valinomycin were stimulated with TPA, a decrease in pH_i was observed. This stimulus-induced acidification is consistent with influx of H^+ through a conductive pathway since, under the conditions used, the electrochemical gradient favors net H^+ uptake (i.e., E_m is more negative than the H^+ equilibrium potential, which approximates −40 mV). However, the observed acidification could also be attributed to residual metabolic H^+ generation triggered by the phorbol ester due to incomplete inhibition of the oxidase or to activation of other, DPI-insensitive metabolic processes. To distinguish between these possibilities, experiments were performed in cells depolarized in high K^+ medium with valinomycin. Because the extracellular pH is more alkaline than the cytosol, activation of a H^+ conductance under these conditions would be expected to result in

‡The absolute magnitude of the depolarization is difficult to assess accurately by the fluorescence method used, since cyanine dyes are known to react with the reduced oxygen metabolites generated by the stimulated cells. Among the cyanines, di-O-C_5(3) is reported to be the least susceptible to this artifact (16). The most reliable data have been obtained using lipid-soluble phosphonium derivatives. After stimulation with TPA, E_m was found by this method to depolarize to −20 to −15 mV (8, 17).

an increased pH_i, which is in the opposite direction and readily distinguishable from the changes expected from metabolic H^+ generation. As shown in Fig. 2*G*, addition of valinomycin to cells suspended in high K^+ solution effectively stabilized E_m near 0 mV both prior to as well as after treatment with TPA. Under these conditions, the phorbol ester was found to induce a sizable cytosolic alkalinization (Fig. 2*H*), consistent with conductive H^+ efflux, since E_m ($\approx$0 mV) is more positive than the H^+ equilibrium potential (≈ -40 mV).

Because the medium is more alkaline than the cytosol, leakage of the fluorescent dye from the cells could be responsible for the apparent alkalosis reported in Fig. 2*H*. This possibility can be discounted because the fluorescence increase caused by the phorbol ester was not dampened by adding to the medium anti-fluorescein antibodies, which are expected to quench the fluorescence of extracellular dye. Alternatively, a change in cell shape could also artifactually increase the fluorescent signal. However, the TPA-induced fluorescence changes were eliminated when pH_i was clamped using the K^+/H^+ exchange ionophore nigericin prior to challenge with the phorbol ester (data not illustrated), ruling out this possibility.

Together with the acidification recorded in NMG^+ medium, the alkalinization observed in depolarized cells suggests that an H^+ conductance was activated by PKC. Consistent with this notion, the effects of the phorbol ester could be mimicked by addition of the exogenous conductive protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP). An acidification was recorded upon addition of CCCP to cells in NMG^+ medium with valinomycin. Conversely, alkalosis was observed in K^+-rich solution when the protonophore was added in the presence, but not in the absence, of valinomycin. In both media, stimulation with TPA after addition of CCCP had little effect on pH_i (data not shown).

Although the data reported above are consistent with activation of an H^+-conductive pathway by TPA, other mechanisms are conceivable and must be ruled out. For instance, the phorbol ester could activate a K^+/H^+ antiport, such as that described in *Amphiuma* red cells and in corneal epithelium (19, 20). Such an electroneutral exchanger would be predicted to acidify pH_i in low K^+ media and to induce alkalinization in high K^+ solution, as observed in Fig. 2. However, several experimental findings argue against the existence of a K^+/H^+ antiport. First, the increase in pH_i induced by TPA in K^+ medium was absent when valinomycin was omitted, and subsequent addition of the ionophore rapidly restored the alkalinizing effect (Fig. 2*I*). This implies that H^+ (equivalent) translocation is rheogenic and limited by the permeability of the counterion. Secondly, TPA was also able to produce an alkalinization in the face of a large outward K^+ gradient, provided that the membrane was depolarized by means of conductive ionophores. These results are illustrated in Fig. 3*A*. Neutrophils were suspended in Na^+-rich medium containing DPI, and the amiloride analog MMPA was added in order to preclude Na^+/H^+ exchange. They were next depolarized by using 200 nM gramicidin D and finally exposed to TPA. As in cells depolarized by K^+ plus valinomycin, the phorbol ester promoted a rapid, pronounced alkalinization. This pH_i change required the presence of gramicidin, as only a slight acidification was recorded when TPA was added in its absence. In the latter case, subsequent addition of gramicidin initiated the alkalinization (data not shown). Together, these observations rule out activation of K^+/H^+ exchange as the mechanism underlying the pH_i changes elicited by TPA in Fig. 2 *F* and *H*.

An electrogenic H^+ pump is believed to exist in the plasma membrane of other phagocytic cells, such as murine macrophages (21). A similar system could, in principle, mediate the

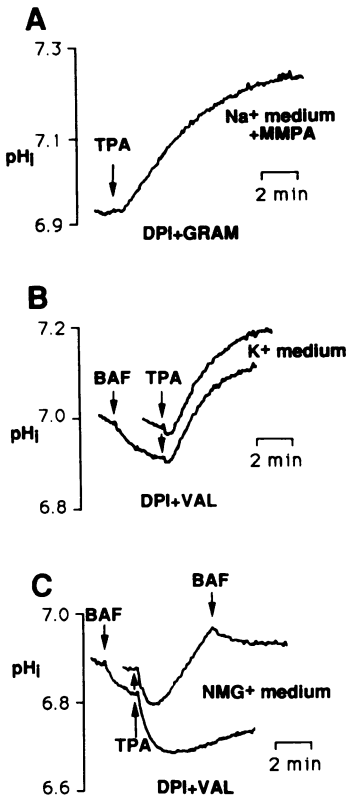

FIG. 3. Assessment of the role of K^+/H^+ exchange and of V-type H^+-ATPases in the TPA-induced changes in pH_i. pH_i was measured fluorimetrically by using BCECF. (*A*) Cells were suspended in Na^+ medium containing 1 μM MMPA to inhibit Na^+/H^+ exchange, 5 μM DPI, and 200 nM gramicidin D (GRAM). (*B*) Cells were suspended in K^+ medium containing 5 μM DPI and 1 μM valinomycin (VAL); 100 nM bafilomycin A_1 (BAF) was added where indicated. (*C*) Cells were suspended in NMG^+ medium containing 5 μM DPI and 1 μM valinomycin; 100 nM bafilomycin A_1 was added where indicated. TPA (50 nM) was added to all traces where noted. The pH of all media was 7.7. Traces are representative of at least three similar experiments.

effects of TPA in neutrophils, accounting for the requirement for a conductive counterion. According to this model, stimulation of the putative pump would lead to alkalinization in depolarized cells. The activity of this pump would be greatly reduced if cells are hyperpolarized, possibly unmasking a metabolic acidification, resulting in a drop in pH_i like that of Fig. 2*F*. To test this hypothesis, the effects of TPA were assessed in the presence of 100 nM bafilomycin A_1. This macrolide antibiotic is the most potent and specific known inhibitor of H^+-ATPases of the vacuolar (V) type (22), including that reported in macrophages. As illustrated in Fig. 3*B*, addition of bafilomycin A_1 induced a rapid but moderate cytosolic acidification. This may reflect a role of plasma membrane H^+ pumps in the maintenance of pH_i, net release of protons from intracellular acidic compartments, or a combination of these events. More importantly, the pump inhibitor had little effect on the TPA-induced alkalinization of depolarized neutrophils (Fig. 3*B*). In four separate experiments, the maximal rates of alkalinization were not significantly different in control (0.10 $\pm$ 0.01 pH unit per min) vs. bafilomycin A_1-treated cells (0.09 $\pm$ 0.01 pH unit per min). The extent of the alkalinization, measured after 5 min, was only marginally affected (control: $\Delta pH = 0.23 \pm 0.01$; bafilomycin A_1-treated: $\Delta pH = 0.20 \pm 0.01$; $0.05 < P < 0.1$). The mitochondrial ATPase inhibitors oligomycin (10 μg/ml) and dicylohexylcarbodiimide (5 μM) were also without effect. The data suggest that the TPA-induced alkalinization in

depolarized neutrophils is not mediated by V-type or F_1F_0-type H^+-ATPases.

Even though bafilomycin A_1 did not affect the alkalosis observed in depolarized cells, significant effects were noted in cells stimulated in NMG^+ medium in the presence of valinomycin (Fig. 3*C*). As illustrated in the top trace, addition of TPA under the latter conditions produced a biphasic pH_i change: a rapid acidification, attributed above to conductive H^+ influx, followed by a slower alkalinization. The latter component was observed reproducibly but was omitted from Fig. 2*F* for simplicity. Addition of bafilomycin A_1 rapidly suppressed the secondary alkalinizing trend, suggesting involvement of V-type ATPases. When the inhibitor was added prior to the phorbol ester, a spontaneous acidification was observed, as described for Fig. 3*B*. Subsequent stimulation with TPA resulted in a more profound acidification, with a pronounced inhibition (70% ± 2%; $n = 5$) of the secondary alkalinization. The latter can therefore be tentatively attributed to active H^+ pumping through a bafilomycin-sensitive ATPase. It is noteworthy that, when the inhibitor was absent, the secondary alkalinization drove pH_i beyond the initial steady-state level (Fig. 3*C*). This implies that activation of the putative pumps was not simply in response to the initial acidification but suggests instead a direct stimulatory effect of the phorbol ester.

Taken together, the data summarized above indicate that neither K^+/H^+ exchange nor H^+ pumping through a V-type ATPase can fully account for the effects of TPA and strongly suggest the induction of a passive H^+ conductive pathway. Activation of this conductance by the phorbol ester TPA is suggestive of the involvement of PKC. However, PKC-independent effects of phorbol esters have been reported (e.g., ref. 23), and the possibility of nonspecific effects of TPA must be considered. To define the involvement of PKC in activating the conductance, a series of structurally unrelated activators of the kinase were tested. As shown in Table 1, in addition to the active isomer of TPA, the diacylglycerol analogue 1,2-dioctanoyl-*sn*-glycerol and the nonphorbol tumor promoter mezerein all activated the H^+ conductance. At the concentrations used, these stimuli are expected to maximally activate PKC in every instance. Accordingly, the effects recorded were comparable in magnitude. Further evidence that PKC mediates the response was obtained by using the inactive 4α isomer of TPA, which failed to activate the conductance. Moreover, in the presence of staurosporine, a potent inhibitor of PKC, the stimulatory form of TPA did not activate the conductive H^+ fluxes (Table 1). The accumulated data clearly point to a role for PKC in the activation of the H^+ permeability pathway.

In summary, human neutrophils appear to have a low basal conductance to H^+ equivalents, thereby minimizing the membrane potential-driven acidification of the cytosol in the resting state. Though depolarization occurs when the cells are stimulated, the change in E_m is by itself insufficient to activate an H^+ conductance. In this regard, neutrophils seem to differ from molluscan neurons (5) and amphibian oocytes (6), which display voltage-gated H^+ channels. Instead, an H^+ (equivalent) conductance appears to be activated when PKC is stimulated in neutrophils. Because the cells tend to acidify and markedly depolarize upon stimulation‡, the combined electrical and chemical gradients (E_m more positive than the H^+ equilibrium potential) can in principle drive a net H^+ efflux through the conductive pathway, possibly accounting for a significant fraction of the Na^+- and HCO_3^--independent efflux of acid equivalents recorded in stimulated cells. Indeed, under the conditions studied ($E_m \approx 0$ mV and $\Delta pH \approx 0.7$; e.g., Figs. 2*H* and 3*B*), TPA induced a maximal conductive H^+ flux averaging 2.8 ± 0.3 milliequivalents per min per liter of cells, calculated using a buffering power of 28 mM per pH unit, determined earlier (4).

Finally, results are also presented suggesting the activation of a bafilomycin-sensitive H^+ extrusion process by phorbol esters. Given the reported specificity of the bafilomycins (22) and the concentration used in our experiments, this process most likely represents a V-type H^+ pump. In cells stimulated in NMG^+ medium, the bafilomycin-sensitive component not only compensated for the TPA-induced acidification, but in fact raised pH_i above the steady state, suggesting direct activation of H^+ pumping by PKC. In accordance with this view, a delayed bafilomycin-sensitive alkalinization can be recorded when K^+-depolarized cells are stimulated in the presence of Zn^{2+}, added to block the H^+ conductance (unpublished observations).

Table 1. Evidence for the involvement of PKC in activating the H^+ conductance

Addition	ΔpH_i in 3 min	*n*
TPA (50 nM)	0.15 ± 0.01	4
Mezerein (100 nM)	0.17 ± 0.02	3
DiC_8 (1 μM)	0.17 ± 0.02	3
α-TPA (50 nM)	0.02 ± 0.01	3
TPA (50 nM) + staurosporine (100 nM)	0.03 ± 0.01	3

pH_i was measured fluorimetrically using conditions identical to those of Fig. 2*H*, except that the indicated compounds were used as stimuli. Numbers are means ± SE of the number (*n*) of determinations indicated. DiC_8, 1,2-dioctanoyl-*sn*-glycerol; α-TPA, 4α-phorbol 12-tetradecanoate 13-acetate.

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